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Associations of gut microbiota features and circulating metabolites with systemic inflammation in children

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ABSTRACT

Objective Gut microbes and microbe-dependent metabolites (eg, tryptophan-kynurenine-serotonin pathway metabolites) have been linked to systemic inflammation, but the microbiota-metabolite-inflammation axis remains uncharacterised in children. Here we investigated whether gut microbiota features and circulating metabolites (both microbe-dependent and non-microbe-dependent metabolites) associated with circulating inflammation markers in children.

Methods We studied children from the prospective Gen3G birth cohort who had data on untargeted plasma metabolome (n=321 children; Metabolon platform), gut microbiota (n=147; 16S rRNA sequencing), and inflammation markers (plasminogen activator inhibitor-1 (PAI-1), monocyte chemoattractant protein-1, and tumour necrosis factor-α) measured at 5–7 years. We examined associations of microbial taxa and metabolites—examining microbe-dependent and non-microbe-dependent metabolites separately—with each inflammatory marker and with an overall inflammation score (InfSc), adjusting for key confounders and correcting for multiple comparisons. We also compared the proportion of significantly associated microbe-dependent versus non-microbe-dependent metabolites, identified a priori (Human Microbial Metabolome Database), with each inflammation marker.

Results Of 335 taxa tested, 149 were associated (q_{FDR}<0.05) with at least one inflammatory marker; 10 of these were robust to pseudocount choice. Several bacterial taxa involved in tryptophan metabolism were associated with inflammation, including kynurenine-degrading *Ruminococcus*, which was inversely associated with all inflammation markers. Of 1037 metabolites tested, 315 were previously identified as microbe dependent and were more frequently associated with PAI-1 and the InfSc than non-microbe dependent metabolites. In total, 87 metabolites were associated (q_{FDR}<0.05) with at least one inflammation marker, including kynurenine (positively), serotonin (positively), and tryptophan (inversely).

Conclusion A distinct set of gut microbes and microbe-dependent metabolites, including those involved in the tryptophan-kynurenine-serotonin pathway, may be implicated in inflammatory pathways in childhood.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ While mouse studies have shown inflammation pathways that are influenced by specific gut microbes and microbial metabolites, including those on the tryptophan-kynurenine-serotonin pathway, studies on this topic are lacking in humans, especially in children.

WHAT THIS STUDY ADDS

⇒ In our community-based cohort of children, we found several gut microbes and circulating metabolites, including short-chain fatty acid-producing taxa and tryptophan metabolites, were associated with one or more inflammation markers (plasminogen activator inhibitor-1, monocyte chemoattractant protein-1, tumour necrosis factor-α, and overall inflammation score), corroborating the existence of a gut microbiota-metabolome-inflammation axis in early childhood. Further, microbial metabolites were proportionally more frequently associated with overall inflammation than non-microbial metabolites.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Understanding the contributions of gut microbes and microbial metabolites to systemic inflammation in childhood may lead to novel interventions and screening approaches.

INTRODUCTION

Systemic inflammation is a common aetiological component of multiple chronic disease outcomes, from cardiovascular[1–3] to neuropsychiatric and neurodegenerative diseases.[4–7] Growing evidence suggests that even in childhood, abnormal levels of inflammatory markers are associated with long-term health consequences.[2 3 8] While the effects of paediatric inflammation throughout the life course remain to be fully described, it is plausible that interventions to prevent systemic inflammation in early life could bring lifelong benefits to health. However, there are few known modifiable factors that can be

targeted for reducing inflammation in childhood, particularly among otherwise healthy children.

The gut microbiota is highly modifiable and affects the initiation and progression of several diseases, with microbial metabolites and microbe-driven inflammation serving as likely mechanistic intermediates of the total effects of the microbiota on human health. Exploring the modulatory effect of gut microbes and their metabolites on systemic inflammation (ie, the microbe-metabolite-inflammation axis) could represent an important advancement in our understanding of the provenance of myriad health outcomes. For example, tryptophan metabolism has been implicated in several inflammation pathways and has been identified as a main mechanistic component of the gut-brain axis, with tryptophan and its microbe-derived metabolites affecting brain health and function directly (eg, serotonin, a neurotransmitter) and indirectly (eg, kynurenine, a modulator of immune, inflammatory, and endocrine responses).[9–12] Unveiling these modifiable causal mechanisms may lead to novel interventions, therapies, and screening.

The preponderance of evidence for the microbe-metabolite-inflammation axis and other relevant associations largely derives from adult populations.[3 13] As major differences in the composition and functional capacity of the microbiome have been observed when comparing children and adolescents to adults,[14–17] it is essential to investigate microbiome-outcome associations in children, separate from adults, to identify whether associations are generalisable to this age group and, if not, to devise age-appropriate interventions. Furthermore, to our knowledge, only one paediatric (average age 10.6±3.7 years) microbiome-inflammation study incorporated data on microbial metabolites (using a targeted platform).[18] While this study made important contributions to understanding the microbe-metabolite-inflammation axis in children, it was limited by its sample size (n=48), study design (clinical population), and use of a targeted metabolite platform (rather than untargeted platform). Thus, an extensive exploration of these associations among a representative, community-based sample of children is warranted.

In the present study, we sought to address these research gaps by jointly examining gut microbiota features and the untargeted plasma metabolome, including microbe-dependent and non-microbe-dependent metabolites, in relation to multiple markers of inflammation in children 5–7 years of age. We measured three markers of inflammation from blood that were previously shown to be involved in immune-mediated aetiologies of insulin resistance, blood glucose dysfunction, and cardiovascular disease: plasminogen activator inhibitor-1 (PAI-1),[19] tumour necrosis factor-α (TNF-α),[20] and monocyte chemoattractant protein-1 (MCP-1).[21] These three inflammation markers were further averaged as an overall inflammation score (InfSc). We hypothesised that we could identify specific gut microbes and microbe-dependent metabolites (those previously identified by the Human Microbial Metabolome Database (MiMeDB)) associated with multiple markers of inflammation in children.

METHODS

Study population and data collection

We used cross-sectional data from the Genetics of Glucose regulation in Gestation and Growth (Gen3G) cohort,[22] a prospective prebirth cohort in the Eastern Townships region of Québec, Canada. From 2010 to 2013, we enrolled adult women in the 5th–16th weeks of gestation and without a history of diabetes. We followed participants from pregnancy until approximately 5 years post partum, as well as their children from birth until approximately 5 years of age. Trained staff collected data through a combination of questionnaires, electronic medical record abstraction, and collection of biospecimens, including blood and stool samples. For the present study, we included all children enrolled in Gen3G with inflammatory blood biomarkers data at the 5-year-old visit who had concurrent metabolome data (n=330). We excluded nine participants due to missing covariate data (one missing drinking water source, fruit/vegetable intake, and dairy intake, four missing fruit/vegetable intake, and four missing dairy intake), resulting in an analytical sample of 321 participants. Among these, faecal (gut) microbiota data were available for 147 participants.

Markers of inflammation

We used child fasting blood plasma samples collected at the 5-year visit and stored at −80°C to measure three unique inflammatory markers using a multiplexed particle-based flow cytometric assay (Luminex Technology, EMD Millipore, USA): PAI-1, TNF-α, and MCP-1. Intra-assay and interassay coefficients of variation were 10% and 15%, respectively. We natural log transformed levels of these inflammation markers to enhance normality. We then created Z-scores for each inflammation marker (among n=330, prior to exclusion due to missing covariate data) and computed an overall InfSc as the average Z-scores between the three measured inflammatory markers. Inflammation markers were also operationalised by tertiles (cut-off values: −0.23, 0.28), computed among n=330 prior to exclusion due to missing covariate data, with the highest tertile representing individuals with higher inflammation. All results reported for the three inflammatory markers used the Z-scores of log-transformed values, except for summary descriptive statistics (eg, cohort characteristics).

Gut microbiota data processing and operationalisation

At the 5-year visit, child stool samples were collected at home with OMNIgene-GUT collection kits (OM-200; DNA Genotek, Canada) and stored at −80°C when received at laboratory. We characterised the paediatric faecal (ie, gut) microbiota through 16S rRNA sequencing of the V4 hypervariable region with primers 515F and 806R.[23] We used the R package DADA2 (V.1.8.0) for quality control of demultiplexed samples and assignment of amplicon

sequence variants (ASVs). A list of ASVs included in our study and their MD5 hash identifiers are shown in online supplemental table 1. Our analyses included samples with a minimum of 1000 reads. We performed rarefaction to 90% of the minimum number of reads (n_{min}=7439) 10 times, averaging them as a single rarefied dataset. We used this rarefied dataset to operationalise alpha diversity (ie, within-subject) as the number of observed ASVs, Chao-1 Index, Shannon Index, Simpson Index, and Faith's Phylogenetic Distance (PD), and beta diversity (ie, between-subject) with weighted UniFrac and unweighted UniFrac. We used non-rarefied data for differential abundance testing, but excluded ASVs present in less than 10% of samples or with average relative abundance across all samples below 0.1%.

Metabolome data processing and operationalisation

We conducted untargeted metabolomics of fasting plasma with Metabolon's multiplatform mass spectrometry, as previously described.[24] Of the 1116 metabolites (857 annotated/known) detected, we excluded 78 detected in less than 50% of participants and EDTA, which is present in the collection tubes as an anticoagulant. For the remaining 1037 metabolites, we imputed undetected abundances as half of their minimum detected abundance across all samples. We log transformed and Pareto scaled (ie, centred around the mean and divided by the square root of the SD) metabolite abundances. We determined microbe-dependent metabolites a priori, using the R package *webchem*[25] to cross-reference PUBCHEM IDs from our data with InChIKey IDs from the MiMeDB,[26] a freely available electronic database, obtained via email from the Wishart Lab (University of Alberta) in February 2024. Of the 1037 metabolites included in our study, 313 were present in the MiMeDB and therefore operationalised as microbe-dependent metabolites.

Covariates

We collected covariate data on initial enrolment, at birth, or concurrently with blood and stool samples at the 5-year visit. At the 5-year post—birth visit, we measured metrics of body habitus (total mass, total fat mass, and trunk fat mass) with dual-energy X-ray absorptiometry (DXA) scanning, as previously described.[27] Concurrently, mothers completed questionnaires about diet and other lifestyle characteristics for their family and the child participants. We defined primary drinking water source as bottled water exclusively, city water (regardless of additional bottled water intake), or well water (regardless of additional bottled or city water intake). Participating mothers reported the child's frequency of vegetable, fruit, or dairy intake per day or other frequencies; if they reported in units of time greater than 1 day (eg, weekly), daily intake was mathematically extrapolated (ie, 1 weekly=1/7 daily). Intake of dairy, vegetables, and fruits was included as covariates as they represent important food groups with known associations with both the microbiome and inflammatory processes. Frequency of vegetable and fruit intake was collected separately and combined as a single variable for simplicity. Inclusion of potential confounders in multivariable models was primarily informed by the literature, but also accounted for the distribution within our data and observed associations with summary metrics of inflammation, microbiome, or metabolome data, evaluated with χ^2 test, two-sided t-test, analysis of variance (ANOVA), or Wilcoxon rank-sum test, as appropriate.

Analytical approaches

We conducted analyses in R (V.4.3.1). We summarised participant characteristics as means (SD) or counts (percentage), as appropriate. We checked the distribution of all variables and transformed distributions as needed (eg, natural log) to improve normality. We investigated whether the frequencies or means of key covariates, including measures of body habitus, varied across tertiles of InfSc (or of other inflammation markers, if warranted) using the χ^2 test, Fisher's exact test, or ANOVA, as appropriate.

We used univariable and multivariable linear regression models to regress Z-scores of each inflammation marker on metabolite abundances and on metrics of alpha diversity. We used principal component analyses and permutational ANOVA (*adonis2* function in R[28]) with 999 permutations to investigate the association of inflammation with microbial community composition, summarised with beta diversity metrics. We performed differential abundance testing across inflammation markers using the Analysis of Compositions of Microbiomes with Bias Correction 2 (ANCOM-BC2), which includes evaluation for robustness of findings to pseudocount choice (ie, robust associations persisted regardless of pseudocount choice).[29 30] We adjusted all models for child age (in years), sex (male, female), drinking water source (bottled, well, city), and intake frequency (per day) of dairy and of fruits and vegetables. When appropriate, we accounted for multiple comparisons with Benjamini and Hochberg's false discovery rate (FDR), deeming a q<0.05 to be statistically significant. For each inflammation marker, we compared the proportion of significantly associated metabolites (q<0.05) between the microbe-dependent and non-microbe dependent metabolite groups, determined a priori, with the two-sample Z-test for proportions or Fisher's exact test (if warranted due to small numbers; ie, <5). We evaluated the correlation between relative abundances of selected ASVs and metabolites using Kendall's tau. Only ASVs and metabolites that were significantly associated with at least one inflammatory metric were included.

RESULTS

Cohort characteristics

We summarised participant characteristics in table 1. The primary analytical cohort consisted of 321 participants who had data on all inflammatory markers and metabolome and had no missing data for key covariates. The

Table 1 Characteristics of study participants (n=321) with metabolomics data, overall and according to tertiles of overall inflammation score

Variables	All (n=321)	Overall inflammation score			P value*
		1st tertile (n=107)	2nd tertile (n=106)	3rd tertile (n=108)	
Mean age at 5-year-old visit (years)	5.27 (0.32)	5.26 (0.37)	5.25 (0.32)	5.3 (0.27)	0.45
Sex					0.54
Female	151 (47.04%)	55 (51.4%)	48 (45.28%)	48 (44.44%)	
Male	170 (52.96%)	52 (48.6%)	58 (54.72%)	60 (55.56%)	
Race/ethnicity					0.04
White	305 (95.02%)	104 (97.2%)	96 (90.57%)	105 (97.22%)	
Non-white	16 (4.98%)	3 (2.8%)	10 (9.43%)	3 (2.78%)	
From the delivery visit or from earliest infancy					
Antibiotic use at delivery†	112 (37.09%)	37 (37.00%)	42 (40.00%)	33 (34.02%)	0.68
Delivery mode†					0.09
Vaginal	269 (84.33%)	86 (81.9%)	96 (90.57%)	87 (80.56%)	
Caesarean	50 (15.67%)	19 (18.1%)	10 (9.43%)	21 (19.44%)	
Birth weight†, g	3406 (487)	3397 (403)	3407 (546)	3414 (505)	0.81
Feeding method					0.32
Breastmilk	127 (39.56%)	41 (38.32%)	37 (34.91%)	49 (45.37%)	
Mixed	152 (47.35%)	50 (46.73%)	58 (54.72%)	44 (40.74%)	
Formula	42 (13.08%)	16 (14.95%)	11 (10.38%)	15 (13.89%)	
Breastfeeding duration (months)†	9.24 (7.23)	8.39 (7.01)	10.12 (7.73)	9.18 (6.89)	0.46
Solid food introduction age (months)†	5.36 (1.61)	5.25 (1.71)	5.36 (1.43)	5.45 (1.69)	0.34
From the 5-year-old visit					
Secondhand smoking exposure†	25 (7.81%)	3 (2.83%)	14 (13.21%)	8 (7.41%)	0.02
Fruit+vegetable intake (frequency/day)	5.22 (1.74)	5.23 (1.80)	5.12 (1.77)	5.31 (1.66)	0.75
Dairy intake (frequency/day)	3.01 (1.22)	3.02 (1.21)	2.94 (1.13)	3.09 (1.32)	0.67
Drinking water source					0.25
Well	78 (24.30%)	29 (27.10%)	26 (24.53%)	23 (21.30%)	
City	221 (68.85%)	74 (69.16%)	74 (69.81%)	73 (67.59%)	
Bottled	22 (6.85%)	4 (3.74%)	6 (5.66%)	12 (11.11%)	
BMI, age and sex-adjusted Z-score	0.21 (0.96)	0.13 (0.94)	0.27 (0.95)	0.21 (0.99)	0.55
PAI-1, ng/mL	9.02 (9.82)	4.78 (2.5)	6.8 (3.02)	15.41 (14.47)	<0.001
TNF-α, pg/mL	5.47 (1.96)	3.87 (1.32)	5.55 (1.12)	6.98 (1.91)	<0.001
MCP-1, pg/mL	70.76 (29.81)	51.45 (15.89)	66.53 (13.7)	94.04 (35.86)	<0.001

Tertiles of the overall inflammation score were computed among n=330, prior to exclusion due to missing data.
*P values derived from analysis of variance (ANOVA) for continuous variables and from χ^2 or Fisher's exact tests for categorical variables.
†Missing data: secondhand smoking exposure=1, antibiotic rounds since birth=5, antibiotic use at delivery=19, delivery mode=2, birth weight=1, breastfeeding duration=42, solid food introduction age=1.
BMI, body mass index; MCP-1, monocyte chemoattractant protein-1; PAI-1, plasminogen activator inhibitor-1; TNF-α, tumour necrosis factor-α.

average age among cohort participants was 5.3 (±0.3) years and there was a similar proportion of males and females (52.8% vs 47.2%, respectively). Among the 321 participants in the primary analytical cohort, 147 had gut microbiota data (online supplemental table 2). Distribution of key covariates did not meaningfully differ between the microbiota subcohort and the metabolome cohort.

Among the 321 children included in analyses, PAI-1 levels ranged from 0.93 ng/mL to 111.87 ng/mL (median=6.13 ng/mL, IQR=4.99), TNF-α ranged from 0.24 pg/mL to 13.72 pg/mL (median=5.38 pg/mL, IQR=2.39), and MCP-1 ranged from 6.45 pg/mL to 283.48 pg/mL (median=67.34 pg/mL, IQR=28.90) (online supplemental figure 1). We observed a significant

moderate positive agreement between each inflammatory marker (online supplemental figure 2A), with the lowest correlation between PAI-1 and TNF-α (r=0.22, p<0.05), followed by PAI-1 and MCP-1 (r=0.29, p<0.05) and TNF-α and MCP-1 (r=0.40, p<0.05). InfSc was strongly correlated with each of the three inflammatory markers (r>0.68, all p<0.05), as expected. Correlation coefficients between inflammatory markers and metrics of alpha or beta diversities ranged from −0.17 to 0.10 (online supplemental figure 2B), with PAI-1 showing stronger correlations with all diversity metrics than other inflammatory markers. PAI-1 was significantly correlated with the first principal component axis of both unweighted and weighted UniFrac (both r=−0.17, p<0.05). No associations were observed among metrics of body habitus (total mass, total fat mass, and trunk fat mass) with metrics of inflammation (online supplemental table 3).

Gut microbiota diversity and inflammation

None of the associations between alpha diversity and inflammation evaluated reached statistical significance in multivariable adjusted models (online supplemental figure 3), although lower inflammation trended with higher metrics of microbiota alpha-diversity (within-sample) richness (observed ASVs, Chao-1) and phylogenetic diversity (Faith's PD) but with lower evenness (Shannon Index, Simpson Index). In terms of microbiota beta (between-sample) diversity, we observed some significant although weak associations: weighted UniFrac was associated with PAI-1 (fully adjusted R^2=0.018, p=0.046) while unweighted UniFrac was associated with PAI-1 (fully adjusted R^2=0.010, p=0.038) and with the InfSc (fully adjusted R^2=0.010, p=0.049). No other significant associations were observed (online supplemental figure 4).

Gut microbiota composition and inflammation

Of 335 ASVs tested in fully adjusted ANCOM-BC2 models, 149 were significantly associated (q<0.05) with at least one inflammatory marker after FDR adjustment (figure 1, online supplemental table 4). Among these, only 10 ASVs had associations robust to pseudocount choice (ie, significance was maintained regardless of the chosen pseudocount). Lower abundance of *Ruminococcus* spp (prevalence=12.9%) was significantly associated with higher inflammation (all markers), but findings were only robust for PAI-1 and InfSc. Similarly, lower abundance of *Blautia luti* (prevalence=20.4%) was significantly associated with higher levels of all inflammation markers, but only findings for TNF-α and InfSc were robust to pseudocount choice. Seven other taxa also had robust inverse associations with at least one marker of inflammation, including *Phascolarctobacterium faecium* (prevalence=63.9%) and two ASVs in the *Oscillibacter* genera (prevalences=55.1%, 38.1%). The only robust, significant association in the positive direction was between *Terrisporobacter* spp (prevalence=44.2%) and MCP-1. Non-robust findings (ie, sensitive to pseudocount choice) are shown in online supplemental figure 5.

Plasma metabolome and inflammation

We regressed each inflammation marker on each of 1037 metabolites, using unadjusted, partially adjusted, and fully adjusted models. Across all models, 104 metabolites were significantly associated (q<0.05) with at least one inflammation marker after FDR correction, of which 87 remained significant after full multivariable adjustment (figures 2 and 3, online supplemental table 5).

The largest number of significant metabolomic associations was for PAI-1 (69 out of 1037, or 6.65% of metabolites tested), followed by the InfSc (n=55, 5.30%), MCP-1 (n=22, 2.12%), and TNF-α (n=7, 0.68%). Importantly, a greater proportion of microbial (ie, microbe-dependent) metabolites versus non-microbe-dependent (ie, other) metabolites were related to the overall InfSc (8.57% vs 3.88%, p=0.002), driven in large part by PAI-1, which was associated with a greater proportion of microbial versus non-microbial metabolites (12.06% vs 4.29%, p<0.0001). A non-significant difference in this same direction was observed for MCP-1 (3.17% vs 1.66%, p=0.12), while no differences were observed for TNF-α (0.63% vs 0.69%, p>0.99). A summary of fully adjusted metabolite-inflammation results is shown in figure 2 and online supplemental table 6.

Adjusted regression coefficients for the 87 metabolites (n=45 microbial) associated with at least one inflammation marker after FDR correction are shown in figure 3. For most metabolites, the directions of association were consistent across inflammatory markers, and no discordant results were observed among significant findings after FDR adjustment. The least overlap in significant hits was observed between TNF-α and the other markers of inflammation, with only four metabolites (palmitoylcholine, arachidonoylcholine, linoleoylcholine, and 1-arachidonoyl-GPI 20:4) being significantly associated with all markers of inflammation after adjustment (all lower levels of metabolites associated with higher inflammation marker levels).

Correlations among gut microbiota, plasma metabolome, and inflammation

We evaluated the correlations between the 87 metabolites and 10 microbial ASVs significantly (and robustly, for ASVs) associated with at least one inflammatory marker (online supplemental figure 6). Although some significant correlation coefficients were observed, they were weak in magnitude, ranging from −0.15 to 0.19. All of the significant metabolite-ASV correlations among the metabolites positively associated with inflammation were inverse (ie, higher abundances of these metabolites were associated with higher levels of inflammation markers but with lower ASV abundances). Both inverse and direct metabolite-ASV correlations were observed among metabolites negatively linked to inflammation.

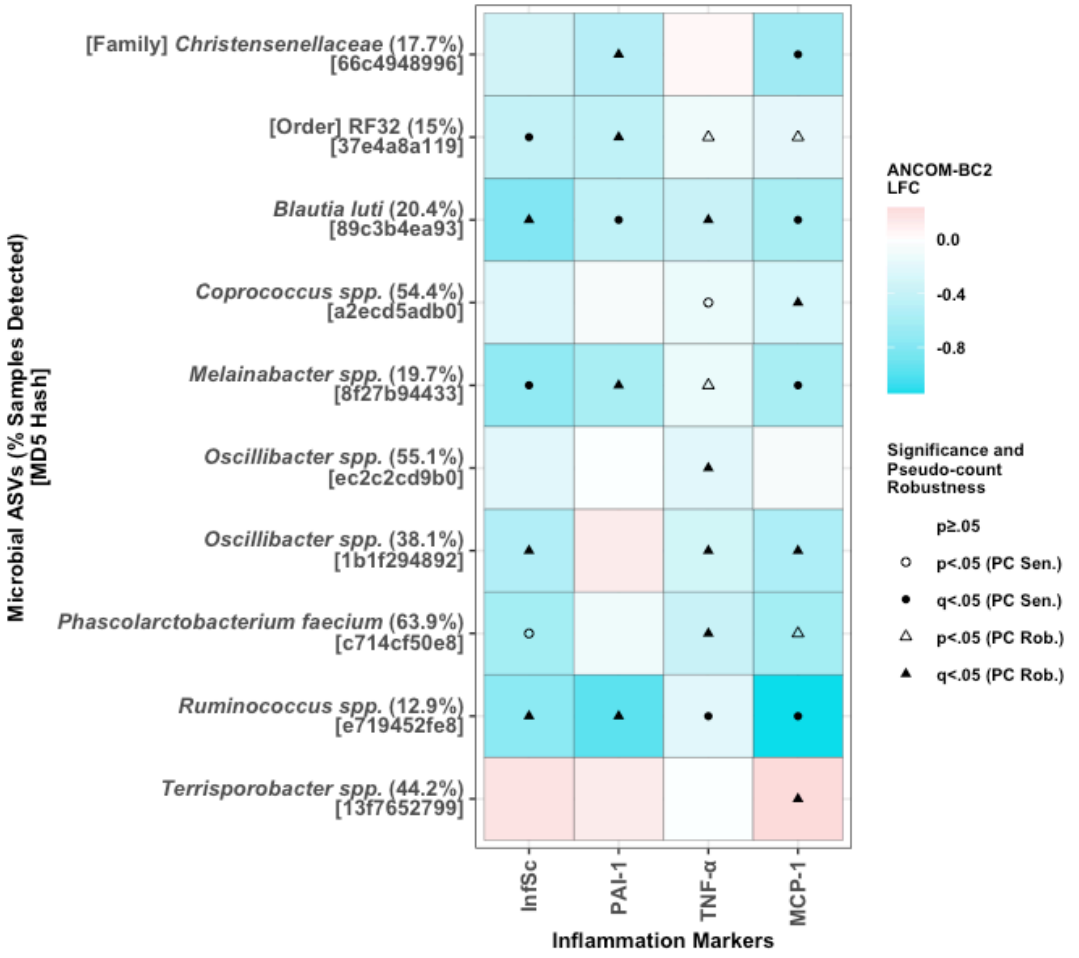

Figure 1 Gut microbial taxa associated with markers of inflammation. Adjusted ANCOM-BC2 results, depicting all amplicon sequence variants (ASVs) significantly and robustly associated (q<0.05, regardless of pseudocount choice) with at least one inflammation marker. Models adjusted for age, sex, drinking water source, dairy intake (frequency/day), and fruit and vegetable intake (frequency/day). Prevalence of each ASV within our data is shown as the percentage of samples with detection (total of 147 samples) following each ASV name. ANCOM-BC2, Analysis of Compositions of Microbiomes with Bias Correction 2; InfSc, inflammation score; MCP-1, monocyte chemoattractant protein-1; PAI-1, plasminogen activator inhibitor-1; PC Rob, pseudocount robust; PC Sen, pseudocount sensitive; TNF-α, tumour necrosis factor-α.

DISCUSSION

In our community-based cohort of 321 children aged 5–7 years from Québec, Canada, multiple gut microbes and circulating metabolites, including those of microbial origin and with relation to tryptophan metabolism, were associated with distinct markers of inflammation and an overall inflammation score. Associations of gut microbiota features and microbial metabolites were strongest for PAI-1, although associations with other inflammation markers were largely in the same direction. Importantly, compared with non-microbial metabolites, microbe-dependent metabolites were proportionally more frequently associated with inflammation markers, suggesting that gut microbes play an important role in systemic inflammation.

To our knowledge, this is the first study to jointly examine the gut microbiota and (untargeted) plasma metabolome in relation to multiple markers of inflammation among healthy children. In the only other paediatric study on this topic, Holle *et al* investigated the gut microbiota, (targeted) plasma metabolome, and markers of inflammation in a clinical paediatric population (n=48; average age 10.6±3.7 years) that included 38 children with chronic kidney disease and 10 controls (ie, normal kidney function).[18] Holle *et al* reported TNF-α to be inversely associated with tryptophan but positively associated with several tryptophan metabolites. We similarly found the TNF-α to be inversely associated with tryptophan and positively associated with three of its metabolites: kynurenate, 3-indoxyl sulfate, and kynurenine—although only the latter was statistically significant after accounting for multiple testing (FDR). In broad terms, both their study and ours detected associations of microbes and/or microbial-related metabolites with inflammatory markers, which are indicative of the modulatory role gut microbes may have on systemic inflammation in children. However, the study by Holle *et al* had limitations, including a small sample size, and only examining targeted metabolites. Furthermore,

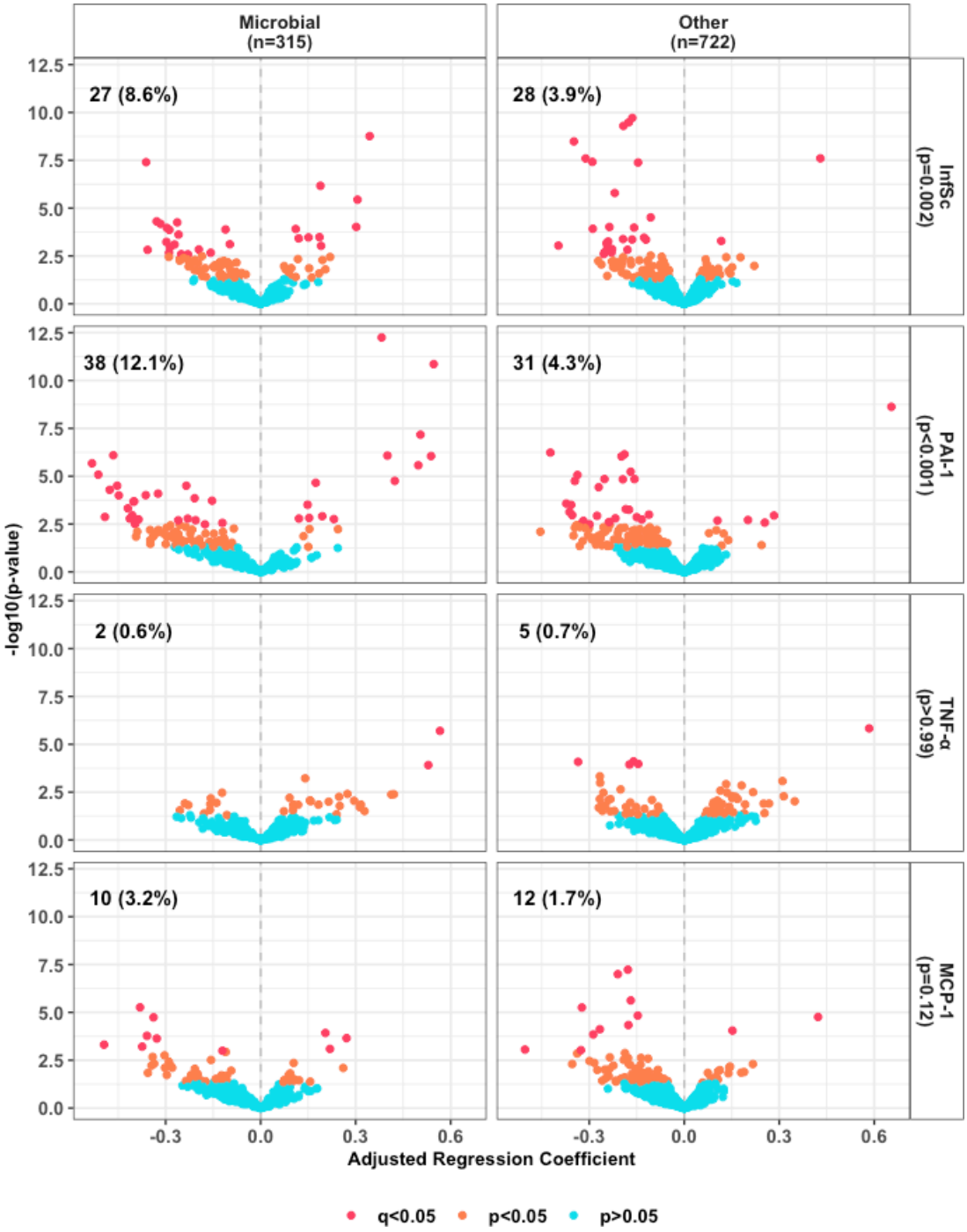

Figure 2 Comparison of proportion of gut microbial metabolites versus non-microbial metabolites associated with markers of inflammation. Volcano plot depicting fully adjusted regression coefficients and p values (–log10 transformed) for the associations of each metabolite with each inflammation marker, panelled by inflammation marker and by metabolite classification as microbial (n=315) or other (n=722), defined a priori with the Human Microbial Metabolome Database. Models are adjusted for age, sex, drinking water source, dairy intake (frequency/day), and fruit and vegetable intake (frequency/day). Associations that are significant after accounting for false discovery rate (FDR) are depicted in red (q<0.05), significant associations prior to FDR adjustment are shown in orange (p<0.05), and non-significant associations in blue (p>0.05). The number of metabolites significantly associated (q<0.05) with each inflammation marker is shown in the upper left corner of each panel. For each inflammatory marker, the proportions of significant hits (relative to number of metabolites tested) among microbial metabolites versus other metabolites were made with two-sample Z-test for proportions or Fisher's exact test, if warranted due to expected counts below 5 (TNF-α only), with p values shown under each inflammatory marker's label. InfSc, inflammation score; MCP-1, monocyte chemoattractant protein-1; PAI-1, plasminogen activator inhibitor-1; TNF-α, tumour necrosis factor-α.

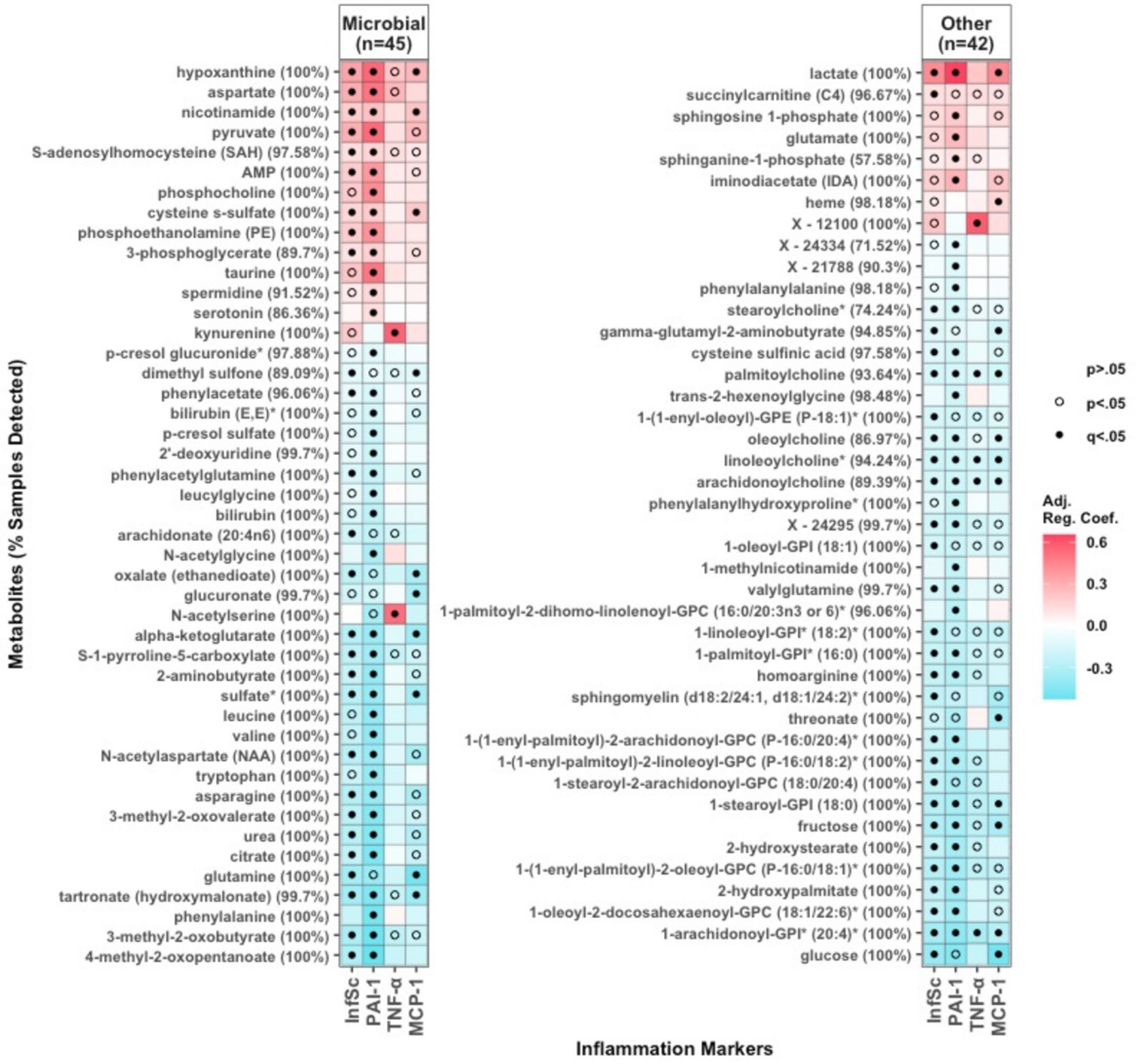

Figure 3 Gut microbial metabolites and non-microbial metabolites associated with markers of inflammation. Results from untargeted metabolomics linear regression analysis among 321 children, depicting metabolites significantly associated (q<0.05) with any inflammation marker in fully adjusted models (age, sex, drinking water source, dairy intake (frequency/day), and fruit and vegetable intake (frequency/day)). Prevalence of each metabolite within our data is shown as the percentage of samples with detection (total of 321 samples), following each metabolite's name. Results panelled by metabolite's classification as having microbial origins (n=315) or not (n=722), defined a priori with the Human Microbial Metabolome Database. InfSc, inflammation score; MCP-1, monocyte chemoattractant protein-1; PAI-1, plasminogen activator inhibitor-1; TNF-α, tumour necrosis factor-α.

their study was in a clinical population that may have limited generalisability to otherwise healthy children because existing chronic kidney disease and medication use may confound associations.[18] Other differences, including age group, methods for assessment and analysis of microbiota and metabolite data, and having only a single overlapping inflammatory marker, TNF-α, making direct comparisons between studies challenging. These caveats withstanding, their findings are similarly supportive of a link between the gut microbiome, bacterial metabolites, and inflammation in children.

In our study, 9 of the 10 bacterial ASVs robustly associated with at least one marker of inflammation (*Blautia, Phascolarctobacterium, Ruminococcus, Oscillibacter,* Christensenellaceae family, *Melainabacter, Coprococcus,* and *Terrisporobacter*)[31–37] are known or suspected producers of short-chain fatty acids (SCFAs), which are small organic

molecules increasingly recognised as anti-inflammatory.[38] Of these nine SCFA-producing ASVs, a positive association was observed only between MCP-1 and *Terrisporobacter*, which has acetate-producing capacity.[37] While unexpected at first, this association could be indicative of hypothesised proinflammatory effects of acetate under certain conditions, given recent work showing that acetate induces MCP-1 production in monocytes in a TNF-α-dependent manner.[39] Unfortunately, we were unable to investigate acetate as a mediator of this association since it was not detected in our metabolomics panel. All other SCFA-producting taxa were inversely linked to inflammation. Of note, the SCFA-producing anaerobe *B. luti* was inversely associated with the overall InfSc and with each inflammation marker in our study, suggesting broad anti-inflammatory properties. This inverse association has been previously documented in children.[40] Benítez-Páez *et al* found *B. luti* to be depleted among children with obesity and/or insulin resistance, and to be inversely linked to TNF-α, interferon-γ, MCP-1, and interleukin-6.[40] The authors further documented anti-inflammatory properties of *B. luti* through in vitro experiments, noting a higher ratio of the anti-inflammatory cytokine interleukin-4 to the pro-inflammatory TNF-α among peripheral blood mononuclear cells exposed to *B. luti* compared with those exposed to a control taxon, *Bacteroides vulgatus*.[40] Beyond *B. luti*, other SCFA-producing bacteria (eg, *Phascolarctobacterium*, *Ruminococcus*, *Oscillibacter*) were also linked to lower inflammation markers; unfortunately, we were unable to directly investigate targeted SCFAs quantifications in relation to inflammation markers. Future studies with targeted SCFA measurements are warranted.

Out of 1037 metabolites tested in our study, 87 were associated with at least one marker of inflammation after full multivariable adjustment. Significant metabolites included amino acids, carbohydrates, lipids, vitamins, xenobiotics, nucleotides, and peptides. Among these 87 metabolites, 45 were determined a priori to be microbe-dependent.[26] Notably, the proportions of microbial metabolites significantly associated with the overall InfSc and with PAI-1 were over two times higher than that of non-microbial metabolites, while no significant differences were observed for TNF-α and MCP-1. These results indicate that some inflammation markers may be more microbially driven than others, highlighting the need for inclusion of more than one inflammatory marker in other microbiota-inflammation studies.

Since we do not have space to provide an in-depth discussion of all 87 plasma metabolites significantly associated with at least one marker of inflammation, we have instead elected to focus on findings pertaining to tryptophan and its metabolites—a group represented in our data by 22 metabolites, of which 16 we determined to have microbial origins. Tryptophan is among the amino acids we found to be inversely associated with an inflammatory marker, PAI-1. While most amino acids are predominately used in anabolic processes, tryptophan is overwhelmingly metabolised into other bioactive compounds by commensal microbes or endogenously, through three main pathways: the kynurenine pathway (primarily), the serotonin pathway, and the indole-3-pyruvate pathway.[9 10] Studies in adults have identified tryptophan metabolism as a key component of the gut-brain axis, as molecules like kynurenine affect brain functioning and have been linked to neuropsychiatric and neurodegenerative diseases, including depression, Parkinson's disease, and Alzheimer's disease.[11 12 41 42]

In our study, kynurenine and serotonin were positively associated with higher levels of PAI-1 and TNF-α, respectively. Since the kynurenine pathway is activated in response to inflammation, and serotonin has known anti-inflammatory and immunomodulatory properties,[9] it is likely that these findings are indicative of upregulation of these pathways in response to chronic inflammation.[10] Our findings on tryptophan and kynurenine are largely consistent with results from the Growing Up in Singapore Towards healthy Outcomes (GUSTO) cohort, which found that the inflammatory marker C reactive protein was positively associated with kynurenine but inversely linked with tryptophan.[43] Similar patterns for inflammation and TNF-α were observed by Holle and colleagues,[18] as discussed above. To our knowledge, there are no other reports on the role of tryptophan and its metabolites on inflammatory processes in children, suggesting this topic warrants further exploration, particularly in relation to the gut microbiome, due to the dual role of gut microbes in producing tryptophan metabolites and depleting bioavailable tryptophan for endogenous processes.[10]

The mechanistic underpinnings driving the microbe-metabolite-inflammation axis and its potential impact on health are still a matter of active investigation. While our study is cross-sectional and not ideal for directly testing such mechanisms, it appears that the associations observed among gut microbiota features, including metabolites, and inflammation in our paediatric cohort were not driven nor accompanied by significant differences in body composition, as evidenced by a lack of association between inflammation markers and DXA-measured metrics of body habitus in our study. These findings could indicate that microbial-driven inflammation precedes changes in body habitus, at least in children. Associations among body habitus, inflammation, and the gut microbiome have been previously observed in adults, suggesting that in adults microbiome-induced inflammation could be an aetiological trigger for obesity and type 2 diabetes.[44 45] Additional studies, ideally with longitudinal or experimental designs, in both children and adults are warranted to offer more expansive insights into the mechanistic underpinnings of the microbe-metabolite-inflammation axis, including the role of and implications for body habitus.

While our study is strengthened by our untargeted investigation of both the gut microbiota and the circulating metabolome, our use of multiple inflammation markers, and the rich covariate data of Gen3G, there are also limitations worth noting. First, our analysis is cross-sectional, so directionality, mediation, and causality cannot be established. Interpretation of results accounted for this, when possible, as highlighted in our discussion of the observed associations between tryptophan metabolites and inflammation. Studies with longitudinal inflammation, microbiome, and metabolome data collection are warranted to better inform the temporality and potential direction of associations. Second, our cohort is largely homogenous in many aspects, including geographical location and race/ethnicity, which may limit the generalisability of our findings. Third, although we adjusted for many factors including diet quality (ie, fruit, vegetable, and dairy intake) and drinking water source as an indicator of urban versus rural dwelling, we cannot rule out the possibility of unmeasured or residual confounding. Finally, instrumental error in the characterisation of the metabolome and microbiome cannot be discarded, even though the methods employed reflect the current standards of a rapidly developing field. Incorporating novel methods with greater validity and approaches that simultaneously incorporate multiple omics data types will be key to advancing our understanding of the effects of the microbiome and microbial-derived metabolites on health and disease.

While the limitations of our study cannot be dismissed, we believe the cross-sectional associations observed among gut microbes, microbial metabolites, and measures of inflammation represent a valuable contribution to the rapidly growing omics literature in paediatric populations and offer critical insights with potential future clinical implications. These findings suggest a nuanced microbe-metabolite-inflammation nexus, where specific microbes and microbe-dependent metabolites may influence certain inflammatory markers (eg, PAI-1) more than others (eg, TNF-α), highlighting the complexity of the gut microbiota's role in immune modulation. Such associations underscore the relevance of gut health in systemic inflammation and disease pathogenesis in early childhood, urging further research to elucidate causal relationships and therapeutic interventions targeting the gut microbiome to mitigate systemic inflammation and its sequelae in children.

In conclusion, in a global investigation of the gut microbiota and circulating metabolome among 5-year-old children, we found several microbes and metabolites that were robustly associated with distinct inflammation markers and overall inflammation. Importantly, a higher proportion of microbial metabolites were associated with overall inflammation than non-microbial metabolites, suggesting a closer relationship between inflammation and microbial metabolites than non-microbial metabolites. While some of the metabolites identified in our study may be on the pathway from altered gut microbiota to systemic inflammation, the opposite is also possible, such as systemic inflammation may alter metabolic pathways, like the tryptophan-kynurenine pathway. These findings corroborate previous research and propound novel hypotheses linking the gut microbes and metabolites to inflammation and preclinical metabolic health status in paediatric populations. Our findings should be explored in future studies across the translational spectrum, particularly with longitudinal or experimental designs and multiomics methods, as identification of microbial and metabolic signatures linked to inflammation holds promise for the development of novel approaches to screening, prevention, and/or therapy in paediatric populations.

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