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The Assessment of Fecal Volatile Organic Compounds in Healthy Infants: Electronic Nose Device Predicts Patient Demographics and Microbial Enterotype

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Abstract

Background: The assessment of fecal volatile organic compounds (VOCs) have emerged as a non-invasive biomarker in many different pathologies. Prior to assessing whether VOCs can be used to diagnose intestinal diseases, including necrotizing enterocolitis (NEC), it is necessary to measure the impact of variable infant demographic factors on VOC signals.

Materials and Methods: Stool samples were collected from term infants at four hospitals in a large metropolitan area. Samples were heated and fecal VOCs assessed by the Cyranose® 320 Electronic Nose. Twenty-eight sensors were combined into an overall smellprint and were also assessed individually. 16s rRNA gene sequencing was used to categorize infant microbiomes. Smellprints were correlated to feeding type (formula vs. breastmilk), sex, hospital of birth, and microbial enterotype. Overall smellprints were assessed by PERMANOVA with Euclidean distances and individual sensors from each smellprint were assessed by Mann-Whitney U tests. $P<0.05$ was significant.

Results: Overall smellprints were significantly different according to diet. Individual sensors were significantly different according to sex and hospital of birth, but overall smellprints were not significantly different. Using a decision tree model, two individual sensors could reliably predict microbial enterotype.

Conclusions: Assessment of fecal volatile organic compounds with an electronic nose is impacted by several demographic characteristics of infants and can be used to predict microbiome

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composition. Further studies are needed to design appropriate algorithms that are able to predict NEC based on fecal VOC profiles

Keywords

Fecal Volatile Organic Compounds; Biomarkers; Necrotizing Enterocolitis; Electronic Nose; Enterotype

Introduction

Volatile organic compounds (VOCs) have recently emerged as a potential non-invasive biomarker in many diseases. VOCs are metabolites that produce a characteristic odor and are produced by both normal and pathophysiologic processes [1]. Clinicians have historically used their own noses to assess VOCs to aid in diagnosis of diabetic ketoacidosis, characterized by an acetone “fruity” odor in a patient’s breath [2]. It did not take long for researchers to use the strong sense of smell of canines to diagnose many diseases, including several different types of cancers and *Clostridium difficile* colitis [3–6]. The analysis of VOCs has become more sophisticated and is now done by several analytical methods, such as electronic nose (eNose) software, gas chromatography, mass spectrometry, and headspace solid-phase microextraction (SPME) [7].

This advancement in the analysis of VOCs has led to several studies in researching their ability to predict disease. Stool analysis of VOCs has been performed to identify gastrointestinal diseases. Fecal VOCs are heterogenous compounds that originate from metabolic byproducts from bacteria. They have been used to diagnose infectious processes such as Clostridium difficile colitis and Campylobacter jejuni enteritis [8]. The host intestinal tract also produces VOCs and have been used to diagnose non-infectious diseases, such as neoplastic and inflammatory processes. There have been several case-control studies utilizing VOCs as a non-invasive biomarker for early detection of colorectal cancer and advanced adenomas [9, 10]. However there was significant variability of technologies used to assess VOCs in these studies [11], and further study is needed to identify specific biomarkers to colorectal cancer that are driving changes in VOCs [12]. Additionally, fecal VOCs have been used for diagnosis of inflammatory bowel disease and irritable bowel syndrome and its response to dietary modification [13, 14].

Given the advancement of VOCs in the diagnosis of gastrointestinal diseases, it is important to consider whether VOC analysis by an eNose device may be used in the diagnosis of necrotizing enterocolitis (NEC), which remains a serious and devastating disease that affects the gastrointestinal tract of the newborn [15]. NEC often presents clinically as abdominal distention, feeding intolerance, and eventual rapid progression to sepsis and surgical intervention requiring resection of necrotic intestine [16, 17]. Despite decades of research, the incidence of NEC remains high at 5–7% of all preterm neonates [18]. Additionally, mortality remains unabated and is high as 42% in very low birth weight infants [19].

While there have been two previous studies that have demonstrated that the eNose device can be used to diagnose NEC, they did not take into account varying patient demographics that could have an impact on VOCs [20, 21]. Prior to the development of a unique “NEC”

VOC signal, it is important to assess whether eNose device analysis will be skewed by certain demographics. We hypothesized that fecal VOC signals produced by the Cyranose® 320 eNose device would vary based upon hospital location, sex, diet, and caloric content in healthy infants. Additionally, we hypothesized that VOC profiles would correlate to the intestinal microbiome and could be used via machine learning to predict microbial enterotype.

Materials and Methods

Patient enrollment

This prospective study was performed from July 2018 to January 2019. This study was approved by our local institutional review board with waiver of informed consent (IRB protocol number 1803568191). Fresh stool was collected from infants in the NICU at four hospitals. Samples from three of the four hospitals were analyzed within one hour of collection. One hospital was located a significant distance away from the site of analysis, and samples from that site were placed on ice prior to being analyzed. Patients eligible for enrollment were term, healthy infants on enteral feeds. Infants who had advanced medical comorbidities or who were on antibiotics were excluded from the study. Demographic data recorded included gestational age, sex, diet (formula, breastmilk, both), caloric content, and hospital location. Caloric density of breastmilk was assumed to be 20 kcal/oz, and caloric density of formula and formula supplements were known and recorded.

VOC Analysis by Electronic Nose

Fresh stool samples were analyzed using the Cyranose® 320 eNose device (Sensigent, Baldwin Park, CA, Figure 1) as previously described [13]. Approximately 1 g of stool was placed in a sealed container and slowly heated to 37 degrees Celsius to enhance VOC signal from the samples and mimic the internal temperature of infants. Researchers were blinded to clinical data when performing analysis. The container was connected to the eNose device using standard oxygen tubing and stopcocks were used to create a closed loop circuit to prevent ambient air from interfering with analysis. Baseline reference signal was created by connecting the eNose device to an organic vapor air filter (Honeywell Part No. 7581P100L). Samples were then analyzed in random order using the Cyranose® 320 eNose device. The Cyranose® 320 uses a NoseChip® array, which contains up to thirty-two polymer nanocomposite carbon sensors. These individual sensors react with VOCs and undergo a change in resistance that is uniquely related to the VOCs biochemical profile, creating a unique “smell-print.” Using advanced pattern recognition, the eNose can be taught to discriminate between samples [22].

16S rRNA Gene Sequencing

Genomic DNA was extracted and the V4 region of the bacterial 16S rRNA gene was amplified using the Shoreline Complete V4 kit (Shoreline Biome). The DNA library was sequenced on an iSeq 100 with 150-bp paired-end reads. Sequences were curated using the mothur software package. Paired-end reads were merged into contigs, screened for sequencing errors, and aligned to the SILVA bacterial SSU reference database. Aligned sequences were screened for chimeras and classified using the Greengenes database.

Samples were binned into community types using kmeans clustering applied to the relative abundances of bacterial families.

Statistical Analyses

All statistical analyses were performed using R (version 3.5.1). Ordinations of smellprints were constructed using Principal Component Analysis. Overall smellprints were associated with clinical variables using a PERMANOVA test as implemented in the adonis function from the vegan R package. Individual sensors were associated with clinical variables using a Mann-Whitney U test for binary variables, Kruskal-Wallis test for categorical variables, and Spearman correlation for continuous variables. Gut community types were associated with individual Cyranose sensors using Kruskal-Wallis tests. The three sensors significantly associated with gut community types were used to construct fast-and-frugal tree models using the FFTrees package in R.

Results

Demographics

A total of 31 infants were enrolled in the study. Demographics of patients are shown in Table 1. Sex distribution of infants enrolled was equivalent across all sites (p=0.832). Infants enrolled at Hospital 4 were older than infants enrolled at other sites (p=0.033). Proportion of infants that received breastmilk vs. exclusive formula was roughly equivalent across all groups (p=0.051).

Diet and Caloric Intake

The overall smellprints were significantly different for infants consuming human breastmilk versus those who were consuming exclusively formula (p=0.032, Figure 2A). Additionally, nine out of twenty-eight individual sensors were significantly different for infants who consumed breastmilk versus formula (Figure 2B). When examining the caloric density of the feeds, overall smellprints did not significantly differ (p=0.22, Figure 2C). Two sensors individually correlated with calories (Figure 2D).

Sex and Hospital of Birth

Overall smellprint analysis by Cyranose® 320 eNose device did not show a significant difference between male and female infants (p=0.3, Figure 2E), however, two individual sensors differed according to infant sex (Figure 2F). Additionally, overall smellprints did not significantly differ for infants based on hospital location of birth (p=0.52, Figure 2G). While a longer amount of time passed between specimen collection and analysis for hospital 1, there was still no significant difference in smellprint between hospital location. Three individual sensors, however, were significantly different based on hospital of birth (Figure 2H).

Microbiome Analysis

Microbiome analysis by 16s rRNA sequencing demonstrated that each infant's microbiome was dominated by one of three distinct families: *Bifidobacteriaceae, Enterobacteriacae, and*

Enterococcaceae. These three families accounted for greater than 75% of all sequences and were observed across different hospital locations (Figure 3A). These observations are consistent with the existence of three infant microbiome enterotypes, which differ greatly from the enterotypes proposed in adults both in terms of the defining bacterial taxa (*Bacteroides*, *Prevotella*, and *Ruminoccus* in adults) and the magnitude of difference between enterotypes [23–25]. Non-metric multidimensional scaling (NMDS) ordination was used to visualize the relationship between microbiomes based on Bray-Curtis dissimilarity of family abundances (Figure 3B). Samples in the ordination clustered into three distinct groups, consistent with the proposed enteroypes. Despite the extreme differences in microbiome composition between enterotypes, they were not significantly associated with any of the infant demographics (Table 2).

Analysis by the Cyranose® 320 eNose device grouped by enterotype showed that three smellprint sensors were significantly associated with certain enterotypes (Figure 4). To further examine the ability of smellprint sensors to differentiate between enterotype, a decision tree model was constructed using the Fast Frugal Trees algorithm (Figure 5). The simple model could differentiate the two most common enterotypes, Enterotype I and Enterotype 2, with 88 percent accuracy using only two sensors, suggesting an electronic nose could be used to rapidly determine the enterotype of an infant's microbiome.

Discussion

Necrotizing enterocolitis is a devastating abdominal disease in infants and to date, there are no adequate biomarkers to aid clinicians in diagnosis. In this study, we found that electronic nose technology could be used to discriminate infants based on certain demographic factors. Overall smellprints differed for infants consuming breastmilk versus those exclusively consuming formula. Additionally, we found that several sensors were affected by variations in calories, the hospital where the infant was born, and by the infant's sex.

There have been two proof of principle studies already completed on whether fecal VOCs can be used to detect NEC. The first study by Garner et al. was a single center study that used frozen stool samples from NICU infants. Stool was heated and VOCs were analyzed by SPME, gas chromatography, and mass spectrometry. This retrospective study demonstrated that a change in VOC profile predated diagnosis of NEC by up to 4 days [20]. The second study was a multicenter study performed in the Netherlands on frozen stool using the same Cyranose® 320 eNose device technology. This study also demonstrated a change in VOC profile predating the diagnosis of NEC when compared to healthy controls [21]. Our study contributes to these findings by highlighting the importance of considering infant demographics when performing fecal VOC analysis. Given that variables such as hospital location and diet can affect eNose sensors, the characteristic "NEC VOC output" may be quite different depending on the geographic location of the patient. Additionally, this same output may be influenced by whether or not the infant is on formula feeds or breastmilk. Adjusting for these variables will be crucial in accurately detecting NEC by VOC analysis, and may involve adjusting the sensitivity of sensors that are affected by these variables, or creating an algorithm that creates a "NEC score" that appropriately weighs the impact of these confounding variables.

Our study found that using a simple decision tree model, the Cyranose® 320 eNose device could reliably differentiate between common stool bacterial communities. These community types, or “enterotypes” represent a distinct stool community type based on relative abundance of bacterial family [23–25]. Distinct enterotypes have been shown to be associated with different pathologies, including obesity and inflammatory bowel disease [26]. Additionally, as our understanding of NEC evolves, it is becoming clear that NEC is preceded by intestinal dysbiosis and there are certain predictable patterns of microbial change that increase the risk of NEC [27–30]. Given that the Cyranose® 320 eNose device can predict certain enterotypes, it may also be able to identify subtle changes in the microbiome that predispose an infant to developing NEC. This potential for early detection of intestinal dysbiosis by VOC analysis is particularly exciting. As this assay is fast and relatively inexpensive, it would allow for earlier cessation of enteral feeds and initiation of antibiotics. Identifying these microbial changes by 16s rRNA sequencing and shotgun metagenomics is often costly and time consuming, which is unfortunately unforgiving in a rapidly evolving and often fatal disease.

A strength of this study is the prospective design that included infants from multiple different centers around the Indianapolis area. Additionally, this study identified major bacterial families in the microbiome of healthy infants by 16s rRNA sequencing and correlated it to fecal VOC analysis. Last, the stool was analyzed fresh and in real time by the Cyranose® 320 eNose device, which closely mimics the proposed translational model where fecal VOCs from a diaper in the NICU could be analyzed quickly by nursing staff to detect NEC.

There are limitations to this study. First, although infants were enrolled from 4 different centers, there were only 31 patients enrolled in the study. Second, the samples were only analyzed in real time and were not analyzed after being frozen. It has been demonstrated that storing fecal samples in different settings can increase variability of fecal VOC analysis [31], and in order to make this study more generalizable and reproducible, it would be helpful to compare fresh versus frozen analysis by the eNose device. Third, for the decision tree model show in Figure 5, there were not enough samples to develop both a validation and training set. Collection of more samples will be necessary to validate the tree model shown in the future. Last, although we are able to establish that different conditions such as consuming breastmilk change the overall smellprint of the eNose device, we are unable to establish *which precise metabolites* caused this change. While the eNose device could theoretically identify a known biomarker in a pure sample, stool from an infant with NEC is heterogenous and has many VOCs, with metabolites both from the host and bacteria, all of which contribute to the smellprint. Concurrent analysis with gas chromatography or stool proteomics to identify and quantify metabolites would be helpful, as it would provide additional insight into the underlying mechanism of why breastmilk and different families of bacteria produce different signals on the polymer naocomposite carbon sensors.

Conclusions

In summary, fecal VOC analysis by the Cyranose® 320 eNose device can be used to predict microbial enterotype and is affected by patient hospital location, sex, and diet. Fecal VOC

analysis by this device is rapid, inexpensive, and may performed at the bedside as a biomarker for the risk of developing NEC. Further studies are needed to design appropriate algorithms that are able to predict NEC based on fecal VOC profiles.

Acknowledgements

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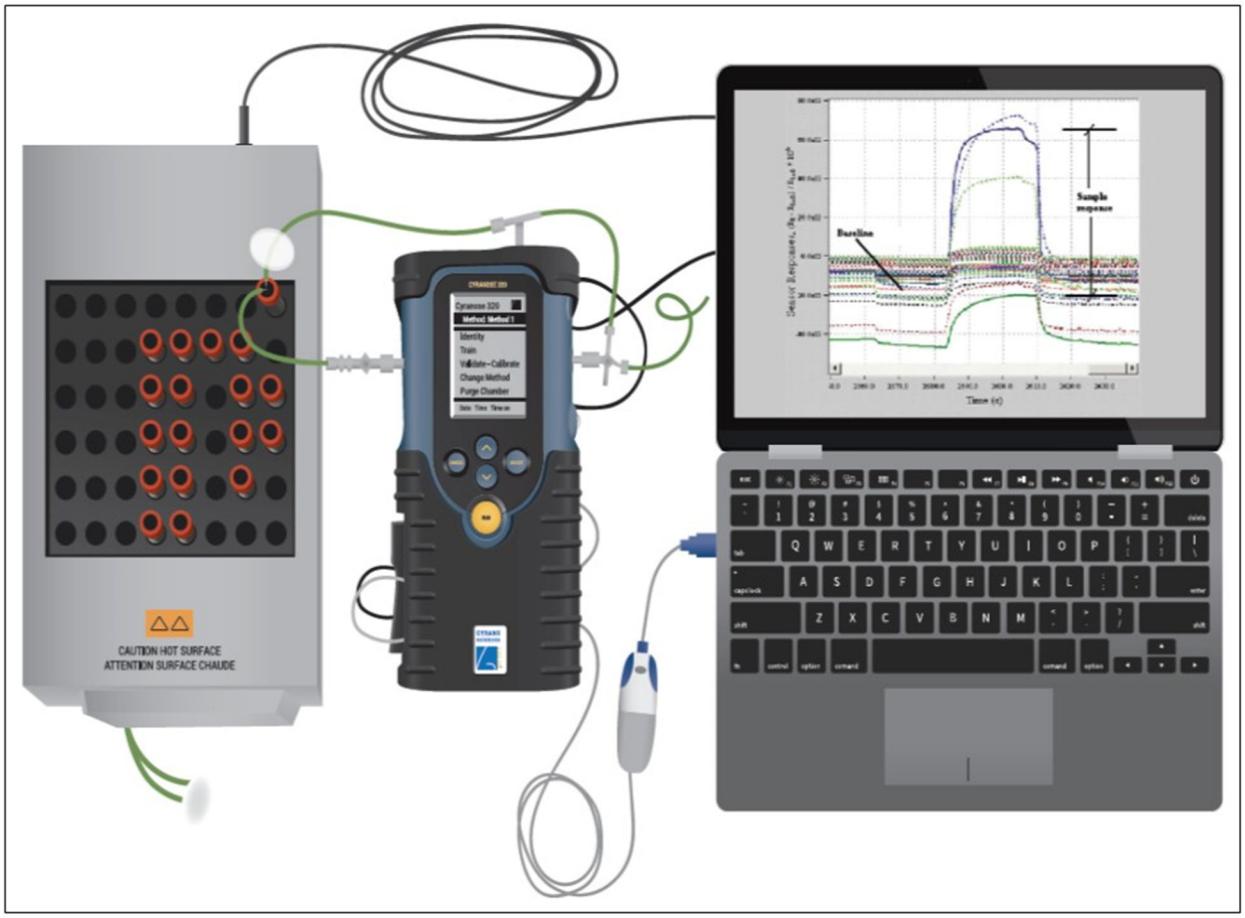

Figure 1.
Cyranose® 320 Electronic Nose device was used to analyze fecal volatile organic compounds from infant stool.

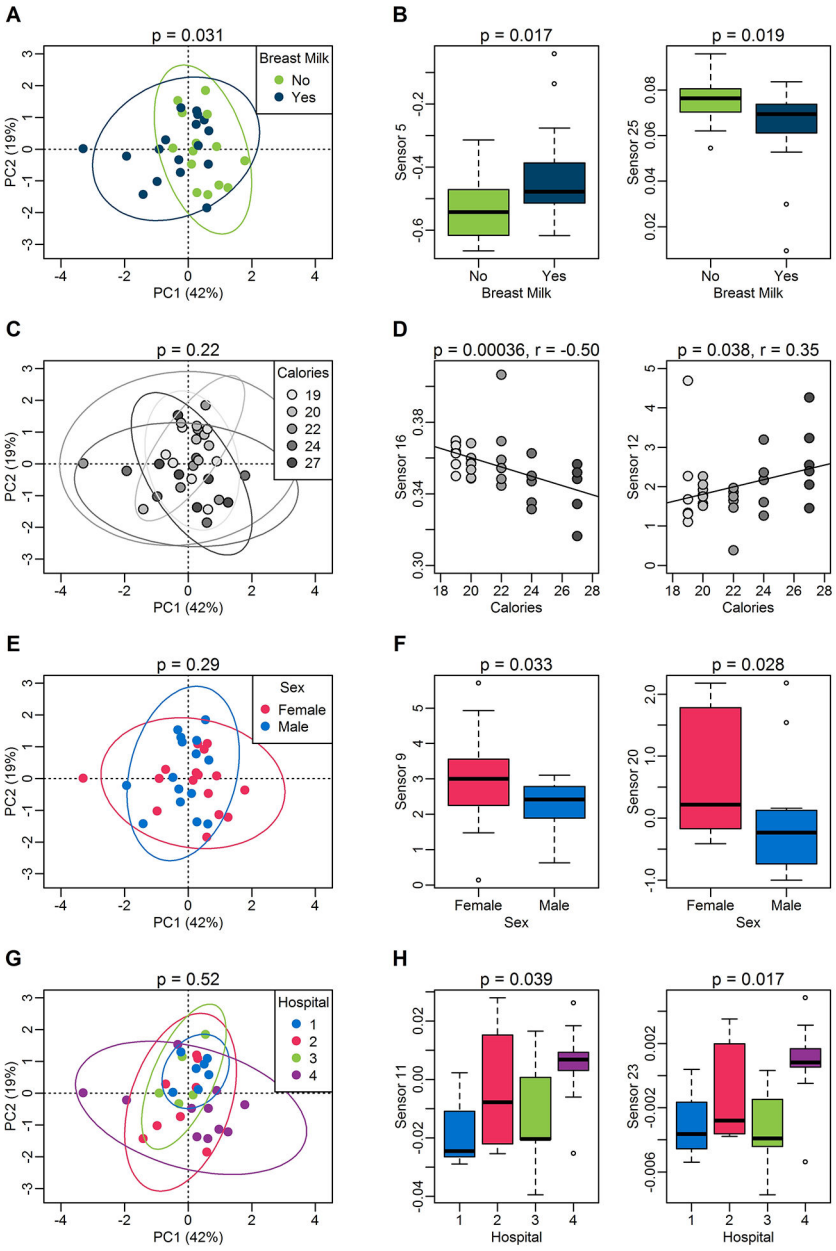

Figure 2.

Differences in fecal VOCs between diet, calories, sex, and hospital location. (A) Overall smellprints were significantly different between infants consuming exclusive human breastmilk (HBM) and infants consuming exclusive formula. (B) Sensor 5 and 25 were the most different. (C) Overall smellprints were not significantly different according to caloric intake. (D) Sensor 16 had the strongest negative correlation, and sensor 12 had the strongest negative correlation between calories. (E) Overall smellprints were not significantly different between male and female infants. (F) Two sensors were significantly different. (G) Overall smellprints were not significantly different between four hospital locations. (H) Sensors 11 and 23 were the most different based on hospital of birth.

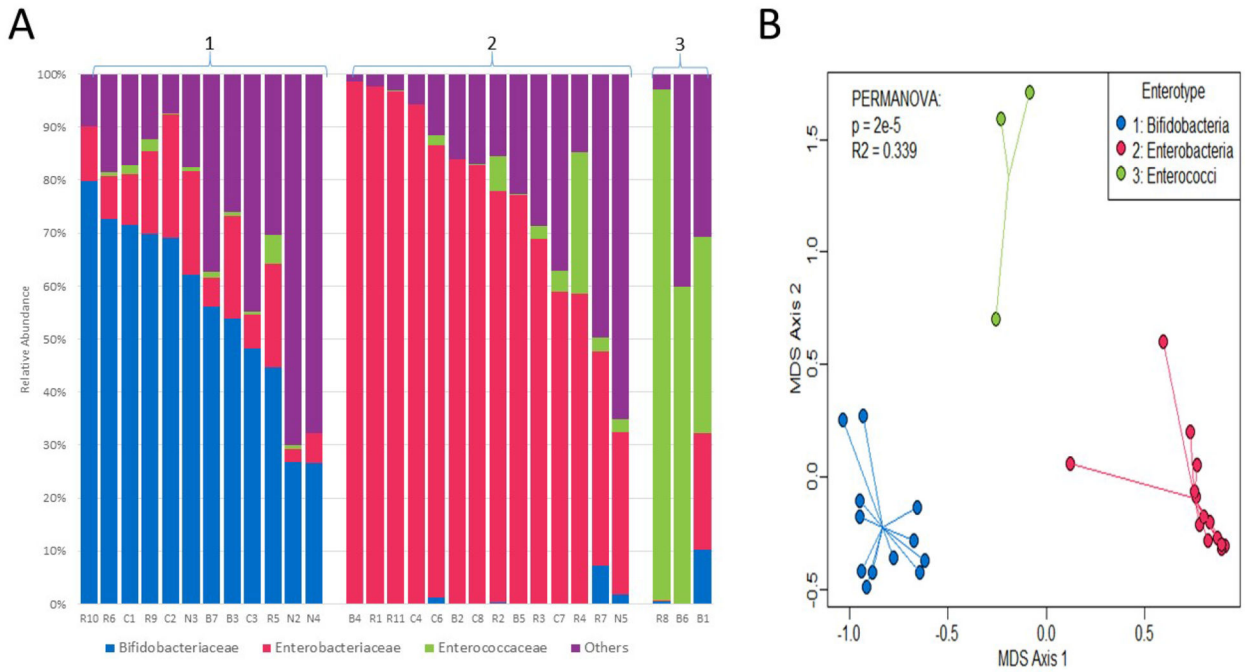

Figure 3.

16s rRNA analysis of stool samples. (A) Relative abundance of bacterial families grouped by enterotype. (B) NMDS Ordinated based on Bray-Curtis dissimilarity of family abundances. The microbiota composition was significantly different between enterotypes (p=0.00002).

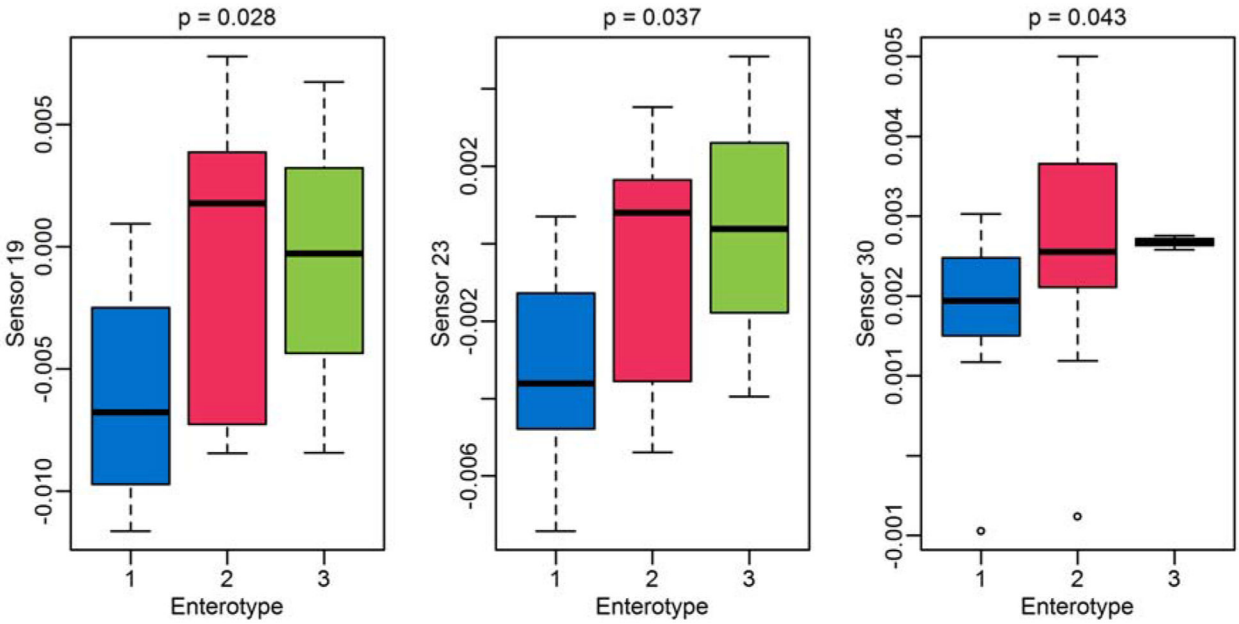

Figure 4.
Three individual sensors were significantly associated with enterotype

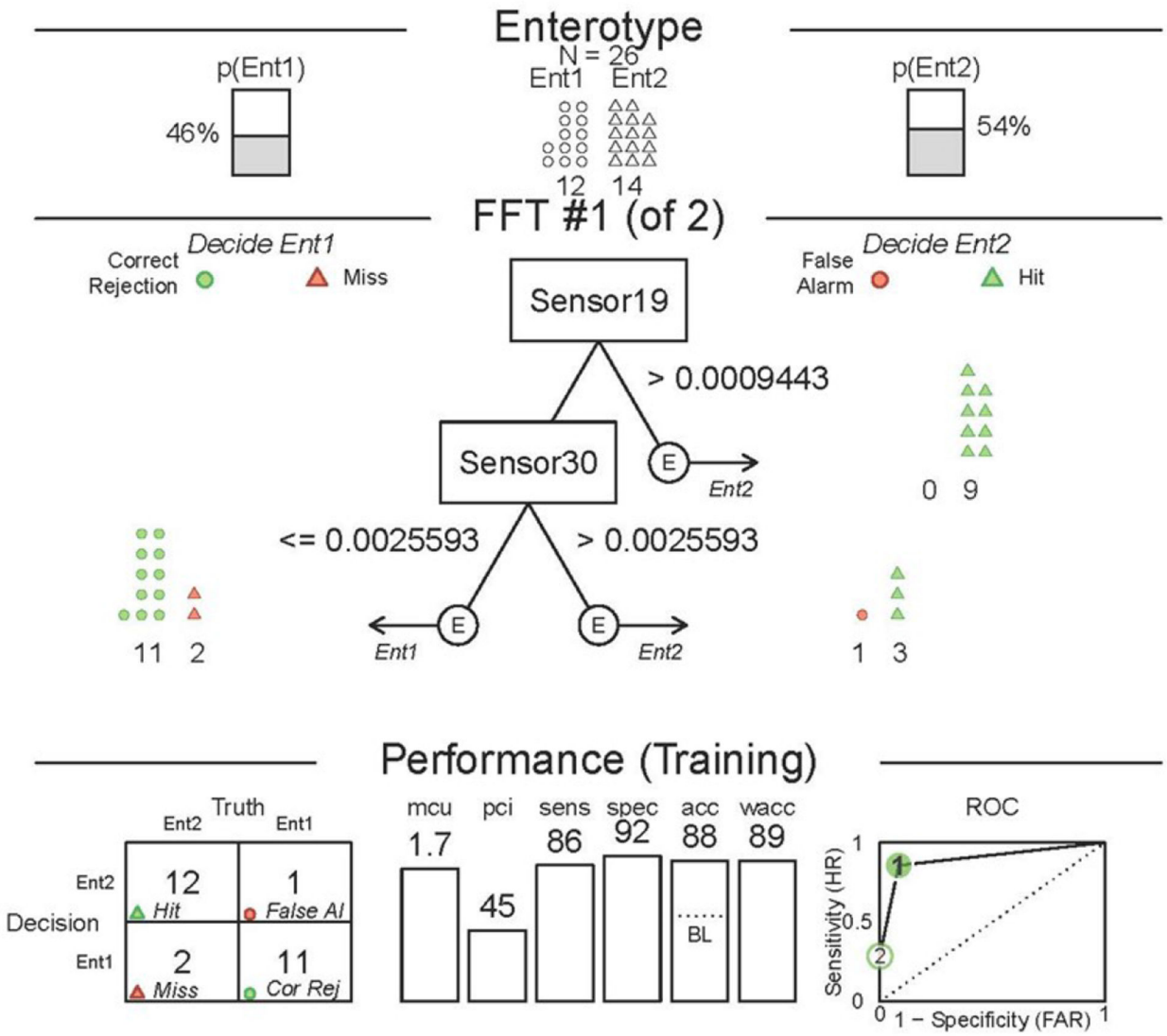

Figure 5.

Decision tree model created by the Fast Frugal Tree algorithm was used to test whether smellprint sensors could be used to predict the two most common enterotypes. The top panel shows the training set population, which consisted of 12 samples with microbiomes belonging to Enterotype 1 (squares) and 14 from Enterotype 2 (triangles). The middle panel shows the structure of the decision tree, which splits samples based on the readings from two smell print sensors. Green shapes indicate correct classifications, while red shapes indicate erroneous classifications. The bottom panel summarizes the performance of the model using a variety of metrics, including the error matrix, sensitivity, specificity, accuracy, and a receiver operator characteristic (ROC) curve.

Table 1.

Demographic data of infants included in the study

Hospital # (n)	**Hospital 1 (7)**	**Hospital 2 (8)**	**Hospital 3 (5)**	**Hospital 4 (11)**	**p-value**
Sex					
Male (n[%])	4 [57]	3 [38]	3 [60]	5 [45]	0.832
Gestational age (median [IQR], wk + d	39+0 [36+4 – 41+4]	39+0 [38+1 – 39+5]	37+1 [36+2 – 37+5]	42+0 [38+3 – 45+0]	0.033
Feeding pattern (n[%])					
Breast Milk +/− Formula	5 [71]	7 [88]	2 [40]	3 [27]	0.051
Exclusive Formula	2 [29]	1 [12]	3 [60]	8 [73]	0.051

Table 2.

Enterotype association with patient demographics

Metadata	p-value
Diet[1]	0.21
Calories[2]	0.58
Sex[1]	0.63
Hospital[1]	0.48
Gestational Age[2]	0.97

[1]Chi-Squared Test

[2]ANOVA