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Longitudinal fecal microbiota and volatile metabolomics preceding necrotizing enterocolitis in preterm infants: a case–control study

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Alterations in fecal microbiota and volatile organic compound (VOC) profiles of preterm infants have been demonstrated before onset of necrotizing enterocolitis (NEC). However, NEC-specific signatures need to be identified before potential application as predictive biomarker in clinical practice. A prospective multicenter case–control study was conducted to identify preclinical fecal microbiota and VOC profiles of infants that developed NEC. Microbiota analysis (PCR-based IS-pro technique) and VOC analysis (gas chromatography-mass spectrometry) were performed on fecal samples collected up to three days before clinical NEC onset. In 112 infants (56 NEC, 56 matched controls), sufficient number fecal samples were collected for either microbiota or VOC analysis. Prior to NEC onset, *Clostridium perfringens* ($p = 0.023$, unadjusted) was more present in infants with NEC, versus controls. VOC analysis showed a clear distinction between fecal profiles of NEC cases and controls (area under the curve = 0.82). Fourteen unique VOC features contributed to this discrimination. Fecal microbiota and VOC profiles may serve as early indicators of NEC, and allow for increased understanding of pathophysiological mechanisms of NEC, but larger validation cohorts are needed before an overarching NEC-specific predictive microbiota-based biomarker can be implemented.

Keywords Metabolomics, Volatile organic compounds, Microbiome, Stool, Necrotizing enterocolitis

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Necrotizing enterocolitis (NEC) is a devastating gastrointestinal disease predominantly affecting preterm infants and is associated with mortality rates of up to 30% in advanced stages[1]. The pathogenesis of NEC is considered multifactorial, including gut immaturity, microbial dysbiosis, and intestinal ischemia by hypoperfusion, resulting in an exaggerated immune response of the gut[2]. However, the exact pathogenesis of NEC remains to be elucidated, limiting the development and implementation of targeted diagnostic and therapeutic strategies. In clinical practice, NEC diagnosis is established on a combination of clinical symptoms, laboratory markers of inflammation, and radiographic findings. However, these parameters lack specificity and typically manifest only in advanced stages of the disease[3–5]. Early recognition of NEC remains challenging and may delay medical intervention, and as a consequent negatively impact clinical outcomes[5].

The consistent observations of microbial dysbiosis preceding NEC propose the potential for microbes to serve as a predictive biomarker[6–11]. However, no NEC-specific microbial signature has yet been identified, largely due to the limited number of inclusions and the lack of standardization of the study design, including the applied microbiota analysis technologies, sampling conditions, and analyzed time-points, limiting the comparison of outcomes[12]. In addition, fecal volatile organic compound (VOC) analysis has been postulated as a potential predictive biomarker for different neonatal morbidities including NEC[13–17]. Fecal VOCs are produced by metabolic processes of the host and gut microbiota and by their mutual interaction, and are therefore suggested to reflect both microbiota composition and their function[18]. However, similarly to microbiota studies, a NEC-specific VOC signature has not yet been identified. This is because the majority of studies utilized eNose technology to describe VOC profiles, which relies on profile recognition rather than the identification of unique chemical molecules. In this study, we aimed to identify microbial and VOC signatures at the molecular level preceding NEC onset, and hypothesized that the preclinical fecal microbiota and VOCs can discriminate infants with NEC from matched controls.

Results

Baseline characteristics

A total of 1002 infants were included during the study period, of whom eighty-three developed NEC (8.3%). Supplementary Table 1 shows the number of infants included in the study per participating center. From 56 infants with NEC, sufficient fecal samples and mass was collected for further analysis. Clinical and demographic data are provided in Table 1. In Supplementary Table 2, the number of analyzed fecal samples per analytical technique and time point is outlined.

Microbiota analysis

There were 53 infants with NEC (NEC IIA, n = 17; NEC IIB, n = 17; NEC IIIA, n = 8; NEC IIIB, n = 11) matched to 53 controls for microbiota analysis. In total, 240 case and control samples were analyzed (Supplementary Table 2). Cumulative IS-pro microbiota profiles of all stage NEC case (n = 120) and control (n = 120) samples were constructed, which demonstrated that *Clostridium perfringens* ($p = 0.023$, adjusted $p = 0.15$) was significantly more present in infants that developed NEC. *C. perfringens* was present in respectively 26% of infants that developed NEC, compared to 9% of the control infants (Fig. 1). Phylum diversity and abundances were assessed between all NEC case (n = 120) and control samples (n = 120) at the three predefined time points. Diversity indices, including all phyla combined and Bacteroidetes, FAFV, and Proteobacteria separate, did not differ between cases and controls (Fig. 2a; Supplementary Table 3). No differences were observed in absolute and relative abundances of cases versus controls for each individual phylum and for all phyla combined (Fig. 3a, b). In addition, for both diversity indices and the absolute and relative abundance, no clear time-associated trend from t_{-3} to t_{-1} was observed (Figs. 2b, 3c).

Subsequently, NEC cases were categorized into subgroups based on NEC staging, and only the last sample obtained before diagnosis was included in the analysis (T_{last}). It was demonstrated that NEC IIIB cases (perforated gut) had a different microbial composition and diversity compared to their matched controls at T_{last} (11 case samples vs. 11 control samples) (Supplementary Table 4). The observed discrimination between NEC IIIB and controls was driven by decreased overall α-diversity ($p = 0.026$) and a lower absolute abundance of FAFV ($p = 0.041$) and Bacteroidetes ($p = 0.018$) in cases.

VOC analyses

For GC–MS analysis, 44 infants with NEC (16 NEC IIA, 12 NEC IIB, 7 NEC IIIA, and 9 NEC IIIB) were matched with 44 controls. In total, 208 samples were analyzed, including 104 NEC samples and 104 control samples. Three control samples were excluded for further analysis due to unreliable measurements (Supplementary Table 2). The number of unique VOCs isolated from the headspace of the fecal samples ranged from 101 to 382 per sample. The majority of these VOCs belonged to aldehyde and *alcohol* subgroups (Supplementary Table 5). Examples of chromatograms at three predefined time points are displayed for one control infant and one NEC case in Supplementary Figure 1.

Similarity analysis of fecal VOC profiles

First, the similarities between all sample pairs in the VOC profiles were assessed (Fig. 4). It was shown that all VOC profiles were moderately comparable to each other, with an overall cosine similarity peak of 0.7 (Fig. 4a). The similarity index between the samples increased to 0.9 when the matched case–control groups were taken into account independently, indicating that case samples were more similar to each other, and control samples were more similar to each other (Fig. 4b). Regarding intra-individual similarity, VOC profiles seemed to be more uniform over time in controls than in NEC cases (Fig. 4c).

	NEC (n = 56)	Controls (n = 56)	*p* value
Gestational age *weeks + days* (mean [SD])	26 + 6 [9]	26 + 6 [9]	0.98
Birth weight *in grams* (mean [SD])	891.1 [225.9]	936.3 [203.8]	0.27
Gender *male* (n[%])	27 [48.2]	36 [64.3]	0.09
Birth mode *vaginal* (n[%])	27 [48.2]	20 [35.7]	0.18
Multiple pregnancy (n[%])	21 [37.5]	19 [33.9]	0.69
Classification of NEC cases[a] (n[%])			NA
NEC IIA	18 [32]	NA	
NEC IIB	17 [30]	NA	
NEC IIIA	9 [16]	NA	
NEC IIIB	12 [22]	NA	
NEC t0 *in days* (median [IQR])	14 [9–20]	NA	NA
Late-onset sepsis preceding or during NEC[b] (n[%])	33 [58.9]	NA	NA
Postnatal age sepsis preceding t0 *in days* (median [IQR])	11 [8–16]	NA	NA
Antibiotics prior t0 *in days* (median [IQR])	7 [4–10]	7 [4–9]	0.61
Antibiotics usage during sampling (n[%])	16 [28.6]	9 [16.1]	0.11
Feeding practice[c] (n[%])			
Human milk	25 [44.6]	28 [50.0]	0.75
Formula milk	7 [12.5]	7 [12.5]	
Combination	21 [37.5]	17 [30.4]	
Line exposure at t0			
None	14 [25.0]	22 [39.3]	*0.01*
Central line	19 [33.9]	19 [33.9]	
Peripheral line	3 [5.4]	9 [16.1]	
Both central and peripheral	20 [35.7]	6 [10.7]	
Ventilation at t0			
None	13 [23.2]	14 [25.0]	*0.01*
Non-invasive	20 [35.7]	33 [58.9]	
Invasive	23 [41.1]	9 [16.1]	
Deceased (n[%])	18 [32.1]	0 [0]	*0.00*
NEC IIA	3 [16.7]	NA	NA
NEC IIB	3 [17.6]	NA	
NEC IIIA	7 [77.8]	NA	
NEC IIIB	5 [41.7]	NA	
Day of life death *in days* (median [IQR])	17 [12–23]	NA	NA

Table 1. Demographics. [a]According to the modified Bell's staging criteria. [b]Sepsis up to three days before diagnosis or during NEC episode. [c]In the NEC group the data regarding feeding practice was missing in three cases, in the control group the data regarding feeding practice was missing in four cases. SD, standard deviation; NEC, necrotizing enterocolitis; IQR, interquartile range; NA, not applicable. Significant values are in italics.

Fecal VOC profile analysis

Second, supervised classification models were applied to discriminate the fecal VOC patterns of NEC samples (n = 104) from control samples (n = 101), resulting in a moderate to low diagnostic accuracy. The best test accuracy was obtained when combining VOC profiles of all three time points (area under curve (AUC) [95% CI]: 0.71 [0.63–0.78]) using GLMVQ (Supplementary Figure 2, Supplementary Table 6).

Single-day and longitudinal fragment and chromatogram feature analysis

The longitudinal course of unique compounds and chromatogram characteristics of the VOC profiles before NEC diagnosis were assessed by analyses including case and control infants with samples analyzed at all three pre-defined timepoints (n = 24 infants with NEC, n = 23 control infants, n = 141 samples). The analysis was performed based on single-day or day-difference fragment and chromatogram features, or a combination of these features, as displayed in Supplementary Table 7 and Table 2. Three classification algorithms were applied to obtain the best performing feature set. The best classification performance was achieved using the logistic regression (LR) classifier by combining all feature sets (F_{FC}) (accuracy = 77.7% and AUC = 0.82) (Table 2, Fig. 5a). In Fig. 5b it is shown that optimal accuracy when combining all feature sets (F_{FC}) was obtained using 14 unique features (AUC = 0.82). The fourteen features that were selected most frequently by LR are displayed in Table 3, of which eleven were significantly different ($p < 0.05$) between NEC samples and controls (Supplemental Figure 3). Eight of these features were from samples obtained 2 days before diagnosis of NEC

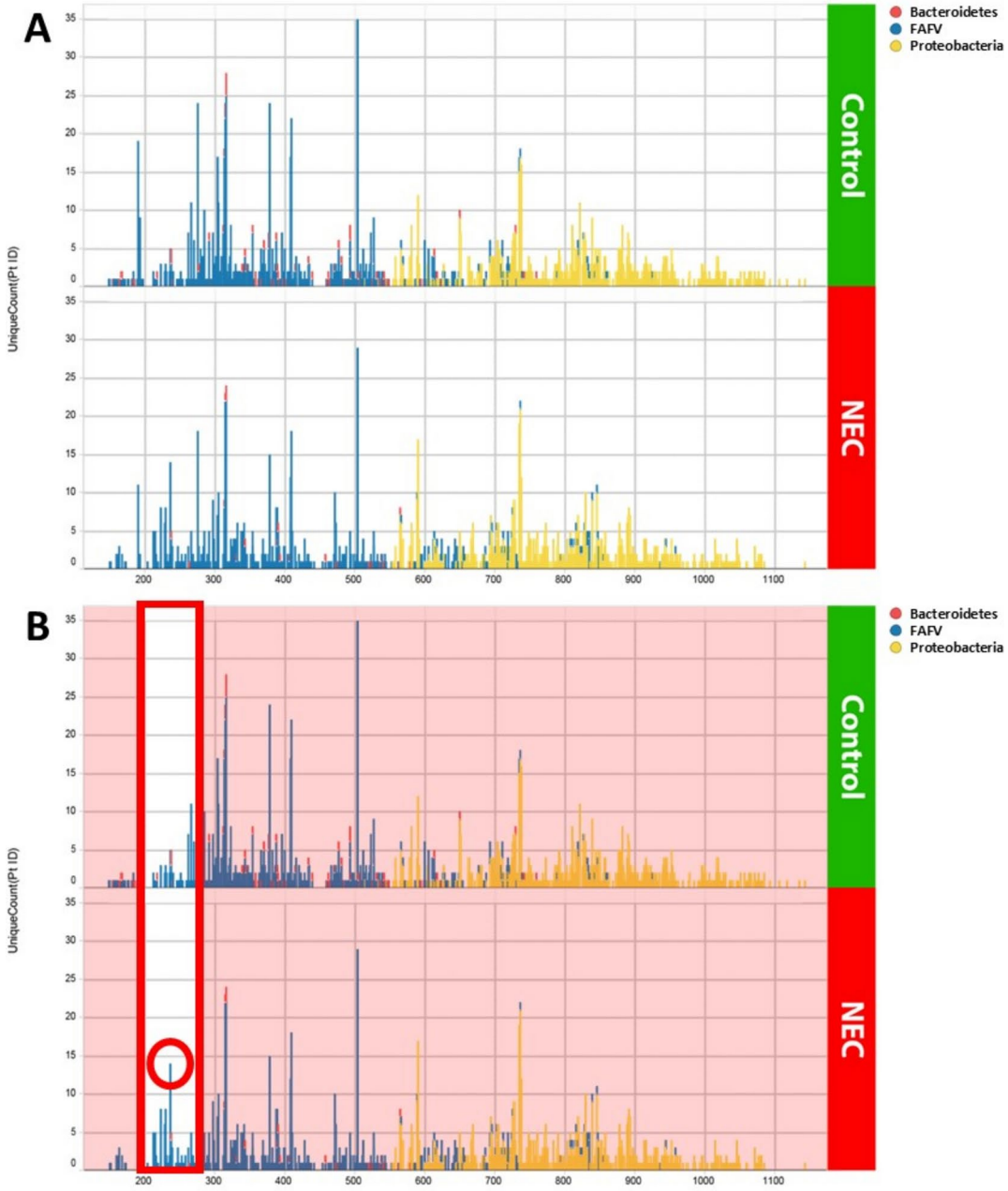

Fig. 1. Cumulative IS-profiles of infants with NEC (all NEC-stages) versus controls. The peak length is displayed on the x-axis and corresponds to IS-fragment length. The peak height on the y-axis reflects the number of infants in which the corresponding peak was found. (**A**) In infants with NEC, *Clostridium perfringens* (peak length 235; $p = 0.023$, FDR adjusted $p = 0.15$) was significantly more observed compared to controls. (**B**) The highlighted version of A. IS, interspace; NEC, necrotizing enterocolitis; FAFV, Firmicutes, Actinobacteria, Fusobacteria and Verrucomicrobia.

(Table 3). Furthermore, eight of these single-day features were identified as unique chemical compounds, namely 3-(methylthio)-propanal ($p = 0.003$, t_{-1}), camphene ($p = 0.017$ and $p = 0.015$, t_{-2}), benzene acetaldehyde ($p = 0.006$, t_{-2}), 3-methylbenzaldehyde ($p = 0.006$, t_{-2}), tert.butoxy-benzene ($p = 0.045$, t_{-2}), 2-ethyl-1,3-butadiene or cyclohexene ($p = 0.067$, t_{-3}), and 2-pentylfuran ($p = 0.025$, t_{-1}), while one feature was unidentified ($p = 0.006$, t_{-2}), (Table 3, Supplementary Figure 3).

Discussion

In this multicenter case–control study, we aimed to identify fecal microbial and volatile metabolic signatures in the three days preceding NEC onset. *C. perfringens* was significantly more abundant in infants with NEC compared to controls. Microbiota composition differed significantly from controls in NEC IIIB cases, which was driven by a decreased overall *α-diversity*, lower absolute abundance of FAFV, and depletion of Bacteroidetes.

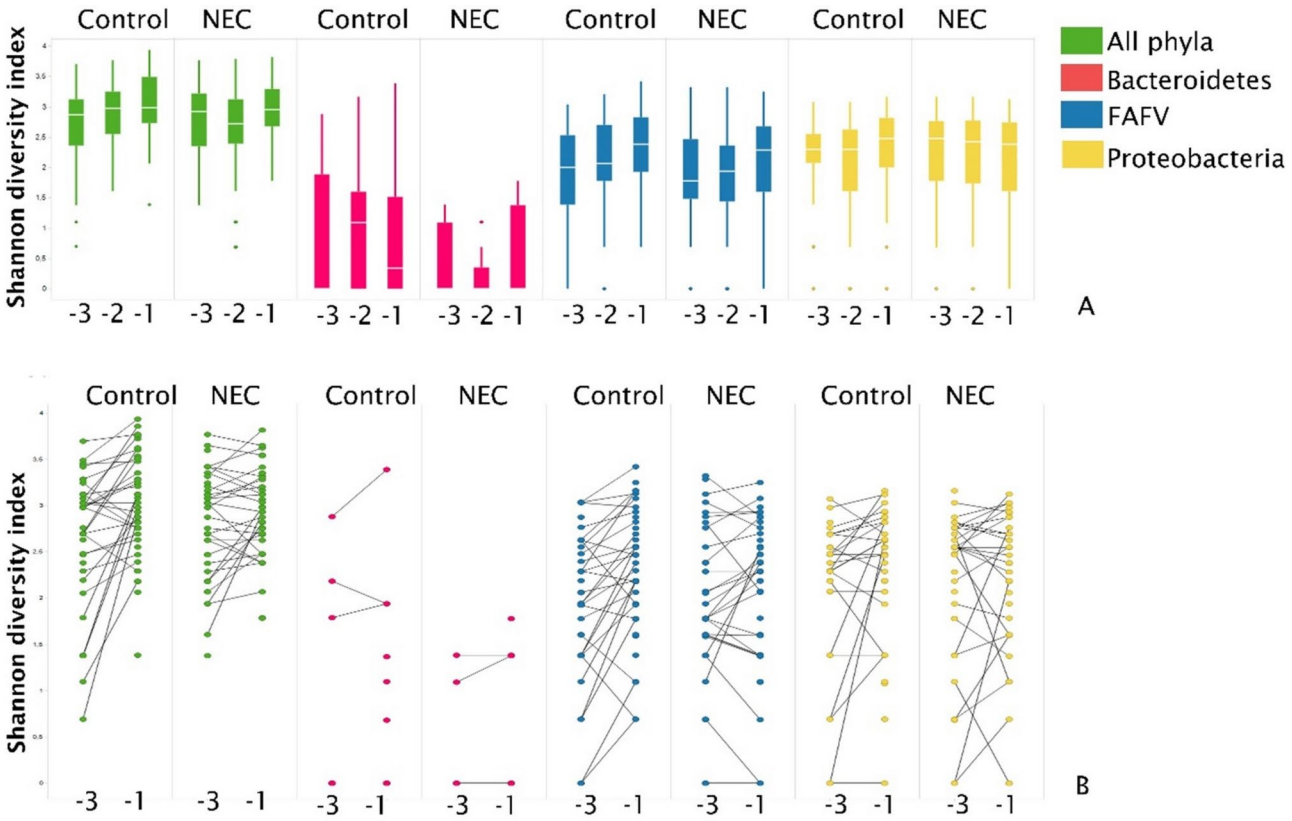

Fig. 2. Shannon diversity index for all phyla between NEC cases and controls. (**A**) The Shannon diversity index is displayed for all phyla combined, and the three phyla (Bacteroidetes, FAFV, and Proteobacteria) separate. The image shows the differences between all NEC cases versus control group for all three time points separately. There were no significant differences observed. (**B**) The difference in Shannon diversity index is displayed over time from three days before diagnosis (t-3) to one day before diagnosis (t-1) for both NEC cases and controls for all phyla combined, and the three phyla separately. Each point resembles a sample. A line is drawn between samples from the same infant. There were no significant differences in Shannon diversity over time. NEC, necrotizing enterocolitis; FAFV, Firmicutes, Actinobacteria, Fusobacteria and Verrucomicrobia; −3, −2, and −1 represent three, two and one day(s) before diagnosis, respectively.

Fourteen VOC features, of which eight unique VOCs differed significantly between cases and controls prior to diagnosis, however, most of these compounds differed only two days prior to clinical onset. Based on the single- and day-difference fragments and chromatogram features combined, NEC could be distinguished from controls with an AUC of 0.82.

Several microbial strains have been proposed to be associated with development of NEC. Here, we demonstrated that *C. perfringens* was significantly more abundant prior to clinical NEC onset compared to controls, with an unadjusted p value of $p = 0.023$. Clostridia species have consistently demonstrated involvement in the development of NEC[11,19–26]. NEC associated with *C. perfringens,* as found in the peritoneal cavity, blood, or stool, is more severe and comprises higher mortality and morbidity[24]. Similar to the current study, a higher abundance of *C. perfringens* in pre-NEC samples was demonstrated in two studies[23,26]. One study showed an increased in *C. perfringens* from birth up to NEC diagnosis (11 NEC cases and 22 matched controls)[23]. Another study identified three pre-NEC taxonomic profiles which were all dominated by well-recognized (opportunistic) pathogens[26]. One of these pre-NEC community types was dominated by *C. perfringens*, amongst other pathogens, with a significant higher abundance of *C. perfringens* in pre-NEC samples versus healthy controls (26.52% and 1.02%, respectively)[26]. Previous research showed that NEC-specific clostridial strains can produce interleukin-8 in Caco-2 cells, suggesting they might induce inflammatory processes involved in the pathogenesis of NEC[27].

We did not observe differences in α-diversity before NEC onset, which is consistent with results from a meta-analysis including 8 studies with 106 NEC and 278 controls[11]. However, a systematic review including 20 studies with 254 NEC cases and 673 controls reported a decrease in α-diversity[10], and Tarracchini et al. reported lower biomass in pre-NEC samples[26]. In these studies, a trend was demonstrated towards an increased relative abundance of Proteobacteria[10,11,26], and decreased levels of Firmicutes and Bacteroidetes in NEC cases[10,11]. We observed a decreased absolute abundance of FAFV and absence of Bacteroidetes in infants with NEC IIIB. The observed microbial differences between the different stages of NEC may suggest the presence of phenotype-specific metabolic and microbial pathways.

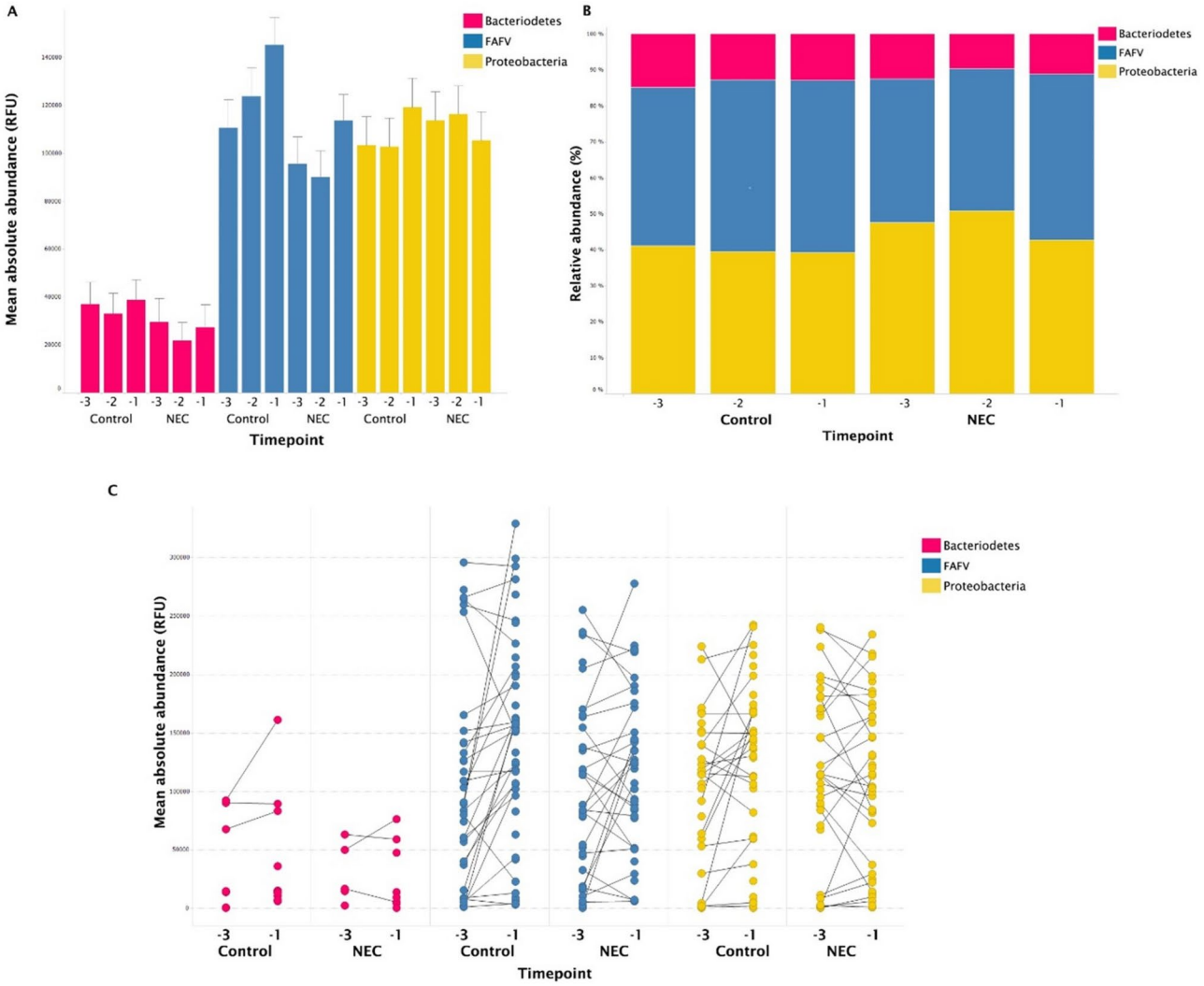

Fig. 3. Mean absolute and relative abundance for all phyla per time point between NEC cases and controls. (**A**) The mean absolute abundance per time point (− 3, − 2, and − 1), phylum (Bacteroidetes, FAFV, and Proteobacteria), and study group (NEC vs controls) is displayed. There were no significant differences. (**B**) This shows relative abundance per time point, phylum and study group. (**C**) The mean absolute abundance is displayed over time from three days before diagnosis (t-3) to one day before diagnosis (t-1) for both NEC cases and controls for the three phyla separately. Each point resembles a sample. A line is drawn between samples from the same infant. There were no significant differences in mean and absolute abundance, and no differences in mean absolute abundance over time. NEC, necrotizing enterocolitis; FAFV, Firmicutes, Actinobacteria, Fusobacteria and Verrucomicrobia; − 3, − 2, and − 1 represent three, two and one day(s) before diagnosis, respectively; RFU, relative fluorescence units.

In the current study the fecal VOC profiles of NEC cases could be distinguished from controls with an AUC ranging from 0.6–0.8, by applying supervised classification models (Supplemental Figure 2). Analyzing the single-day and day-difference fragment and chromatogram features revealed that eight unique compounds belonging to *aldehydes* and *alcohols*, strongly contributed to the observed discrimination between NEC and controls. Comparable to our results, Probert et al. (32 NEC cases vs. 70 controls) showed several unique compounds belonging to aldehydes and alcohols resulted in the differentiation of NEC from controls with AUCs of 0.75–0.76 up to four days prior to clinical onset[14]. One of these VOCs, 2-pentylfuran, also contributed to the discrimination of cases versus controls in the current study. Contrary, Garner et al. (6 NEC cases vs. 7 controls) demonstrated differences in the group of esters in fecal samples of infants with NEC, up to four days prior to clinical onset[15].

One of the discriminative aldehydes detected in this study, 3-methylthiopropanal, is synthesized by specific bacteria belonging to Firmicutes, including *Lactococcus lactis*[28]. This compound is a prostaglandin antagonist that possibly mitigates the inflammatory effects of prostaglandins[29]. We demonstrated that a reduced absolute abundance of FAFV is associated with the development of NEC IIIB, and thus hypothesize that lower 3-methylthiopropanal levels may result from the reduced abundance of Firmicutes. Other aldehydes, including

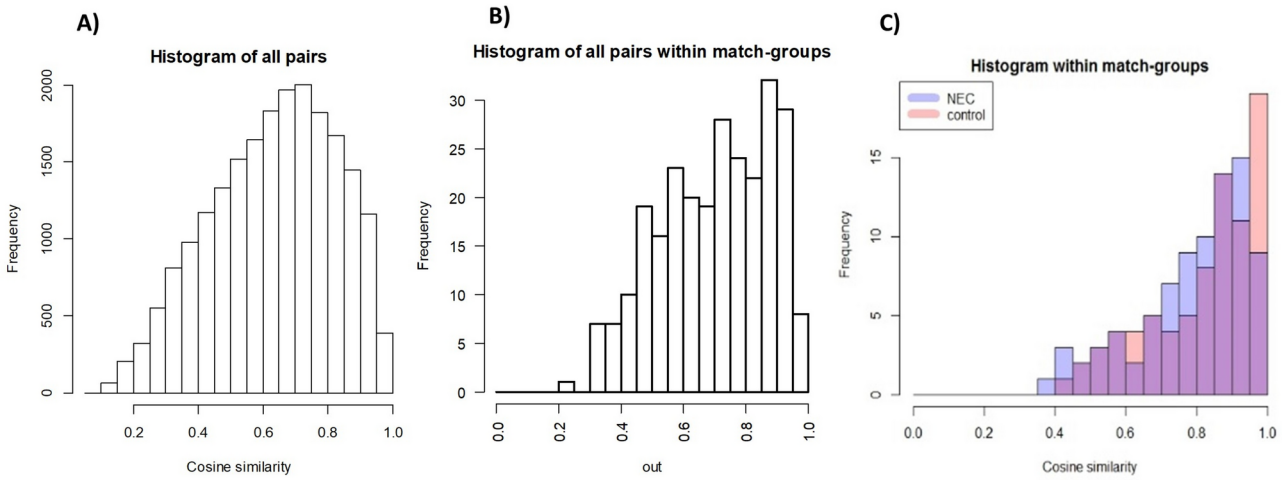

Fig. 4. Similarities between samples as measured by cosine similarity measure. Similarity values range between zero and one. Higher cosine similarity values demonstrate an increase in sample similarity. (**A**) Includes all sample pairs irrelevant of disease state. This demonstrates that all samples are moderately similar (similarity index 0.7). (**B**) Shows the similarity between study groups, indicating that samples within the NEC and within the control group are more similar to each other (similarity index 0.9). (**C**) Demonstrating intra-individual similarity. This shows that control samples are more stable over time compared to NEC samples. NEC, necrotizing enterocolitis.

Feature set		Modality	Diagnostic Accuracy (%)		AUC	
			Single run	Multiple runs (average ± SD)	Single run	Multiple runs (average ± SD)
$F_{s\text{-}frag}$	Single-day fragment features	MS	70.1	70.5 ± 7.6	0.79	0.77 ± 0.11
$F_{d\text{-}frag}$	Day-difference fragment features	MS	68.5	68.9 ± 2.7	0.72	0.73 ± 0.04
$F_{s\text{-}Chro}$	Single-day chromatogram features	GC	60.6	62.4 ± 5.1	0.57	0.61 ± 0.07
$F_{d\text{-}Chro}$	Day-difference chromatogram features	GC	67.2	67.2 ± 3.2	0.68	0.69 ± 0.02
F_{frag}	Fragment features combined	MS	70.3	70.7 ± 8.1	0.79	0.78 ± 0.08
F_{Chro}	Chromatogram features combined	GC	69.9	70.3 ± 2.4	0.75	0.74 ± 0.03
F_{FC}	All feature sets combined	MS, GC	78.9	77.7 ± 5.3	0.84	0.82 ± 0.05

Table 2. Performance of classification model on different feature sets by logistic regression. All features sets that were tested using logistic regression are displayed. Feature sets are based on single-day or day-difference fragments and chromatogram features. Each feature set was run multiple times with different training set arrangement, here the average and standard deviation are outlined. From these multiple runs, we selected a single run with similar classification performance as the pooled results. $F_{s\text{-}frag}$, fragment feature including single day features (e.g. one of the three time points); $F_{d\text{-}frag}$ fragment feature differences between two time points (e.g. between day one and two or day two and three); $F_{s\text{-}chro}$, chromatogram feature including single day features; $F_{d\text{-}chro}$, chromatogram feature differences between two time points; F_{frag} fragment feature in which all time points and differences between time points are included; F_{chro}, chromatogram feature in which all time points and differences between time points are included. F_{FC}, all fragment and chromatogram features were included in the classification model.

benzene-acetaldehyde and 3-methylbenzaldehyde, have been associated with the development of Crohn's disease[30]. It has been hypothesized that aldehydes are products of lipid peroxidation, which is thought to occur due to epithelial inflammation[30]. The presence of these compounds could serve as early markers of enterocolitis. Further studies are required to confirm these hypotheses.

Based on an extensive literature and mVOC 4.0 (Microbial Volatile Organic Compounds) database search, the observed set of 14 VOC features could not specifically be linked to *C. perfringens*[31]. It is important to note that fecal VOCs, in addition to originate from metabolic activity of microbes, also have been described to originate from host metabolism and host-environmental factors, but the precise contribution of each factor on VOC composition remains incompletely understood[32]. These multiple VOC sources complicates the interpretation of findings in VOC studies and should be taken into account in future research and clinical applications.

Although we demonstrate differences in VOC features in fecal NEC samples versus controls, our findings on VOCs as predictive marker for NEC are not completely consistent with findings of earlier studies[13–15], hampering validation for use as tool in daily clinical practice. Interhospital differences influencing fecal VOC

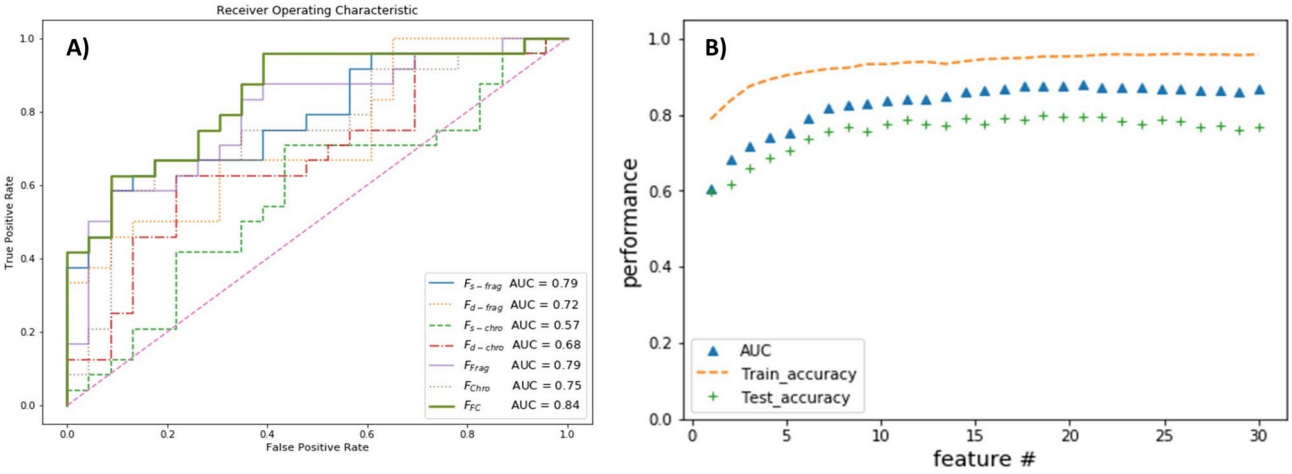

Fig. 5. **Receiver-operating-characteristic curves and features performance by logistic regression classification.** (**A**) Receiver-operating-characteristic curves for the different feature sets as obtained by logistic regression classification (n = 44 infants with NEC vs. 44 controls). Diagonal dashed line represents a random guess. (**B**) Influence of the number of features on the performance of the logistic regression classification model. A plateau is reached when 14 features are included in the classification model. Including more than 15 features did not result in an improved diagnostic accuracy in differentiating infants with NEC from controls (n = 44 infants with NEC vs. 44 controls). AUC, area under curve; $F_{s\text{-}frag}$, fragment feature including single day features (e.g. one of the three time points); $F_{d\text{-}frag}$ fragment feature differences between two time points (e.g. between day one and two or day two and three); $F_{s\text{-}chro}$, chromatogram feature including single day features; $F_{d\text{-}chro}$, chromatogram feature differences between two time points; F_{frag} fragment feature in which all time points and differences between time points are included; F_{chro}, chromatogram feature in which all time points and differences between time points are included. F_{FC}, all fragment and chromatogram features were included in the classification model.

#	Selected features	Specific compound (molecular weight (g/mol))	Number of times selected for fivefold cross-validation	Modality
1	Day-1-35@905	3-(Methylthio)-propanal (104)	4	MS
2	Day-2-77@953	Camphene (136)	4	MS
3	Day-2-117@1006	Unknown	4	MS
4	Day-2-65@953	Camphene (136)	4	MS
5	Day-2-119@978	Benzeneacetaldehyde (120)	3	MS
6	Day-2-119@996	3-Methylbenzaldehyde (120)	3	MS
7	Day-2-66@998	Tert.butoxy-benzene (150)	3	MS
8	Day-3-67@823	2-Ethyl-1,3-butadiene or cyclohexene (82)	3	MS
9	Day-1-126@956	2-Pentylfuran (138)	2	MS
10	Day-2-gc_seg_ratio3	NA	3	GC
11	Diff23-SampEn	NA	3	GC
12	Day-1-DiffEn	NA	2	GC
13	Diff23-Cos_sim	NA	2	GC
14	Day-2-SampEn	NA	2	GC

Table 3. The fourteen features included to obtain most optimal classification model using fivefold cross validation with logistic regression. The name of the feature is build up by the selected day for the feature, thus, Day 1 is t_{-1}, and Diff23 stands for the difference between day 2 and 3 (t_{-2} and t_{-3}). For the features build up by MS data, the second part of the feature name is build up by the m/z@rt, where m/z stands for the mass-to-charge ratio and rt stands for the retention time. MS, Mass spectrometry; GC, Gas chromatography; SampEn, Sample entropy; DiffEn, Differential entropy; Cos_Sim, cosine similarity; gc_seg_ratio, gas chromatography segment ratio; NA, not applicable.

composition could play a role, but variations in sampling and analysis methods between studies need to be considered as possible explanation for the apparent differences in VOC outcomes as well[33,34]. Therefore, uniform, publicly available standard operating procedures (SOPs) should be applied in future studies, allowing for better inter-study comparisons. Such a proposal has been described in a previous study in which optimal sampling conditions for fecal VOC analysis by GC–MS were studied and applied in the current study[35]. Next to the need

for a SOP, the well-known difficulties in the diagnosis and staging of NEC may also complicate classification of NEC in studies[36,37]. NEC has a multifaceted clinical presentation and typical signs of NEC such as pneumatosis are often not overtly present in the most preterm infants. Therefore, despite our best efforts, there is a risk of misclassification of NEC[38,39], particularly in the lower stage cases with no surgical and pathologic confirmation. Even a sporadic misclassification of NEC may negatively impact the predictive value of NEC biomarkers. The higher accuracy in the NEC IIIB cases may, next to the pathophysiological processes, possibly be result from more certain classification of these specific cases.

The major strength of this study is its prospective multicenter study design, allowing for the inclusion of a large number of NEC cases, enabling the assessment of different techniques for their potential as non-invasive preclinical diagnostic biomarkers. Furthermore, a uniform collection method was applied for all infants regarding clinical data and sample collection, the latter minimizing the risk of bias by sampling variation. Moreover, the large dataset allowed for a strict matching procedure based on gestational age and center of birth, limiting these two factors as possible confounders[40,41]. VOC analysis was done according to an optimized sampling protocol[35]. Finally, we applied a novel PCR-based microbiota analytical technique that enables high-throughput analyses and with the potential to generate outcomes at the species level within five hours after sampling[42]. Consequently, clinicians may utilize this technique in their clinical decision-making process.

One of the main limitations was the limited fecal sample mass available per included infant. Hence, simultaneous analyses of microbiota and VOCs on all fecal samples was not feasible. It was therefore not possible to combine VOC and microbiota outcomes, limiting the insight into the functional roles of the bacteria in the development of NEC. In addition, the observed difference in the presence of *C. perfringens* between infants with NEC and controls did not remain significant after correcting for multiple testing. However, this study is explorative and hypothesis-generating, aimed at identifying potential microbes associated with NEC. Given that *Clostridia* spp. have previously been linked to NEC, we chose to report these results. We acknowledge that future studies are essential to further validate this microbial signature and confirm its association with NEC.

In conclusion, we demonstrated differences in fecal microbiota and VOC composition up to 3 days before NEC onset in cases versus matched controls. Our findings underline the potential of fecal microbiota and VOC profiles as early diagnostic biomarkers of NEC, but illustrate that future studies with uniform SOPs for collection and analysis and flawless classification of NEC, by only including the more severe stages of NEC, are needed to validate the current results. Ideally, future studies should use a multi-omics approach to integrate the microbiota composition and fecal VOC profiles, gaining more insight into the gut microbiota function in NEC pathophysiology. This could aid in the development of a fecal microbiota-based non-invasive biomarker for identification of infants at risk of NEC that can be used in daily clinical practice.

Methods

Subjects

In this prospective case–control study infants born ≤ 30 weeks of gestation in nine participating neonatal intensive care units (NICUs) in the Netherlands and Belgium (Supplementary Table 1) were eligible to participate[13,43]. Infants that developed NEC (Bell's stage ≥ IIA) in the first 28 days of life were included in the study[44]. All NEC cases were independently reviewed and staged based on clinical, radiographic, laboratory, and, if present, pathological data by two expert clinicians (HN and TM), in which consensus was reached in all cases. Case and control infants were excluded in case of major congenital gastrointestinal diseases, isolated spontaneous intestinal perforation (SIP), early-onset sepsis (EOS), abdominal surgery unrelated to NEC, or insufficient fecal sample mass (< 75 mg per sample). Late-onset sepsis (LOS) was an additional exclusion criteria, however, infants with NEC that developed LOS within 3 days before or during NEC onset were not excluded. To avoid duplicate publication, infants were excluded for VOC analysis if previously analyzed[13].

Sample analysis

Sample and data collection

Fecal samples were collected from the diaper, daily from birth up to 28 days postnatally, transferred into a stool container (Stuhlgefäß 10 mL, Frickenhausen, Germany), and stored at − 20 °C until further handling. Probiotics were not administered during the sampling period across participating centers. For analysis, fecal samples from infants with NEC were selected one, two, and three days (t_{-1}, t_{-2}, and t_{-3}, respectively) prior to clinical onset (t_0). Infants with NEC were matched to controls based on gestational age (± 2 days), center of birth, and sample availability at postnatally age-matched days (e.g. every sample from an infant with NEC was postnatally age-matched to a control sample). In case of insufficient fecal sample mass (< 150 mg per sample) to perform both analyses, the case–control sample pairs were randomly selected for microbiota or VOC analysis.

Clinical and demographic data were prospectively collected from patient records (Table 1). Feeding practices were categorized into three subgroups: (1) human milk (all days before t0 consisted of a daily intake of > 75% human milk) (2) formula milk (all days before t0 consisted of a daily intake of > 75% formula milk) , and (3) a combination of human milk and formula milk (the days before t0 varied between days with > 75% human milk, > 75% formula milk, or 25–75% human milk supplemented with formula milk).

Microbiota analyses

Sample preparation, DNA extraction and microbiota analysis

A detailed description of the applied IS-pro technique, a molecular microbiota profiling method with capacities to generate data on microbiota composition at the species level, generating outcome within several hours following sampling, has been published previously[42,45]. IS-pro analyzes the entire microbial community in a sample without targeting specific organisms. A summary of sample preparation, DNA extraction, and microbiota analysis is provided in Appendix A. In short, three different fluorescent-labelled PCR primers were used to

amplify the phyla Bacteroidetes and Proteobacteria separately, and Firmicutes, Actinobacteria, Fusobacteria, and Verrucomicrobia (FAFV) combined. The interspacer length of the 16S-23S rDNA region, expressed as the number of nucleotides, was used to identify the bacteria at the species level.

Fecal VOC analyses

Sample preparation and analysis

Fecal VOC analysis was performed using gas chromatography–time-of-flight mass spectrometry (GC-ToF–MS) (Agilent 7890 B GC-TOF, LECO Pegasus 4D system, LECO, St. Joseph, MI, USA). A detailed description of the sample preparation and device settings is provided in Appendix B and is in accordance with a previously published study[35]. The software used for the operation of the equipment and data analysis included LECO Chromatof (LECO, St. Joseph, MI, USA) and the National Institute of Standards and Technology (NIST) mass spectral library version 2.0 build 2012 (NIST, Gaithersburg, MD, USA).

Data preprocessing

The measured chromatograms were processed according to the method described by Smith et al., as was the execution of peak detection and alignment[46]. To compensate for the small drifts in the GC–MS output, the chromatograms were normalized using hexafluoroisopropanol as the internal standard (ISTD). Because the ISTD can be influenced by the VOC content in the fecal samples, the ISTD in the quality control samples was used for normalization at the start and end of each batch.

Statistical analyses

Clinical and demographical data

SPSS® (version 26.0, IBM, NY) was utilized for statistical analyses of clinical and demographic data. Independent samples t-test, Chi-squared test, or Mann–Whitney U test were applied when appropriate. Data distribution was assessed through histogram shape, employing the Shapiro–Wilk test in case of uncertainty.

Microbiota analyses

Standard IS-pro proprietary software (Fragment Analyser V0.22.3) was used for the statistical analysis which is elaborated on in Appendix C[47]. In short, α-diversity was determined using the Shannon-diversity index, differences in absolute and relative abundance were assessed using the Mann–Whitney U-test, and intra-individual progression was evaluated by comparing abundance and diversity from t_{-3} to t_{-1}.

VOC data analyses

The data analysis regarding VOC analysis is described in detail in Appendix C. The similarity analysis and supervised classification were performed using R (version 3.3.3) with the R–studio interface[48]. In short, the similarity between the samples was estimated by calculating the cosine similarity measure. To discriminate NEC VOC profiles from controls, the supervised classification methods sparse partial least squares discriminant analysis (sPLS-DA), least absolute shrinkage and selection operator (LASSO), and generalized matrix learning vector quantization (GLMVQ) using tenfold cross validation were applied[49–52].

Next, infants with samples collected at all three predefined time points were included in an additional analysis to include fragment and chromatogram differences over time (e.g. difference between t_{-1} and t_{-2}), as well as single-day (e.g. t_{-1}, t_{-2}, or t_{-3}) chromatogram and fragment features (Supplementary Table 7). Different types of features sets were established for these analyses (Appendix C). Optimal classification models were constructed using these selected feature sets, including logistic regression, k-nearest neighbors, and support vector machines.

Data availability

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to containing information that could compromise the privacy of research participants.

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Author contributions
D.B., M.X., and S.e.M.e.H. designed the study, collected data, and drafted the initial manuscript. T.N., H.K., H.W., H.N., T.d.M., L.W., M.v.W., A.v.K., V.C., C.P., R.v.L., C.v.H., D.c.V., W.d.B., B.K., A.B., M.B., and N.d.B. collected the data and critically reviewed the manuscript for important intellectual content. X.L. contributed to the design of the study, supervision, and critically reviewed the manuscript for important intellectual content. N.M.F. critically reviewed the manuscript for important intellectual content. T.d.M., T.N., H.K., and H.N. conceptualized and designed the study and data collection, collected data, supervised data collection, and critically reviewed the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

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Declarations

Competing interests
The authors declare no competing interests.

Ethics statement
The study was approved by all local Medical Ethical Review Boards (protocol number A2016.313) and written informed consent was obtained from the legal guardians of all included infants. All experiments were performed in accordance with relevant guidelines and regulations.

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