

Clinical Communications

Fecal short-chain fatty acids in pregnancy and offspring asthma and allergic outcomes

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Clinical Implications

- Mothers with higher fecal acetic acid, compared with other short-chain fatty acids, during pregnancy were less likely to have offspring with atopic asthma. If replicated, findings support use of microbiome-targeted treatments during pregnancy to increase fecal acetic acid.

TO THE EDITOR:

Asthma is the most common chronic disease in children and comorbid environmental allergies have prognostic relevance.¹ Short-chain fatty acids (SCFA), including acetic acid, butyric acid, and propionic acid, are metabolites produced by intestinal microbes through fermentation of dietary fiber.² Immune-modulating properties of SCFA are numerous and include increasing T regulatory cell differentiation.³ Fecal SCFA during infancy are associated with reduced risk of asthma with allergic sensitization later in life.⁴⁻⁶ In mouse models, SCFA supplementation during pregnancy leads to reduced allergic airway disease in offspring,^{7,8} and in 40 mother-child pairs, low maternal serum acetic acid was associated with increased cough or wheeze in infant offspring.⁷ Other than this, the association of SCFA during pregnancy and offspring risk of asthma and/or allergy has not been reported.

We hypothesized that SCFA during pregnancy, measured via mass spectrometry, would be associated with reduced asthma and allergy in offspring. [Methods](#) are detailed in this article's Online Repository at www.jaci-inpractice.org. Subjects were 150 mother-child pairs who participated in the Vitamin D Antenatal Asthma Reduction Trial (VDAART), a randomized controlled trial of vitamin D during pregnancy for prevention of asthma (NCT00920621, [Tables E1-E3](#), available in this article's Online Repository at www.jaci-inpractice.org). The study protocol was approved by the institutional review boards at each participating institution and at Brigham and Women's Hospital. All participants provided written informed consent.

Participating mothers provided stool samples during the third trimester of pregnancy in which SCFA were measured by liquid chromatography with tandem mass spectrometry. We examined both absolute concentrations of SCFA and relative

concentrations of each SCFA as a proportion of all measured SCFA. In general, the fecal acetic acid:butyric acid:propionic acid ratio is approximately 60:20:20. Deviations from this ratio reflect differences in bacterial composition and other exposures. For example, relative acetic acid has repeatedly been associated with breastfeeding and associated gut microbes.⁹ In addition, abundance of one SCFA could influence the functions of others via mechanisms such as competition at common G-protein coupled receptors² and effects on bioavailability. So, relative proportions may be more important in some contexts than absolute concentrations.

Clinical outcomes in offspring were ascertained at age 6 years. Asthma/wheeze was based on parental report of physician-diagnosed asthma, recurrent wheeze, and asthma medication use. Allergic sensitization was defined by serum specific IgE ≥ 0.35 kU/L to at least one environmental or food allergen. Hay fever was defined by parental report of physician-diagnosed allergic rhinitis. Seventy (47%) children had asthma/wheeze, 38 (48%) had allergic sensitization, and 54 (40%) had hay fever. Seventeen (21%) had both asthma/wheeze and sensitization and 33 (24%) had both asthma/wheeze and hay fever.

In logistic regression analyses, neither absolute nor relative maternal fecal SCFA were associated with the individual outcomes of asthma/wheeze, sensitization, or hay fever ([Figure 1](#) and [Table E4](#), available in this article's Online Repository at www.jaci-inpractice.org). However, relative, but not absolute, acetic acid concentration was inversely associated with asthma/wheeze with sensitization (odds ratio [OR] for a 10% increase in relative acetic acid concentration 0.46, 95% confidence interval [CI] 0.22-0.94, $P = .036$) ([Figure 2](#), [Table E4](#)). A similar nonsignificant inverse association was seen between maternal fecal relative acetic acid and asthma/wheeze with hay fever (OR, 0.63; 95% CI, 0.37-1.05, $P = .077$) ([Figure 2](#)). Permutation testing yielded similar results ([Table E5](#), available in this article's Online Repository at www.jaci-inpractice.org).

As SCFA are products of microbial fermentation of dietary fiber, we investigated the maternal diet and fecal microbiome, hypothesizing that these factors correlate with fecal SCFA and offspring outcomes. Maternal dietary fiber intake was estimated from food frequency questionnaires from the third trimester. In adjusted linear regression analyses, fiber intake was positively associated with total SCFA (beta for a 1-unit increase in log-transformed dietary fiber nutrient density = 0.26; 95% CI, 0.02-0.44, $P = .035$) and with absolute concentrations of acetic acid (beta = 0.30; 95% CI, 0.07-0.53, $P = .011$) and propionic acid (beta = 0.26; 95% CI, 0.01-0.51, $P = .045$) ([Table E6](#), available in this article's Online Repository at www.jaci-inpractice.org). Fiber intake was positively but nonsignificantly associated with relative acetic acid (beta = 0.03; 95% CI, -0.0004 to 0.07, $P = .052$). However, fiber intake during pregnancy was not associated with offspring outcomes ([Table E7](#), available in this article's Online Repository at www.jaci-inpractice.org), and this may be because dietary fiber contributes to offspring disease risk only in combination with relevant intestinal microbes.

Bacterial 16S rRNA sequencing was performed on the same stool samples used for SCFA profiling in 114 of the 150

Associations of Maternal Fecal Short-Chain Fatty Acids with Offspring Clinical Outcomes

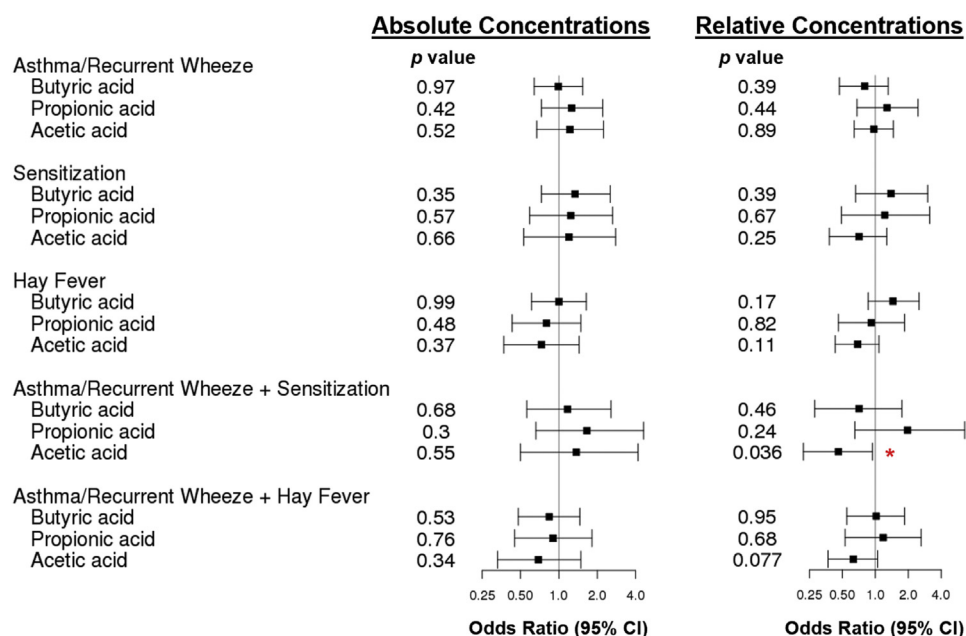


FIGURE 1. Associations of maternal absolute and relative fecal SCFA and offspring clinical outcomes. Odds ratios are for crude logistic regression analyses. Absolute SCFA concentrations were log-transformed before analysis. For relative concentrations, odds ratios pertain to change in odds of the outcome associated with a 10% increase in SCFA relative concentrations. SCFA, Short-chain fatty acids.

Association of Fecal Relative Acetic Acid Concentration During Pregnancy with Clinical Outcomes in Offspring

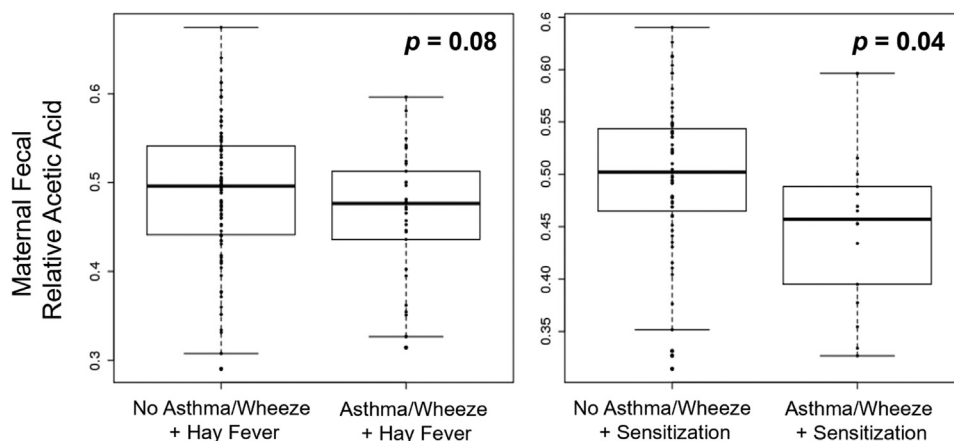


FIGURE 2. Box plots summarize maternal fecal relative acetic acid for subjects with asthma/wheeze with sensitization ($n = 17$) versus without asthma/wheeze with sensitization ($n = 63$); and for subjects with asthma/wheeze with hay fever ($n = 33$) versus without asthma/wheeze with hay fever ($n = 102$).

participating mothers. Shannon index, an alpha diversity metric, was not associated with SCFA (Table E8, available in this article's Online Repository at www.jaci-inpractice.org). Bray-Curtis dissimilarity, a beta diversity measure of taxonomic composition, was associated with relative and absolute concentrations of all 3 SCFA (all $P < .05$, Table E8), providing evidence that specific taxa contribute to production of SCFA. However, Bray-

Curtis dissimilarity was not associated with offspring outcomes (Table E9, available in this article's Online Repository at www.jaci-inpractice.org).

In negative binomial regression analyses, 4 taxa were associated with relative concentrations of all 3 SCFA (false discovery rate < 0.20 ; Table E10, available in this article's Online Repository at www.jaci-inpractice.org). Of these, *Eubacterium*

dolichum and *Streptococcus anginosus* demonstrated directionally consistent associations with offspring asthma/wheeze with sensitization (log2-fold change 1.7 and -1.2 ; $P = .002$ and $.045$, respectively; Table E11 and Figure E1, available in this article's Online Repository at www.jaci-inpractice.org). These species belong to genera known to produce SCFA,² though given the broad array of species that produce SCFA, likely represent a small subset of those with relevance to offspring health. From these analyses, we concluded that specific taxa may impact relative acetic acid in the gut during pregnancy, which may in turn modify risk of atopic asthma/wheeze in offspring.

In summary, we found that the relative concentration of the SCFA acetic acid during pregnancy is associated with reduced asthma/wheeze with allergic sensitization in offspring. A similar nonsignificant association was seen between relative acetic acid during pregnancy and asthma/wheeze with hay fever in offspring. Although prior literature suggests that maternal fecal SCFA may influence child outcomes via mechanisms including epigenetic modification, it is possible that maternal fecal SCFA also correlate with child diet and microbiome composition and the latter may have more causal significance. If findings are replicated, this study supports the use of microbiome-targeted therapies, potentially in combination with dietary fiber, to increase SCFA production as therapeutic strategies to prevent asthma and atopy in offspring.

This analysis is subject to limitations. Food frequency questionnaire data are subject to presumably nondifferential measurement bias, though the significant correlations we saw between fiber intake and fecal SCFA provide some degree of validation for both measures. Both dietary intake and relevant elements of the gut microenvironment may have varied over pregnancy, and we assessed only 1 time point in the third trimester. Our sample size was limited as data were not available for all VDAART participants, and though our findings are biologically plausible, they require replication in an independent sample.

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Conflicts of interest: J. Lasky-Su is a consultant to Metabolon, Inc. R. S. Zeiger is a consultant for AstraZeneca, DBV Technologies, Genentech, Inc., GlaxoSmithKline, Novartis, Patara Pharma, Regeneron, TEVA Pharmaceuticals, and Theravance Biopharma, and has received research support from Aerocrine, AstraZeneca, Genentech, Inc., GlaxoSmithKline, National Heart, Lung, and Blood Institute, MedImmune, and Merck. G. T. O'Connor is a coinvestigator on a grant from Janssen Pharmaceuticals to Boston University that funds a study of the pathogenesis of chronic obstructive pulmonary disease. L. B. Bacharier participates on the Data Safety Monitoring Board of DBV Technologies. A. Beigelman holds stock from DBV Technologies. S. T. Weiss has received royalties from UpToDate, Inc. A. A. Litonjua has received author royalties from UpToDate, Inc. and consultant fees from AstraZeneca, LP. The rest of the authors declare that they have no relevant conflicts of interest.

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ONLINE REPOSITORY

METHODS

Study design and participants

Subjects were mother and offspring participants in the Vitamin D Antenatal Asthma Reduction Trial, a randomized controlled trial of vitamin D supplementation during pregnancy for prevention of asthma in offspring conducted in the United States (NCT00920621).^{E1} The study protocol was approved by the institutional review boards at each participating institution and at Brigham and Women's Hospital. All participants provided written informed consent. All participants were asked to provide a stool sample during the third trimester of pregnancy for measurement of short-chain fatty acids (SCFA) and other assays, and 150 mothers elected to do so. A flow diagram is displayed in Figure E1. There were no statistically significant differences in offspring clinical outcome distributions by SCFA data availability; study site was the only characteristic that differed between groups, with subjects from Boston more likely to have available SCFA and those from San Diego less likely to have available SCFA data (Table E1).

Measurement of short-chain fatty acids (SCFA)

One hundred and fifty mothers provided stool samples during the third trimester of pregnancy for measurement of SCFA. Eight SCFA were measured by liquid chromatography with tandem mass spectrometry by Metabolon, Inc. (Morrisville, NC): acetic acid, propionic acid, butyric acid, isobutyric acid, 2-methyl-butiric acid, isovaleric acid, valeric acid, and hexanoic acid. Although multiple SCFA measurements throughout pregnancy would have been ideal, this was not available and a single sample was felt to be sufficient for several reasons: (1) we saw correlations between relatively long-term estimates of fiber intake (from food frequency questionnaires asking about diet over the prior month) and spot fecal SCFA measured by mass spectrometry; (2) studies of dietary interventions on fecal SCFA show no significant changes in control arms^{E2}; and (3) the gut metabolome is closely linked with the gut microbiome,^{E3} which is personally distinct and relatively stable over time in the absence of major perturbations.^{E4,E5} Stool samples were spiked with stable labeled internal standards and were homogenized and subjected to protein precipitation with an organic solvent. After centrifugation, an aliquot of the supernatant was derivatized to form the corresponding SCFA hydrazides. The reaction mixture was diluted, and an aliquot injected onto an Agilent 1290/AB Sciex QTrap 5500 liquid chromatograph with a tandem mass spectrometry system equipped with a C18 reversed phase ultrahigh-performance liquid chromatography column. The mass spectrometer was operated in negative mode using electrospray ionization. The peak area of the individual analyte product ions was measured against the peak area of the product ions of the corresponding internal standards. Quantitation was performed using a weighted linear least-squares regression analysis generated from fortified calibration standards prepared immediately before each run. Raw data were collected and processed using AB SCIEX software Analyst 1.6.2. Data reduction was performed using Microsoft Excel 2013. Four levels of quality control (QC) samples were used: QC Low, QC Medium, QC High, and QC Endogenous with all analytes at the endogenous levels, except hexanoic acid, which was spiked to approximately Standard D level. QC performance was assessed using the corresponding QC

replicates in the sample run. QCs met acceptance criteria at all levels for all analytes (targeted acceptance criteria: at least 50% of QC samples at each concentration level per analyte should be within $\pm 20.0\%$ of the running mean, and at least two-thirds of all QC samples per analyte should fall within $\pm 20.0\%$ of the corresponding running mean). Samples that fell below the limit of quantitation are labeled "BLOQ." In the case of acceptable data, extrapolated values are given for BLOQ samples. There were 3 samples BLOQ without extrapolated values for valeric acid and isovaleric acid and 2 for isobutyric acid and 2-methylbutyric acid; otherwise there were no missing values. Values BLOQ without extrapolated values were replaced by one-half of the lower limit of quantification. SCFA concentrations were reported in $\mu\text{g/g}$ feces (fresh weight). Relative SCFA concentration was defined by the absolute quantity of an SCFA divided by the sum of the absolute quantities of all measured SCFA. Absolute SCFA concentrations were right-skewed and were log-transformed before analysis. Relative SCFA concentrations were approximately normally distributed. Means (standard deviations) for absolute acetic acid, butyric acid, and propionic acid concentrations were 3247 (1548), 1455 (1018), and 1175 (663) $\mu\text{g/g}$, respectively. Means (standard deviations) for relative acetic acid, butyric acid, and propionic acid concentrations were 0.48 (0.08), 0.20 (0.07), and 0.18 (0.05), respectively.

Clinical outcomes ascertainment

Clinical outcomes in offspring were ascertained at age 6 years. The outcome of asthma/wheeze has been described previously^{E6} and is based on parental report of physician-diagnosed asthma, recurrent wheeze, and asthma controller medications. Hay fever is based on parental report of physician-diagnosed hay fever or allergic rhinitis. Parents were queried about these outcomes by telephone every 3 months and at annual in-person visits for the first 6 years of life, and data throughout follow-up were used. Allergic sensitization was defined by serum specific IgE ≥ 0.35 kU/L to at least one common environmental or food allergen. Tested allergens included *Alternaria alternata*, *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, German cockroach, cat dander, dog dander, grass pollen mix, tree pollen mix, egg white, walnut, milk, peanut, soybean, and wheat. Of the 150 offspring of mothers with available fecal SCFA during pregnancy, asthma/wheeze status was known for all 150 offspring; hay fever status was known for 135 offspring, and serum-specific IgE for sensitization ascertainment was available for 80 offspring.

Ascertainment of other covariates

Other characteristics analyzed included child sex, child and mother race/ethnicity, study center, maternal education, and number of living children previously born to mothers. Data collected at or shortly after birth included maternal antibiotics during delivery or child antibiotics after delivery and mode of delivery. Parental questionnaire responses were used to determine whether the child was exclusively breastfed for the first 4 months of life and presence of a pet dog in the home during infancy. Maternal body mass index (BMI) was based on study visit measurements at the first study visit (between weeks 10 and 18 of gestation). Maternal BMI at the first study visit and pre-pregnancy BMI were highly correlated (Spearman rho = 0.97, $P < .001$), and BMI at the first study visit was used because fewer data were missing on this variable (5 vs 16).

Maternal dietary fiber intake estimation

All 150 mothers with available fecal SCFA measurements completed a validated, abbreviated food frequency questionnaire during the third trimester of pregnancy asking them how often they ate each of 18 foods or food groups over the prior year.^{E7} Mothers were asked how often they added salt to their food, and how often they ate the following foods or food groups in the past year:

- Dark green leafy vegetables
- Cruciferous vegetables (broccoli, cabbage, cauliflower, etc)
- Carrots
- Other vegetables
- Citrus fruits
- Other fruits
- Whole milk food products
- Low fat milk products
- Whole eggs
- Stick margarine
- Whole grain foods
- Pasta, rice, or noodles
- Baked products (doughnuts, etc)
- Beef, pork, or lamb (main dish)
- Processed meats
- Fish/seafood (not fried)
- Deep fried foods

Possible responses included “Less than once per week,” “Once per week,” “2 to 4 times per week,” “Nearly daily or daily,” and “Twice or more per day.” Of note, only total dietary fiber was estimated, and this is a limitation as fiber types vary in SCFA yield. Dietary fiber was analyzed as a nutrient density, and all analyses of dietary fiber were adjusted for total calorie intake as previously described.^{E8,E9}

Maternal bacterial microbiome profiling

Mothers were asked to collect a 0.5 teaspoon-sized sample during the third trimester of pregnancy in the 48 hours before a study visit and store the sample in a home freezer before transport with a freezer pack to the study visit. Stool was not collected if participants had used antibiotics in the past 7 days. After delivery to the study site, stool samples were immediately stored at -80°C . DNA extraction and sequencing of the bacterial 16S V4 hyper-variable region using the Illumina MiSeq platform (San Diego, Calif) were performed at Partners Healthcare Personalized Medicine (Boston, Mass). Approximately 200 mg of stool per sample was resuspended in 1 mL InhibitEX Buffer from the Qiagen QIAamp Fast DNA Stool Mini Kit (catalogue number 51604; Qiagen, Venlo, The Netherlands) in tubes containing 0.1-mm silicon beads (catalogue number 31-212S1; Genesee Scientific, Morrisville, NC). Samples were disrupted using a Mini-Beadbeater 24 from Biospec Products for 3 minutes. Extraction continued on the contents of the entire tube using the Qiagen QIAamp Fast DNA Stool Mini Kit automated on a Qiagen QIAcube. Resulting DNA QC was evaluated using a PicoGreen assay (Quant-iT dsDNA assay kit, catalogue number P7589; ThermoFisher, Waltham, Mass) on a Gemini XP spectrophotometer from Molecular Devices.

Input into library construction was 15 ng DNA using the NEXTflex 16S V4 Amplicon-Seq kit 2.0 (catalogue number 4203-04; Bioo Scientific, Austin, Tex). Finished libraries were normalized to 10 nM using PicoGreen quantitation, and sizing

information was gathered using TapeStation D100 screen tapes and reagents (catalogue numbers 5067-5582 and 5067-5583; Agilent, Santa Clara, Calif). Final QC was carried out to establish the amount of library containing ligated Illumina adaptors using KAPA Library Quantification Kits (catalogue number KK4824; KAPA Biosystems, Wilmington, Mass). Up to 288 libraries were pooled to provide equimolar amounts of each library in the pool, which was then run on Illumina MiSeq using the MiSeq Reagent Kit v3 600 cycle kit (MS-102-3003). Each pool contained a 20% spike-in of PhiX Control library v3 (catalogue number FC-110-3001; Illumina, San Diego, Calif) to increase diversity of the library sequence. FASTQ files were generated on the MiSeq instrument.

Filtering, trimming, and chimera checking were performed as previously described.^{E10,E11} We used closed reference operational taxonomic unit (OTU) classification in Qiime to group sequences according to sequence similarity and match to taxonomy.^{E4} Additional processing of microbiome data was performed using the Phyloseq package for R.^{E5} Specifically, samples with read counts of less than 1000 and OTUs that were not seen at least once in at least 5% of samples were excluded. OTUs with the same species annotation were merged using the `tax_glom` function.

Statistical analyses

Statistical analyses were conducted using R version 3.5.0 (R Foundation for Statistical Computing). Baseline characteristics were tabulated and Fisher's exact tests were used to test for differences by offspring clinical outcome. Analyses of clinical outcomes were adjusted for potential confounders that were associated with at least one clinical outcome and at least one maternal fecal SCFA in univariable analyses. The distribution of demographic variables was similar between offspring with and without clinical outcomes (Table E2). However, children with asthma/wheeze were more likely to have had a dog during infancy (23% vs 10%, $P = .047$) and less likely to have been breastfed (17% vs 42%, $P = .002$), and hay fever was more frequent among black subjects (57% vs 36%, $P = .032$), those with mothers with higher early pregnancy BMI (mean 30.7 vs 27.2, $P = .006$), and also varied by study center ($P = .021$). Breastfeeding and early pregnancy BMI were associated with at least one maternal SCFA level ($P < .05$), whereas dog ownership, race/ethnicity, and study center were not associated with any maternal SCFA (Table E3). Accordingly, outcome analyses were adjusted for early pregnancy BMI and breastfeeding. This confounder selection strategy was used because of the relatively low number of subjects with some outcomes (eg, asthma/wheeze with sensitization) to prevent overfitting models with all theoretical potential confounders.

Analyses of maternal dietary fiber included continuous variables where a larger number of covariates could be included in models. So, they were adjusted for potential confounders selected *a priori*: maternal daily total energy intake, race/ethnicity, education, early pregnancy BMI, and study center. Logistic regression was used to determine the associations of maternal fecal SCFA, dietary fiber, and microbiome alpha diversity with offspring clinical outcomes. Linear regression was used to determine associations of maternal dietary fiber with maternal fecal SCFA and microbiome alpha diversity. Given limited sample size, where regression models showed significant associations between maternal fecal SCFA and offspring clinical

outcomes, we further tested the null hypothesis of no association with permutation testing of P values, resampling 1000 times with the Monte Carlo algorithm using the R package `pgirmess` (function `PermTest`). This generates P value estimates based on comparison of observed P values with P values generated by random shuffling of the clinical outcome for each subject.

For analyses of microbiome data, the function `diversity` in the R package `vegan`^{E12} was used to calculate alpha diversity (Shannon index) based on OTU-level data. Spearman correlations between SCFA concentrations and Shannon index, which was approximately normally distributed, were calculated. For analyses of beta diversity, Bray-Curtis dissimilarities were

calculated using the function `ordinate` in R package `Phyloseq` after scaling reads to the minimum sample read count (7274). Permutational multivariate analysis of variance using the `adonis` function in R package `vegan` was used to compare Bray-Curtis beta diversity by SCFA concentrations and by offspring outcome. R packages `Phyloseq` and `DESeq2` were used to conduct negative binomial regression analyses of associations of species-level taxa with SCFA and offspring outcomes. The method of Benjamini and Hochberg^{E13} was used to correct P values for multiple testing in analyses of taxonomic differences only. For all other analyses, raw P values are reported given the targeted nature of this analysis.

Association of Maternal Fecal *Eubacterium Dolichum* and *Streptococcus anginosus* with Asthma/Wheeze with Sensitization in Offspring

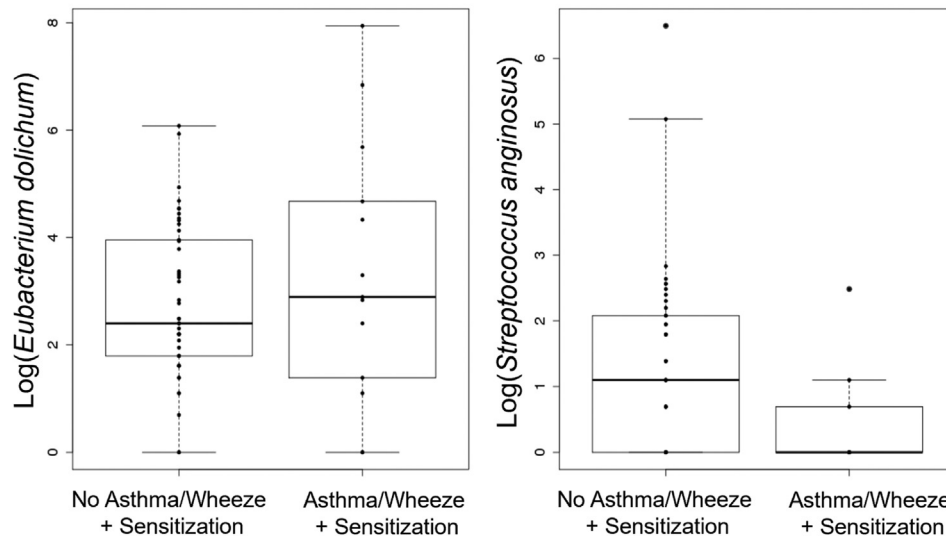


FIGURE E1. Box plots summarize maternal fecal *Eubacterium dolichum* and *Streptococcus anginosus* for offspring with ($n = 13$) and without ($n = 50$) asthma/wheeze with allergic sensitization. *Streptococcus anginosus* was absent in maternal fecal samples for 5 of 13 (62%) children with asthma/wheeze with sensitization at age 6 years versus 18 of 50 (36%) subjects without asthma/wheeze with sensitization.

TABLE E1. Baseline demographic variables of subjects with vs without available maternal fecal SCFA concentrations

Demographic variable	SCFA available (n = 150)	SCFA unavailable (n = 656)	<i>P</i> value
Male sex, n (%)	87 (64)	334 (51)	.12
Race/ethnicity, n (%)			.44
Black, non-Hispanic	66 (44)	251 (38)	
White, non-Hispanic	27 (18)	134 (20)	
Hispanic or other	57 (38)	271 (41)	
VDAART treatment group, n (%)			.42
4400 IU/d vitamin D	80 (53)	325 (50)	
400 IU/d vitamin D	70 (47)	331 (50)	
Study center, n (%)			5e-6
Boston	67 (45)	173 (26)	
St. Louis	53 (35)	239 (36)	
San Diego	30 (20)	244 (37)	
Maternal education, n (%)			.71
High school or less	61 (41)	280 (43)	
Some college or more	89 (59)	376 (57)	
Birth by cesarean section, n (%)	50 (33)	189 (29)	.32
Perinatal antibiotics, n (%)	55 (37)	282 (43)	.17
Pet dog during infancy, n (%)	25 (17)	143 (23)	.10
Older sibling(s), n (%)	82 (55)	353 (54)	.86
Exclusive breastfeeding for first 4 mo of life, n (%)	43 (30)	204 (34)	.33
Early pregnancy BMI, mean (SD)	28.6 (7.0)	29.0 (7.8)	.56
Maternal dietary fiber density, mean (SD)	0.01 (0.004)	0.01 (0.004)	.50
Asthma/wheeze at age 3 y, n (%)	40 (28)	178 (29)	.76
Sensitization at age 3 y, n (%)	51 (51)	202 (46)	.38
Asthma/wheeze with sensitization at age 3 y, n (%)	17 (17)	68 (15)	.76
Asthma/wheeze at age 6 y, n (%)	70 (47)	290 (44)	.59
Sensitization at age 6 y, n (%)	38 (48)	175 (52)	.46
Asthma/wheeze with sensitization at age 6 y, n (%)	17 (21)	93 (28)	.26

BMI, Body mass index; *SCFA*, short-chain fatty acids; *SD*, standard deviation; *VDAART*, Vitamin D Antenatal Asthma Reduction Trial.

Asthma/wheeze missing for 58 subjects at age 3 years. Sensitization missing for 265 and 392 subjects at ages 3 and 6 years. Mode of delivery and perinatal antibiotics missing for 3 subjects. Dog ownership missing for 49 subjects. Breastfeeding missing for 68 subjects. Early pregnancy BMI missing for 43 subjects. Fiber intake missing for 30 subjects. *P* values are for the *t* test for early pregnancy BMI and for logistic regression adjusted for total energy intake for maternal dietary fiber intake. *P* values for all other comparisons are for Fisher's exact test. Significant results are bolded.

TABLE E2. Associations of baseline demographic variables with clinical outcomes

Demographic variable	All children (n = 150)	Asthma/wheeze (n = 70)	No asthma/wheeze (n = 80)	P value	Sensitized (n = 38)	Not sensitized (n = 42)	P value	Hay fever (n = 54)	No hay fever (n = 81)	P value
Sex, n (%)				.32			.37			.07
Male	87 (58)	44 (63)	43 (54)		24 (63)	22 (52)		26 (48)	52 (64)	
Female	63 (42)	26 (37)	37 (46)		14 (37)	20 (48)		28 (52)	29 (36)	
Race/ethnicity, n (%)				.23			1.00			.032
Black, non-Hispanic	66 (44)	35 (50)	31 (39)		17 (45)	19 (45)		31 (57)	29 (36)	
White, non-Hispanic	27 (18)	9 (13)	18 (22)		5 (13)	5 (12)		10 (19)	17 (21)	
Hispanic or other	57 (38)	26 (37)	31 (39)		16 (42)	18 (43)		13 (24)	35 (43)	
VDAART treatment group, n (%)				.33			1.00			.72
4400 IU/d vitamin D	80 (53)	34 (49)	46 (58)		22 (58)	25 (60)		31 (57)	43 (53)	
400 IU/d vitamin D	70 (47)	36 (51)	34 (43)		16 (42)	17 (40)		23 (43)	38 (47)	
Study center, n (%)				.79			.96			.021
Boston	67 (45)	29 (41)	38 (48)		16 (42)	18 (43)		17 (31)	37 (46)	
St. Louis	53 (35)	26 (37)	27 (34)		12 (32)	12 (29)		29 (54)	24 (30)	
San Diego	30 (20)	15 (21)	15 (19)		10 (26)	12 (29)		8 (15)	20 (25)	
Maternal education, n (%)				.15			.52			.24
<High school	25 (17)	14 (20)	11 (29)		6 (16)	7 (16)		9 (17)	11 (14)	
High school or technical school	36 (24)	18 (26)	18 (35)		11 (29)	7 (16)		16 (30)	15 (19)	
Some college	48 (32)	25 (36)	23 (23)		15 (39)	17 (40)		18 (33)	27 (33)	
College graduate or higher	41 (27)	13 (19)	28 (14)		6 (16)	11 (26)		11 (20)	28 (35)	
Birth by cesarean section, n (%)	50 (33)	24 (34)	26 (32)	.86	12 (32)	15 (36)	.81	21 (39)	23 (28)	.26
Perinatal antibiotics, n (%)	55 (37)	30 (43)	25 (31)	.17	11 (29)	19 (45)	.17	22 (41)	28 (35)	.47
Pet dog during infancy, n (%)	25 (17)	18 (23)	7 (10)	.047	7 (18)	7 (17)	1.00	9 (17)	14 (18)	1.00
Older sibling(s), n (%)	82 (55)	43 (61)	39 (49)	.14	17 (45)	22 (52)	.51	33 (61)	42 (52)	.38
Exclusive breastfeeding for first 4 mo of life, n (%)	43 (30)	12 (17)	31 (42)	.002	12 (32)	14 (36)	.81	12 (22)	30 (38)	.06
Early pregnancy BMI, mean (SD)	29 (7)	29 (7)	28 (7)	.63	28 (7)	28 (7)	.90	30.7 (7)	27.2 (6)	.006
Maternal dietary fiber density, mean (SD)	0.01 (0.004)	0.01 (0.004)	0.01 (0.004)	.54	0.01 (0.004)	0.01 (0.005)	.80	0.01 (0.005)	0.01 (0.004)	.79

Demographic variable	All children (n = 150)	Asthma/wheeze with sensitization (n = 17)	Asthma/wheeze without sensitization (n = 63)	P value	Asthma/wheeze with hay fever (n = 33)	No asthma/wheeze without hay fever (n = 102)	P value
Sex, n (%)				.28			.23
Male	87 (58)	12 (71)	34 (54)		16 (48)	62 (61)	
Female	63 (42)	5 (29)	29 (46)		17 (52)	40 (39)	
Race/ethnicity, n (%)				.51			.041
Black, non-Hispanic	66 (44)	6 (35)	30 (48)		21 (64)	39 (38)	
White, non-Hispanic	27 (18)	3 (18)	7 (11)		5 (15)	22 (22)	
Hispanic or other	57 (38)	8 (47)	26 (41)		7 (21)	41 (40)	
VDAART treatment group, n (%)				.28			.69

(continued)

TABLE E2. (Continued)

Demographic variable	All children (n = 150)	Asthma/wheeze with sensitization (n = 17)	Asthma/wheeze without sensitization (n = 63)	<i>P</i> value	Asthma/wheeze with hay fever (n = 33)	No asthma/wheeze without hay fever (n = 102)	<i>P</i> value
4400 IU/d vitamin D	80 (53)	8 (47)	39 (62)		17 (52)	57 (56)	
400 IU/d vitamin D	70 (47)	9 (53)	24 (38)		16 (48)	45 (44)	
Study center, n (%)				.036			.006
Boston	67 (45)	4 (24)	30 (48)		8 (24)	46 (45)	
St. Louis	53 (35)	4 (24)	20 (32)		21 (64)	32 (31)	
San Diego	30 (20)	9 (53)	13 (21)		4 (12)	24 (24)	
Maternal education, n (%)				.88			.19
<High school	25 (17)	2 (12)	11 (17)		7 (21)	13 (13)	
High school or technical school	36 (24)	5 (29)	13 (21)		9 (27)	22 (22)	
Some college	48 (32)	7 (41)	25 (40)		12 (36)	33 (32)	
College graduate or higher	41 (27)	3 (18)	14 (22)		5 (15)	34 (33)	
Birth by cesarean section, n (%)	50 (33)	4 (24)	23 (37)	.39	14 (42)	30 (29)	.20
Perinatal antibiotics, n (%)	55 (37)	6 (35)	24 (38)	.89	16 (48)	34 (33)	.15
Pet dog during infancy, n (%)	25 (17)	2 (12)	12 (19)	.72	4 (12)	19 (19)	.44
Older sibling(s), n (%)	82 (55)	8 (47)	31 (49)	1.00	21 (64)	54 (53)	.32
Exclusive breastfeeding for first 4 mo of life, n (%)	43 (30)	5 (29)	21 (35)	.78	6 (18)	36 (36)	.06
Early pregnancy BMI, mean (SD)	29 (7)	28 (6)	29 (8)	.84	30 (7)	28 (7)	.24
Maternal dietary fiber density, mean (SD)	0.01 (0.004)	0.01 (0.004)	0.01 (0.004)	.46	0.01 (0.005)	0.01 (0.004)	.43

BMI, Body mass index; SD, standard deviation; VDAART, Vitamin D Antenatal Asthma Reduction Trial.

Sensitization missing for 70 subjects. Hay fever missing for 15 subjects. Dog ownership missing for 3 subjects. Breastfeeding status missing for 6 subjects. Early pregnancy BMI missing for 5 subjects. *P* values are for the *t* test for early pregnancy BMI and for logistic regression adjusted for total energy intake for maternal dietary fiber intake. *P* values for all other comparisons are for Fisher's exact test. Significant results are bolded.

TABLE E3. Associations of potential confounders with maternal SCFA

Potential confounders	Dog		Breastfeeding		Study center		Early pregnancy BMI		Race/ethnicity	
	<i>t</i>	<i>P</i> value	<i>t</i>	<i>P</i> value	<i>F</i>	<i>P</i> value	Rho	<i>P</i> value	<i>F</i>	<i>P</i> value
Absolute acetate	−1.5	.15	2.1	.042	1.5	.23	0.30	2e-4	2.0	.23
Absolute butyrate	−1.6	.11	1.7	.09	0.3	.74	0.33	5e-5	1.7	.74
Absolute propionate	−1.4	.16	1.1	.27	1.3	.27	0.27	.001	1.0	.27
Relative acetate	0.8	.43	1.5	.15	0.1	.93	−0.04	.62	1.8	.93
Relative butyrate	−0.6	.53	1.5	.14	1.7	.19	0.23	.005	0.9	.19
Relative propionate	1.0	.33	−0.3	.74	2.8	.06	−0.02	.85	0.2	.06

BMI, Body mass index; *SCFA*, short-chain fatty acids.

Potential confounders were initially identified based on univariable associations with asthma/wheeze, sensitization or hay fever. Absolute concentrations of maternal SCFA were log-transformed before analysis. Results are for *t* tests for dog ownership and breastfeeding, ANOVA for study center and race/ethnicity, and Spearman correlation for early pregnancy BMI. *P* values <.05 are bolded.

TABLE E4. Associations of maternal fecal short-chain fatty acids with clinical outcomes at age 6 years

Outcome	Asthma/wheeze (n = 150)				Sensitization (n = 80)				Hay fever (n = 135)			
	None		Maternal BMI, breastfeeding		None		Maternal BMI, breastfeeding		None		Maternal BMI, breastfeeding	
Covariates	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Acetate												
Absolute	1.22 (0.67, 2.24)	.52	1.22 (0.57, 2.20)	.75	1.19 (0.49, 2.97)	.70	1.24 (0.54, 2.92)	.62	0.73 (0.37, 1.44)	.37	0.47 (0.21, 1.01)	.058
Relative	0.97 (0.64, 1.46)	.89	0.91 (0.59, 1.42)	.69	0.69 (0.35, 1.29)	.25	0.72 (0.38, 1.32)	.30	0.69 (0.43, 1.08)	.11	0.65 (0.39, 1.06)	.088
Butyrate												
Absolute	0.99 (0.64, 1.54)	.97	0.95 (0.58, 1.58)	.85	1.36 (0.71, 2.70)	.36	1.40 (0.76, 2.65)	.29	1.00 (0.61, 1.64)	.99	0.78 (0.44, 1.35)	.38
Relative	0.80 (0.47, 1.31)	.39	0.78 (0.45, 1.32)	.35	1.40 (0.64, 3.14)	.40	1.55 (0.71, 3.51)	.28	1.45 (0.86, 2.51)	.17	1.29 (0.73, 2.32)	.39
Propionate												
Absolute	1.26 (0.73, 2.21)	.42	1.31 (0.72, 2.44)	.38	1.35 (0.61, 3.05)	.46	1.28 (0.61, 2.79)	.52	0.80 (0.43, 1.49)	.48	0.62 (0.31, 1.23)	.18
Relative	1.28 (0.68, 2.45)	.44	1.49 (0.77, 3.02)	.25	1.55 (0.60, 4.35)	.38	1.29 (0.50, 3.48)	.61	0.92 (0.46, 1.85)	.82	1.04 (0.49, 2.17)	.92
Outcome	Asthma/wheeze with hay fever (n = 135)				Asthma/wheeze with sensitization (n = 80)							
	None		Maternal BMI, breastfeeding		None		Maternal BMI		Breastfeeding			
Covariates	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Acetate												
Absolute	0.69 (0.33, 1.49)	.34	0.62 (0.27, 1.40)	.25	1.37 (0.50, 4.18)	.55	1.38 (0.48, 4.57)	.57	1.35 (0.50, 4.06)	.56		
Relative	0.63 (0.37, 1.05)	.077	0.61 (0.35, 1.04)	.071	0.46 (0.22, 0.94)	.036	0.48 (0.22, 0.99)	.052	0.45 (0.21, 0.93)	.036		
Butyrate												
Absolute	0.84 (0.48, 1.46)	.53	0.80 (0.44, 1.47)	.48	1.17 (0.56, 2.56)	.68	1.13 (0.52, 2.60)	.76	1.18 (0.57, 2.54)	.66		
Relative	1.02 (0.55, 1.85)	.95	1.00 (0.54, 1.86)	1.00	0.71 (0.28, 1.75)	.46	0.67 (0.26, 1.67)	.39	0.74 (0.29, 1.81)	.51		
Propionate												
Absolute	0.90 (0.45, 1.82)	.76	0.90 (0.43, 1.92)	.79	1.66 (0.66, 4.62)	.30	1.67 (0.64, 4.90)	.32	1.67 (0.68, 4.55)	.28		
Relative	1.18 (0.53, 2.62)	.68	1.30 (0.59, 2.92)	.52	1.97 (0.65, 6.56)	.24	2.08 (0.68, 7.12)	.21	2.12 (0.70, 7.38)	.20		

BMI, Body mass index; *CI*, confidence interval; *OR*, odds ratio; *SCFA*, short-chain fatty acids.

Results are from logistic regression models. Absolute SCFA concentrations were log-transformed before analysis. For relative concentrations, OR pertain to change in odds of outcome associated with a 10% increase in SCFA relative concentration. Analyses of asthma/wheeze with sensitization were adjusted for each covariate individually to prevent overfitting models. Significant results are bolded.

TABLE E5. Associations of maternal fecal acetic acid with offspring asthma/wheeze with sensitization

	Model covariates	OR (95% CI)	Logistic regression <i>P</i> value	Permutation testing <i>P</i> value
Asthma/wheeze with sensitization at age 6 y	None	0.46 (0.22, 0.94)	.036	.033
	Maternal BMI	0.48 (0.22, 0.99)	.052	.050
	Breastfeeding	0.45 (0.21, 0.93)	.036	.047

BMI, Body mass index; *CI*, confidence interval; *OR*, odds ratio.

Results from both logistic regression models and permutation testing (*n* = 1000 Monte Carlo resamples per model) are shown. Significant results are bolded.

TABLE E6. Associations of maternal dietary fiber intake and fecal SCFA (*n* = 150)

	Beta (95% CI)	<i>P</i> value
Total SCFA	0.26 (0.02, 0.44)	.035
Acetic acid		
Absolute	0.30 (0.07, 0.53)	.011
Relative	0.03 (−0.0004, 0.07)	.052
Butyric acid		
Absolute	0.22 (−0.08, 0.53)	.15
Relative	0.007 (−0.02, 0.04)	.62
Propionic acid		
Absolute	0.26 (0.01, 0.51)	.045
Relative	0.003 (−0.02, 0.03)	.77

BMI, Body mass index; *CI*, confidence interval; *SCFA*, short-chain fatty acids.

Results are from linear regression analysis adjusted for estimated total energy intake, maternal race/ethnicity, study center, maternal education, and BMI. Dietary fiber was analyzed as log-transformed nutrient density. Absolute concentrations of SCFA were log-transformed before analysis.

P values <.05 are bolded.

TABLE E7. Associations of maternal dietary fiber during pregnancy and clinical outcomes in offspring

Asthma/wheeze (n = 150)		Sensitization (n = 80)		Hay fever (n = 135)		Asthma/wheeze + sensitization (n = 80)		Asthma/wheeze + hay fever (n = 135)	
OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
0.87 (0.38, 1.98)	.74	1.04 (0.34, 3.17)	.94	0.67 (0.28, 1.61)	.38	1.92 (0.50, 8.20)	.35	0.57 (0.20, 1.56)	.28

CI, Confidence interval; OR, odds ratio.

Dietary fiber was analyzed as log-transformed nutrient density and all models including total energy intake as a covariate. Results are from logistic regression models and were not significantly changed when potential confounders (study center, breastfeeding) were added to models.

TABLE E8. Associations of SCFA with alpha and beta diversity metrics (n = 114)

SCFA	Associations with Shannon diversity		Associations with Bray-Curtis dissimilarity
	Spearman rho	P value	P value
Acetic acid—absolute	0.026	.78	.001
Acetic acid—relative	0.066	.49	.016
Butyric acid—absolute	0.068	.47	.001
Butyric acid—relative	0.130	.17	.001
Propionic acid—absolute	0.001	.99	.005
Propionic acid—relative	−0.146	.12	.004

SCFA, Short-chain fatty acids.

Results are from Spearman correlations for Shannon index (alpha diversity), and permutational multivariate analysis of variance (Adonis test) for Bray-Curtis dissimilarity (beta diversity). Absolute SCFA concentrations were log-transformed before analysis.

P values <.05 are bolded.

TABLE E9. Associations of maternal fecal beta diversity (Bray-Curtis dissimilarity) with offspring clinical outcomes

Outcome	Covariates	P value
Asthma/wheeze (n = 110)	None	.15
	Maternal BMI + breastfeeding	.18
Sensitization (n = 61)	None	.88
	Maternal BMI + breastfeeding	.94
Hay fever (n = 104)	None	.085
	Maternal BMI + breastfeeding	.079
Asthma/wheeze + hay fever (n = 104)	None	.55
	Maternal BMI + breastfeeding	.52
Asthma/wheeze + sensitization (n = 61)	None	.32
	Maternal BMI	.29
	Breastfeeding	.29

BMI, Body mass index.

P values are for permutational multivariate analysis of variance (Adonis test).

TABLE E10. Maternal intestinal bacterial taxa associated with relative concentrations of fecal SCFA during pregnancy (n = 114)

Species	Relative acetic acid			Relative butyric acid			Relative propionic acid		
	Log2-fold change	P value	Adjusted P value	Log2-fold change	P value	Adjusted P value	Log2-fold change	P value	Adjusted P value
<i>Eubacterium dolichum</i>	−9.0	8.0e-8	1.3e-5	−9.4	4.6e-6	2.3e-4	13.1	2.1e-8	1.5e-6
<i>Streptococcus anginosus</i>	5.5	.001	.07	−4.7	.025	.16	−7.2	.002	.023
<i>Veillonella dispar</i>	4.9	.004	.10	10.3	5.9e-7	4.5e-5	−13.5	1.3e-8	1.5e-6
<i>Anaerofustis</i> spp	−5.0	.003	.10	−5.5	.008	.099	9.8	3.4e-5	9.4e-4

SCFA, Short-chain fatty acids.

Results are from negative binomial regression. P values were adjusted using the method of Benjamini and Hochberg.

TABLE E11. Associations between relative SCFA-associated species with asthma/wheeze with sensitization at age 6 years (n = 63)

Species	Crude		Adjusted for maternal BMI		Adjusted for breastfeeding	
	Log2-fold change	P value	Log2-fold change	P value	Log2-fold change	P value
<i>Eubacterium dolichum</i>	1.7	.002	2.8	6e-8	3.3	2e-8
<i>Streptococcus anginosus</i>	−1.2	.045	−1.6	.005	−1.3	.046
<i>Veillonella dispar</i>	0.3	.57	0.1	.88	1.4	.020
<i>Anaerofustis</i> spp	0.1	.91	0.7	.21	0.2	.78

BMI, Body mass index; SCFA, short-chain fatty acids.

Results are from negative binomial regression models. Though *Veillonella dispar* is associated with asthma/wheeze with sensitization, the direction of association is not consistent with taxa/outcome associations with relative acetate. Covariates were considered one at a time to prevent model overfitting.

P values <.05 are bolded.

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