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ARTICLE

Metabolomic Screening of Fecal Samples as an Alternative Method to Initial Clinical Trials of Allergy to Cow's Milk Protein on Infants

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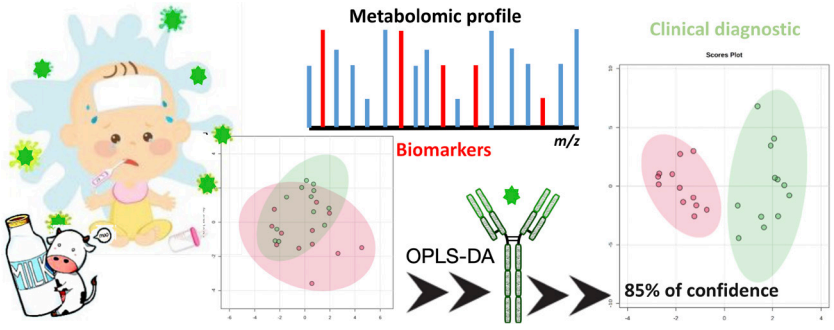

Cow's Milk Protein Allergy (CMPA) is one of the most recurrent pediatric conditions of food allergies, which occur in early childhood. The diagnosis is not easy and demands oral food challenge tests (OFC). 24 metabolomic profiles of fecal samples from infants suspected of CMPA were assessed while searching for a possible intestinal metabolite that can be used as a biomarker of CMPA. The children were previously diagnosed with an open OFC. Feces samples were extracted and directly analyzed by ultra-high resolution mass spectrometry (HRMS) using an FT-Orbitrap mass spectrometer. Metabolomic profiles were initially treated by principal component analysis (PCA) which was not efficient in distinguishing the samples to propose a diagnosis. Then, the metabolomic profile of a specific *m/z* range obtained in the negative mode of analysis was successfully subjected to orthogonal partial least squares discriminant analysis (OPLS-DA) which separated the two groups with and without CMPA. The model fit was $R^2 = 0.88$, with a predictive capability of $Q^2 = 0.52$ in the test with 2000 permutations and significance p-value <0.05. In this preliminary study, the model obtained using the metabolomic profile showed significant validation values, indicating the potential to distinguish between the two groups of interest, suggesting its use as a possible diagnostic tool for CMPA patients.

Keywords: allergy, cow's milk, metabolomics, microbiota

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INTRODUCTION

The intestinal microbiota comprises a broad and dynamic ecosystem of microorganisms of distinct and complex species, responsible for establishing a mutually beneficial relation with the host, performing fundamental functions in communication, modulation and maintenance of metabolism and the immune system.[1,2] The development of the microbiota occurs rather simply in the first 3 years of life with a rapid increase in its diversity, making the microbiota more complex and more stable during adulthood.[3] The compositional pattern exhibited in early life colonization of the intestine has a lasting impact on the microbiota and largely contributes to the individual's relation between intestinal health and disease (dysbiosis).[4] Currently, the intestinal microbiota and its metabolite products generated by symbiotic bacteria in the intestine have been shown to have a potential effect on intestinal pathogenesis and food allergy, generating an immunological response during exposure to these antigens in the process of intestinal dysbiosis.[4] This can result in the interruption of mucosal immunological tolerance and trigger allergic and inflammatory processes.[5–7]

Cow's Milk Protein Allergy (CMPA) is one of the most recurring and early pediatric conditions of reproducible food allergies (FA), which occurs in early childhood.[8,9] It is linked with the breakdown of immunological tolerance to food antigens, causing significant impairment in the quality of life. It currently has an estimated prevalence of 2% to 3%, and with a significant global increase.[8,9] In CMPA, the most common clinical manifestations are gastrointestinal, skin, and respiratory symptoms, which may include abdominal pain, bloody diarrhea, abdominal distension, and vomiting, and reaching the most severe symptoms such as anaphylaxis.[10]

Diagnostic investigation and treatment have become a challenge due to the degree of difficulty in the accuracy and reliability of results.[11] CMPA has a negative impact on the quality of life of babies and their families, affecting health conditions and financial situation.[12] Currently, the diagnosis is made based on clinical history, laboratory tests, the measurement of specific IgE to the suspected food *in vivo* and *in vitro* and oral food challenge test (OFC).[13] Double-blind, placebo-controlled oral food challenge (DBPCOFC) is the gold standard for confirming the diagnosis of CMPA.[14] Open OFC is easier to perform and, therefore, remains the most used in clinical practice, especially in children under 3 years old.[15] However, an adequate hospital environment with trained healthcare professionals is necessary, due to the risk of some serious reactions such as anaphylaxis.[16]

Metabolomics emerges as an alternative for diagnosing and staging diseases in a safe way. Through the analysis of biofluids, such as serum or feces, metabolic changes in response to internal and external stimuli in biological systems can be characterized and investigated.[17] Microbial metabolites have a signature that allow us to identify and broadly measure the metabolic activities of the system, which are important information about the host's health status and the interactions produced microbially.[18]

The analytical strategy used in the analyzes depends mostly on the type of sample and the focus of the study. Mass spectrometry (MS) has been a high-performance analytical technique widely used in the identification of small molecules from biological body fluids in the diagnosis and treatment of diseases[19] such as asthma, allergic rhinitis, immunodeficiencies and food allergies.[20]

Applying the metabolomic study, this work aims to analyze the profile of intestinal metabolites in the fecal samples from symptomatic infants with and without CMPA, evaluated by OFC, and propose a diagnostic screening model using ultra-high resolution mass spectrometry (HRMS) using a FT-Orbitrap mass spectrometer for earlier and safety diagnosis of CMPA.

MATERIALS AND METHODS

This investigation was submitted and approved by the ethics committee of the Federal University of Sergipe under number CAAE 92776518.3.0000.5546 and Decision number: 2,778,235 on July 20, 2018, for the prospective phase of data collection and by the PPGCS- Postgraduate Program in Health Sciences - UFS. The inclusion occurred upon signature of the Free and Informed Consent Form (TCLE) by parents or legal guardians. They received explanations about the study's objectives, as well as the risks and benefits for eligible children.

Type of study

This is an analytical cohort study of a comparative nature. Fecal samples were obtained from infants between zero and six months old, who showed symptoms of gastrointestinal disorders, which led to the suspicion of CMPA. The collections were performed during the appointments at the Reference Center for Food Allergy Center of Sergipe (RCFAS), Federal University of Sergipe (in consultations and OFCs), as well as through active search during the period of the SARS-Covid -19 pandemic. 24 samples were used and divided into two groups: with and without CMPA (CMPA and w/o CMPA), in order to obtain the diagnosis through OFC.

Sample collection

Fecal samples were collected at the hospital prior to the OFC whenever possible or by the parents, who were given a kit containing spatulas, sterile containers, and a sheet with instructions on storage and immediate transportation in a thermal box containing Gelox®. Each fecal sample was immediately sent to the laboratory, where an aliquot of approximately 329 mg taken and subsequently stored in Eppendorfs containing 750 µL of phosphate-buffered saline (PBS). All samples were identified with the research participant's code and then refrigerated in a freezer at -80 °C until extraction and metabolomic analysis.

Preparation and extraction of fecal metabolites

Sample preparation and extraction were adapted as described in the previous study.[21] To separate microbial cells from the fecal matrix, the sample was thawed and 1,000 µL of (PBS) was added, homogenized by vortexing for 1 min, and placed in a centrifuge at 1,000 rpm for 1 min. Then, 600 µL of supernatant was transferred to a 2 mL Eppendorf, centrifuged again at 13,000 rpm for 5 min, and the supernatant was removed. For the methanol (MeOH) extract, 1,200 µL of MeOH conditioned at -80 ºC was added to the supernatant and vortexed for 10 seconds. It was then sonicated for 30 s at 15 W (in ice water at 25 ºC and repeated 2 more cycles with 5 min storage on ice between cycles), centrifuged again at 12,000 rpm for 10 min and the supernatant (1,000 µL) was stored at -80 ºC. For the H_2O extract, the final pellet was resuspended in 1,200 µL of Ultra-pure HPLC grade H_2O at 4 ºC, sonicated for 20 s at 15 W (in ice water at 25 ºC for 3 cycles, with 2 min incubation on ice between cycles) and then centrifuged at 12,000 rpm for 10 min at 4 ºC, removing the supernatant (1,000 µL). For protein precipitation: 1,000 µL of MeOH extract was added to 1,000 µL of H_2O extract and 20 µL of this was removed and added to an Eppendorf containing 200 µL of acetonitrile, and then centrifuged to remove possible impurities, at 13,000 rpm for 10 min and remove the supernatants. Using a 0.2 µm nylon syringe filter, 200 µL was transferred to the analytical vial for extract analysis.

Preparation of quality controls (QCs) and carryover verification

A blank sample was prepared using the same conditions and reagents as the samples mentioned above for quality control (QC) over the 3 days of the extraction procedure, to exclude any possible contaminants that may appear during the analysis.[21] In this way, the potential carryover was assessed through the injection of blank QC samples, excluding the possibility of any impurities or cross-contamination during the analysis. Mass spectra were acquired in the range of *m/z* 100 to 1000 with the accumulation of 100 micro scans and a resolution of 140,000 FWHM *(m/z* 200). The final average mass spectrum from the accumulated spectrum was obtained by subtracting the blank spectrum from the sample mass spectrum, guaranteeing the homogeneity and accuracy of the data.

Analysis of extracts by HRMS with FT-Orbitrap

The samples were analyzed by direct infusion, without prior dilution, using an Exactive HCD Plus system (Thermo Scientific, Bremen, Germany) equipped with an Ion Max API ionization source with HESI probe (Heated Electrospray Ionization – HESI) using a syringe pump model Fusion 101 (Chemyx, Stafford, TX) with a 500 µL syringe (Thermo Scientific, NJ, USA) at a flow rate of 10 µL min^{-1}. The analysis conditions

used in HESI(+) mode were: spray voltage 3.0 kV, heating of the vaporization region 50 ºC, capillary temperature 300 ºC, sheath gas 15 ua (arbitrary unit), auxiliary gas 0 ua and swep gas 5 ua. In HESI(-) mode, the conditions used were: spray voltage 3.5 kV, heating of the vaporization region 50 ºC, capillary temperature 300 ºC, sheath gas 15 ua, auxiliary gas 10 ua and swep gas 5 ua.

The data was processed using Xcalibur 3.0.63 software (Thermo-Fisher Scientific, Inc.). The molecular formula assignments for the ions were only considered valid when the mass error between the experimental and theoretical *m/z* values was less than or equal to 3 ppm, the list of ions was exported to then perform the statistical treatment and generate a prediction model.

Data analysis

24 mass spectra were obtained in HESI(+) mode and 24 in HESI(-) mode. The 24 samples in each mode were divided into two classes and named according to OFC (+ or -), in groups with CMPA and without-CMPA. The data was imported into MATLAB and a matrix was constructed for each ionization mode. The variables consisted of the absolute intensities of the ions.

The Metaboanalyst 5.0 platform was used for data analysis. The data went through some pre-processing steps: filtering missing values, excluding variables that had at least 20% of zeros; normalization by sum and logarithmic transformation. Then, using statistical tools, the following was performed: Principal Components Analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA), and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA).

A first analysis (PCA and PLS-DA) was performed using the complete matrix for both positive (24 x 22003) and negative (24 x 16113) modes. Next, two new matrices were generated, now with the 50 most relevant variables of projection importance values (VIP) in PLS-DA, and, from these matrices, all ions with a charge mass ratio of up to *m/z* 400 were considered. Finally, the negative mode matrix had 34 variables, while the positive mode had 31, so PCA and OPLS-DA, presented in this work, were performed. The variables with VIP values >1 underwent metabolite assignment using the CEU Mass Mediator online platform.

RESULTS AND DISCUSSIONS

Evidence indicates that the intestinal microbiota is closely linked with the rate of allergic reactions. Understanding the impacts caused by early microbial exposures during the development of CMPA and microbiota-host interactions that can be elucidated by “omics” sciences.[19] In this study, we propose a diagnostic model through undirected metabolomic analysis, in fecal samples from symptomatic infants with OFC+ and OFC-, then diagnosed as with and without CMPA respectively. Metabolomics has currently become an important tool in identifying potential biomarkers for the diagnosis of diseases.[21] Considering that the current diagnostic method, the OFC for CMPA, has operational difficulties and possible reactions during the evaluation,[22] alternative methods that can make this differentiation are very welcome. Extracts of fecal samples from 24 infants (between 0 and 6 months of age) who were symptomatic for CMPA were analyzed (12 diagnosed with CMPA and 12 without CMPA) by a high-resolution mass spectrometry by direct infusion, using the HESI ionization source in the positive and negative modes of analysis.

From the principal components analysis in Figure 1 (a) and (b), both positive and negative mode data show a tendency to group in the classes of interest (with CMPA and without-CMPA). In negative mode, this is observed by the samples’ projection on PC 2 and PC 3, which explain the 31.2% of the dataset variance. In the case of the positive mode, it is observed on PC 3 and PC 5, explaining 14.7% of the variance. Considering the maximum of eight principal components, around 88.3% of the total variance was explained in the negative mode and 85.3% in the positive mode (Figures S1 and S2 – supplementary material).

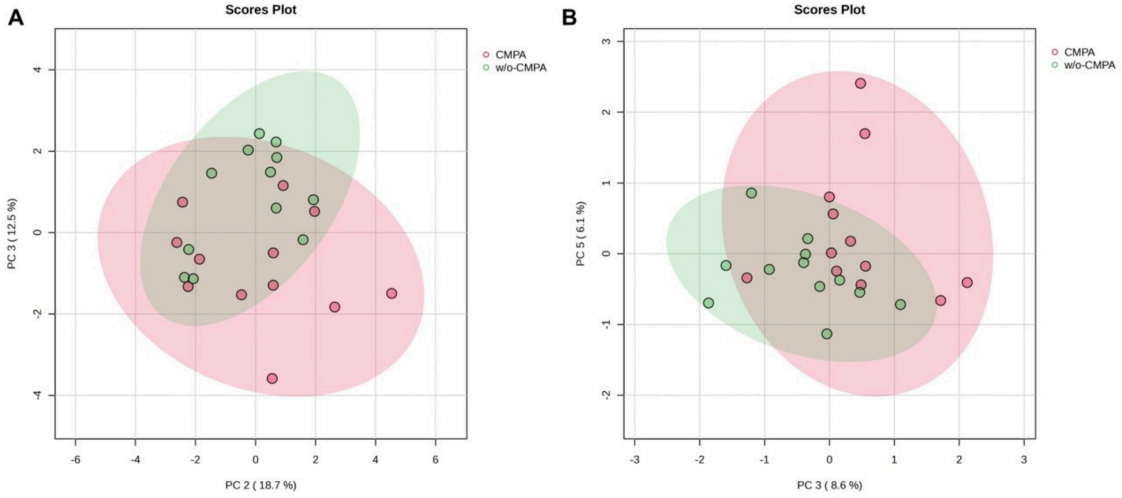

Figure 1. Graph of sample scores of the principal components (PC), HESI reading mode A) negative PC2 and PC3, B) positive PC3 and PC5. Explained variations are shown in parentheses.

In general, our analyses of the fecal extract samples revealed a significant variability in the mass spectra profiles, which can be attributed to factors such as microbial diversity, compositional changes during dysbiosis, dietary patterns, and medication use, among other unaccounted variables. However, this variability underscores the richness of fecal material as a matrix for exploring host health and its interactions with the microbiome.[23] To address this, we have meticulously standardized pre-analytical conditions including sample collection, storage, and preparation, recognizing the sensitivity of the employed technique. By maintaining rigorous standards throughout these steps, we aimed to minimize variability and reduce potential biases, ensuring the robustness and reliability of our findings.[23]

Through the model built with OPLS-DA (Figure 2), it was possible to obtain a considerable number of metabolites capable of differentiating both groups of interest, with CMPA and without-CMPA, in the diagnostic screening between samples. The model presented an acceptable predictive ability with Q^2 significant values obtained when it comes to diagnosing CMPA. Nevertheless, no work was found regarding the metabolomics approach applied to the investigation of CMPA in infants, and we could only find works about FA in general.[24]

The models built with OPLS-DA were able to distinguish samples from patients with CMPA and without CMPA, as shown in Figure 2 (A) and (B). The negative mode model presented better results, presenting a better fit to the data (R^2 = 0,882) than the positive mode model (R^2 = 0,874), Figure 2 (C) and (D). The models' Q^2 values indicate satisfactory predictive ability (0.523) in negative mode, whereas the value was below zero in positive mode. Therefore, the positive model is not predictive, and its results were not further interpreted.

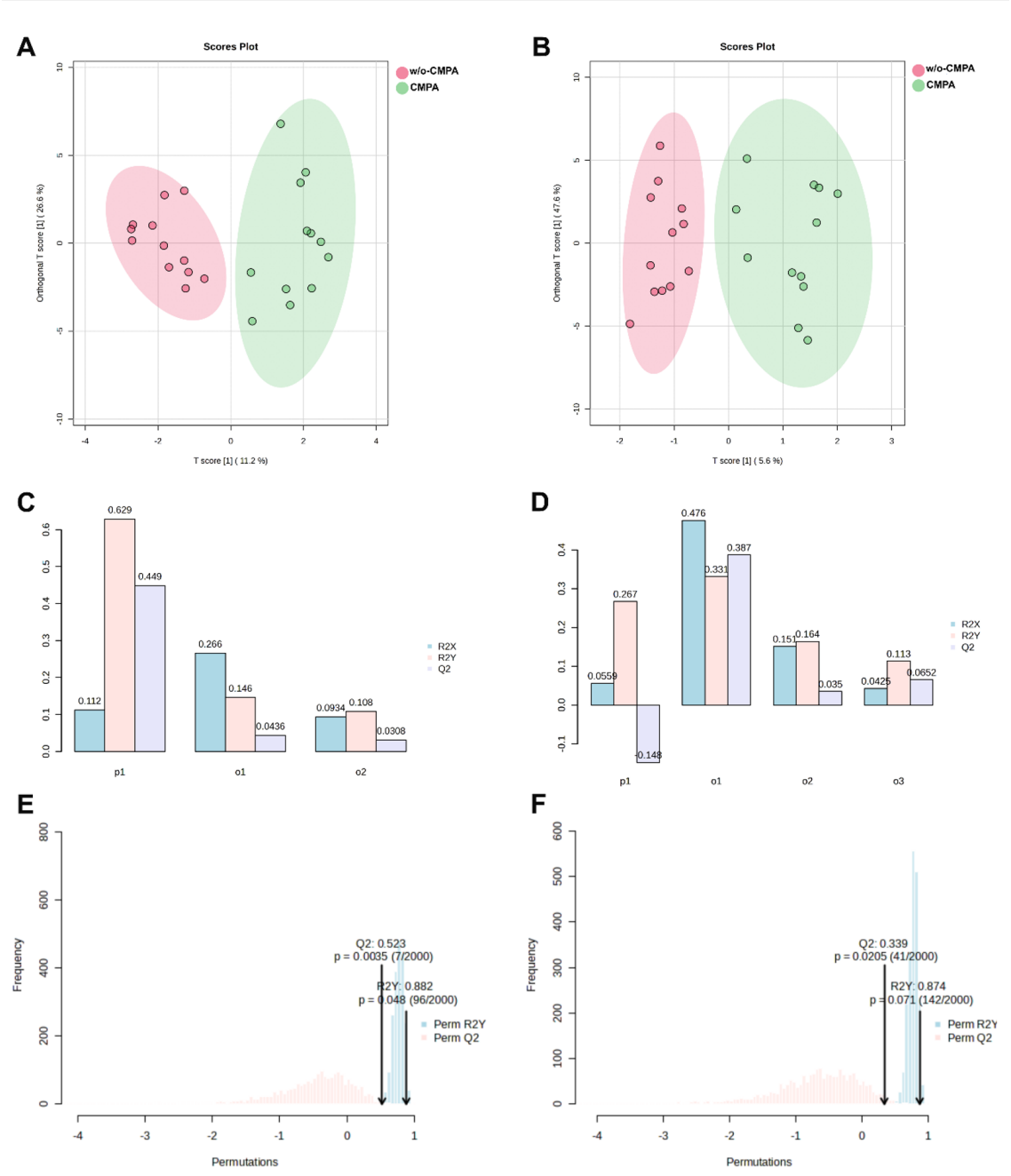

Figure 2. OPLS-DA results. A) Score Plot in negative mode; B) Score Plot in positive mode; Model performance estimated with leave-one-out cross-validation; C) negative mode; D) positive mode; E) Permutation test in negative mode with 2000 permutations, showing p-value = 0.0035 for Q^2 and p-value = 0.048 for R^2; F) Permutation test in positive mode with 2000 permutations, showing p-value = 0.0205 for Q^2 and p-value = 0.071 for R^2.

The test with 2000 permutations, Figures 2 (E) and (F), to evaluate the statistical significance of the model, presented a p-value <0.05 for both R^2 and Q^2 in negative mode, providing reliability to the proposed model. Therefore, the modeling results using OPLS-DA indicate that when using MS data in positive mode, a low predictive capacity occurred, as evidenced by the obtained Q^2 values. Hence the reason for using the results obtained with MS data in the negative mode.

Table I presents the confusion matrix, and the figures of merit were calculated. Sensitivity, specificity, accuracy, and positive and negative predictive values are 85.7%, 100%, 91.7%, 100%, and 83.3%, respectively. These values confirm the robustness of the model and its ability to correctly classify the samples and distinguish between the classes investigated. Furthermore, these results are consistent with literature.[14,24]

Table I. Confusion matrix – OPLS-DA model

	CMPA	w/o-CMPA
CMPA	12	0
w/o-CMPA	2	10

Even so, in the work of Li, et al. (2018)[14] to demonstrate a better performance in the diagnosis of FA, it was obtained a predictive capacity between 0.800 – 0.957, while the model adjustment was 92.6% and specificity of 72.9%, for the discrimination for CMA and without-CMA of better accuracy in infants and young children using other mathematical models. In the work of Laha et al. (2022)[24] performed in India using the Klemans model, they also obtained a high level of sensitivity, with a predictive capacity of 97% and an accuracy of 98% for milk allergy.

From the OPLS-DA model, the variables with higher VIP values have the greatest weight in the construction of the model, therefore, corresponding to the ions of the metabolites that are crucial for the distinction of the classes of interest. In the HESI(-) reading mode (Figure 3), we obtained a total of 17 relevant ions. Among them, four ions with greater intensity in the group with CMPA and 13 ions with greater intensity in the group without CMPA (Table SI).

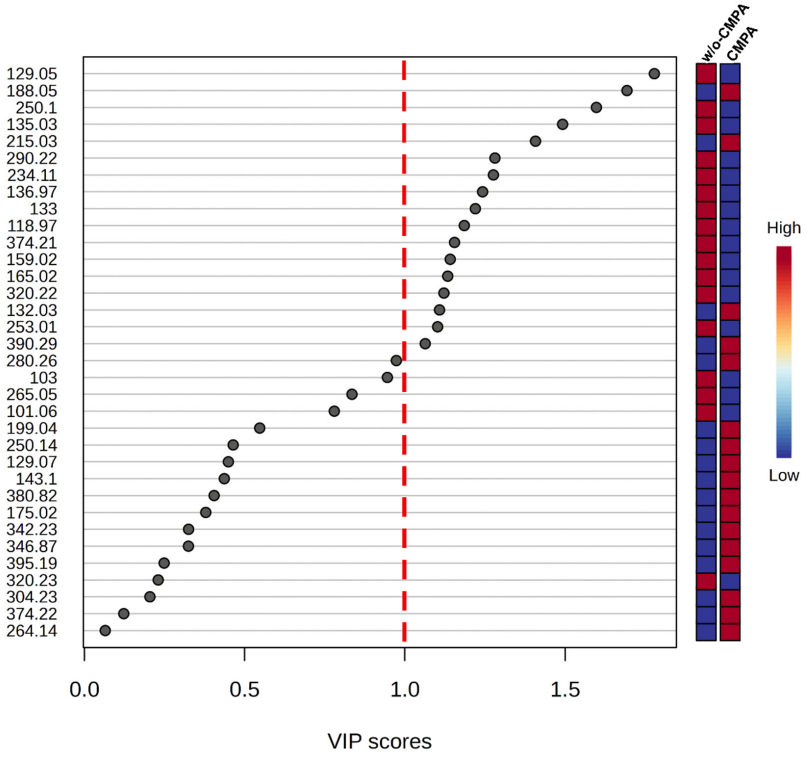

Figure 3. Variables that are most important in the OPLS-DA model for the HESI negative mode analysis mode.

Furthermore, in the findings of this study, through the analysis of VIPs (Figure 3), it was possible to identify significant differences in the metabolites that make up and differentiate the two classes of interest for the diagnosis of CMPA through fecal samples, highlighting ions with greater weight in the model construction and greater intensity that are responsible for discriminating the investigated classes.

The ion with *m/z* 280.264, belonging to the CMPA group, have been assigned to the molecular formula $C_{18}H_{34}O_1N_1$. In its neutral state, this ion has the formula $C_{18}H_{35}O_1N_1$, with proposed identification as oleamide, a fatty acid amide originating from oleic acid and ammonia established by the enzymatic amidation of bacteria.[25] Reported in previous studies of different pathologies as a possible anti-inflammatory site with non-specific location, and a response pattern not yet explored, also present in dairy products, with an important connection with the intestinal microbiota.[26–28] In the VIP score, the value linked with Oleamide was very close to 1. Even below this threshold, this metabolite plays an important role in discriminating between classes, highlighting its relevance in the analyzed context.

Previous studies point to the mediation of metabolites, such as SCFAs (acetate, propionate, and butyrate) and metabolite byproducts arising from the fermentation of dietary fibers not digested by bacteria, as responsible for modulating cell proliferation, histone acetylation, gene expression, all considered immunomodulators and allergy protectors in the intestinal microbiota.[29–31] As well as valeric acid and the balance of Clostridium species, which are attributed to the health pattern of the intestinal microbiota.[31] It was observed that without-CMA infants do present an increase in the production of butyrate from Clostridium strains present in fecal samples, pointing to a strong correlation as a biomarker of protection for food allergies.[32] The complexity surrounding the intestinal microbiota emphasizes the importance of developing methods that can identify and characterize its compositional pattern and its relationship with CMPA.

The metabolite profile obtained exhibited significant values in the validation of the model, and it is possible to discriminate the classes investigated with CMPA and without-CMPA, presenting distinct characteristics that distinguish both groups. The negative mode presented the most robust preliminary model in the identification of metabolite ions in the diagnostic screening of infants suggestive of CMPA, with the possibility of improving its adjustment by increasing the number of samples.

Even though this study presented a model with significant values in cross- validation, and that it is possible to discriminate an individual with symptomatic CMPA from another without CMPA, the spectra obtained did not show homogeneity between classes, which made the entire process of construction of models from the data pre-processing stage difficult. Considering that they are samples with similar behavior, we expected that the spectra related to each class would have a similar profile, which was not observed. Another limitation found was the identification of metabolite ions on the available platforms, which hindered broad comparison and their imputation as biomarkers for CMPA.

We have strong evidence that fecal metabolomic analysis can help identify patients at risk of CMPA in a timely and safe manner. While this study does not yet establish a definitive diagnostic tool, the results suggest that this approach could reduce adverse events during the diagnostic process with current methods, such as exposure to allergenic antigens. Furthermore, it could overcome the limitations of conventional methods, promoting a more efficient and less invasive diagnosis for infants suspected of having CMPA.

CONCLUSIONS

As a preliminary study, the model using untargeted metabolomic data corroborates and presents itself as a possible safe tool for the diagnosis of patients with CMPA. This may overcome and even replace the current diagnostic method, the OFC, which requires direct exposure to the antigen and specific care for potential clinical reactions that may be manifested. Furthermore, the profile of metabolites found in fecal samples is highly diverse and has distinct characteristics, which can be differentiated and identified as possible biomarkers of this pathology.

Conflicts of interest

The authors declare that there is no conflict of interest regarding financial or potential sources of bias such as affiliations, funding sources and financial or management relationships which may constitute a conflict of interest.

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SUPPLEMENTARY MATERIAL

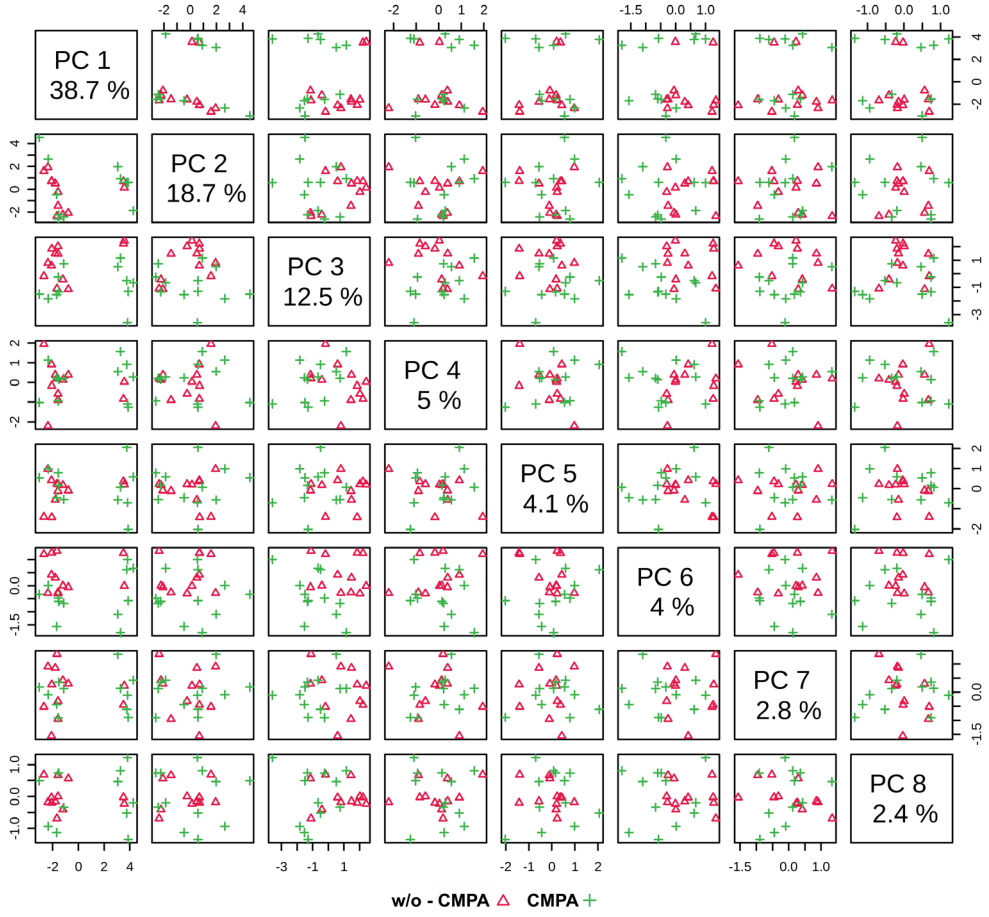

Figure S1. PCA Negative mode.

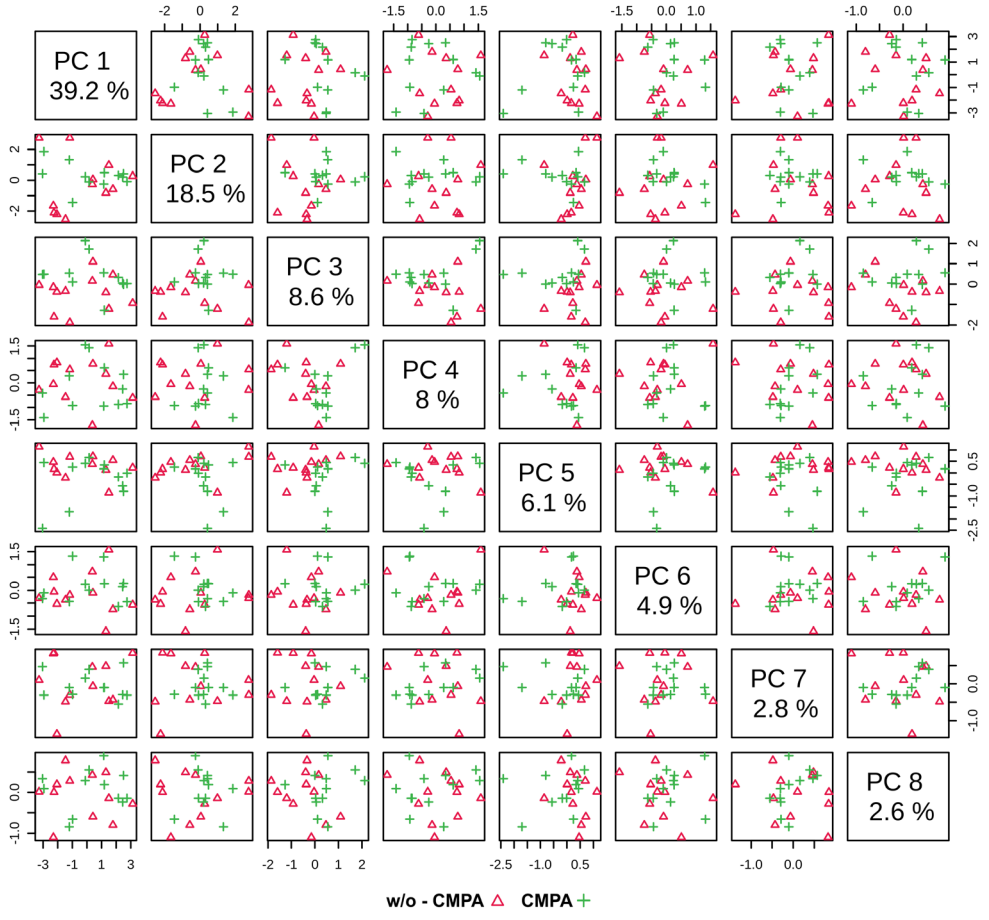

Figure S2. PCA Positive mode.

Table S1. VIP score OPLS-DA HESI Negative mode

R *m/z**	*m/z*	Attribution	Chemical Formula	Analysis Mode	With/Without CMPA
188.05	188.05636		$C_7 H_{10} O_5 N_1$	Negative	With
215.03	215.03217			Negative	With
132.03	132.03989			Negative	With
390.29	390.29075			Negative	With
280.26	280.26438	Oleamide	$C_{18}H_{35}O_1N_1$	Negative	With
190.05	190.05287			Negative	Without
250.10	250.09617			Negative	Without
135.03	135.03467			Negative	Without
290.22	290.22000			Negative	Without

(continued on next page)

Table S1. VIP score OPLS-DA HESI Negative mode (continuation)

R *m/z**	*m/z*	Attribution	Chemical Formula	Analysis Mode	With/Without CMPA
234.11	234.11520			Negative	Without
136.97	136.95691			Negative	Without
133,00	133.00440		$C_6 H_2 O_2 N_2$	Negative	Without
118.97	118.95718			Negative	Without
374.21	374.22065		$C_{19}H_{29}O_3N_5$	Negative	Without
159.02	159.02737		$C_{10} H_7 S_1$	Negative	Without
165.02	165.02088			Negative	Without
320.22	320.22358		$C_{19} H_{30} O_3 N_1$	Negative	Without
253.01	253.01457		$C_{14} H_5 O_5$	Negative	Without
103.00	103.01300			Negative	Without
Total		**19**			

*Rounded *m/z* values for statistical data analysis.