Fecal microbiota and metabolite composition associates with stool consistency in young children

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ABSTRACT

Stool consistency explains a large proportion of inter-individual variation in gut microbiota and represents a useful measure of gut transit time, which is pivotal for bacterial metabolism and *vice versa*. However, in infants and small children, a detailed understanding of how gut microbiota and the metabolome associate with stool consistency is lacking. We analyzed both stool microbiota and metabolome of 2.5 (n=360), 6 (n=229), 14 (n=274), and 30-month-old children (n=169) from the FinnBrain Birth Cohort Study using 16s rRNA sequencing and mass spectrometry-based targeted techniques. We used stool water content and parent-reported stool consistency as proxies for stool consistency. We found that microbiota community composition as well as the relative abundance of specific genera such as *Clostridium*, *Hungatella*, and *Lactobacillus* were associated with stool consistency. Of the metabolites, lower concentrations of conjugated bile acids and acetic acid were associated with having constipation and lower water content. Branched short-chain fatty acid concentrations were negatively associated with stool water content. The findings reflect a shift in bacterial saccharolytic to proteolytic metabolism in the gut. Parental crude reports agreed with the objective measure of child stool consistency, and associated with similar features in the stool metabolome. Overall, our findings support the concept that stool consistency and total water content associate with gut microbiota composition and metabolic activity in infants and young children.

Introduction

Emerging literature, including our own work, suggests that the gut microbiota in early life plays a pivotal role in host health and the development of various diseases across the lifespan [1, 2] . There is a growing awareness that the impact of microbes on various physiological processes including initiation and pathogenesis of various diseases may be mediated by microbially-regulated metabolites [3-6]. Current microbiota research related to human health and disease has shifted its focus from characterizing mere community composition towards understanding the intricate interplay between the gut microbiota and microbiota-dependent metabolites. Efforts have been made to enhance the identification of potentially confounding factors affecting gut microbiota and metabolite composition, including diet, antibiotic use, gut transit time, and disease status [7-11]. Accounting for these confounding factors is crucial if one is to identify robust disease markers in human microbiota studies [12]. Particularly high intra-individual variance in gut microbiota community composition is explained by proxies for gut transit time [13] . However, measuring the gut transit time is often inconvenient due to logistic challenges and its invasive nature [11, 14]. Stool consistency measurement represents a useful proxy for gut transit time [15]; hard stools suggest slow transit while loose stools are linked with fast transit through the colon. Nevertheless, stool consistency is often neglected in human microbiota studies.

The concept of assessing stool consistency and its link with gastrointestinal pathophysiology dates back over a century [16]. These days, stool consistency is generally assessed using the Bristol Stool Scale. Recent reports suggest that stool consistency is strongly associated with gut microbiota richness, composition, and microbial metabolism [9, 17-19] . Additionally, pathological conditions related to alterations in stool consistency or defecation frequency, such as chronic constipation, are associated with gut microbiota composition in children [20].

To the best of our knowledge in children, there are no studies investigating stool metabolites in relation to the stool consistency. Several human studies from both healthy and pathological conditions have identified associations with gut microbiota composition, stool consistency, or transit time measurement in adults [12]. Although

understanding in adult populations is increasing, there is a dearth of studies examining the impact of the gut microbiota and metabolome on both stool consistency and total water content of feces or *vice versa* in children [21]. Here, we aim to characterize associations between stool consistency, total water content of stool, stool microbiota composition, and metabolite levels in infants and small children. We used stool samples and complementary markers for gut transit time: stool water content and parental reports of crude stool consistency from 2.5-, 6-, 14-, and 30-month-old children.

Results

Longitudinal measurements

We measured the gut microbiota, metabolome, stool consistency, and water content from 618 stool samples (Figure 1). We obtained measurements at four time points of 2.5 months (n=360), 6 months (n=229), 14 months (n=274), and 30 months (n=169) of age. The distribution of participants providing samples was as follows: those who provided only one sample (326), two samples (190), three samples (82), and all four samples (20). We performed targeted metabolomics analysis of six short-chain fatty acids (SCFAs; acetic, propionic, iso-butyric, butyric, isovaleric, and valeric acids), and 33 bile acids (BAs; including both taurine and glycine conjugates). We also detected metabolites panel including a wide range of chemical classes, such as amino acids, carboxylic acids (mainly free fatty acids and other organic acids), hydroxy acids, phenolic compounds, alcohols, and sugar derivatives in an untargeted metabolomics assay. Parents reported on crude stool consistency. Stool water content was measured as the percentage of water present in the stool sample. Table 1 shows clinical characteristics of the study subjects and Table S1 shows the sample sizes for available data types including measured metabolites, stool consistency and water content analyses.

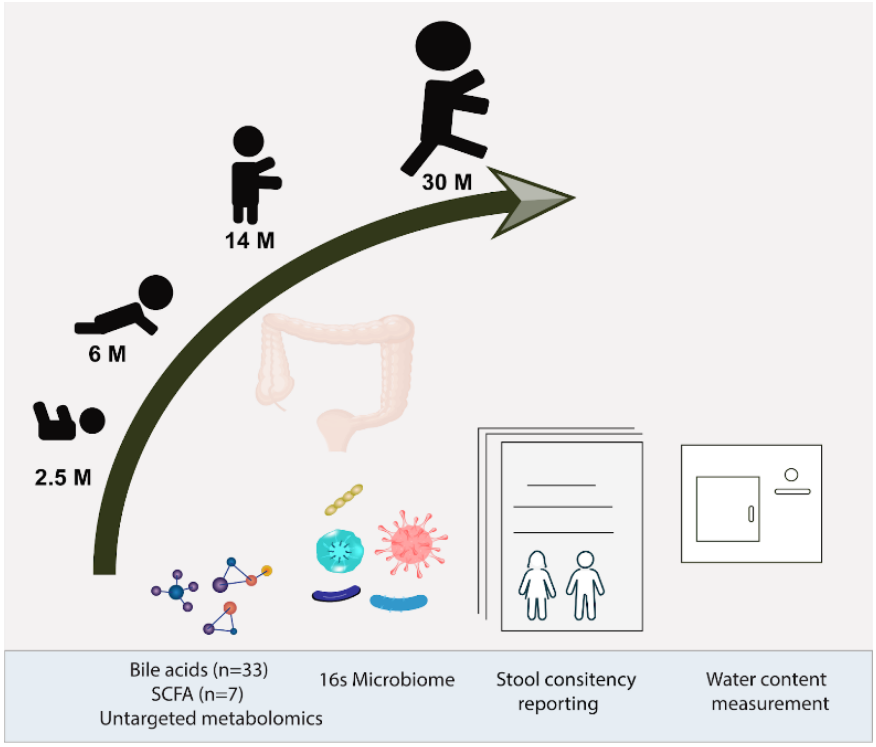

Figure 1. An overview of the study setting. We obtained longitudinal gut microbiota (16S rRNA), metabolome (targeted (bile acid, SCFAs) and untargeted (polar metabolites)), stool consistency, and water content measurements at four time points of 2.5 months (n=360), 6 months (n=229), 14 months (n=274), and 30 months (n=169) of age. Short-chain fatty acids (SCFAs). The human silhouette in this figure is adapted from our previous work [2, 22].

Table 1. Characteristics of the study subjects who had either stool consistency or water content reported.

Timepoint	2.5	6	14	30	Overall
Sample sizes	360	229	274	169	1032
Stool consistency					
constipation	23 (6.4%)	31 (13.5%)	9 (3.3%)	10 (5.9%)	73 (7.1%)
loose	32 (8.9%)	4 (1.7%)	14 (5.1%)	2 (1.2%)	52 (5.0%)
normal	189 (52.5%)	89 (38.9%)	105 (38.3%)	80 (47.3%)	463 (44.9%)
other or gas	58 (16.1%)	8 (3.5%)	4 (1.5%)	4 (2.4%)	74 (7.2%)

missing	58 (16.1%)	97 (42.4%)	142 (51.8%)	73 (43.2%)	370 (35.9%)
Water content					
mean (SD)	79.3 (5.70)	77.4 (6.43)	75.4 (5.50)	75.0 (5.86)	76.6 (6.06)
median [Min, Max]	79.3 [60.8, 98.6]	78.4 [47.2, 90.9]	76.2 [60.5, 87.3]	75.7 [53.6, 86.3]	77.2 [47.2, 98.6]
missing	236 (65.6%)	42 (18.3%)	30 (10.9%)	23 (13.6%)	331 (32.1%)
Delivery mode					
C-section	57 (15.8%)	37 (16.2%)	45 (16.4%)	23 (13.6%)	162 (15.7%)
vaginal	298 (82.8%)	191 (83.4%)	226 (82.5%)	145 (85.8%)	860 (83.3%)
missing	5 (1.4%)	1 (0.4%)	3 (1.1%)	1 (0.6%)	10 (1.0%)
Gestational age					
mean (SD)	40.1 (1.36)	40.0 (1.38)	39.8 (1.52)	39.9 (1.36)	40.0 (1.41)
median [Min, Max]	40.1 [34.6, 42.3]	40.0 [34.1, 42.3]	40.1 [32.9, 42.3]	40.0 [35.6, 42.3]	40.1 [32.9, 42.3]
Antibiotic treatment prior 6 months					
no	300 (83.3%)	128 (55.9%)	111 (40.5%)	75 (44.4%)	614 (59.5%)
yes	2 (0.6%)	4 (1.7%)	22 (8.0%)	21 (12.4%)	49 (4.7%)
missing	58 (16.1%)	97 (42.4%)	141 (51.5%)	73 (43.2%)	369 (35.8%)
Current feeding					
full breastfeeding	220 (61.1%)	9 (3.9%)	0 (0%)	0 (0%)	229 (22.2%)
no breastfeeding	7 (1.9%)	1 (0.4%)	3 (1.1%)	1 (0.6%)	12 (1.2%)
partial breastfeeding	50 (13.9%)	145 (63.3%)	32 (11.7%)	1 (0.6%)	228 (22.1%)
ceased breastfeeding	10 (2.8%)	53 (23.1%)	217 (79.2%)	155 (91.7%)	435 (42.2%)

missing	73 (20.3%)	21 (9.2%)	22 (8.0%)	12 (7.1%)	128 (12.4%)
Maternal age					
mean (SD)	30.8 (4.42)	30.7 (4.52)	31.1 (4.33)	31.0 (4.29)	30.9 (4.39)
median [Min, Max]	31.0 [19.0, 45.0]	30.0 [19.0, 42.0]	31.0 [19.0, 45.0]	30.0 [21.0, 45.0]	30.0 [19.0, 45.0]
Primiparity					
no	158 (43.9%)	105 (45.9%)	137 (50.0%)	74 (43.8%)	474 (45.9%)
yes	202 (56.1%)	124 (54.1%)	137 (50.0%)	95 (56.2%)	558 (54.1%)
Sex					
boy	189 (52.5%)	125 (54.6%)	150 (54.7%)	98 (58.0%)	562 (54.5%)
girl	171 (47.5%)	104 (45.4%)	124 (45.3%)	71 (42.0%)	470 (45.5%)

Demographic factors and stool characteristics

We investigated the association between demographic factors including age, breastfeeding status, delivery mode, recent antibiotics intake and premature birth (born < 37 gestational weeks) on stool consistency and water content. As expected, individuals with parent-rated constipation exhibited lower overall water content (Figure 2A, Kruskal-Wallis test, Dunn's test q=0.003), particularly at 6 and 14 months (Figure 2A, Kruskal-Wallis test, Dunn's test q = 0.0324, q= 0.017, respectively). Our analysis revealed a declining trend in water content with increasing age (Figure 2B, Linear mixed model, p = 0.00000005). Moreover, subjects who were breastfed demonstrated higher water content compared to those who were not (Figure 2C, Wilcoxon signed-rank test q = 0.0000008).

Overall, breastfeeding associated with stool consistency (χ^2 q = 0.00110), but delivery mode, antibiotic intake or prematurity at any specific time point or overall did not have an association. However, we observed a distinct pattern between stool consistency and age (Figure 2D). Specifically, we noted fewer occurrences of loose

stool at 6 months compared to 2.5 months (Figure 2D, Dunn's test q = 0.028). Additionally, there were more reports of gas at 2.5 months compared to 6, 14, or 30 months (Figure 2D, Dunn's test q = 2.3×10^{-6}, q = 8.8×10^{-11}, q = 1.2×10^{-7}, for 6, 14, 30 months, respectively). The prevalence of constipation was higher at 6 months compared to 2.5, 14, and 30 months (Figure 2D, Dunn's test q = 0.0000003, q = 0.000008, q = 0.0026 for 2.5, 14, and 30 months, respectively. Furthermore, reports on normal stool consistency were more common at 14 and 30 months compared to 2.5 months (Figure 2D, Dunn's test q = 0.0017, q = 0.0017 for 14 and 30 months, respectively).

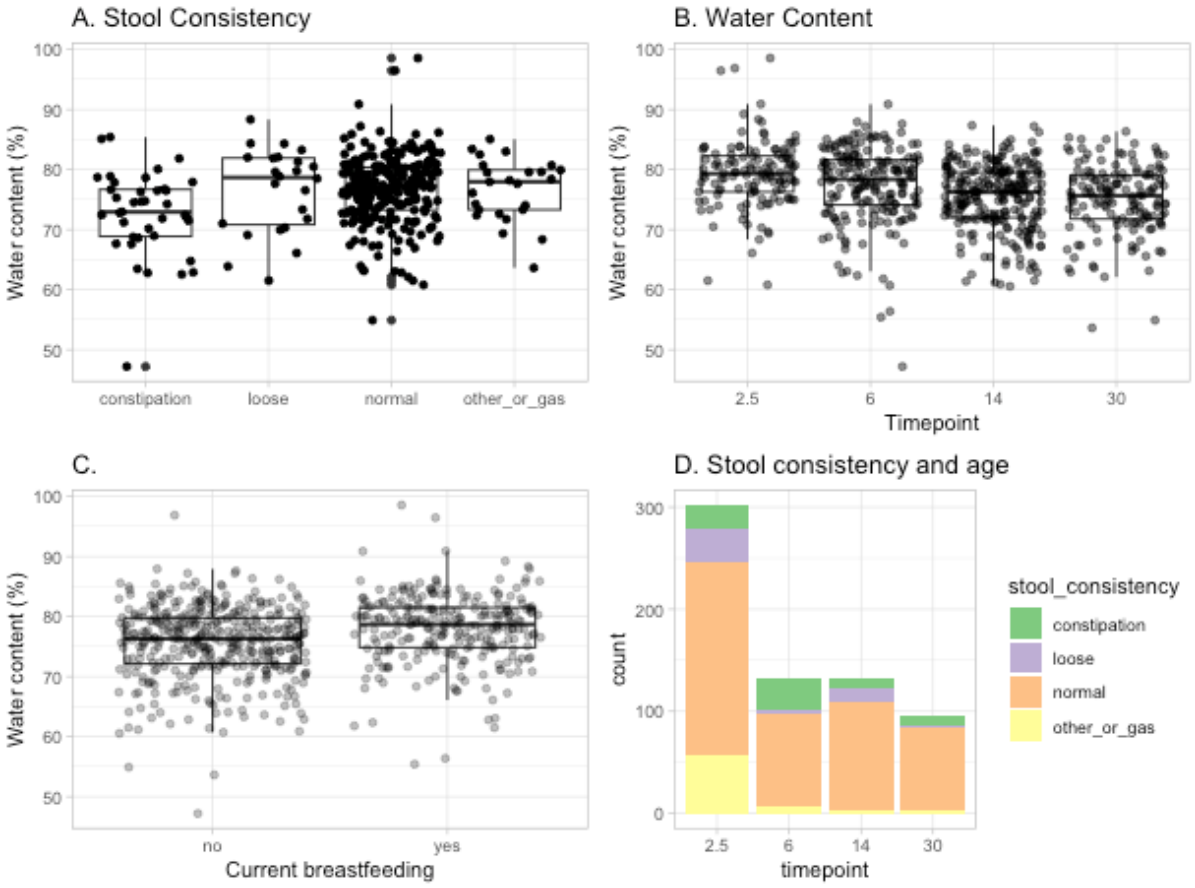

Figure 2. A. Constipated subjects had lower water content. B. Water content gradually decreased as the child aged, and C. consequently water content was associated with breastfeeding. D. There were more loose stools and reports of gas and other complaints at 2.5 months, whereas constipation was reported more at 6 months.

Taxonomic richness, diversity and composition associate with water content and stool consistency

We observed that both the Shannon index and observed richness at 6 months (Figure 3, Spearman ρ = -0.29, q = 0.003) and observed richness at 30 months (Figure 3, Spearman ρ= -0.2, q = 0.039) associated with stool water content. These associations were significant also after adjusting for breastfeeding (q = 0.0001 for Shannon index at 6 months, q = 0.0001 for observed richness at 6 months, q = 0.0166 for observed richness at 30 months). Moreover, in a mixed model, both observed richness and Shannon index associated (Table S2) with water content. However, neither observed richness nor Shannon index were associated with stool consistency across time points or longitudinally (Table S2).

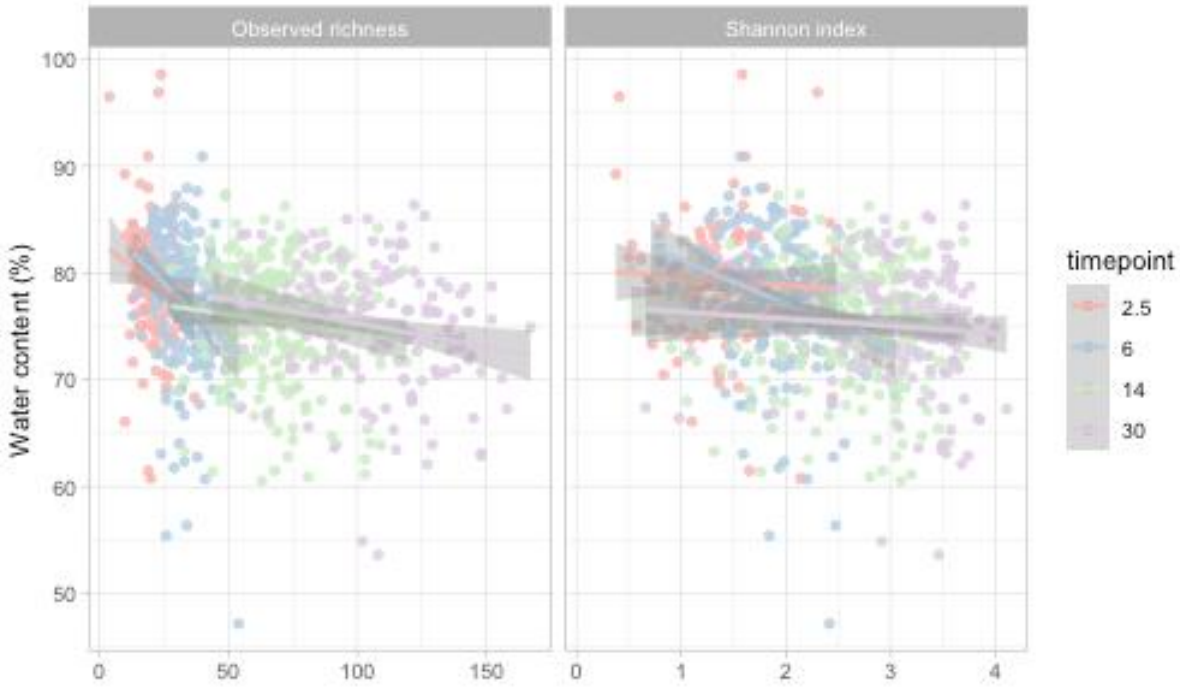

Figure 3: Taxonomic richness (observed richness) and diversity (Shannon index) and water content.

Community composition was associated with water content in distance-based redundancy analysis (Figure 4, Table S3, db-RDA PERMANOVA q = 0.0070) at both 6 (Table S3, q = 0.0070) and 30 months (Table S3, q = 0.0093). This association persisted after adjusting for breastfeeding in the entire dataset and at 6 months. None of the

subjects were breastfed at 30 months. At 6 months, variance in water content was positively associated with amplicon sequence variants (ASVs) annotated to *Clostridium, Escherichia,* and *Veillonella*, while it was inversely associated with *Bifidobacterium*. ASVs in *Bacteroides* and *Enterobacteriaceae* showed loading in both directions. Conversely, in 30-month-olds, the relative abundance of *Veillonella, Sutterella,* and *Ruminococcus* were associated with lower water content, while *Bacteroides, Bifidobacterium,* and *Enterobacteriaceae* were associated with higher water content.

Community composition was associated with overall stool consistency (Figure4A, Table S3, PERMANOVA q = 0.0084), even after adjusting for breastfeeding (Table S3, PERMANOVA q = 0.0220). While in age-wise analysis, the association of stool consistency with community composition remained insignificant, there was differential dispersion in stool consistency groups (Figure 4G. PERMDISP2 p = 0.003), with constipated subjects showing a smaller distance to the centroid. Therefore, the statistical significance found in the db-RDA test may be due to heterogeneity in the group dispersions.

Next, we aimed to determine the link between individual genus abundances and stool consistency and water content, conducting both longitudinal analyses with Linear models for differential abundance analysis (LinDA) and age-wise analyses with ANOVA-Like Differential Expression (ALDEx2). Longitudinal analysis revealed a higher abundance of *Terrisporobacter* and *Varibaculum* in constipation compared to normal stool when adjusted for breastfeeding (Table S5). Multiple genera such as *Clostridium*, *Hungatella*, and *Lactobacillus* were indirectly associated with water content, with most associations persisting after adjusting for breastfeeding (Table S5, Figure S1). However, associations regarding stool consistency or water content were not observed in age-wise analysis (Table S4, S6).

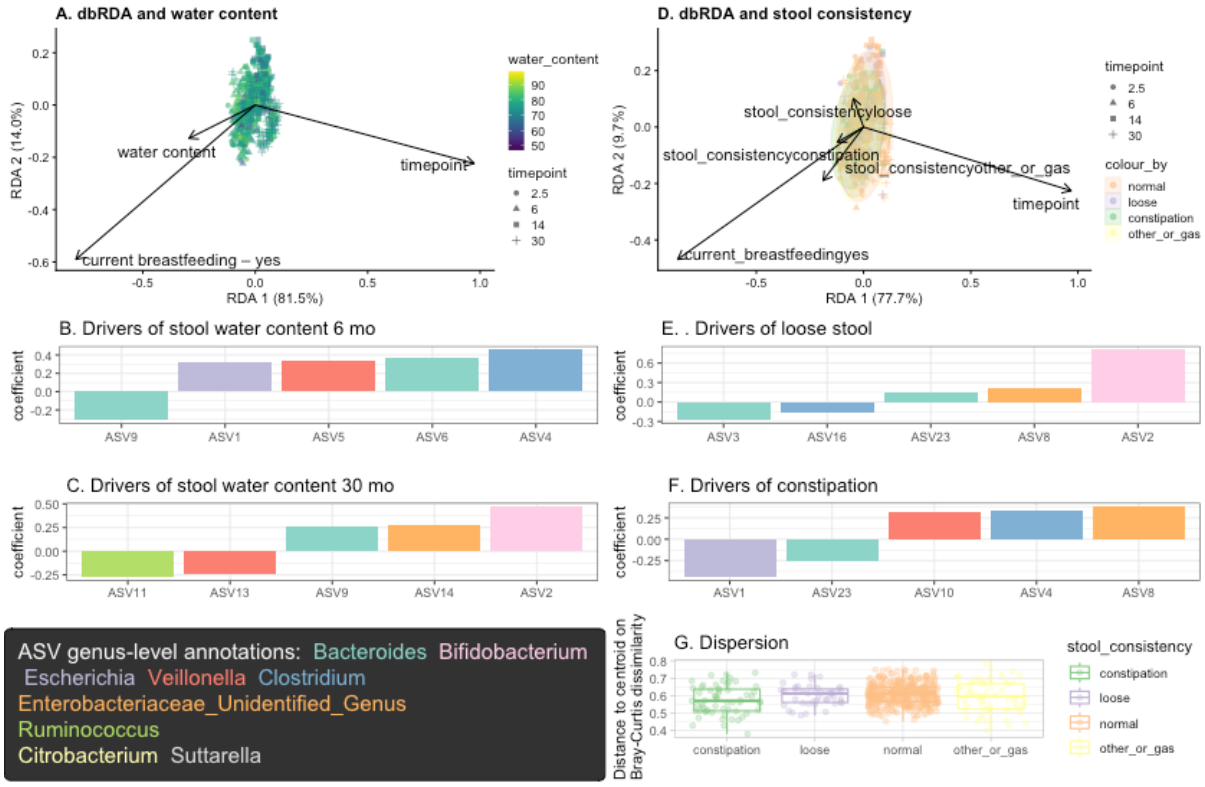

Figure 4: Stool consistency and community composition. db-RDA on A. water content and D. stool consistency. B, C, E, F show the ASVs that have the largest absolute coefficients in the RDA models. The bar colors represent the genus-level annotation of the ASV. G. shows the distances to group centroid in stool consistency groups in Bray-Curtis dissimilarity.

Stool metabolite concentrations and stool consistency and water content

Next, we sought to investigate whether stool metabolites were associated with stool consistency and water content (Figure 5A-B). We found that constipated subjects had lower levels of tauro- and glycoconjugated bile acids compared to subjects with normal or loose stools at both 2.5 and 14 months (Figure 5, Table S8, S9, Kruskal-Wallis test, q= 0.020 for tauroconjugated BA at 2.5 months, q = 0.029 for glycoconjugated BA at 14 months, q = 0.037 for tauroconjugated BA at 14 months < 0.05). Acetic acid concentration was also lower in constipated subjects compared to normal subjects at 6 months (Figure 5, Figure S2, Table S2 Kruskal-Wallis test q = 0.019). Similarly, sum of all SCFA concentrations seemed to be lower in constipated individuals at 6 months (Figure 5C-D, Kruskal-Wallis test q =0.085, Dunn's test q =

0.042). Additionally, several polar metabolites, including fructose, fucose, glycerol, pentadecanoic acid 15, pinitol, and palmitoleic acid, were lower in constipated subjects (Table S88, Figure S9).

We also investigated whether the stool metabolite content was associated with the water content (Figure 5A, Table S3, Figures S6-S9). Consistent with microbiota findings, multiple metabolite associations remained in age-wise analysis (n = 157 associations, Table S8). Most of these associations remained unchanged even after adjusting for breastfeeding (n = 118 associations). Conjugated bile acids were positively associated with water content across all time points (Figure S6). Primary bile acids were also positively associated with water content at 14 and 30 months, although with low estimates (estimate 0.04 and 0.03, respectively, Table S3). On the other hand, acetic acid was positively associated with water content at 6, 14, and 30 months (Figure S8). Butyric acid was positively associated at 6 months (Figure S8). Branched SCFAs, particularly isobutyric and isovaleric acids, were negatively associated with water content at 6, 14, and 30 months (Figure S8). The sum of all SCFA concentrations was directly correlated with water content at 6, 14 and 30 months (Figure 5C, Table S10). Furthermore, fucose, fructose, glycerol, and pinitol were positively associated with water content, consistent with the findings from constipated subjects (Figure S9).

In the longitudinal analyses with mixed models, conjugated bile acids inversely associated with constipation when compared to normal stool consistency after adjusted for age and further with breastfeeding (Figure 5B, Table S11). Moreover, we observed in linear mixed models that tauroconjugated bile acids, primary bile acids, acetic acid, fucose, pinitol and several other polar metabolites were positively associated with water content, whereas isobutyric acid and isovaleric acid were negatively associated, both adjusted and unadjusted for breastfeeding (Figure 5A, Table S12). Additionally, water content associated directly and constipation associated indirectly with the sum of all SCFA (Figure 5C-D, linear mixed-model, $p = 0.9 \times 10^{17}$ for water content, $p = 0.047$ for constipation).

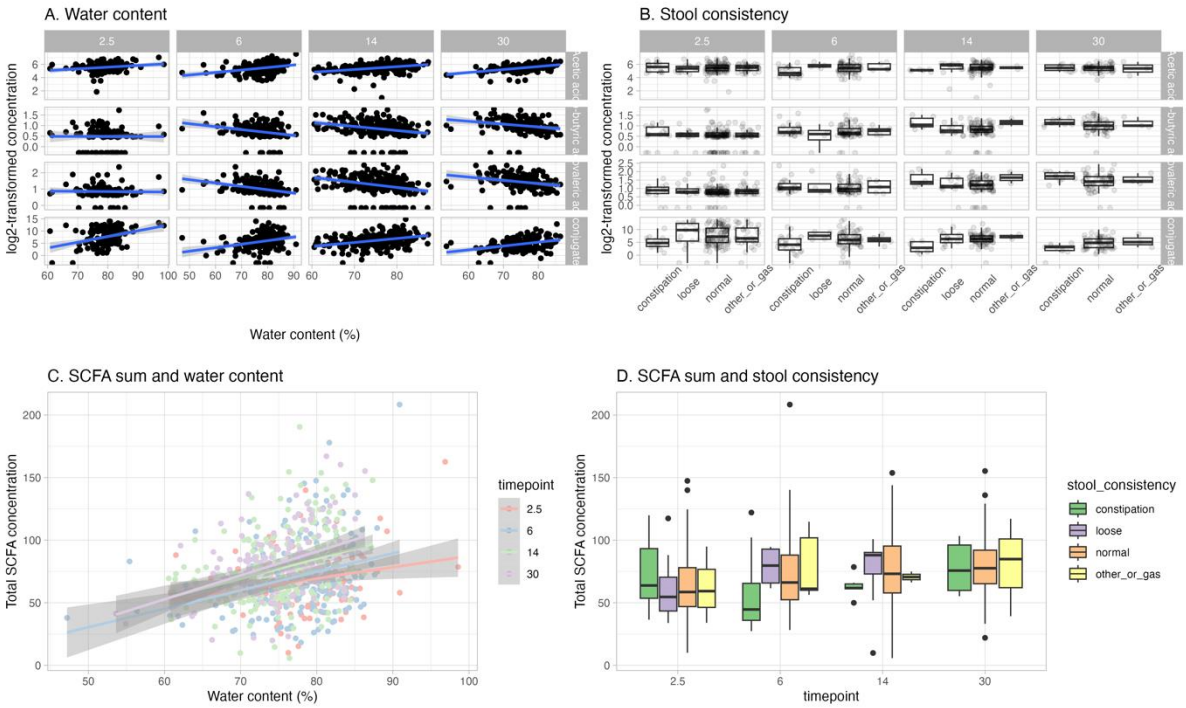

Figure 5. Metabolite concentrations associated with SCFA and conjugated BAs. A. Water content and B. stool consistency, particularly reports of constipation, associate with a part of categorized bile acids (BAs) and short-chain fatty acids (SCFA) across ages and longitudinally. The only study subject with loose stools at 30 months was removed from the figure. C. Water content associate directly with overall SCFA concentrations, and D. The constipated individuals had lower total SCFA at 6 months.

Discussion

We show in infants and small children that both stool microbiota and metabolome link with the stool consistency and the total water content. This corroborates earlier findings indicating that stool consistency and gut transit time correlate with microbiota and metabolism in adults [8, 9, 23],. However, data especially from infants and with longitudinal study design are scarce. Our findings underscore the potential of using the total water content as an objective measure in microbiota studies, particularly during early infancy when stool consistency vary considerably. We also

found agreement between the total stool water content and parental crude reports of constipation.

Fluctuations in stool consistency correlate with microbiota taxonomic richness, and community composition[9, 12, 23, 24], yet this factor is often overlooked in microbiota studies. Vandeputte et al. (2016) found that firmer stools in Bristol stool form scale were associated with a higher Bacteroidetes:Firmicutes ratio and elevated levels of *Akkermansia* and *Methanobrevibacter* whereas loose stools were associated with lower taxonomic richness in adults [9]. In line, our study in infants and small children reveals that stool consistency associates with the bacterial community composition, particularly through variations in *Bacteroides, Bifidobacterium, Ruminococcus,* and *Clostridium* abundances. We also found an ASV in *Bacteroides* genus associated with the water content-related community composition positively at 30 months and negatively at 6 months. However, a previous study reported a different direction in the association between stool consistency and microbiota composition during infancy and childhood [21]). In the longitudinal analysis, genera including *Clostridium*, *Prevotella*, and *Eggarthella* associated negatively with water content. Many of these genera are known as dietary fiber fermenters, specifically *Clostridium* and *Prevotella*. Apart from fiber fermentation, both *Clostridium* and *Eggarthella* contain species implicated in the bile-acid metabolism [25, 26]. Intriguingly, Kwiatkowska *et al.* 2024 reported that functional constipation in three-year-olds was associated with depletion of rare genera, such as butyrate-producer *Subdoligranulum* [27]We observed that constipated individuals had more homogenous community composition compared to other stool consistency groups, and the lack of rare taxa could partly explain our observation, however, we did not directly assess prevalence or abundance of rare taxa. Overall, these findings align with previous research on the relationship between stool consistency and microbiota, although differences in associated taxonomic groups may result from gut microbiota alterations across different life stages [28].

Regarding the metabolic features, we show that the associations between metabolites and low stool water content and parent-report of constipation are qualitatively similar:

children with constipation and lower water content also showed decreased concentrations of conjugated bile acids (glycine and taurine-bound). Gut microbes regulate human bile acid metabolism [29]. Primary bile acids are synthesized and conjugated in the liver, stored in the gallbladder, secreted in the intestine after meal consumption, and later modified (de-conjugated or again re-conjugated) by microbes in the gut before reabsorption (95%) or excretion (5%) [30]. Thus, the decreased level of conjugated BAs in egested constipated stool suggests that longer transit time allows for more extensive bacterial metabolism of bile acids in the gut[23]. This might have health implications, since biliary excretion of xenobiotics is an important elimination route, and deconjugation might delay clearance of substances such as estrogens [31].

Besides bile acids, constipation and lower water content were associated with less SCFA, specifically acetic acid, while branched short-chain fatty acids (BCFAs) concentrations were inversely associated with stool water content at 14 and 30 months. In fact, SCFAs, primarily acetate, propionate, and butyrate, are end products of fermentation of complex carbohydrates such as dietary fiber [32, 33], while increased BCFAs reflect ongoing putrefaction or/and proteolytic activities in the colon. We hypothesize that prolonged colonic transit time depleted available carbohydrates for fermentation which consequently induced proteolytic activity, regarded as an alternative pathway for carbon and nitrogen, and an energy source for the microbes in the colon. Interestingly, another study showed that by reducing gut transit time with drugs, the concentrations of total SCFAs could be increased [34]. Our finding is consistent with similar works in adults, where lower water content or longer gut transit time showed to shift in colonic metabolism towards proteolytic activity [8, 23, 34-36]. Roager *et al*. found that longer transit time was associated with higher levels of protein-derived molecules in the urine such as p-cresol-sulfate and phenylacetylglutamine, indicating a shift from saccharolytic to proteolytic activity in the colon [8]. Also, Zhang *et al*. found that the Bristol stool form scale scores were found to negatively associate with the levels of fecal amino acids, suggesting increased protein catabolism in irritable bowel syndrome patients [37]. In adults, metabolites from protein catabolism have been implicated in atherosclerotic cardiovascular diseases and colon cancer [38-40]. Hence our observation that higher BCFA concentrations were

associated with constipation might have further health-implications, although this has not yet been studied in infants or small children.

We expect that our findings may reflect the influence of longer colonic transit time on stool microbiota activity and levels of microbial-regulated metabolites. However, other mechanisms may underline the links between microbiota and gut motility. Early life microbiota may affect the development of enteric nervous system and consequently gut motility and secretions [41-44].Furthermore, our results indicate a need to study whether similar microbiota and microbially-regulated metabolite signatures could be seen *i.e.* in functional gastrointestinal disorders in older children [27, 45-47].

Our findings of parent-reported stool consistency, water content, child age and breastfeeding are in line with earlier literature and clinics. First, the information that parent-reported constipation associated with objective measure of lower water content may be clinically relevant to support future research to avoid invasive examination techniques of colonic transit time [48, 49]. Second, the association between breastfeeding and stool consistency and water content is in line with general clinical observation that breastfed infants seldom suffer from constipation [50], and prolonged breastfeeding may protect from functional constipation[51]. Moreover, parental reports of gas were prevalent at 2.5-months-of-age when over half of the infants were breastfed and they typically swallow air during breastfeeding which leads to gas problems [50] . Third, constipation turns more prevalent at 6 months that aligns well with the introduction of solid foods and reduction of breastfeeding. Our observation that water content gradually decreases by age likely reflects the normative prolonging of gut transit time by age [52, 53].

Our study has limitations. We used crude parental report of child stool consistency, which is not as refined as the Bristol stool scale, and this may have implications on comparability or generalizability of our results but still, it was associated with the objective measure of total stool water content. Stool water content is different from *e.g.* gastrointestinal transit time assessment with radio-opaque markers, but we studied vulnerable study subjects of healthy infants and young children, where utilizing invasive measuring techniques has ethical constrains. Utilizing stool samples

does not allow proper inference of absorption of the microbiota-produced metabolites in whole gut. Serum or urinary metabolome analysis may have given greater insight into the systemic alterations related to the saccharolytic *vs.* proteolytic fermentation and enterohepatic circulation. Moreover, one can speculate that extremely low water content in the faeces may alter the DNA extraction success, which may subsequently lead to changes in the microbiota composition. However, we are not aware of methodological studies addressing this.

In summary, our findings in infants and small children support the concept that stool consistency and total water content associate with gut microbiota composition and metabolic activity. We found microbiota community composition to be associated with stool consistency, and these varied partly by age. Our result suggests metabolic shifts from saccharolytic to proteolytic activity and decreased concentrations of conjugated bile acids related to constipation and lower water content, providing insights into gut health and function. Our findings indicate that stool consistency could be considered as a confounder in microbiota studies, especially for establishing robust and reproducible microbiota-based markers of disease. Parental crude reports agreed with the objective measure of child stool consistency which may have clinical implication in avoiding invasive measuring techniques of gastrointestinal transit time.

Methods

Cohort and Questionnaire Data

The study subjects are children from the FinnBrain Cohort Study [54]that is a general population birth cohort study located in the southwestern Finland. The Ethical Committee of Southwestern Finland approved the study. Parents provided informed consent on behalf of their children. STORMS guideline was used for reporting the methods and materials[55].

The sample used in the current study were a subset of children that participated in a study visit at 2.5, 6, 14 and 30 months postpartum. The fecal samples were collected from May 2013 to May 2018. The samples were collected in plastic tubes, and parents were instructed to store the sample in a refrigerator, and bring the sample to the laboratory within 24 h. The sample collection time was reported.

Information on breastfeeding was collected with questionnaires to the mother at 3, 6, 12, and 24 months postpartum and during study visits 2.5, 6, 14, and 30 months. Information on delivery mode and gestational age was collected from the hospital records (The Wellbeing Services County of Southwest Finland, VARHA). Breastfeeding was categorized as any current breastfeeding (yes vs. no). At the sample collection, the parent reported the child stool consistency as: normal, loose, constipated, gas or other.

DNA Extraction, Sequencing and Data Processing

The samples were divided into cryotubes and freezed in -80C within 2 days after arriving at the laboratory. Samples were kept at +4C before freezing. Only samples that were freezed within 48 h of sample collection were sequenced. DNA was extracted using a semi-automatic extraction instrument Genoxtract with DNA stool kit (HAIN life science, Germany). The DNA extraction was performed according to the manufacturer's protocol except that 1 ml of lysis buffer was added and the samples were homogenized with glass beads 1000 rpm / 3 min. The samples were centrifuged at high speed (> 13000 rpm) for 5 min. The lysate (800µL) was then transferred to tubes and the extraction proceeded according to the manufacturer’s protocol. DNA yields were measured with Qubit fluorometer using Qubit dsDNA High Sensitivity

Assay kit (Thermo Fisher Scientific, USA). Bacterial community composition was determined by sequencing the V4 region of 16S rRNA gene using Illumina MiSeq platform (Illumina, USA). The sequence library was constructed with an in-house developed protocol as described in [56, 57].Positive control (DNA 7-mock standard) and negative control (PCR grade water) were included in library preparation and sequencing runs.

DADA2-pipeline (version 1.14) was used to preprocess the 16S rRNA gene sequencing data to infer exact amplicon sequence variants (ASVs) (PMID: 27214047). The reads were truncated to length 225 and reads with more than two expected errors were discarded (maxEE = 2). SILVA taxonomy database (version 138) and RDP Naive Bayesian Classifier algorithm ref were used for the taxonomic assignments of the ASVs [58].

Stool Water Content

To quantify the water content, stool samples underwent freeze-drying. Prior drying, samples were weighted and briefly centrifuged in the bottom of the tubes. Subsequently, they were transferred to a custom freeze-drier equipped with a vacuum pump. The samples were left to dry overnight and then reweighed to determine their dry weight.

Metabolite Analyses

The BAs, SCFA and untargeted metabolome were previously described and adapted for this publication[56].

Targeted BAs measurement. The BAs were measured in fecal samples as described previously. Only samples frozen within 24 h of sample collection were included in the metabolome analyses. The order of the samples was randomized before sample preparation. Two aliquots (50 mg) of each fecal sample were weighed. An aliquot was freeze-dried prior to extraction to determine the dry weight. The second aliquot was homogenized by adding homogenizer beads and 20 µL of water for each mg of dry weight in the fecal sample, followed by samples freezing to at least -70 °C and homogenizing them for five minutes using a bead beater. The BAs analysed were

Litocholic acid (LCA), 12-oxo-litocholic acid(12-oxo-LCA), Chenodeoxycholic acid (CDCA), Deoxycholic acid (DCA), Hyodeoxycholic acid (HDCA), Ursodeoxycholic acid (UDCA), Dihydroxycholestanoic acid (DHCA), 7-oxo-deoxycholic acid (7-oxo-DCA), 7-oxo-hyocholic acid (7-oxo-HCA), Hyocholic acid(HCA), β-Muricholic acid (b-MCA), Cholic acid (CA), Ω/α-Muricholic acid (w/a-MCA), Glycolitocholic acid (GLCA), Glycochenodeoxycholic acid (GCDCA), Glycodeoxycholic acid (GDCA), Glycohyodeoxycholic acid (GHDCA), Glycoursodeoxycholic acid (GUDCA), Glycodehydrocholic acid (GDHCA), Glycocholic acid (GCA), Glycohyocholic acid (GHCA), Taurolitocholic acid (TLCA), Taurochenodeoxycholic acid (TCDCA), Taurodeoxycholic acid (TDCA), Taurohyodeoxycholic acid (THDCA), Tauroursodeoxycholic acid (TUDCA), Taurodehydrocholic acid (TDHCA), Tauro-α-muricholic acid (TaMCA), Tauro-β-muricholic acid (TbMCA), Taurocholic acid (TCA), Trihydroxycholestanoic acid (THCA) and Tauro-Ω-muricholic acid (TwMCA). BAs were extracted by adding 40 µL fecal homogenate to 400 µL crash solvent (methanol containing 62,5 ppb each of the internal standards LCA-d4, TCA-d4, GUDCA-d4, GCA-d4, CA-d4, UDCA-d4, GCDCA-d4, CDCA-d4, DCA-d4 and GLCA-d4) and filtering them using a Supelco protein precipitation filter plate. The samples were dried under a gentle flow of nitrogen and resuspended using 20 µL resuspenstion solution (Methanol:water (40:60) with 5 ppb Perfluoro-n-[13C9]nonanoic acid as in injection standard). Quality control (QC) samples were prepared by combining an aliquot of every sample into a tube, vortexing it and preparing QC samples in the same way as the other samples. Blank samples were prepared by pipetting 400 µL crash solvent into a 96-well plate, then drying and resuspending them the same way as the other samples. Calibration curves were prepared by pipetting 40 µL of standard dilution into vials, adding 400 µL crash solution and drying and resuspending them in the same way as the other samples. The concentrations of the standard dilutions were between 0.0025 and 600 ppb. The LC separation was performed on a Sciex Exion AD 30 (AB Sciex Inc., Framingham, MA) LC system consisting of a binary pump, an autosampler set to 15 °C and a column oven set to 35 °C. A waters Aquity UPLC HSS T3 (1.8µm, 2.1×100mm) column with a precolumn with the same material was used. Eluent A was 0.1 % formic acid in water and eluent B was 0.1 % formic acid in methanol. The gradient started from 15 % B and increased to 30 % B over 1 minute.

The gradient further increased to 70 % B over 15 minutes. The gradient was further increased to 100 % over 2 minutes. The gradient was held at 100 % B for 4 minutes then decreased to 15 % B over 0.1 minutes and re-equilibrated for 7.5 minutes. The flow rate was 0.5 mL/min and the injection volume was 5 µL. The mass spectrometer used for this method was a Sciex 5500 QTrap mass spectrometer operating in scheduled multiple reaction monitoring mode in negative mode. The ion source gas1 and 2 were both 40 psi. The curtain gas was 25 psi, the CAD gas was 12 and the temperature was 650 °C. The spray voltage was 4500 V. Data processing was performed on Sciex MultiQuant.

Quantification of SCFA. We adapted and modified the targeted SCFA analysis from previous work [59]. Fecal samples were homogenized by adding water (10 µL per mg of dry weight as determined for the BA analysis) to wet feces, the samples were homogenized using a bead beater. Analysis of SCFA was performed on fecal homogenate (50 µL) crashed with 500 µL methanol containing internal standard (propionic acid-d6 and hexanoic acid-d3 at 10 ppm). Samples were vortexed for 1 min, followed by filtration using 96-Well protein precipitation filter plate (Sigma-Aldrich, 55263-U). Retention index (RI, 8 ppm C10-C30 alkanes and 4 ppm 4,4-Dibromooctafluorobiphenyl in hexane) was added to the samples. Gas chromatography (GC) separation was performed on an agilent 5890B GC system equipped with a Phenomenex Zebron ZB-WAXplus (30 m × 250 µm × 0.25 µm) column a short blank pre-column (2 m) of the same dimensions was also added. A sample volume of 1 µL was injected into a split/splitless inlet at 285°C using split mode at 2:1 split ratio using a PAL LSI 85 sampler. Septum purge flow and split flow were set to 13 mL/min and 3.2 mL/min, respectively. Helium was used as carrier gas, at a constant flow rate of 1.6 mL/min. The GC oven program was as follows: initial temperature 50°C, equilibration time 1 min, heat up to 150°C at the rate of 10°C/min, then heat at the rate of 40°C/min until 230°C and hold for 2 min. Mass spectrometry was performed on an Agilent 5977A MSD. Mass spectra were recorded in Selected Ion Monitoring (SIM) mode. The detector was switched off during the 1 min of solvent delay time. The transfer line, ion source and quadrupole temperatures were set to 230, 230 and 150°C, respectively. Dilution series of SCFA standards of acetic, propionic,

butyric, valeric, hexanoic acid, isobutyric, and iso-valeric acid were prepared in concentrations of 0.1, 0.5, 1, 2, 5, 10, 20, 40, and 100 ppm for the construction of standard curves for quantification. SCFAs were quantified using Agilent Masshunter.

Polar metabolites were extracted in methanol. The method was adapted from the method used by [60]. Fecal homogenate (60 µL) were diluted with 600 µL methanol crash solvent containing internal standards (heptadecanoic acid (5 ppm) valine-d8 (1 ppm) and glutamic acid-d5 (1 ppm)). After precipitation the samples were filtered using Supelco protein precipitation filter plates. One aliquot (50 µL) was transferred to a shallow 96-well plate to create a QC sample. The rest of the sample volume was dried under a gentle stream of nitrogen and stored in -80 °C until analysis. After thawing the samples were again dried to remove any traces of water.
Derivatization was carried out on a Gerstel MPS MultiPurpoe Sampler using the following protocol: 25 µL methoxamine (20 mg/mL) was added to the sample followed by incubation on a shaker heated to 45 °C for 60 minutes. N-Methyl-N-(trimethylsilyl) trifluoroacetamide (25 µL) was added followed by incubation (60 min). After that, 25 µL retention index was added, the sample was allowed to mix for one min followed by injection. The automatic derivatization was carried out using the Gerstel maestro 1 software (version 1.4). Gas chromatographic (GC) separation was carried out on an Agilent 7890B GC system equipped with an Agilent DB-5MS (20 m x 0,18 mm (0,18 µm)) column. A sample volume of 1 µl was injected into a split/splitless inlet at 250°C using splitless mode. The system was guarded by a retention gap column of deactivated silica (internal dimensions 1.7 m, 0.18 mm, PreColumn FS, Ultimate Plus Deact; Agilent Technologies, CA, USA). Helium was used as carrier gas at a flow rate of 1.2 ml/min for 16 min followed by 2 mL/min for 5.75 min. The temperature programme started at 50°C (5 min), then a gradient of 20°C/min up to 270°C was applied and then finally a gradient of 40°/min to 300°C, where it was held stable for 7 min. The mass spectrometry was carried out on a LECO Pegasus BT system (LECO). The acquisition delay was 420 sec. The acquisition rate was 16 spectra/sec. The mass range was 50 – 500 m/z and the extraction frequency was 30 kHz. The ion source was held at 250 °C and the transferline heater temperature was 230 °C. ChromaTOF software (version 5.51) was used for data acquisition. The samples were run in 9 batches, each consisting

of 100 samples and a calibration curve. In order to monitor the run a blank, a QC and a standard sample with a known concentration run between every 10 samples. Between every batch the septum and liner on the GC were replaced, the precolumn was cut if necessary and the instrument was tuned. The retention index was determined with ChromaTOF using the reference method function. For every batch a reference file was created. The reference file contained the spectras and approximate retention times of the alkanes from C10 to C30 as determined manually). A reference method was implemented for every sample in order to determine the exact retention time of the alkanes. Text files with the names and retention times of the alkanes were then exported and converted to the correct format for MSDIAL using an in-house R script. The samples were exported from ChromaTOF using the netCDF format. After this they were converted to abf files using the abfConverter software (Reifycs). Untargeted data processing was carried out using MSDIAL (version 4.7). The minimum peak height was set to an amplitude of 1000, the sigma window value was 0.7 and the EI spectra cut off was 10. The identification was carried out using retention index with the help of the GCMS DB-Public-kovatsRI-VS3 library provided on the MSDIAL webpage. A separate RI file was used for each sample. The RI tolerance was 20 and the m/z tolerance was 0.5 Da. the EI similarly cut off was 70 %. The identification score cut off was 70 % and retention information was used for scoring. Alignment was carried out using the RI with an RI tolerance of 10. The EI similarity tolerance was 60 %. The RI factor was 0.7 and the EI similarity factor was 0.5. The results were exported as peak areas and further processed with excel. In excel the results were normalized using heptadecanoic acid as internal standard and the features with a coefficient of variance of less than 30 % in QC samples were selected. Further filtering was carried out to remove alkanes and duplicate features. The IDs of the features which passed the CV check were further checked using the Golm Metabolome Database.

Statistical analyses

All of the analyses were conducted, and figures created in R (version 4.3.2) using packages mia (version 1.10.2), miaViz (version 1.10.2), scater (version 1.30.1) (PMID: 2808876), vegan (version 2.6.2004), nlme (version 3.1.164) and tidyverse (version 2.0.0) [61-64]. The markers of stool consistency were modeled with: 1. water content as

continuous variable, 2. stool consistency as a 4-class variable. All analyses were conducted with all the timepoints as well as separately for each age group.

To describe the longitudinal pattern of reported stool consistency, binary variables of a. loose vs no loose, b. constipation vs no constipation, c. gas and other complaints vs no were created. Subsequently, differences between timepoints were tested with Kruskal-Wallis and Dunn's posthoc test. To inform the interconnectedness of the transit time markers as well as background factors, Spearman correlation, Wilcoxon signed rank test as well as Kruskal-Wallis test were used.

Taxonomic diversity, i.e. alpha diversity, was estimated with Shannon index and richness with observed richness (number of observed species) from non-rarefied data. The associations were calculated with Spearman correlation for water content and Wilcoxon Signed rank test and Kruskal-Wallis test for the stool consistency variables. The longitudinal analyses were conducted with mixed models with alpha diversity as the dependent variable, breastfeeding and water content or stool consistency as the independent variables and subject as the random effect.

Community composition i.e. beta diversity, was tested with distance-based redundancy analysis (db-RDA) with non-rarefied relative abundance ASV-level data and Bray-Curtis dissimilarity.

Differential abundance testing was conducted with ALDEx2 glm-module for each timepoint [65]. In addition, the longitudinal data was tested with LinDA with following formula: abundance ~ water content + current breastfeeding + age, 1 | subject [66] .

Metabolome data was log2-transformed with half of the smallest value as a pseudocount. Associations with water content were tested with Spearman correlation. Associations with stool consistency were tested with Kruskal-Wallis test. The longitudinal data was tested with linear mixed model with metabolite concentration as the dependent variable, stool consistency or water content as fixed effects, and subject as the random effect. P-values were corrected for multiple testing with

Benjamini-Hochberg, and we consider $q<0.05$ as statistically significant. The R srcipts used to generate the statistical analyses are in: doi:10.5281/zenodo.11222841

Ethics declarations

Ethical issues have been considered and there is a research permit for the project. FinnBrain has a permit from VSSHP ethical committee (ETMK: 57/180/2011), which has approved Cohort profile and research protocol. FinnBrain parents have signed a consent form about their children's participation in research and given permission to use their samples for scientific purposes. Samples went through the laboratory process anonymously with research code to protect participants' privacy.

Competing Interests

The authors declare no competing interests.

Acknowledgments

Finnbrain Birth cohort Study (HK) has been funded by Research Council of Finland (grant numbers 253270, 134950), Jane and Aatos Erkko Foundation, as well as Signe and Ane Gyllenberg Foundation. LK was funded by the Research Council of Finland (grant number 308176 and 325292), Yrjö Jahnsson Foundation (6847, 6976), Signe and Ane Gyllenberg Foundation, Finnish State Grants for Clinical Research (P3654), Jalmari and Rauha Ahokas Foundation, and Waterloo Foundation (2110-3601). AKA was supported by Yrjö Jahnsson Foundation, Psychiatry Research Foundation, Emil Aaltonen Foundation, Brain Foundation, Instrumentarium Science Foundation, Signe and Ane Gyllenberg Foundation, Duodecim Finnish Medical Society, Juho Vainio Foundation and the Research Council of Finland (grant number 347640). SL was supported by the Academy of Finland postdoctoral grant (323171). LL was supported by Research Council of Finland (grant number 330887). AMD has been funded by the Waterloo foundation and Research Council of Finland (347924). Further support was received by the "Inflammation in human early life: targeting impacts on life-course

health" (INITIALISE) consortium funded by the Horizon Europe Program of the European Union under Grant Agreement 101094099 (to MO), and InFLAMES Flagship Programme of the Research Council of Finland (decision number: 337530). AKA and HI were supported by the Signe and Ane Gyllenberg Foundation (grant no. 6273). HI received funding from the Finnish Cultural Foundation (grant no. 00230482), the Finnish Foundation for Cardiovascular Research, the Diabetes Research Foundation, and the Doctoral Program in Clinical Research at the University of Turku. We thank the FinnBrain Birth Cohort Study participants and personnel, Turku Metabolomics Center for the assistance and resources in the analyses of metabolites.

Author Contributions

Conception or design of the work: AKA, SL, AMD. Acquisition, data analysis, or interpretation of data: LK, HK, HI, MK, LL, ML, MO, AMD, AKA, SL, EM. SL and AKA wrote the manuscript. All authors critically reviewed and approved the final manuscript. All authors have approved the submitted revision.

Data and code availability

Due to Finnish national legislation, the metadata cannot be made available online, but data can potentially be shared with Data Transfer Agreement as part of research collaboration. Requests for collaboration can be sent to the Board of the FinnBrain Birth Cohort Study; please contact Linnea Karlsson (linnea.karlsson@utu.fi). The metabolomics datasets generated in this study is available at the NIH Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench (https://www.metabolomicsworkbench.org) where it has been assigned with DataTrackID 4779. The 16s rRNA gene sequencing data with key metadata for this study are available in the European Nucleotide Archive with accession code PRJEB73815. The R scripts for data analyses can be found in Zenodo doi:10.5281/zenodo.11222841

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