Insights into the early-life chemical exposome of Nigerian infants and potential correlations with the developing gut microbiome

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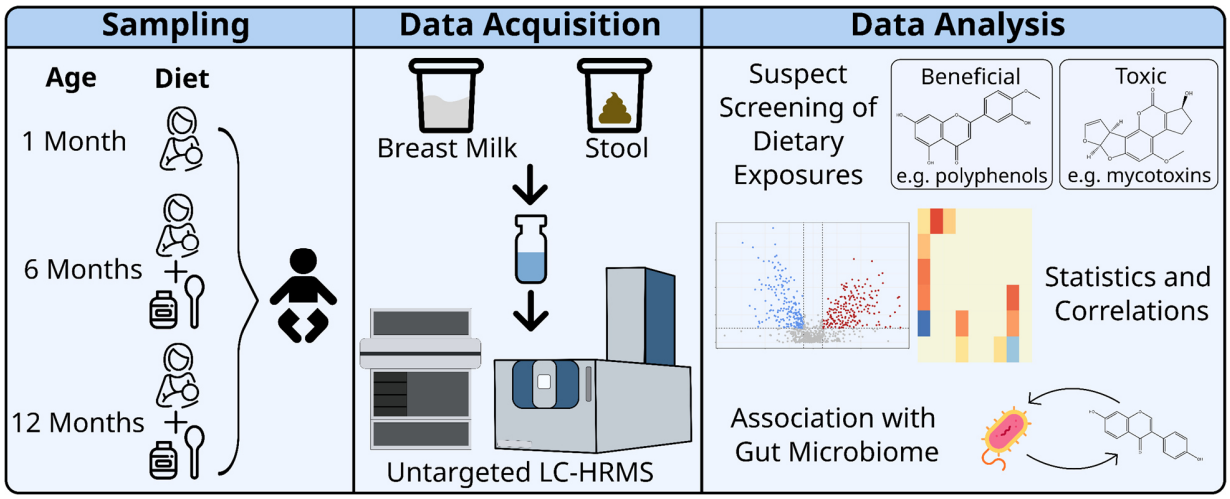
Abstract

Early-life exposure to natural and/or synthetic chemicals can impact acute and chronic health conditions. Here, a suspect screening workflow anchored on high-resolution mass spectrometry was applied to elucidate xenobiotics in breast milk and matching stool samples collected from Nigerian mother-infant pairs (n = 11) at three-time points. Potential correlations between xenobiotics and the gut microbiome, as determined by 16S rRNA gene amplicon sequencing, were subsequently explored. Overall, 12,192 and 16,461 features were acquired in the breast milk and stool samples, respectively. Following quality control and suspect screening, 562 and 864 features remained, respectively, with 149 of these features present in both matrices. Taking advantage of 242 authentic reference standards measured for confirmatory purposes of both, potentially beneficial and adverse xenobiotics, 34 features in breast milk and 68 features in stool were unambiguously identified and subsequently semi-quantified. Moreover, 51 and 78 features were annotated with spectral library matching, as well as 416 and 652 with *in silico* fragmentation in breast milk and stool, respectively. Despite that the workflow was originally optimized for polyphenols, a diverse range of other chemical classes were simultaneously identified including mycotoxins, endocrine-disrupting chemicals (EDCs), antibiotics, plasticizers, perfluorinated alkylated substances, and pesticides. Spearman rank correlation of the identified features revealed significant correlations between chemicals of the same classification such as polyphenols. One-way ANOVA and differential abundance analysis of the data obtained from stool samples revealed that features of plant-based origin were elevated when complementary foods were introduced to the infants' diets. Features in the stool deemed significant by ANOVA, such as tricetin, positively correlated with the genus *Blautia*. Moreover, vulgaxanthin negatively correlated with *Escherichia-Shigella*. Despite the limited sample size, this exploratory study provides high-quality exposure data of matched biospecimens obtained from mother-infant pairs in sub-Saharan Africa, and showed potential correlations between the chemical exposome and the gut microbiome.

Keywords

Non-targeted screening/analysis, Exposomics, Infant stool, Human breast milk, Food bioactives, Toxicants

Graphical abstract



1. Introduction

In 2005, the "exposome", an idea that expands and brings together the already established "-ome" research fields, was introduced by Wild (2005). The concept can be summarized as investigating the totality of chemicals that an individual is exposed to during their lifetime, and the consequent health-related effects (Miller and Jones 2014). The main focus of current exposome research lies on xenobiotics and their biotransformation products, and can be further complemented with other -omic techniques, e.g. proteomics or metagenomics, to characterize the biological impact of these exogenous chemicals (Vitale et al. 2021, Kalia et al. 2022).

Exposure to various xenobiotics during one´s lifetime, especially during the first thousand days, significantly influences human health and the development of chronic diseases (Heindel et al. 2015). For example, exposure to endocrine disrupting chemicals, such as bisphenol A, have been associated with an increased risk of diseases like obesity and neurodevelopmental disorders (Braun 2017). Early-life exposure to mycotoxins, such as aflatoxins and fumonisins, can adversely influence child growth (IARC 2015, Rasheed et al. 2021). Conversely, exposure to other xenobiotic classes, for instance polyphenols, can have potentially beneficial health effects such as reduced risk for breast cancer due to soy isoflavones consumption in early life (Wu et al. 2008). The diet of infants is a major source of exposure to both potentially toxic or beneficial xenobiotics. For example, consumption of complementary foods rather than breast milk can expose infants to higher levels of mycotoxins (Krausová et al. 2023).

Exposome research has mainly focused on adverse exposures and often does not include molecules that might be able to mitigate these actions. One vital class of natural xenobiotics is polyphenols, which are prevalent in plant- and fungi-based foods. They can impact the microbiome by, for example, promoting the growth of beneficial bacteria (Gowd et al. 2019). Additionally, polyphenols are bioactive compounds in humans, exhibiting properties that have shown to be either positive, like antimicrobial or anti-inflammatory properties (Pandey and Rizvi 2009), or potentially negative, such as estrogenic effects of isoflavones (Křížová 2019). Polyphenols can also reduce the toxicity of other xenobiotics, as seen with mycotoxins (Rasouli et al. 2022). Therefore, assessing human exposure to polyphenols is essential. However, this is a challenging task as polyphenols are an immense class of molecules that, once ingested, yield a variety of biotransformation products, from both human and microbial metabolism (Oesterle et al. 2021).

Since xenobiotics, including polyphenols, contain chemically diverse molecules, assessing human exposure to these chemicals requires holistic and cutting-edge exposomic workflows anchored on mass spectrometry (MS) (Bocato et al. 2019, Flasch et al. 2022b, Gu et al. 2023). Untargeted approaches, compared to targeted approaches, are advantageous as they allow the exploration of a broader spectrum of analytes rather than simply those with an available reference standard (Oesterle et al. 2021). However, identification of the analytes in untargeted approaches is challenging as only features, defined as a detected signal intensity and an associated *m/z* and retention time value, are acquired. Therefore, at least the fragmentation spectra of the features are required. One untargeted approach to acquire both features and their fragmentation spectra simultaneously is sequential window acquisition theoretical hold (SWATH) data-independent acquisition (DIA), which involves fragmenting all ions in sequential windows of monoisotopic masses (Bonner and Hopfgartner 2019). Though deconvolution of the acquired data is more complex than data-

dependent acquisition (DDA), SWATH DIA yields fragmentation spectra for all features present rather than specified features (Guo and Huan 2020), allowing retrospective feature annotation. As untargeted approaches generate a very high number of features, suspect screening can be employed to filter the features for analytes of interest thought to be present in the samples (Pourchet et al. 2020).

Besides xenobiotics, the gut microbiome has a major and highly complex impact on human health (Hou et al. 2022). The composition of the gut microbiome can be modulated through diet (Claesson et al. 2012, David et al. 2014, Wilson et al. 2020) and environmental exposures (Claus et al. 2016). Conversely, the microbiome can influence xenobiotics (Collins and Patterson 2020), leading to a bi-directional relationship between the gut microbiome and xenobiotics (Clarke et al. 2019). Xenobiotics can contribute to, or drive, dysbiosis during early-life (Chi et al. 2021, Ayeni et al. 2022), especially as the microbiome of infants is not yet fully established (Wopereis et al. 2014). Thus, research efforts have been geared towards understanding xenobiotic and early-life microbiome interactions.

Several studies have reported on metabolomic profiles of healthy children (Chen et al. 2019, Holzhausen et al. 2023), children with severe acute malnutrition (McMillan et al. 2016), or comparing breastfed infants with infants fed formula (Brink et al. 2020, Silner et al. 2021). However, to our knowledge, there is limited data on the longitudinal exposomic/metabolomic profiles of neonates and infants via breast milk and matching stool samples, which is necessary for making correlations with the gut microbiome. Thus, the objectives of this study were to apply a recently developed untargeted exposomic biomonitoring workflow (Oesterle et al. 2023) with SWATH DIA on breast milk and matching stool collected longitudinally from Nigerian mother-infant pairs. This workflow allowed us to 1) elucidate exposure profiles of polyphenols and other potential beneficial or toxic xenobiotics present in the samples, 2) investigate changes and correlations of xenobiotics as complementary foods are introduced in the diet, and 3) correlate the xenobiotics detected in the infants' stool to the gut microbiome.

2. Materials and methods

2.1. Study design, sampling, and ethical approval

The longitudinal pilot study involved human breast milk and matched infant stool samples. Details of study location and sample collection were previously described by Ayeni et al. (under review). The same set of samples was used before to investigate specific exposure classes: polyphenols in the breast milk samples (Berger et al. submitted), and mycotoxins in the stool samples (Krausová et al. 2022) and breast milk samples (Ayeni et al. under review). Briefly, samples were provided by eleven Nigerian infant-mother pairs from Ilishan-Remo, Ogun state. Breast milk and infant stool samples were collected at the same time point by the mothers, and temporarily stored at 4°C prior to further cold storage. Samples were then stored at -20°C until analysis. Food frequency questionnaires were administered to the mothers to ascertain dietary patterns and health status of the infants (unpublished data). Ethical approval was obtained from the Ethical Committee of Babcock University (BUHREC 421/21R, BUHREC 466/23). All mothers were properly informed before providing their written consent to be included in the study.

2.2. Reagents and chemicals

A total of 242 authentic reference standards were used in this study, allowing for Level 1

identifications of relevant xenobiotics, benchmarking the approach, and enabling absolute quantitation for a high number of relevant environmental and food-related exposures. The selection represented different xenobiotics, both synthetic and natural, containing, for example, mycotoxins, plasticizers, antibiotics, and all the main polyphenol classes. These standards were acquired at the highest possible purity and their acquisition information and molecular class are listed in Table A.1. Acetonitrile (ACN) and methanol (MeOH), LC-MS grade, were acquired from Honeywell. Water (H_2O), LC-MS grade, was acquired from VWR, and formic acid (FA), UPLC-MS Optigrade, was acquired from Bartelt. Anhydrous magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) were acquired from Sigma-Aldrich. The stock solutions were prepared by diluting the solid standards (Table A.1) in either MeOH (polyphenols) or ACN (other standards) and used to make various working mixes to enrich the samples.

2.3. Sample preparation

The breast milk samples were prepared following a protocol optimized by Berger et al. (submitted) for diverse polyphenols. In brief, 200 µL aliquots of breast milk were diluted 1:2 with acidified ACN (1% v/v FA) in a micro-reaction tube. The samples were vortexed for 3 min. Then, 4 mg of $MgSO_4$ and 1 mg of NaCl per 10 µL of matrix were added and the samples were again vortexed for 3 min. Next, the samples were centrifuged for 10 min at 4°C and 2,000 x g, and the supernatant was transferred to a new micro-reaction tube and placed at -20°C for 2 h. Following protein precipitation, the samples were centrifuged for 2 min at 4°C and 18,000 x g. The supernatant was transferred again to a new micro-reaction tube and diluted 1:1 with acidified H_2O (1% v/v FA). Lastly, the samples were centrifuged again for 5 min at 4°C and 18,000 x g, and the supernatant was transferred to an amber LC vial.

The infant stool samples were prepared following the method of Krausová et al. (2022) with minor modifications. In brief, approximately 80 mg of the wet infant stool was weighed in a micro-reaction tube and dried in a vacuum concentrator (Labconco). H_2O was then added to the samples at 40 µL per 20 mg of dried stool, followed by 160 µL per 20 mg of dried stool of ACN:MeOH (1:1) + 1% v/v FA. The samples were then vortexed and ultrasonicated on ice for 15 min, and subsequently placed at -20°C overnight to allow for protein precipitation. After, they were centrifuged for 10 min at 4°C and 18,000 x g. The supernatant was transferred to a new micro-reaction tube and diluted 10 times with ACN:H_2O (1:1) + 1% v/v FA, and finally the samples were passed through a PTFE filter into an amber LC vial. The quantities of wet stool and dry stool for each sample is listed in Table A.2.

2.4. LC-HRMS instrumentation and parameters

A UHPLC-ESI-QTOF-HRMS system consisting of an Agilent 1290 Infinity II UHPLC and a *Sciex* ZenoTOF 7600 MS was used to analyze the samples. The LC parameters used were previously optimized (Oesterle et al. 2022). In brief, a *Waters* Acquity HSS T3 (2.1 x 100 mm, 1.8 µm) column with a *Waters* Vanguard precolumn at a temperature of 30°C, an autosampler temperature of 4°C, and an injection volume of 5 µL was used. The eluents were composed of H_2O with 0.1% v/v FA as eluent A and ACN with 0.1% v/v FA as eluent B, at a flow rate of 0.6 mL/min, and the gradient given in Table A.3.

The MS was operated in negative polarity, and the source parameters consisted of a CAD gas of 9 arb. unit, a curtain gas of 35 psi, an ion source gas 1 and 2 of 50 psi, a source temperature of

550°C, and a spray voltage of -4500 V. The TOF MS was operated with a scan window of *m/z* 100 to 1000, an accumulation time of 0.25 s, a declustering potential of -70 V, and a collision energy of -10 V. The SWATH parameters used involved scanning for fragments from *m/z* 100 to 1000 with 10 windows, a declustering potential of -70 V, an accumulation time of 0.05 s, and different collision energies for each window. The SWATH windows were optimized for each matrix separately using the total ion chromatogram from the injection of a pooled quality control and are listed in Table A.4 along with the collision energies applied to each window.

2.5. Data processing of the acquired LC-HRMS data

The acquired raw data files were first converted to ABF file format with Reifycs Analysis Base File Converter (v2011-2020), before they were further processed in MS-Dial (v4.9.221218) (Tsugawa et al. 2015). MS-Dial was used for feature pre-processing, e.g., building extracted ion chromatograms, and for feature annotation with a spectral library created by MS-Dial that combines various databases such as GNPS and MassBank (Tsugawa et al. 2015). The parameters used in MS-Dial for the two biological matrices are listed in Table A.4. Features were further annotated with *in silico* fragmentation using MS-Finder (v3.52) (Tsugawa et al. 2016). Prior to feature annotation, the feature lists were filtered by MS^1 matching with the Exposome-Explorer (Neveu et al. 2020) and Phytohub (Giacomoni et al. 2017) databases. R (v4.3.1) (R Core Team 2023) was used for MS^1 matching and feature clean-up with the process blank and pooled quality control (QC) samples.

The identification levels of the feature annotation were given based on the levels previously defined by Schymanski et al. (2014). In brief, features identified with authentic reference standards were labeled as Level 1 and features annotated with spectral libraries as Level 2a. Level 3 was then split in a similar manner as described in Oesterle et al. (2023), and features annotated by in silico fragmentation were labeled as Level 3a, and features putatively annotated by their MS^1 with the two databases as Level 3b. The chemical classes of the features were also determined using the ChemRICH MeSH prediction tool (Barupal and Fiehn 2017), the classes listed in MS-Finder and MS-Dial, and the classes listed in the entries of the two online databases (PhytoHub and Exposome-Explorer).

2.6. Quality control of the LC-HRMS measurements

QC samples were prepared for each biological matrix. The stool QC was prepared by pooling aliquots of each infants' wet stool in respective quantities (Table A.2). The breast milk QC was prepared by combining 10 µL of each sample. The QC samples were processed following the same procedure as the experimental samples. For each biological matrix, the respective QC was used to condition the LC column prior to the acquisition of the experimental samples. Moreover, after column conditioning, three technical replicates of the QCs were measured. The QCs were routinely analyzed after every five experimental samples to continuously check the reliability of the instrument as well as to correct for any signal drifts that may occur within the acquisition batch. In addition, for each QC, a five-point serial dilution series with a constant dilution factor of four was prepared with the respective dilution solvent of the biological matrix's sample preparation procedure. Moreover, matrix-matched calibration curves for the 242 reference standards were created with the QCs (Table A.5). For each biological matrix, along with the samples, a process blank was prepared by leaving the micro-reaction tube empty rather than taking

an aliquot of the biological matrix. For the stool protocol, the process blank was assumed to contain 20 mg of dry weight, therefore 40 µL of H_2O and 160µL of ACN:MeOH (1:1) + 1% v/v FA were added. Both process blanks were measured in triplicates.

Features with an average chromatographic peak area in the process blank measurements greater than one-third of the average in the samples were removed (Kirwan et al. 2014). In addition, features were removed unless they were detected in at least two of the triplicate measurements of the QCs and their peak area in the QC triplicate measurements had a relative standard deviation <30% (Dudzik et al. 2018). Features with an average signal-to-noise value of less than three, as calculated by MS-Dial, were also removed. Finally, the QC dilution series was developed to assess the reliability of extracted features, meaning a feature's signal in the QCs should decrease as the QC is diluted. Therefore, Spearman rank correlation was applied to the QC dilution series for assessing the association between a feature´s signal and the overall dilution factor, whereby features with a correlation value less than 0 were removed.

2.7. 16S rRNA gene amplicon sequencing of the infant stool

Gut microbiome data of the infants' stool samples obtained at month 1, 6 and 12 post-delivery were retrieved from Ayeni et al. (under review). Detailed procedure of DNA extraction, polymerase chain reaction and 16S rRNA gene amplicon sequencing applied to the infants' stool was previously published (Pjevac at al. 2021). Sequencing was done at the Joint Microbiome Facility of the University of Vienna and the Medical University of Vienna. Raw sequence reads of the infants' microbiome are available under the BioProject accession number PRJNA1013123.

2.8. Statistical analysis

Following data pre-processing and annotation, MetaboAnalyst (v5.0) (Pang et al. 2022) was used for statistical analysis. Prior to analysis in MetaboAnalyst, the chromatographic peak areas of the features extracted from the stool samples were normalized by their sample dry weight (Table S2). After importing the data into MetaboAnalyst, the features in each matrix were both normalized by median and applied $\log_{10}$ transformation. Principal Component Analysis (PCA) of the features in the breast milk and stool was applied to investigate clustering of the samples. Then, for the stool samples, one-way Analysis of Variance (ANOVA) was applied to determine significant features over time, while volcano plots (fold change versus T-tests) were used to determine fold changes between the time points. Heatmaps with Ward hierarchical clustering of the annotated features were created to compare the distribution in each matrix.

ChemRICH (v4.0) (Barupal and Fiehn 2017) was used to generate chemical enrichment plots of the stool samples with the statistical analysis results from MetaboAnalyst. Boxplots of the quantification results were made with the *ggplot2* (v3.4.3) (Wickham 2016) and *cowplot* (v1.1.1) (Wilke 2020) packages. Spearman rank correlation between the features in both matrices was calculated using *stats* (v3.6.2) package in R (v4.3.1) (R Core Team 2023). The *pheatmap* package (v1.0.12) (Kolde 2018) was applied to generate heatmaps of the correlation results. Benjamini-Hochberg was applied for multiple testing (Benjamini and Hochberg 1995).

Microbiome data were loaded into R (v4.3.1) and filtered using the *ampvis2* package (v2.8.3) (Andersen et al. 2018). Spearman rank correlations with Benjamini-Hochberg correction between features and the microbiome was done using the *stats* (v3.6.2) package and visualized as heatmaps with the *pheatmap* (v1.0.12) package.

To further visualize the correlations between the features and the microbiome, a network was created in Cytoscape (v3.9.1) (Shannon et al. 2003).

3. Results and discussion

3.1. Suspect screening of breast milk and stool samples

Overall, the utilization of the LC-HRMS methods in negative mode yielded a total of 12192 features acquired in the breast milk samples and 16461 features in the stool samples. After quality control, 4347 and 6905 features remained in breast milk and stool, respectively. A suspect screening workflow optimized for polyphenols but also capable of covering many other xenobiotics and endogenous metabolites (Oesterle et al. 2023) was then used to extract features of interest in the biological matrices. This workflow involved a suspect list from two databases: PhytoHub, an online database containing 2268 phytochemicals, mainly polyphenols (Giacomoni et al. 2017), and Exposome-Explorer, a database containing 1262 chemicals known to be biomarkers for exposure to environmental and lifestyle factors such as diet, pollutants, or contaminants (Neveu et al. 2020). The application of this workflow resulted in a total of 542 matched features in the breast milk and 864 in the stool samples. From the 542 breast milk features, 34 were identified as Level 1, 51 annotated as Level 2a, 416 as Level 3a, and 41 as Level 3b. While from the 864 features in the stool, 68 were identified as Level 1, 78 annotated as Level 2a, 652 as Level 3a, and 66 putatively annotated as Level 3b. The annotated features are listed in Table A.6 for breast milk and Table A.7 for stool.

In the breast milk, many of the features detected were fatty acids, peptides, saccharides, or amino acids (Figure 1a). This was expected as breast milk is rich in nutrients, especially lipids, proteins, and carbohydrates, required for infant growth and development (Boudry et al. 2021). The breast milk also contained polyphenols such as flavonols, flavones, and isoflavones. Several studies have shown that polyphenols are abundant in human breast milk and they could be beneficial to the infants (Lu et al. 2021, Song et al. 2013, Carregosa et al. 2023). In contrast, toxic xenobiotics, such as mycotoxins, were detected at a low prevalence in breast milk. This class of xenobiotics are typically found at low concentrations in breast milk (Adejumo et al. 2013, Braun et al. 2020, Braun et al. 2022). Moreover, the heatmap of the features detected in the breast milk (Figure 1b) depicts that the features cluster in two main groups, though there is no clear separation of the types of features in each of the two groups.

More diverse chemical classes, predominantly of plant-based origin, were present in the stool (Figure 2a). One of the main chemical classes detected were polyphenols, which was expected as the LC-MS method was originally optimized for these analytes (Oesterle et al. 2022, Oesterle et al. 2023). A wide range of different polyphenol classes were detected, including 55 flavonols, 32 flavones, 29 isoflavones, four chalcones, and 27 hydroxycinnamic acids. Besides polyphenols, several potentially toxic xenobiotics were also detected. For instance, one feature (m/z 254.952, retention time: 5.8 min, Level 3b) was putatively annotated as a polychlorinated biphenyl-16. It was previously reported that prenatal exposure to PCBs were associated with adverse effects on birth weight (Govarts et al. 2020). In addition, naphthalene epoxide (Level 3a), a metabolite of naphthalene, was detected. Naphthalene exposure has been associated with various negative health implications, especially for respiratory health (Cakmak et al. 2014). Several fatty acids were also detected in the stool samples, including arachidonic acid (Level 2a)

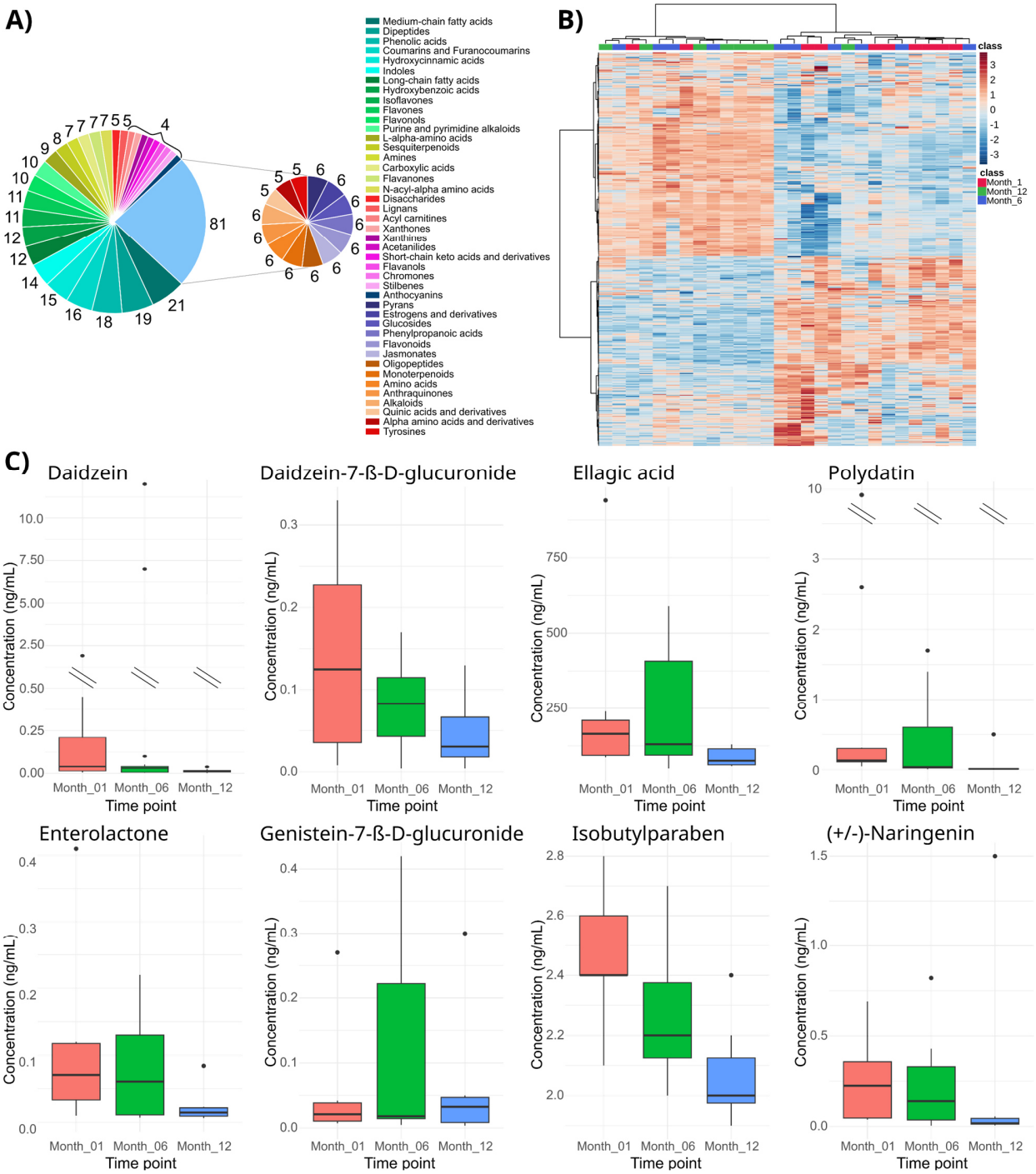


Figure 1. A) Pie chart representing the major chemical classes of the features identified and annotated in the breast milk samples obtained from Nigerian mothers at three different time points. **B)** Heatmap with Ward clustering displaying the features detected in all the breast milk samples. **C)** Box plots showing quantitative differences (Table A.8) over time for selected analytes identified at Level 1 that represent both potentially beneficial and toxic xenobiotics in the breast milk samples. The boxplots of all the identified features are shown in Figure B.2.

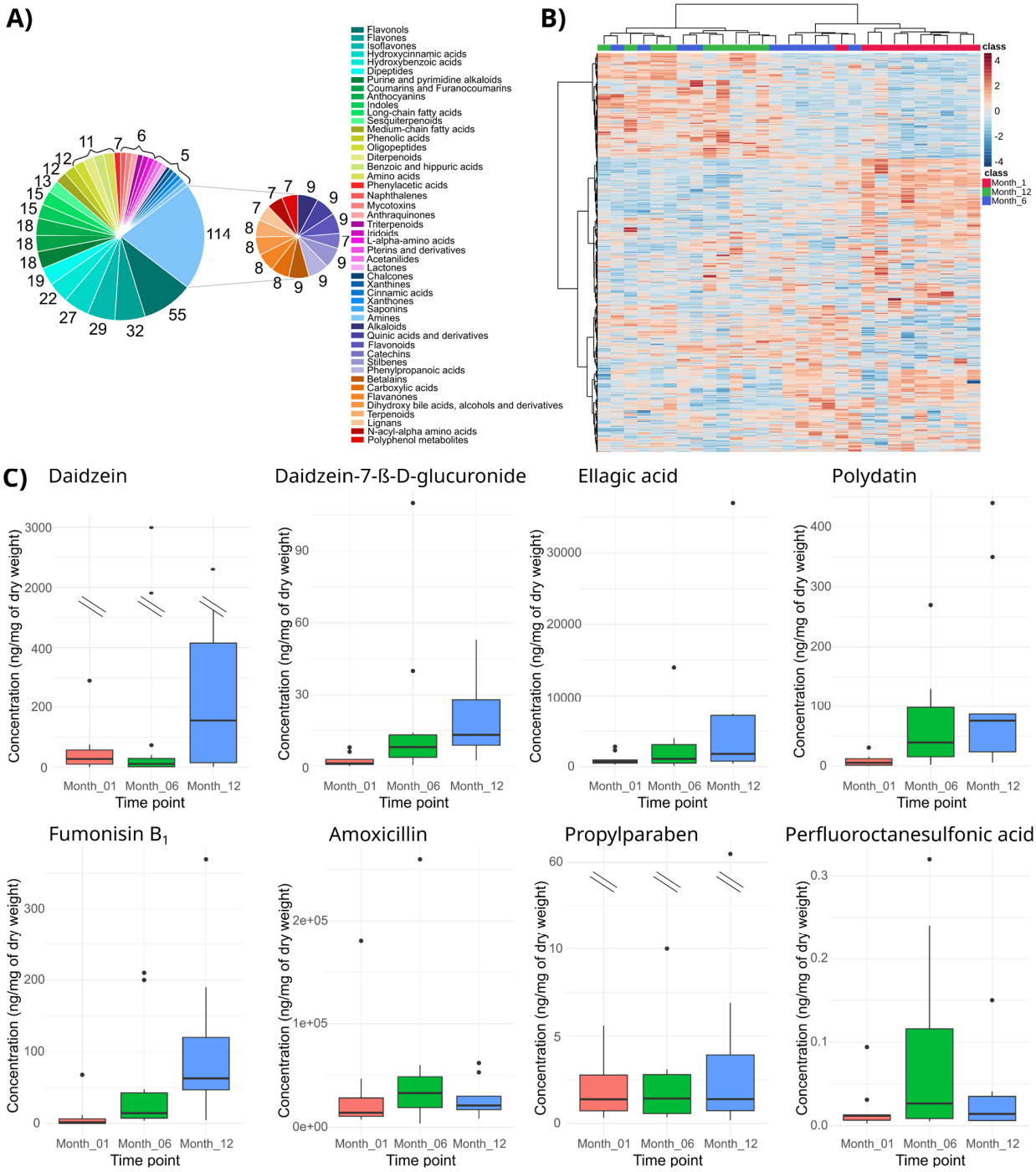


Figure 2. A) Pie chart representing the major chemical classes of the features identified and annotated in the stool samples from Nigerian infants at three different time points. **B)** Heatmap with Ward clustering displaying the features detected with the suspect screening workflow in the stool samples. **C)** Box plots of the semi-quantification (Table A.8) at the three distinct infant ages of several identified features (Level 1) that represent both potentially beneficial and toxic xenobiotics in the stool samples. These plots show that complementary foods increase exposure to various xenobiotics, and breast feeding keeps exposure levels low, despite potential lactational transfer. The box plots of all the identified features are shown in Figure B.3.

and eicosapentanaenoic acid (Level 3a). The levels of these two fatty acids were previously reported to be higher in stool of infants that were breastfed compared to infants fed that were formula-fed (Sillner et al. 2021).

Similar to the breast milk heatmap, the features in the stool (Figure 2b) also form two clusters, with one cluster from the samples during the time of breastfeeding (month 1) and the other for those acquired after the introduction of complementary foods (months 6 and 12). In addition, many of the features detected in the stool samples were conjugated with sugar moieties. This could be attributed to phase II metabolism, as it was previously reported that metabolites of genistein included hexose and pentose conjugates in cells (Flasch et al. 2022a); or to their low intestinal absorption, as, for example, seen with the bioavailability of large polyphenols like proanthocyanidins (Scalbert et al. 2002). Moreover, the bioavailability in infants may be further reduced as metabolic pathways, e.g. phase I and II metabolism (Lu and Rosenbaum 2014), or their microbiome (Wopereis et al. 2014) are not yet fully developed.

The features from each biological matrix were then compared with each other with a retention time deviation of 0.1 min and a mass error of 20 ppm. This resulted in a total of 149 features that were detected in both matrices, of which 32, 24, 91, and four were annotated as Levels 1, 2a, 3a, and 3b, respectively. One of the most prevalent chemical classes in both biological matrices was phenolic acids. Phenolic acids are a polyphenol class comprising of many human and microbial metabolites, including metabolites from other larger polyphenols such as anthocyanins (de Ferrars et al. 2014). Besides phenolic acids, several other biotransformation products were detected in both matrices including caffeic acid-3-ß-D-glucuronide, daidzein-7-ß-D-glucuronide (Figure 3b), and genistein-7-ß-D-glucuronide. This observation suggests that the chemicals were either transferred directly from the breast milk to the infant and the corresponding stool samples or that the infant's metabolism conjugated the parent compounds in the colon or liver by UDP-glucuronosyltransferases (Rowland et al. 2013). The overlap in features between the two matrices showed that xenobiotics, for example polyphenols, are transferred from the mother to the infant. Though scant data exist, the lactational transfer of various xenobiotics have been previously described in humans. This includes persistent organic pollutants (Haddad et al. 2015), ellagitannins and their metabolites (Henning et al. 2022), and pharmaceutical drugs and environmental pollutants (Dubbelboer et al. 2023). In addition, the heatmap (Figure B.1) of all the features detected in both matrices showed that the two matrices are highly different, as only 149 from the 1259 total features are present in both matrices. This further underscores the differences previously seen in the types of chemical classes found in each matrix (Figure 1a and 2a).

3.2. Semi-quantification of Level 1 identified features in breast milk and stool

A total of 242 authentic reference standards representing different xenobiotics were utilized for identification purposes. These standards included air pollutants, disinfection by-products, endogenous estrogens, food processing by-products, industrial side-products, pesticides, mycotoxins, perfluorinated alkylated substances, personal care product / pharmaceuticals, phytotoxins, plasticizer / plastic components, antibiotics, and polyphenols (Table A.1). In the breast milk, 34 features were identified that consisted of one antibiotic, one estrogen, one mycotoxin, one personal care product / pharmaceutical, and 30 polyphenols. While in the stool samples, 68 features were identified that consisted of five antibiotics, three mycotoxins,

one perfluorinated alkylated substance, one pesticide, one plasticizer / plastic component, three personal care product / pharmaceuticals, and 54 polyphenols. Polyphenols were more abundantly detected in the samples as they are typically found in higher concentrations than other xenobiotics in human biofluids (Rappaport et al. 2014, Achaintre et al. 2018) and also the applied workflow was originally optimized towards the balanced and decent performance in detection of polyphenols.

The identified features (Level 1) in the breast milk (34) and stool (68) were semi-quantified (Table A.8) with calibration curves created from the reference standards (Table A.5). To approximate signal suppression and enhancement effects, the calibration curves were matrix-matched with the pooled QC of the respective biological matrix. However, standard addition was required for quantification since all the Level 1 identified features already had a chromatographic peak present in the pooled QCs. Each identified feature in breast milk and stool samples at the three different time points was represented in (Figure 1 and B.2) and (Figure 2 and B.3), respectively.

Overall, there was no clear pattern in the concentration of features detected in the breast milk at the three time points, but a decrease over time was observed for daidzein, daidzein 7-ß-D-glucoronide, estradiol 17-glucuronide, isobutylparaben and naringenin (Figure 1c). For instance, daidzein was detected at concentration ranges between 0.0035 to 12 ng/mL (Table A.8, Figure 1c). The concentration of daidzein was similar to the reported concentrations from a longitudinal study of an Austrian mother (Jamnik et al. 2022). Moreover, compared to the Austrian study, higher concentrations were recorded for of other polyphenols including enterodiol (range: 0.024 - 23 ng/mL) and enterolactone (range: 0.0079 to 0.41 ng/mL) (Table A.8). In addition, the breast milk samples from this cohort were previously analyzed with a targeted LC-MS/MS workflow focusing on polyphenols (Berger et al. submitted). Despite the targeted assay detecting higher quantities, similar analytes and patterns were observed with both assays. Besides polyphenols, only two potentially toxic xenobiotics, alternariol monomethyl ether and isobutylparaben, were identified at Level 1 in the breast milk samples. Alternariol monomethyl ether was previously reported as a prevalent mycotoxin in breast milk from a Nigerian cohort (Braun et al. 2022, Ezekiel et al. 2022). While there is scarce reported data on parabens in breast milk from Nigeria, these xenobiotics have been reported in breast milk from other regions (Fisher et al. 2017, Kim et al. 2023). Lastly, in the breast milk samples, an antibiotic, azithromycin, was detected in only one sample at a concentration of 1100 ng/mL (Table A.8). Interestingly, the participant who donated this sample used azithromycin for medical treatment purposes (unpublished data). Moreover, azithromycin was detected at a high concentration (140000 ng/mg of dry weight) in the corresponding infant stool sample (Table A.8). It should be noted that the concentrations are semi-quantitative and would therefore require absolute quantification with targeted assays. However, this observation suggests that azithromycin can be transferred from mother's breast milk to infant (Roca et al. 2015).

In contrast, patterns could be observed in the features identified and subsequently quantified in the stool samples. Many of the features belonging to catechins, chalcones, flavanones, flavones, flavonols, isoflavones, phenolic acids, stilbenes, and mycotoxins increased in concentration from month 1 to 12. Moreover, the concentrations of the identified features in stool changed when complementary foods were introduced to the infants at 6 months showing a substantially higher quantity at month 6 compared to month 1 (Figure 3). This observation suggests that complementary foods have a considerable

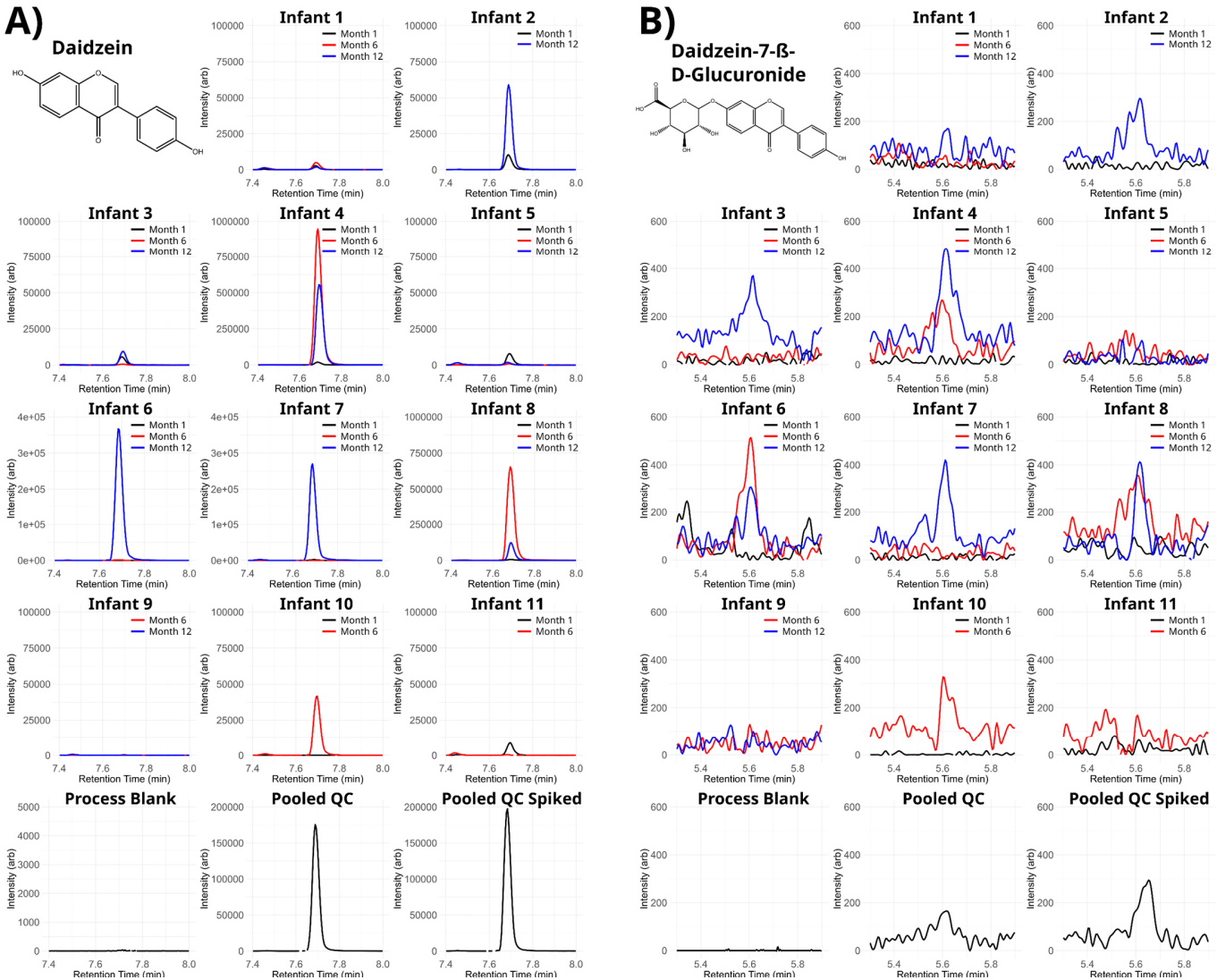


Figure 3. Extracted ion chromatograms of daidzein (*m/z* 253.050, retention time: 7.7 min, Level 1) (**A**) and daidzein-7-ß-d-glucuronide (*m/z* 429.079, retention time: 5.6 min, Level 1) (**B**) in all the infants at the three distinct time points. In addition, the process blank, pooled QC, and pooled QC spiked with the authentic reference standards (14 ng/mL for daidzein and 1.1 ng/mL for daidzein-7-ß-D-glucuronide) are depicted. These chromatograms illustrate that xenobiotics of plant origin significantly increase in concentrations as complementary foods are introduced in the infants' diets.

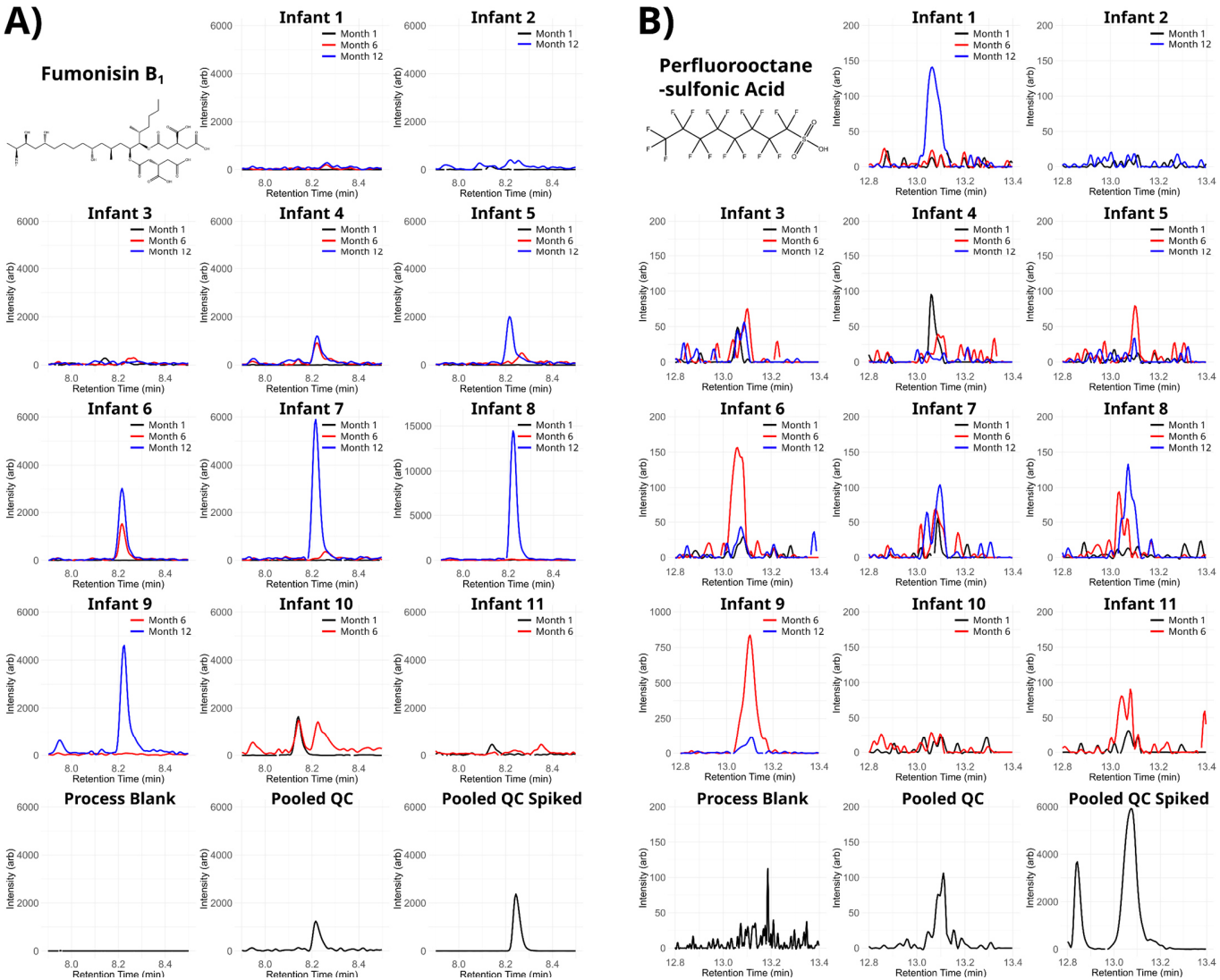


Figure 4. Extracted ion chromatograms of fumonisin B_1 (*m/z* 720.379, retention time: 8.2 min, Level 1) (**A**) and perfluorooctanesulfonic acid (*m/z* 498.929, retention time: 13.1 min, Level 1) (**B**) in all the infants at the three distinct time points. In addition, the process blank, pooled QC, and pooled QC spiked with the authentic reference standards (6.3 ng/mL for fumonisin B1 and 0.38 ng/mL for perfluorooctanesulfonic acid) are depicted. These chromatograms illustrate that exposure levels of adverse xenobiotics during exclusive breastfeeding were typically low and an increase was observed as complementary foods were introduced.

influence on the features in stool. Moreover, the presence of various features when the infants were one-month old showed that bioactive compounds, e.g. polyphenols, are transferred from the breast milk to the infants. For instance, one analyte that is most likely transferred via breast milk due to its stable concentrations over time (Figure B.3) is urolithin A, a microbial metabolite that showed properties beneficial to health (D'Amico et al. 2021).

Some of the features detected in the infant stool were toxic xenobiotics, such as the mycotoxins fumonisin B_1 (Figure 4a) and nivalenol. Fumonisin B_1 has been previously associated with neural tube defects and the etiology of esophageal cancer in humans (Marasas et al. 2004, Missmer et al. 2006). Moreover, this mycotoxin was previously found in stool of the same cohort using a targeted LC-MS/MS-based assay (Krausová et al. 2022, Ayeni et al. under review), and the longitudinal pattern of occurrence was comparable (Ayeni et al., under review). These observations add an extra layer of confidence to the results herein and highlight that targeted and non-targeted LC-MS/MS approaches can be complementary (Flasch et al. 2023). The detection of antibiotics such as amoxicillin, azithromycin, oxytetracycline and trimethoprim in the stool (Figure B.3) can be attributed to the infants taking these antibiotics for medical treatment, or transferred via breastmilk. Florfenicol, a veterinary drug, decreased in concentration in the stool from month 1 to 12, suggesting exposure to the drug was from breast milk. Other xenobiotics exclusively detected in the stool were personal care products (e.g., methylparaben, propylparaben and triclosan), pesticides (e.g., p-nitrophenol), and plasticizer (e.g., mono-2-ethylhexyl phthalate). In addition, perfluorooctanesulfonic acid, a toxic perfluorinated alkylated substance, was detected (Figure 2c and 4b). For all infants, except one, the concentrations were lower during exclusive breastfeeding (month 1 to months 6) compared to when complementary foods were introduced (months 6 to 12). Moreover, perfluorooctanesulfonic acid was detected in Ogun river (>10 ng/L) (Ololade et al. 2018), a river in the region were the samples were collected. While many of the identified toxicants came from dietary sources, others may be attributed to other routes of exposure such as air, water, or cosmetics.

3.3. Correlating xenobiotics within and between each biological matrix

As xenobiotics can exhibit synergistic or antagonistic effects, correlations of the features in the breast milk and stool samples were explored. Hence, Spearman rank correlation (p. adj < 0.05) was applied among the 34 identified features in the breast milk (Table A.9, Figure 5a), and the 68 in the stool (Table A.10, Figure 5b). In the breast milk samples, many of the polyphenols showed moderate to strong correlations (ρ = 0.37 - 0.88) (Table A.9). For example, the two microbial metabolites of matairesinol, enterolactone and enterodiol, strongly correlated with each other as expected (ρ = 0.67). Additionally, polyphenols also correlated with other xenobiotics, e.g. ellagic acid and dihydrocaffeic acid showed moderate correlation with alternariol monomethyl ether (ρ = 0.38 and 0.38, respectively). Estradiol-17-glucuronide also showed moderate to strong correlations with numerous polyphenols, including enterolactone (ρ = 0.41), 4-hydroxybenzoic acid (ρ = 0.58), and salicylic acid (ρ = 0.70). It was previously observed in mice that enterolactone activates estrogen-sensitive reporter gene expression (Penttinen et al. 2007). Hippuric acid showed negative correlation with other phenolic acids, such as benzoic acid (ρ = -0.73), which is most likely due to hippuric acid being a metabolite of benzoic acid (Lees et al. 2013).

Figure 5. A) Spearman rank correlation (p. adj < 0.05) heatmap between the 34 identified features (Level 1) in the breast milk samples. **B)** Spearman rank correlation (p. adj < 0.05) heatmap between the 67 identified features (Level 1) in the stool samples.

All the identified features in the stool, except for one combination, positively correlated with one another, especially features within the same chemical class. For example, two lignan metabolites, enterodiol and enterolactone, moderately correlated with each other ($\rho = 0.52$); and two isoflavone metabolites, daidzein 7-ß-D-glucuronide and genistein 7-ß-D-glucuronide, showed strong correlation ($\rho = 0.82$). Additionally, phloretin strongly correlated with diosmetin ($\rho = 0.87$), kaempferol ($\rho = 0.81$), and quercetin ($\rho = 0.81$). Besides polyphenols, the potentially toxic xenobiotics: nivalenol, amoxicillin, mono-2-ethylhexyl-phthalate, alternariol monomethyl ether, trimethoprim, p-nitrophenol, and florfenicol, showed moderate to strong correlations with one another ($\rho = 0.40$ - 0.90) (Table A.10). Of the xenobiotics, the highest correlation was recorded between mono-2-ethylhexyl phthalate and p-nitrophenol ($\rho = 0.90$). Apart from individual effects, mixture toxicity of these xenobiotics can lead to more severe health consequences especially during early-life (Hamid et al. 2021, Krausová et al. 2023). The only negative correlation was between alternariol monomethyl ether, an *Alternaria* mycotoxin, and genistein-7-sulfate, a metabolite of genistein ($\rho = -0.41$). Previously, it was observed *in vitro* that genistein had antagonistic effects on the genotoxicity of alternariol, another *Alternaria* mycotoxin (Aichinger et al. 2017).

Spearman rank correlation was further applied to explore possible correlations between the identified features in breast milk and infant stool. Only samples obtained at month 1 were selected because the infants predominantly consumed breast milk at this time point. No significant correlations were found (p. adj > 0.05) (Table A.11 and Figure B.4) which may be attributed to the low sample size ($n = 10$). However, with the raw p-value ($p < 0.05$), many of the polyphenols in the breast milk positively correlated with polyphenols in the stool, especially between phenolic acids. For example, benzoic acid in breast milk strongly correlated with 4-hydroxybenzoic acid ($\rho = 0.79$) in the infant stool. Alternariol monomethyl ether in the breast milk did not correlate with alternariol monomethyl ether in the stool, but showed strong negative correlation with nivalenol ($\rho = -0.72$). Interestingly, alternariol monomethyl ether in the breast milk had strong negative correlations with several polyphenols in the stool. For example, alternariol monomethyl ether strongly negatively correlated with (-)-epicatechin ($\rho = -0.98$) and urolithin A ($\rho = -0.69$). Urolithin C, also a metabolite of ellagitannins and structurally similar to urolithin A, was shown to influence the metabolism of alternariol *in vitro* (Crudo et al. 2021). Overall, further investigations are necessary to access the toxicological potentials of many of the observed correlations in *in vitro* models.

3.4. The impact of age and diet on the infants' chemical exposome

The influence of age on the features detected in breast milk and stool was investigated by PCA plots (Figures 6a and b). The breast milk samples showed no clear grouping at the three different time points (Figure 6a). On the contrary, the infant stool samples showed clear grouping, though there was some overlap between the clusters at months six and twelve (Figure 6b). The results of both PCAs are in agreement with the hierarchical clustering in the feature heatmaps (Figure 1b and 2b).

One-way ANOVA was applied on the features detected in the infant stool, yielding 325 features that showed significance across all time points (Table A.7). To further investigate the changes between each time point, volcano plots (fold change versus t-test p values) were generated (Figure 6c-e), with the significant features (fold change > 2, $p < 0.05$) of each

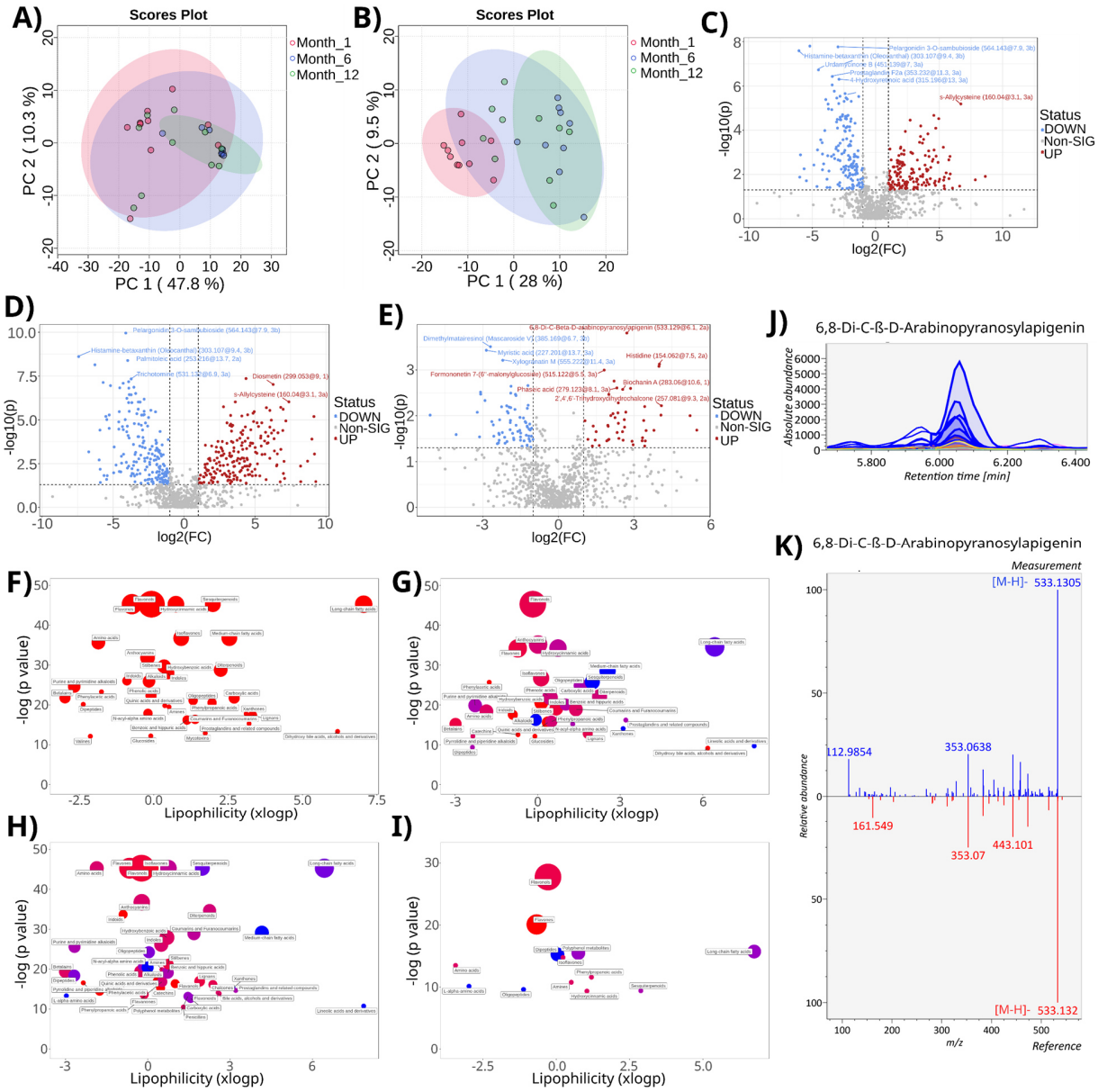


Figure 6. A) Principal component analysis (PCA) plot of the breast milk samples demonstrates that the milk composition did not cluster, indicating no major overall changes of the breast milk composition over time. This makes the study design suitable to explore the impact of solid food intake introduced during early life. **B)** PCA plot of the infant stool samples shows grouping by age with a clear separation of the first month after delivery. **C)** Volcano plot ($p < 0.05$, fold change > 2) of the features between the samples from infants at one month old and six months old. **D)** Volcano plot ($p < 0.05$, fold change > 2) of the features between the samples from infants at one month old and twelve months old. **E)** Volcano plot ($p < 0.05$, fold change > 2) of the features between the samples from infants at six months old and twelve months old. **F)** Chemical enrichment (ChemRICH) plot of the significant features from ANOVA **G)** ChemRICH plot of the significant features from C) with the chemical classes in blue are down-regulated and those in red are up-regulated. **H)** ChemRICH plot of the significant features from D). **I)** ChemRICH plot of the significant features from E). **J)** The overlapping extracted ion chromatograms from 6,8-Di-C-ß-D-arabinopyranosylapigenin which showed high fold change and significance in E). **K)** Mirror plot of the experimental MS^2 and the MS^2 from the spectral library match of the feature from J).

volcano plots listed in Table A.7. A total of 303 features were significant between months one and six, with 151 showing an increase and 153 a decrease. Then from six to twelve months, 118 were significant of which 48 increased and 70 decreased. Finally, from one to twelve months, there were 388 significant features, with 197 increasing and 191 decreasing. A greater number of features were significant between the ages of one to six months and one to twelve months than from six to twelve months, showing the impact of complementary foods. Overall, significant features from ANOVA were similar to the majority of the significant features from the volcano plots, especially between months one and twelve.

ChemRICH plots generated from the volcano plot and ANOVA results (Figure 6f-i) depicted clusters of chemical classes that were either up- or down-regulated. Months one to six and months one to twelve showed up-regulation in chemical classes from plant-origin such as polyphenols, e.g. flavonols or flavones (Figure 6f-h). Additionally, a down-regulation of chemical classes, such as amino acids and fatty acids, were observed. Fatty acids that were down-regulated over time included arachidonic and eicosapentanaenoic acid, which is most likely related to the infants' reduced consumption of breast milk (Sillner et al. 2021). Conversely, no clear pattern with the chemical classes were observed for months six to twelve (Figure 6i), which may be due to the large variance of phytochemicals in foods. These variances may be based on several factors, such as duration of food storage and/or cooking practices (Arfaoui 2021).

3.5. Correlations between significant features and the developing infant gut microbiome

Spearman rank correlation was applied to explore correlations between the features that showed significance through ANOVA and the stool microbiome. It should be noted that samples from all the time points were included, thus other confounding factors, such as age and dietary changes, may influence the correlations. Besides creating a heatmap from the correlation matrix (Figure 7a), a network was generated to better visualize the correlations (Figure 7b). Several significant correlations between some taxa and features in the stool (p. adj < 0.05) were found (Table A.12, Figure 7). Specifically, 58 microbe-feature pairs had positive correlations, while 12 microbe-feature pairs had negative correlations. The correlated features consisted mainly of phytochemicals, such as polyphenols and terpenoids. For example, the flavone tricetin showed a strong positive correlation with *Blautia* ($\rho = 0.69$). *Blautia* has been shown to biotransform flavonoids e.g., polymethoxyflavones into several demethylated flavones (Kim et al. 2014, Liu et al. 2021). Flavonols, e.g. kaempferol-4'-methylether and quercetin-3-arabinoside, showed strong correlations with *Clostridium sensu stricto* 1 ($\rho = 0.69$ and 0.67, respectively) (Figure 7). Members of *Clostridium* are known to degrade flavonols (Zhang et al. 2014, Steed et al. 2017). Fumonisin B_1 showed strong correlation with *Streptococcus* ($\rho = 0.75$). Previously, it was observed that *Streptococcus* can bind to fumonisin B_1 (Niderkorn et al. 2006). Several flavonoids, including kaempferol and biochanin A, strongly correlated with *Romboutsia* ($\rho = 0.70$ and 0.68, respectively). An extract containing flavonoids was previously shown to increase relative abundance of *Romboutsia* in mice (Wang et al. 2022).

Vulgaxanthin I showed a strong negative correlation with *Escherichia-Shigella* ($\rho = -0.70$). Extracts containing vulgaxanthin I exhibited antibacterial activities against Gram-negative bacteria including *Escherichia* (Vulić et al. 2013). The alkaloid, 1,3-dimethylurate, had strong negative correlations with *Clostridioides*

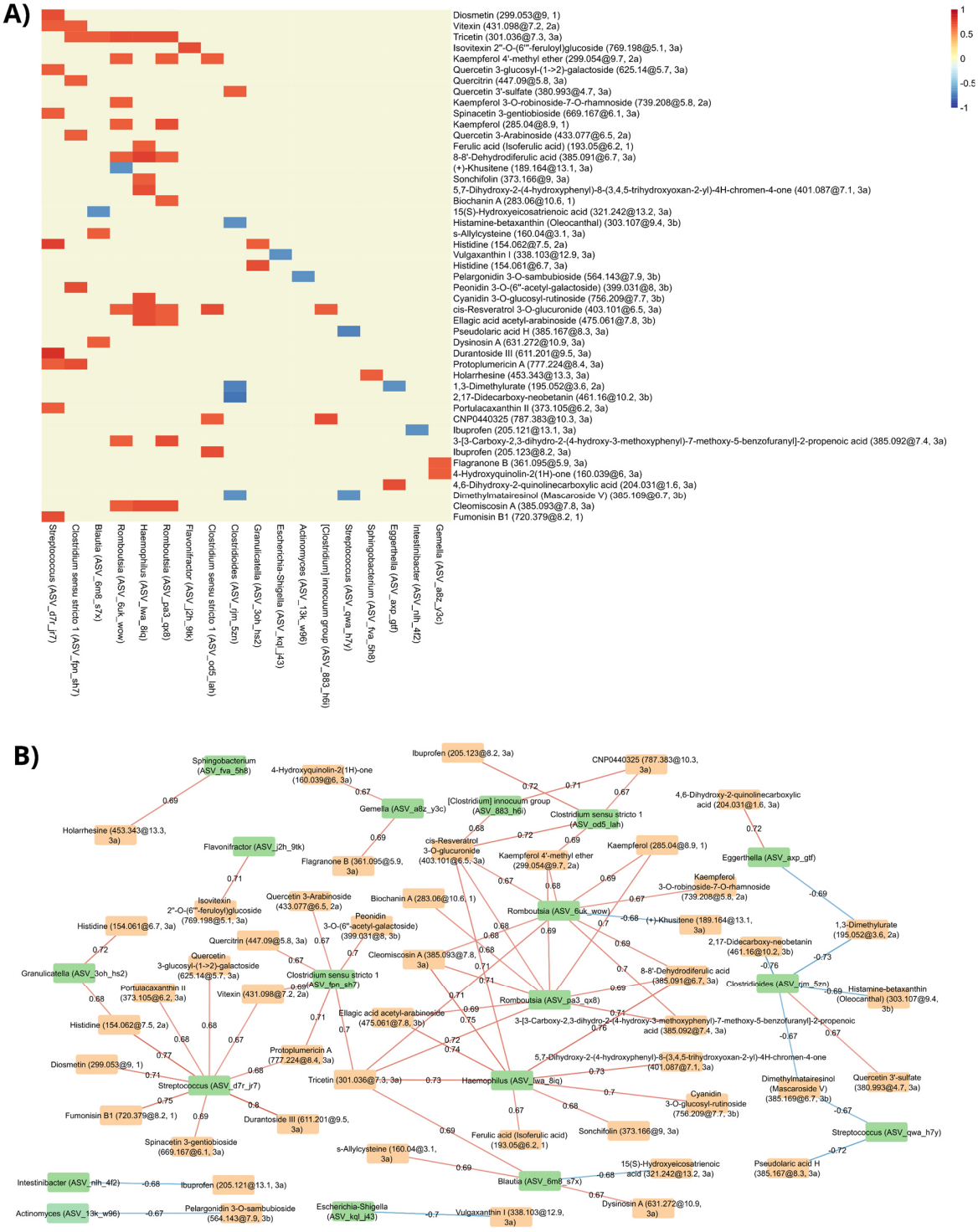


Figure 7. A) Spearman rank correlation (p.adj < 0.05) heatmap between the microbes detected by 16S rRNA gene amplicon sequencing (with their species and ASV identifier given) and the features that showed significance by ANOVA (with their *m/z*, retention time, and identification level given). **B)** A network of the Spearman rank correlation results with microbes (with their genus-level classification and ASV identifier given) colored in green and features (with their *m/z*, retention time, and identification level given) in orange.

(ρ = -0.73) and *Eggerthella* (ρ = -0.69). Similarly, the terpenoids (+)-khusitene negatively correlated with *Romboutsia* (ρ = -0.68) and pseudolaric acid H with *Streptococcus* (ρ = -0.72). The negative correlations observed for alkaloids and terpenoids may be attributed to their antibacterial properties (Yang et al. 2020, Cushnie et al. 2014). While the causal link between the features derived from xenobiotics and members of the infant gut microbiome remains to be elucidated, the results highlight the need to explore xenobiotic-microbiome interactions in less complex *in-vitro* models and its consequent health implications.

3.6. Limitations

Several limitations that are essential to consider have become apparent in the applied analytical workflow and the overall study design. Many of the analytes investigated, especially polyphenols, have a wide range of isomers, making annotation of the features a complex task. This is reflected in the suspect screening results where many features (Table A.6 and A.7) have the same annotation though they were distinct analytes. Additionally, DIA MS^2 spectra typically have more noise than DDA MS^2 spectra (Guo and Huan 2020), further complicating annotation. The MS^2 spectra with increased noise have a strong influence on *in silico* fragmentation, hence a high number of features annotated at level 3a is reported. The uncertainty in feature annotation also impacts the biological relevance of the statistical and correlation analyses. In addition, the number of participants in the study was small (n = 11) and as such, the correlation results must be interpreted with caution. Moreover, due to the low sample size, all time points were included in the correlations between the infant stool and gut microbiome. Therefore, the correlations may reflect indirect drivers such as the dynamic development of the microbiome in early life or changes in diet with age. A larger sample size and comparison among cohorts from other geographical settings would be needed for a more comprehensive analysis. Data is sparse on longitudinal metabolomic/exposomic profiles of healthy Nigerian mother-infant pairs anchored on high-resolution mass spectrometric analysis of breast milk and matching stool samples. Thus, the data herein gives an important snapshot of the chemical exposome in biofluids of a selected population that has been considered to be at a relatively high exposure levels to many beneficial and adverse xenobiotics.

4. Conclusion

The influence of natural and/or synthetic chemicals in early life is known to have a considerable impact on the development of humans. This study provides a comprehensive overview of potentially beneficial as well as potentially toxic xenobiotics in breast milk and stool from Nigerian mother-infant pairs. Several xenobiotics detected in the breast milk were also present in the corresponding stool samples, although the stool samples contained, as expected, a higher number of different xenobiotics. Correlations were observed between xenobiotics and certain members of the gut microbiome of the infants. Exposure to xenobiotics and their impact on the health of the infants significantly increased with the introduction of complementary foods. However, the toxicological relevance of these correlations needs to be further explored in larger cohorts and validated in *in vitro* models. Despite the limited sample size, the longitudinally study design and the advanced exposomic/metabolomic workflow applied allowed for the detailed assessment of the chemical exposome in breast milk and stool. The next steps should be the application of such workflows in larger cohorts and in different populations, especially in long-term studies, to better characterize the influence that exposure to

various chemicals and their impact on health and microbiome development have.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgment

The authors would like to thank the mothers and their infants for providing the samples. They would also like to thank and acknowledge all the members of their working groups, the Warth and Rompel labs, for their help, support, and feedback. The authors would like to express their gratitude to the Mass Spectrometry Center of the Faculty of Chemistry at the University of Vienna for technical support during the measurements, and to the Joint Microbiome Facility of the University of Vienna and the Medical University of Vienna. This work was supported by the University of Vienna through the Exposome Austria Research Infrastructure (B.W.), the Austrian Federal Ministry of Education, Science and Research (project DigiOmics4AT, B.W.), the Austrian Federal Ministry for Climate Protection, Environment, Energy, Mobility, Innovation and technology (BMK, B.W.), and the Austrian Science Fund (FWF) grant P32326 (A.R.).

Data availability

LC-MS raw data files have been submitted to the MetaboLights data repository (MTBLS8792). The 16S rRNA gene amplicon data is available on the BioProject accession number PRJNA1013123.

Appendix

Supplementary file A (Excel) is given that contains all of the tables mentioned in the text, e.g. the suspect screening results for breast milk and stool.

Supplementary file B (PDF) is given that contains various figures, including boxplots of the semi-quantification results and heatmaps from the correlation analysis.

CRediT author contributions

Ian Oesterle: Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Kolawole I. Ayeni:** Conceptualization, Software, Formal analysis, Writing - Review & Editing. **Chibundu N. Ezekiel:** Writing - Review & Editing, Resources. **David Berry:** Writing - Review & Editing. **Annette Rompel:** Writing - Review & Editing, Supervision, Funding acquisition, Resources. **Benedikt Warth:** Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition, Resources.

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