Associations of Gut Microbiota Features and Circulating Metabolites with Systemic Inflammation in Children

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SUPPLEMENTAL MATERIALS

Supplemental Table 1: Complete list of gut microbial taxa examined in this study. List of the 335 amplicon sequence variants (ASVs) included in this study. MD5 hash values provided for each ASV in both long (32 characters) and short (10 characters) formats. Prevalence (i.e., proportion of samples with non-zero counts) of each ASV shown relative to 147 samples.

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Supplemental Table 2: Characteristics of study participants (n=147) with gut microbiome data.

Variables	**All (N=147)**	**1st Tertile (N=35)**	**2nd Tertile (N=57)**	**3rd Tertile (N=55)**	**p-value^**
Mean Age at 5-year-old Visit (Years)	5.3 (0.25)	5.31 (0.29)	5.24 (0.25)	5.35 (0.2)	0.27
Sex					0.09
Female	65 (44.22%)	21 (60%)	21 (36.84%)	23 (41.82%)	
Male	82 (55.78%)	14 (40%)	36 (63.16%)	32 (58.18%)	
Race/Ethnicity					0.58
White	140 (95.24%)	34 (97.14%)	53 (92.98%)	53 (96.36%)	
Non-white	7 (4.76%)	1 (2.86%)	4 (7.02%)	2 (3.64%)	
From the Delivery Visit or from Earliest Infancy					
Antibiotic Use at Delivery*	48 (35.56%)	11 (35.48%)	21 (37.5%)	16 (33.33%)	0.91
Delivery Mode*					0.25
Vaginal	123 (84.25%)	26 (76.47%)	51 (89.47%)	46 (83.64%)	
Caesarean	23 (15.75%)	8 (23.53%)	6 (10.53%)	9 (16.36%)	
Birthweight*, g	3396 (469)	3380 (463)	3432 (467)	3369 (481)	0.83
Feeding Method					0.63
Breastmilk	57 (38.78%)	12 (34.29%)	22 (38.6%)	23 (41.82%)	
Mixed	72 (48.98%)	19 (54.29%)	30 (52.63%)	23 (41.82%)	
Formula	18 (12.24%)	4 (11.43%)	5 (8.77%)	9 (16.36%)	
Breastfeeding Duration (Months)*	9.46 (6.45)	8.99 (5.79)	10.01 (7.6)	9.16 (5.49)	0.99
Solid Foods Introduction Age (Months)*	5.24 (1.5)	5.33 (1.81)	5.1 (1.17)	5.32 (1.6)	0.93
From the 5-year-old Visit					
2nd Hand Smoking Exposure*	10 (6.85%)	2 (5.88%)	6 (10.53%)	2 (3.64%)	0.34
Fruit + Vegetable Intake (Frequency/Day)	5.17 (1.46)	5.04 (1.49)	5.03 (1.45)	5.4 (1.45)	0.21
Dairy Intake (Frequency/Day)	3.04 (1.21)	3.08 (1.18)	2.86 (1.04)	3.19 (1.39)	0.54
Drinking Water Source					0.047
Well	34 (23.13%)	12 (34.29%)	13 (22.81%)	9 (16.36%)	
City	101 (68.71%)	20 (57.14%)	43 (75.44%)	38 (69.09%)	
Bottled	12 (8.16%)	3 (8.57%)	1 (1.75%)	8 (14.55%)	
BMI, Age & Sex Adjusted Z-score	0.23 (0.89)	0.17 (0.8)	0.23 (0.9)	0.26 (0.95)	0.65
PAI-1, ng/mL	10.83 (12.48)	5.48 (3.24)	7.07 (3.4)	18.13 (17.73)	<.0001
TNF-α, pg/mL	5.55 (2.06)	3.48 (1.49)	5.43 (1.01)	6.99 (2.03)	<.0001
MCP-1, pg/mL	72.82 (27.9)	51.15 (16.02)	66.87 (14.22)	92.77 (31.41)	<.0001
Observed # ASVs	199.8 (39.91)	205.1 (44.3)	200.0 (42.5)	196.2 (34.2)	0.31
Chao-1 Index	245.7 (52.0)	254.4 (55.8)	246.3 (55.9)	239.5 (45.0)	0.19
Shannon Index	3.7 (0.6)	3.6 (0.6)	3.6 (0.6)	3.7 (0.5)	0.29
Simpson Index	0.9 (0.07)	0.9 (0.1)	0.9 (0.1)	0.9 (0.1)	0.12
Faith's PD Index	26.0 (4.5)	26.9 (5.1)	25.9 (4.8)	25.5 (3.9)	0.17

Tertiles of InfSc were computed among n=330 participants included in this study, prior to exclusion due to missing data (including microbiome).

*** Missing Data:** 2nd Hand Smoking Exposure = 1, Antibiotic Use at Delivery = 12, Delivery Mode = 1, Breastfeeding Duration = 18, Solid Food Introduction Age = 1.

^ p-values derived from ANOVA for continuous variables and from Chi Squared or Fisher's Exact Tests for categorical variables.

Abbreviations: BMI = body mass index; PAI-1 = plasminogen activator inhibitor-1; TNF-α = tumor necrosis factor-α; MCP-1 = monocyte chemoattractant protein-1; ASVs = amplicon sequence variants; PD = phylogenetic distance.

Supplemental Table 3: Association between metrics of inflammation and body habitus.
Mean (standard deviation) of DXA-measured total mass, total fat mass, and trunk fat mass across tertiles of the inflammation summary score, PAI-1, TNF-α, and MCP-1, among 189 participants with DXA data. P-values were computed with type III ANOVA, comparing any differences in means between tertiles groups.

A. Tertiles of Inflammation Z-score				
DXA Variables Mean (SD)	**Tertile 1 (n=107)** [-2.61, -0.23 SDu]	**Tertile 2 (n=106)** [-0.23, 0.28 SDu]	**Tertile 3 (n=108)** [0.28, 2.39 SDu]	**p-value** (any diff.)
Total Mass (g)	18841.08 (2786.12)	18886.16 (3019.13)	19079.67 (3249.8)	0.59
Total Fat Mass (g)	5830.86 (1483.43)	5810.1 (1640.48)	5939.29 (1696.12)	0.65
Trunk Fat Mass (g)	2109.6 (565.19)	2146.3 (731.65)	2212.61 (691.14)	0.29
B. Tertiles of PAI-1				
DXA Variables Mean (SD)	**Tertile 1 (n=107)** [0.93, 5.11 ng/mL]	**Tertile 2 (n=108)** [5.12, 7.82 ng/mL]	**Tertile 3 (n=106)** [7.84, 111.87 ng/mL]	**p-value** (any diff.)
Total Mass (g)	18788.71 (2783.63)	18959.95 (2800.31)	19051.53 (3449.31)	0.55
Total Fat Mass (g)	5765.04 (1502.09)	5838.63 (1543.79)	5973.83 (1768.26)	0.37
Trunk Fat Mass (g)	2088.38 (581.7)	2159.17 (693.95)	2218.92 (713.24)	0.18
C. Tertiles of TNF-α				
DXA Variables Mean (SD)	**Tertile 1 (n=104)** [0.24, 4.54 pg/mL]	**Tertile 2 (n=107)** [4.57, 6.00 pg/mL]	**Tertile 3 (n=110)** [6.08, 13.72 pg/mL]	**p-value** (any diff.)
Total Mass (g)	18777.59 (2552.14)	18828.03 (3307.05)	19188.55 (3143.94)	0.34
Total Fat Mass (g)	5876.96 (1399.46)	5722.98 (1649.6)	5973.44 (1746.6)	0.67
Trunk Fat Mass (g)	2147.62 (559.48)	2089.6 (623.78)	2227.85 (788.77)	0.40
D. Tertiles of MCP-1				
DXA Variables Mean (SD)	**Tertile 1 (n=107)** [6.54, 59.09 pg/mL]	**Tertile 2 (n=106)** [59.10, 75.36 pg/mL]	**Tertile 3 (n=108)** [75.41, 283.48 pg/mL]	**p-value** (any diff.)
Total Mass (g)	18656.44 (2692.37)	19361.05 (3630.92)	18808.1 (2626.17)	0.70
Total Fat Mass (g)	5757.72 (1373.47)	5993.9 (1973.11)	5834.12 (1423.4)	0.73
Trunk Fat Mass (g)	2096.59 (511.39)	2220 (863.79)	2155.93 (583.13)	0.52

Abbreviations: DXA = dual-energy x-ray absorptiometry; InfSc = Inflammation Score, PAI-1 = plasminogen activator inhibitor-1, TNF-α = tumor necrosis factor-α, MCP-1 = monocyte chemoattractant protein-1; SDu = Standard Deviation units

Supplemental Table 4: Direction and significance of associations and robustness to pseudo-count choice for all 335 microbial ASVs examined in relation to inflammation markers. In our analyses we used ANCOM-BC2 with full adjustment for age, sex, drinking water source, dairy intake frequency/day, and fruits and veggies intake frequency/day (Model 2). [Abbreviations: InfSc = Inflammation Score, PAI-1 = plasminogen activator inhibitor-1, TNF-α = tumor necrosis factor-α, MCP-1 = monocyte chemoattractant protein-1].

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Supplemental Table 5: Direction and significance of associations and robustness to pseudo-count choice for all 1037 metabolites examined in relation to inflammation markers. Associations are from models that included full adjustment for age, sex, drinking water source, dairy intake frequency/day, and fruits and veggies intake frequency/day (i.e., Model 2). Metabolites are organized by super- and sub-pathways, as provided by Metabolon. [Abbreviations: InfSc = Inflammation Score, PAI-1 = plasminogen activator inhibitor-1, TNF-α = tumor necrosis factor-α, MCP-1 = monocyte chemoattractant protein-1].

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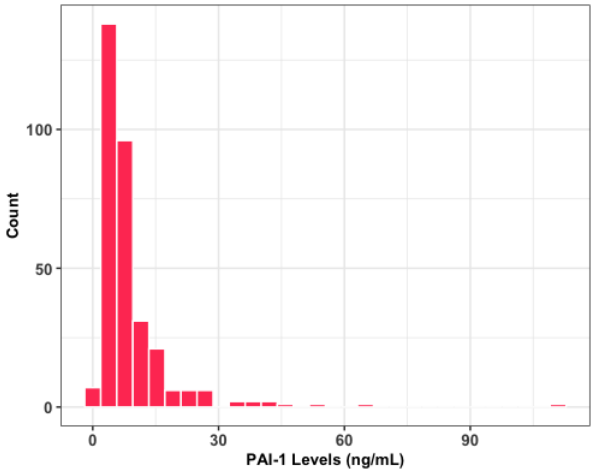
Supplemental Table 6: Counts and proportion of microbial and non-microbial metabolites significantly associated (q<0.05) with each inflammation marker after full adjustment. Full adjustment (i.e., Model 2) included adjustment for age, sex, drinking water source, dairy intake, and fruits and vegetables. Proportions were relative to number of metabolites tested overall and stratified by microbial vs other origin. Comparison of the proportions of significant hits among microbial metabolites vs other metabolites compared with two sample Z-test for proportions or Fisher's Exact Test, if warranted due to expected counts below 5.

Inflammation Marker	All Metabolites (n = 1037)	Microbial Metabolites (n = 315)	Other Metabolites (n = 722)	p-value
InfSc	55 (5.30%)	27 (8.57%)	28 (3.88%)	0.002[A]
PAI-1	69 (6.65%)	38 (12.06%)	31 (4.29%)	<0.0001[A]
TNF-α	7 (0.68%)	2 (0.63%)	5 (0.69%)	>0.99[B]
MCP-1	22 (2.12%)	10 (3.17%)	12 (1.66%)	0.12[A]

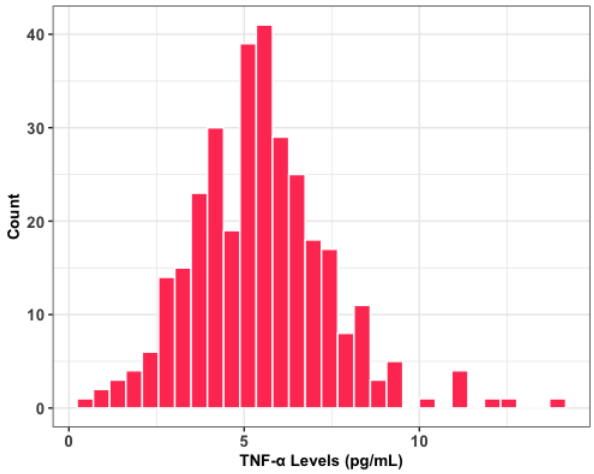
[A]Two sample Z-test for proportions
[B]Fisher's Exact Test, due to expected counts below 5

Supplemental Figure 1: Distribution of inflammation markers. Distribution of inflammatory marker levels in blood, prior to standardization, and the summary Inflammation Z-score among 321 children in the Gen3G cohort included in this study. [Abbreviations: InfSc = Inflammation Score, PAI-1 = plasminogen activator inhibitor-1, TNF-α = tumor necrosis factor-α, MCP-1 = monocyte chemoattractant protein-1].

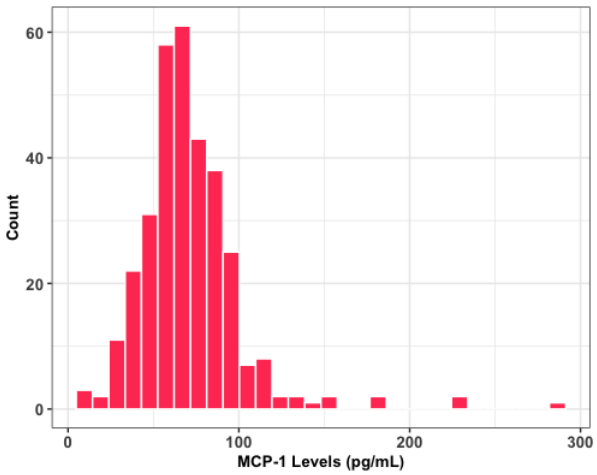
1A) PAI-1



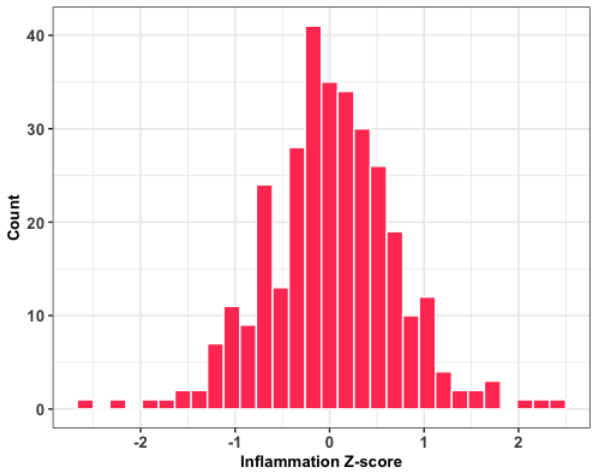
1B) TNF-α



1C) MCP-1

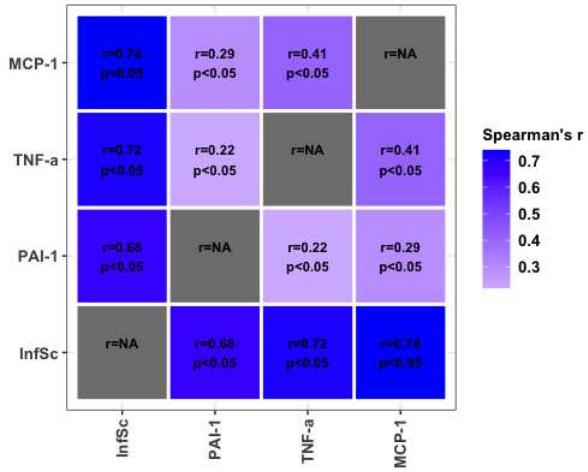


1D) Inflammation Z-score (InfSc)

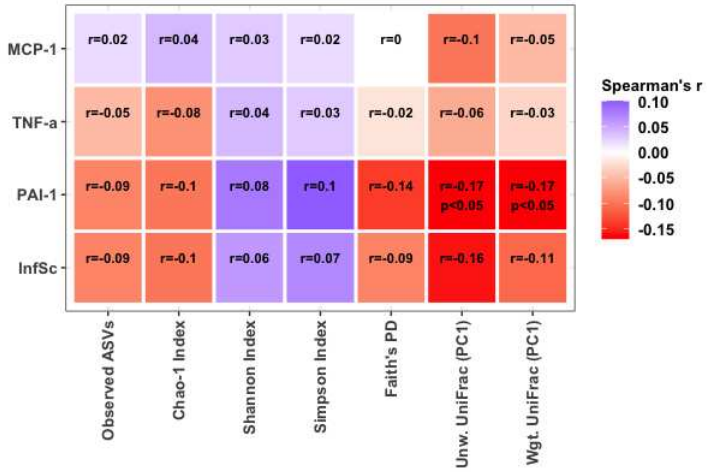


Supplemental Figure 2: Correlation between key variables. Correlation between markers of inflammation (A) and between inflammation markers and metrics of alpha and beta diversity (B), computed with Spearman's method. Beta diversity metrics are summarized through the first and second principal component axes (PC1, PC2), which don't have inherent directionality. All alpha diversity metrics and weighted UniFrac were derived from rarefied data. [Abbreviations: InfSc = Inflammation Score, PAI-1 = plasminogen activator inhibitor-1, TNF-α = tumor necrosis factor-α, MCP-1 = monocyte chemoattractant protein-1, ASV = amplicon sequence variance, PD = phylogenetic distance, Wgt = Weighted, Unw = Unweighted, PC = principal component axis].

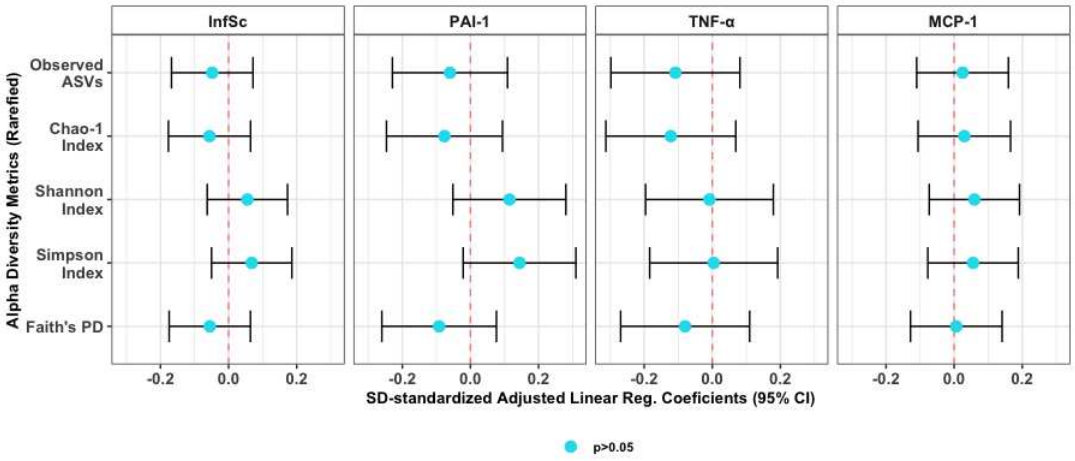
2A) Spearman's Correlation between Inflammation Markers (n = 321)



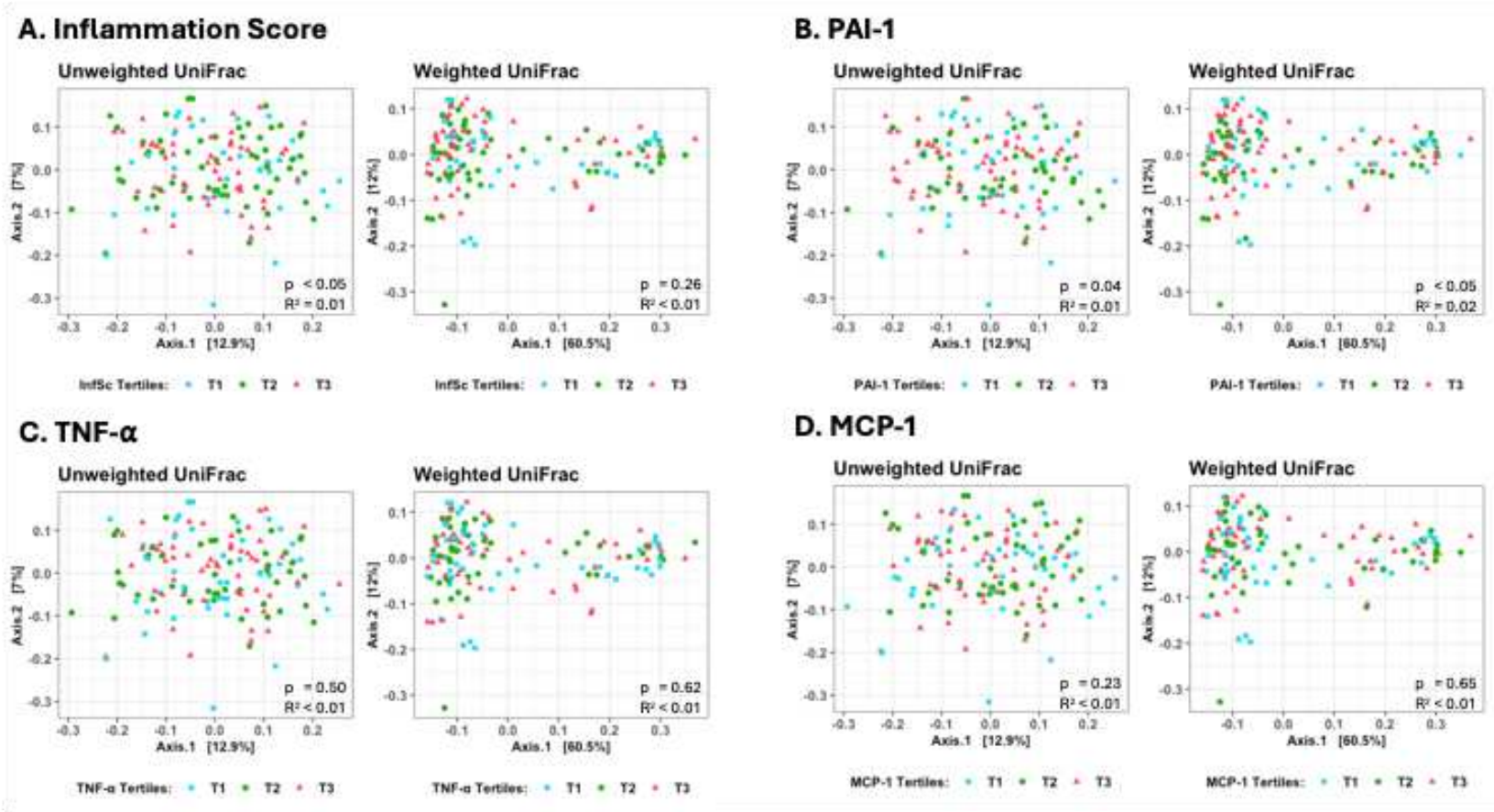
2B) Spearman's Correlation between Inflammation Markers and metrics of Alpha and Beta Diversity (n = 147)



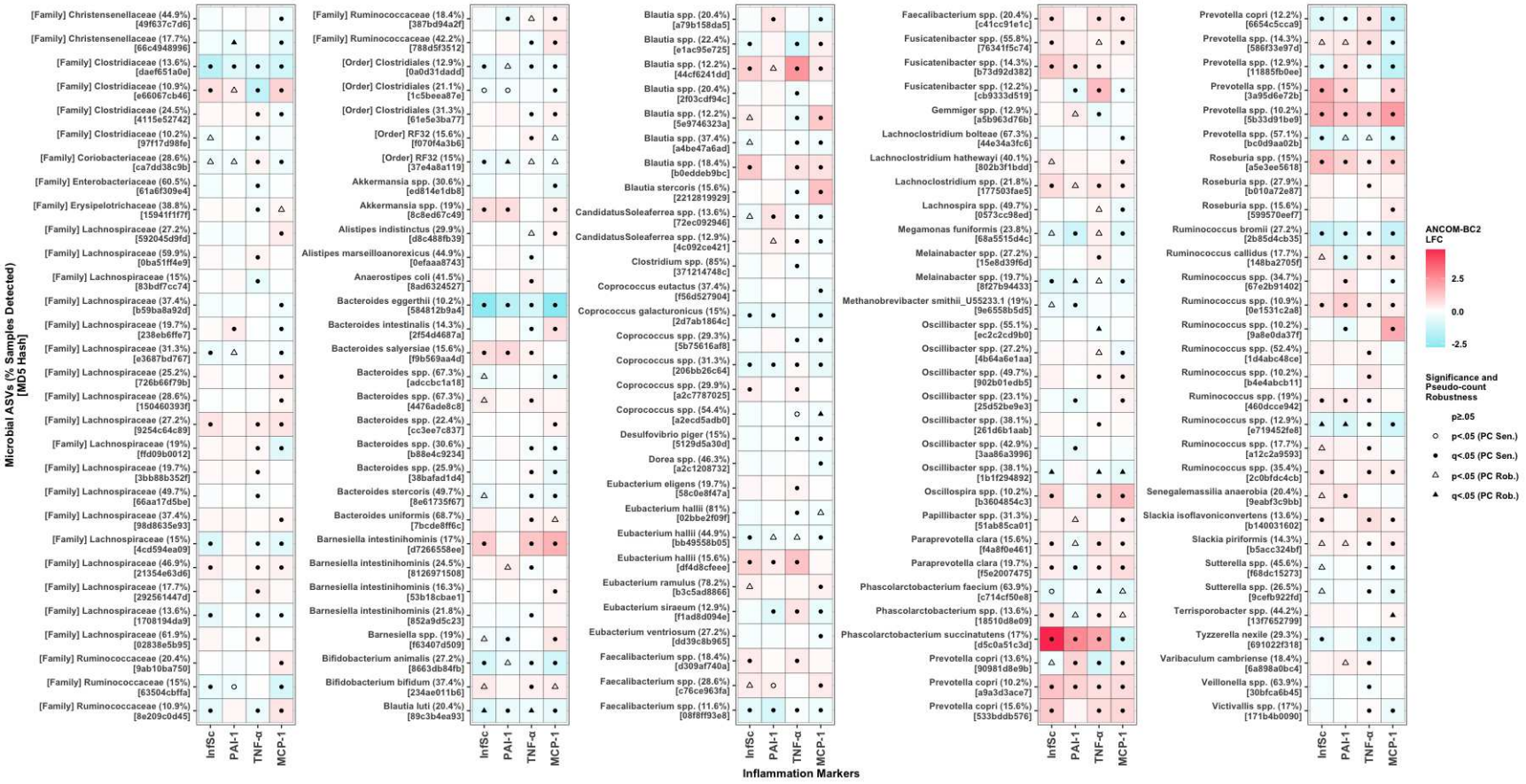
Supplemental Figure 3: Microbial Alpha Diversity and Inflammation. Associations between rarefied metrics of gut microbiome alpha (within-sample) diversity and inflammation markers in fully adjusted models (Model 2: adjusted for age, sex, drinking water source, dairy intake [frequency/day], and fruits and veggies intake [frequency/day]), summarized as regression coefficients and 95% confidence intervals standardized by the independent variable standard deviation. [Abbreviations: InfSc = Inflammation Score, PAI-1 = plasminogen activator inhibitor-1, TNF-α = tumor necrosis factor-α, MCP-1 = monocyte chemoattractant protein-1].



Supplemental Figure 4: Microbial Beta Diversity and Inflammation.
Associations between inflammation markers and microbial community composition, summarized with beta (between-sample) diversity indices: unweighted UniFrac and weighted UniFrac. Tables show R^2 and p-values from adjusted PERMANOVA/adonis for each beta diversity index and inflammatory markers, modeled as a continuous variable, with adjustment for age, sex, drinking water source, dairy intake (frequency/day), and fruits and veggies intake (frequency/day). [Abbreviations: InfSc = Inflammation Score, PAI-1 = plasminogen activator inhibitor-1, TNF-α = tumor necrosis factor-α, MCP-1 = monocyte chemoattractant protein-1].



Supplemental Figure 5: All gut microbial taxa associated with at least one marker of inflammation. Adjusted (M2) ANCOM-BC2 results, depicting all ASVs significantly (q<.05) associated with at least one inflammation markers. Models adjusted for age, sex, drinking water source, dairy intake frequency/daily, and fruits and veggies intake frequency/daily. Paneling done for ease of reading only. [Abbreviations: InfSc = Inflammation Score, PAI-1 = plasminogen activator inhibitor-1, TNF-α = tumor necrosis factor-α, MCP-1 = monocyte chemoattractant protein-1; PC Sen. = pseudo-count sensitive; PC Rob. = pseudo-count robust].



Supplemental Figure 6: Correlations between gut microbial taxa and metabolites significantly associated with inflammation markers. Heatmap with Kendall's Tau for the correlation between the relative abundance of selected taxa and metabolites (pareto-scaled). Selected taxa and metabolites had a significant association with at least one marker of inflammation.