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RESEARCH

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Pediatric migraine is characterized by traits of ecological and metabolic dysbiosis and inflammation

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Abstract

Background Recently, there has been increasing interest in the possible role of the gut microbiota (GM) in the onset of migraine. Our aim was to verify whether bacterial populations associated with intestinal dysbiosis are found in pediatric patients with migraine. We looked for which metabolic pathways, these bacteria were involved and whether they might be associated with gut inflammation and increased intestinal permeability.

Methods Patients aged between 6 and 17 years were recruited. The GM profiling was performed by the 16S rRNA metataxonomics of faecal samples from 98 patients with migraine and 98 healthy subjects. Alpha and beta diversity analyses and multivariate and univariate analyses were applied to compare the gut microbiota profiles between the two group. To predict functional metabolic pathways, we used phylogenetic analysis of communities. The level of indican in urine was analyzed to investigate the presence of metabolic dysbiosis. To assess gut inflammation, increased intestinal permeability and the mucosal immune activation, we measured the plasmatic levels of lipopolysaccharide, occludin and IgA, respectively.

Results The α-diversity analysis revealed a significant increase of bacterial richness in the migraine group. The β-diversity analysis showed significant differences between the two groups indicating gut dysbiosis in patients with migraine. Thirty-seven metabolic pathways were increased in the migraine group, which includes changes in tryptophan and phenylalanine metabolism. The presence of metabolic dysbiosis was confirmed by the increased level of indican in urine. Increased levels of plasmatic occludin and IgA indicated the presence of intestinal permeability and mucosal immune activation. The plasmatic LPS levels showed a low intestinal inflammation in patients with migraine.

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Conclusions Pediatric patients with migraine present GM profiles different from healthy subjects, associated with metabolic pathways important in migraine.

Keywords Gut microbiota; migraine; tryptophan, Occludin, Lipopolysaccharide, Indican, IgA, Dysbiosis, Inflammation, Gut permeability

Introduction

Migraine affects approximately 10% of the pediatric population and can significantly interfere with the child's activities and worsen the quality of life of patients and their family [1]. Migraine causes intense pain typically accompanied by nausea and/or vomiting, photophobia and phonophobia (ICHD-3 criteria) [25] and some subjects may be resistant to available pharmacological treatments [56].

Though still incompletely known, migraine pathophysiology involves inherited alteration of brain excitability, vascular phenomena and sensitization of the trigemino-vascular system (TVS), and consequential structural and functional changes in genetically susceptible subjects [54]. The identification of new therapeutic targets may be aided by a greater understanding of migraine pathophysiological mechanisms.

Recently, scientific interest has been raised by the possibility of a link between gut microbiota (GM) and migraine. The bidirectional communication between brain and gut is commonly referred as microbiome-gut-brain (MGB) axis [55, 63]. The connection is achieved through a complex network involving the nervous system (vagus nerve and enteric nervous system) and immune, vascular, and hormonal signals [45]. The MGB axis and intestinal dysbiosis have been implicated in various neurological conditions, including autism spectrum disorder, cerebrovascular disease, Alzheimer's disease, and Parkinson's disease [6, 16, 59, 61]. In patients with migraine, GM profiles are very different from those of healthy subjects [21, 24, 47, 61]. The intestinal dysbiosis observed in migraineurs might lead to the production of pro-inflammatory substances such as TNF-α, INF-γ, nitric oxide (NO) cytokines and lipopolysaccharide (LPS) [20, 26]. These substances are associated with inflammatory hyper nociception at sensory afferent endings [68]. The regulation of migraine pain is greatly influenced by bacterial metabolites in the gut, particularly short-chain fatty acids (SCFAs), γ-aminobutyric acid (GABA) and tryptophan [4]. An increase in intestinal permeability could lead to these substances passing through the systemic circulation and act on the nociceptors of the TVS, leading to the onset of migraine [82]. Despite the possible role of MBG axis dysfunction in migraine, there are few functional studies showing profiles of GMs involved in NO metabolism, kynurenine degradation and tryptophan production [36, 47, 60].

The study of the relationships between GM and migraine in pediatric age offers the advantage of observing these conditions at an earlier stage, when the impact of environmental factors and comorbidities is lower. Despite this there are only two recent studies dealing with GM in children with migraine [7, 36]. They showed a gut microbial dysbiosis consisting of shifts in gut microbial diversity and relative abundance of probiotic versus pathogenic bacteria. Particularly, an overall lower diversity in the GM [11] and a higher abundance of inflammation-related bacteria (e.g., *Eggerthella, Sutterella, and Eubacterium*) have been found [7, 36]. Unfortunately both studies had two important limitations: 1) they did not consider the impact of probiotics and antibiotics, and 2) data on GM came from digital datasets and the criteria for the diagnosis of migraine were not specified [7, 36].

Here we present the results of the "Migraine Microbiota" project, carried out at the Bambino Gesù Children's Hospital, which aimed to study GM in a population of pediatric migraine patients. This is the first prospective study exploring both the composition and functions of GM in a pediatric population with migraine. The study focused on examining the presence of GM dysbiosis in migraine patients and the potential impact of intestinal bacteria's metabolic pathways on this disease. We also investigated whether the intestinal permeability, inflammation and the mucosal immune activation might be related to migraine.

Materials and methods

Patients

We prospectively enrolled patients visited from 2021 to 2023 at the Headache Center of the Bambino Gesù Children's Hospital in Rome, Italy. Diagnosis of migraine with or without aura was performed by pediatric neurologists according to third version of the International Classification of Headache Disorder (ICHD-3). We referred to the migraine patients in this study with the acronym MIMIC (Migraine-Microbiota).

Healthy subjects (controls, CTRLs) were enrolled during an epidemiological survey carried out at the Microbiome Unit of Bambino Gesù Children's Hospital (BBMRI Human Microbiome Biobank, OPBG) to generate a

reference biobank of samples from healthy subjects. MIMIC and CTRL subjects were matched according to age, sex and BMI.

For both MIMIC and CTRL groups we considered the following inclusion criteria: aged between 7 and 17 years; eating style that follows a mediterranean diet. The following exclusion criteria were considered for all subjects: history of chronic inflammatory diseases of the gastrointestinal (GI) tract; history of allergies; intake of antibiotics and/or pre/probiotics in the last 3 months; history of gastroenteritis and/or parasitosis in the last 3 months; particular dietary regimes (vegetarian, vegan diet, etc.); BMI $\leq$ 18.4 or BMI > 24.9; history of other neurological, gastrointestinal and psychiatric pathologies.

In the MIMIC group, pediatric neurologists excluded: an history of chronic migraine; and/or medication overuse headache; taking prophylactic drugs or any other pharmacological treatment at the time of sample collection. In the CTRL group, pediatric neurologists excluded history of migraine or any other type of primary headache according to ICHD-3 criteria.

For each MIMIC and CTRL subject, a stool sample was requested. The sample collection should not have been carried out less than 24 h after a possible migraine attack or the intake of a rescue drug.

MIMIC subjects were divided into subgroups based on the number of headache days per month (monthly migraine days, MMD) (low frequency if MMD < 4 and high frequency if MMD $\geq$ 4), migraine onset (early and late onset before and after 10 years, respectively), presence of nausea/vomiting and/or photophobia/phonophobia, gender, and presence of aura.

The study was approved by the Ethical Committee of the Bambino Gesù Children's Hospital, IRCCS (protocol No. 596_OPBG_2021; protocol No. 2590_OPBG_2021; healthy subjects: protocols No. 1113_OPBG_2016 and No.2839_OPBG_2022) and was conducted in accordance with the Principles of Good Clinical Practice and the Declaration of Helsinki. Written informed consent was obtained from either parents or legal representative of the children. From each subject in these cohorts, a single faecal sample was collected and stored at -80 °C until further analyses.

16S rRNA-based gut microbiota profiling

DNA was extracted from 200 mg of stools by QIAmp Fast DNA Stool mini kit (Qiagen, Germany), following the manufacturer's instructions. The 16S rRNA V3-V4 regions (~460 bp) were amplified following the MiSeq rRNA Amplicon Sequencing protocol (Illumina, San Diego, CA). The bacterial libraries were obtained by DNA amplifications, cleaning and barcoding using Illumina Nextera adaptor-primers (Illumina, San Diego, CA). The final library was quantified by Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA). Samples were sequenced on an Illumina MiSeq™ platform according to the manufacturer's specifications to generate paired-end reads of 300 base-length [15].

Pre-Processing of fastq Files with QIIME2

A total of 422 fastq files (211 paired-end fastq files) were obtained by four distinct sequencing experiments and imported into QIIME2 v2023.2 [19]. With DADA2 plugin [8], paired-end reads were denoised, filtered from chimeras and joined into Amplicon Sequences Variants (ASVs). The sequences were taxonomically assigned by querying the Greengenes nucleotide sequence database v2022.10 [46] by greengenes2 plugin. The phylogenetic tree was built with the phylogeny align-to-tree-mafft-fasttree method. Finally, the ASV tables, the taxonomic data frames and phylogenetic trees were imported in R v4.3.2 to perform statistical analyses.

Batch Normalization and Statistical Analyses

The ASVs' matrices were joined with the phyloseq R package v1.40.0, obtaining a single matrix characterized by 1,690 ASVs. To reduce the batch effect introduced by the sequencing steps, the matrix was normalized with the Conditional Quantile Regression (CQR) method, applied with ConQur R package v2.0 [34].

Age was evaluated as confounding factor by means of Microbiome Multivariable Association with Linear Model 2 (MaAsLin2) algorithm [43].

Prior to perform the α- and β-diversity analyses the ASV matrix was normalized with the rarefaction method based on the minimum sample size. Statistical analyses on α-diversity indices were performed using the non-parametric Mann–Whitney test. PERMANOVA test was applied on β-diversity matrices.

The ASV matrix was normalized by the cumulative sum scaling (CSS) method and filtered out retaining ASVs present in at least the 25% of the total samples and with relative abundance > 0.01, prior to perform statistical analyses. For the univariate analysis (Linear Discrimant Effect Size [LEfSe]), the α value of 0.05 and an effect size threshold of 3 were used to identify the significant bacterial biomarkers [64]. For the multivariate analysis (Partial Least Square Discriminant Analysis [PLS-DA]), the *plsda* function of the *MixOmics* package was used and the algorithm was a regression model. To evaluate the performance of the model and the overfitting phenomenon, a cross validation was performed with 200 random permutations, using the *Bioconductor ropls package*. The score plots and loading plots were generated through the *mixOmics package*, representing

in the *Loadingplot* only the taxa with a loading coefficient between 0.1 and 0.3 in absolute value. The loadings with VIP score < 1 were filtered out.

The Root Mean Square Error (RMSE), Q^2, R^2 and the Area Under the Receiver Operating Characteristics (AUROC) parameters were calculated to evaluate the performance of the PLS-DA. Then the Principal Component Analysis (PCA) was performed with the *prcomp* function and the score plot and the biplots were visualized through the *Factoextra package of R* the data [57].

All p-values were adjusted for multiple testing with the Benjamini–Hochberg procedure.

Functional and network analyses

To predict functional pathways, the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) [18] software was used exploiting the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs database. LEfSe was used to identify statistically significant biochemical pathways (α value of 0.05 and a logarithmic LDA score threshold of 3.0). Correlations between microorganisms and KEGG pathways were performed by using Spearman's correlation method by means of Hmisc package v4.7–1, and networks were obtained with igraph package v1.3.5.

Statistical analyses

IBM SPSS Statistics 21 software (IBM Corp., NY, U.S.A.) was used for statistical analysis of the patients' anthropometric and clinical data. To test whether our dataset was normally distributed we used the Shapiro–Wilk test. Student's t test (t test) and analysis of variance (ANOVA), was used for comparison of normally distributed variables. The Mann–Whitney U test and the Kruskal–Wallis test were used for non-parametric statistical comparisons. Categorical variables were analyzed by the chi-square test or Fisher's exact test. The relationship between two variables was tested by the Spearman's rank correlation test.

To estimate the sample size, it was assumed that a difference in diversity indices (α and β indexes) equal to 0.4 points would be observed between MIMIC and CTRL. In a previous work we found a standard deviation of the biodiversity score of 0.6 points [62]. Therefore, with alpha set at 5% and a power of 80%, a minimum number of 36 patients per group was obtained. Since from the same series a drop-out rate of approximately 15% was observed, the sample size was set at minimum 42 patients per group. Considering the subgroup analyses, we therefore considered a total population of approximately 90–100 subjects [12].

Intestinal inflammation, permeability, mucosal immune activation and dysbiosis markers

In the migraine patients we measured the levels of lipopolysaccharide (LPS), occludin and IgA in the plasma to estimate the state of inflammation, intestinal permeability and mucosal immune activation, respectively. We also measured the levels of indican (indoxyl sulphate) in the urine to verify the state of metabolic dysbiosis.

LPS levels were measured by an enzyme-linked immunosorbent assay (ELISA) kit (concentration range of 0.04–10.0 EU/mL) (Hycult Biotechnology, Uden, The Netherlands). The absorbance was measured in a microplate reader at 405 nm.

The levels of occludin and IgA were measured by ELISA kits (concentration ranges: IgA, 25-200 ng/ml; occludin, 0.156–10 ng/ml) (Wuhan Fine Biotech Co, Wuhan, China), according to the product instructions. The absorbance for both tests was measured at 450 nm, using a microplate reader.

Indican was quantitatively measured by QuantyChrom TM Indican Assay Kit (detection range is 0.2–20 mg/dL) (Biossay Systems, CA, USA), following manufacturer's instructions. The absorbance was measured in a microplate reader at 480 nm.

Results

Characteristics of the migraine cohort

A total of 98 MIMIC subjects were enrolled, 64 females and 34 males, mean age of 12.24 years (range 6–17) at the time of enrolment. The mean age of migraine onset was 9.35 years (median 9, SD 3.2, range 6–17). The most common diagnosis was migraine without aura (N 91, 92.9%).

Features of MIMIC subjects are reported in Table 1.

Characterization of the gut microbiota

The characteristics of the GM were investigated by 16S rRNA analysis of 195 fecal samples from 97 MIMIC and 98 CTRL subjects. We obtained a total of 1848 ASV, filtered and grouped into 75 bacterial genera.

To exclude gender and age as confounding factors in the gut microbiota analyses, we performed a β-diversity analysis on the faecal microbiota composition of the overall cohort, grouping subjects by gender and median age (11 years). No differences in the distribution of gut microbiota taxa were observed for gender (PERMANOVA p-value = 0.25) (Supplementary Fig. 1, A).

On the contrary, age resulted a confounding factor (PERMANOVA p-value = 0.001) (Supplementary Fig. 1, B).

Table 1 Features of migraine cohort (MIMIC)

Total Patients:	98
Age: mean (median ± SD; *range)*	12.24 years (12.5 ± 2.87; 6–17)
Female: male (%)	64:34 (females 65.3%—males 34.7%)
Headache diagnosis	
Migraine without aura	91 (92.9%)
Migraine with aura	7 (7.1%)
Migraine Features	
Mean (median ± SD; range) age at onset	9.35 (9 ± 3.2; 6–17)
Early Onset (≤ 10 years of age)	61 (62%)
Late Onset (> 11 years of age)	37 (38%)
MMD	
Low frequency (< 4/m)	54 (55.1%)
High Frequency (> 4/m)	44 (44.9%)
Nausea/Vomiting	70 (71.4%)
Photophobia	74 (75.5%)
Phonophobia	74 (75.5%)

We tried to identify a specific gut microbiota signature associated to migraine by removing the potential effect of latent confounding variable (age). Consequently, after performing a general linear model, we could identify that *Bifidobacterium* 38,775 and *Eggerthella* were specifically associated to age and not to case–control matching *(*Supplementary Fig. 2).

Gut bacterial dysbiosis in patients with migraine

The α-diversity analysis revealed a statistically significant increase of all three indexes in the MIMIC group compared to CTRL (Shannon index: p-value = 5×10^{-7}, Simpson index p-value = 0.0006; Chao 1 p-value = 0.001) (Fig. 1A). The PERMANOVA test, performed on the Bray–Curtis distance matrix (β-diversity analysis), highlighted significant differences in the gut microbiota profiles of the two groups (p-value = 0.001) (Fig. 1B), indicating a gut dysbiosis in children with migraine.

To highlight the differences in gut microbiota composition between MIMIC and CTRL, we applied multivariate (unsupervised and supervised analysis and loading variable plots) and univariate statistical approaches (Fig. 2 and Supplementary Fig. 3). In all types of analysis, bacteria providing a statistically significant profile between MIMIC and CTRL samples were: *Gemmiger*, *Phocaeicola*, *Roseburia*, *Escherichia*, ER4, *Acetatifactor*, *Alistipes*_A_871400, *Dorea*_A and *Rombustia* for MIMIC subjects, and *Eggerthella*, *Alistipes*_A_871404, *Clostridium*, *Collinsella*, *Parabacteroides*, *Erysipelatoclostridium*, *Akkermansia* and *Faecalibacillus* for CTRLs. *Bifidobacterium*_388775 was also identified as marker of CTRLs, but was excluded in the previous confounding analysis because associated with age. The area under the ROC curve (AUROC) was 0.969, indicating that the applied model had high accuracy in group classification.

Finally, we tested the influence of anthropometric and clinical characteristics in shaping the gut microbiota, but we did not observe any statistical differences in patients' group by α- and β-diversity analyses.

Metabolic inferred profile of migraine

To assess the microbial metabolic pathways, inferred by 16S rRNA sequences, we performed the prediction of pathways of the two cohorts, shown in the LDA plot (Fig. 3).

Thirty-seven pathways, belonging to 17 defined metabolic classes, have been associated with the MIMIC profile. Of these, the following pathways were increased in MIMIC: metabolism of phenylalanine, ascorbate, aldarate, glyoxylate, dicarboxylate, propanoate, pyruvate, nitrogen, glycerophospholipid, porphyrin, riboflavin, beta-alanine, cyanoamino acid, and glutathione; degradation of lysine, geraniol, benzoate, dioxin, ethylbenzene, toluene and xylene; biosynthesis of penicillin, cephalosporin, fatty acids, ansamycins and siderophore group non-ribosomal peptides; pentose and glucuronate interconversions; bacterial chemotaxis; flagellar assembly; biofilm formation (*Vibrio cholerae*); carbon fixation pathways in prokaryotes; plant-pathogen interaction; protein processing in endoplasmic reticulum; sulphur relay system; bacterial secretion system; two-component system; RNA polymerase; RNA transport; drug metabolism (other enzymes). These pathways were decreased in MIMIC: metabolism of cysteine, methionine, glycine,

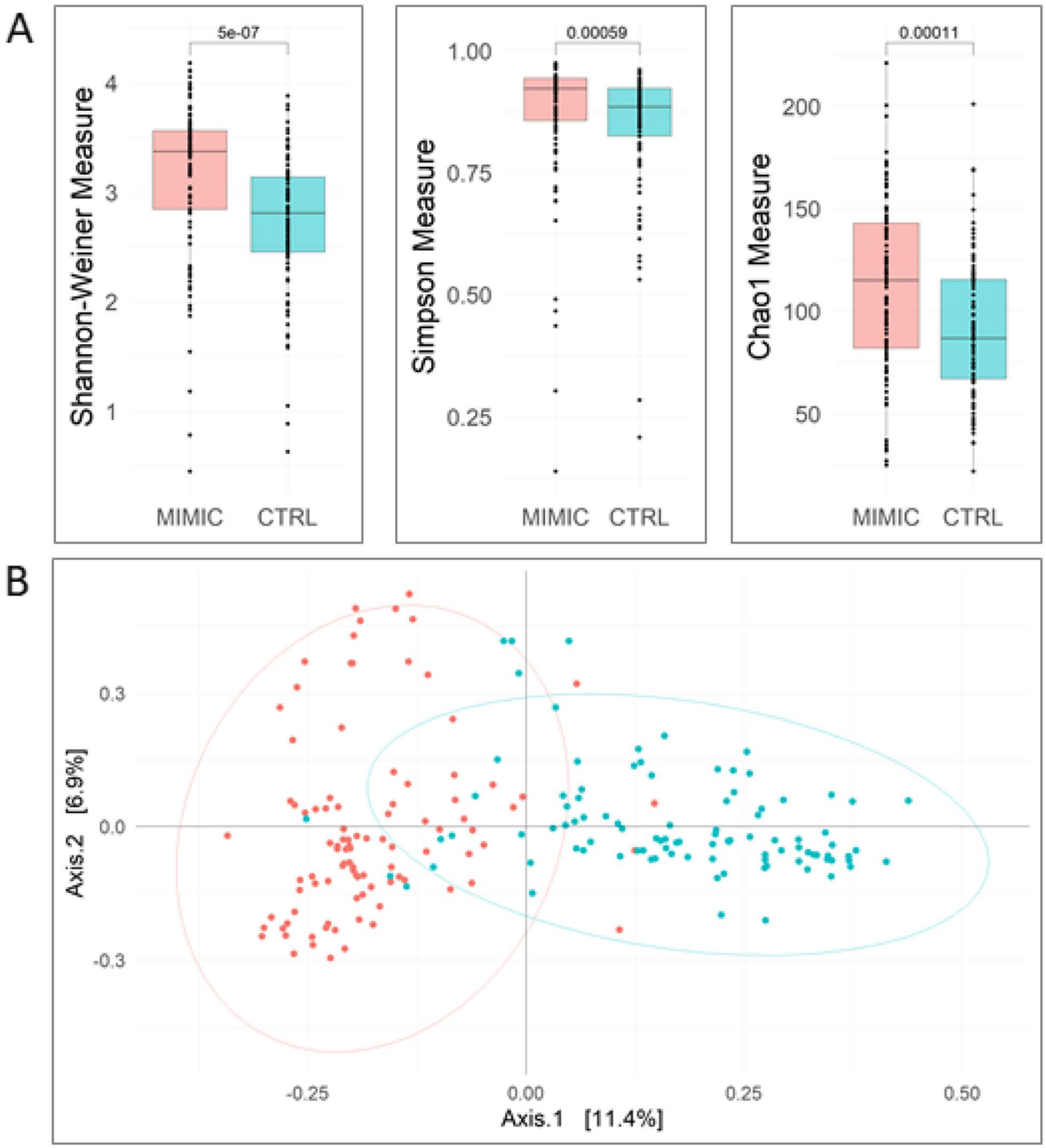

Fig. 1 Gut microbiota biodiversity. **A** α-diversity analysis based on the Shannon-Weiner, Simpson and Chao1 indexes. The Mann–Whitney test was applied for the comparisons between migraine patients (MIMIC) and control subjects (CTRL). **B** β-diversity was calculated by the Bray–Curtis algorithm and presented by PCoA plots. PERMANOVA test results are statistically significant, *p*-value = 0.001

serine, threonine, amino sugars, nucleotide sugars, C5-Branched dibasic acids, fructose, mannose, galactose, inositol phosphate, linoleic acid, nicotinate, nicotinamide, thiamine, seleno-compound and purine; biosynthesis of lysine, phenylalanine, tyrosine, tryptophan, valine, leucine, isoleucine, novobiocin, pantothenate, Coenzyme A, terpenoid backbone, aminoacyl-tRNA; degradation of RNA, bisphenol, chloroalkane and chloroalkene; glycolysis/gluconeogenesis;

cell cycle (*Caulobacter*); adipocytokine signalling pathway; one carbon pool by folate; base and nucleotide excision repair; DNA replication and ribosome pathway.

Network analysis between bacteria and metabolites

The link between bacteria and inferred pathways was highlighted by network analysis (Fig. 4). Remarkably, in MIMIC group (Fig. 4A), only *Escherichia* 71,083, *Faecalibacterium*, *Bacteroides* H, *Phocaeicola*_A_858004 and *Dorea*_A were linked to metabolic pathways. In particular, *Escherichia* showed only positive correlations with 33 metabolic pathways; *Faecalibacterium* had 6 negative and 2 positive correlations with metabolic pathways; *Bacteroides* had 8 negative and 1 positive correlations with metabolic pathways; *Dorea_A* and *Phocaeicola* had 2 and 1 positive correlations, respectively. The metabolic pathways of biosynthesis of siderophore group non-ribosomal peptides, galactose metabolism, fructose and mannose metabolism, beta-Alanine metabolism were shared between *Escherichia* and *Faecalibacterium*, whit inverse sign of correlation.

Interestingly, the CTRL network analysis highlighted correlations amongst metabolic pathways and *Escherichia*_710834, *Faecalibacterium*, *Bacteroides*_H, *Alistipes*_A_871400, *Blautia*_A_141781, *Bifidobacterium*_388775, and *Akkermansia* (Fig. 4B). In particular, Escherichia 710,834 showed positive correlations with 18 metabolic pathways, of which 16 were the same reported for MIMIC group. Moreover, one of these, penicillin and cephalosporin biosynthesis was in common with *Faecalibacterium.* The pathway Geraniol degradation was in common between *Bacteroides* H (positive correlation) and *Blautia* A 141781 (negative correlation). The latter was linked by Chloroalkane and chloroalkene degradation with *Bifidobacterium* 388,775. Bacteroides was linked to *Alistipes* A 871400 by the positive correlation with Protein processing in the endoplasmic reticulum.

Finally, *Akkermansia* showed negative correlation with 9 metabolic pathways.

Gut permeability, inflammation, metabolic dysbiosis and mucosal immune activation in migraine

In the MIMIC group, we examined plasma levels of LPS, occludin and IgA, markers of systemic inflammation, microbial translocation and mucosal immune system activation, respectively. We also tested patients' urinary indican levels to indirectly measure gut dysbiosis levels [41].

It was previously reported that the plasma concentration of LPS ranges from undetectable levels up to 0.2 ng/ml [42, 52], while occludin ranges from 0.5 to 1.7 ng/ml [79]. In MIMIC subjects, the mean value of LPS was 0.26 ± 0.17 ng/ml and occludin was 4.87 ± 4.21 ng/ml (Fig. 5). The mean value of systemic IgA was 76.91 ± 72.47 g/L. The IgA range between 0.90–1.13 g/L was reported in healthy children from 6 to 10 years [29].

The mean value of indican urinary level in the MIMIC group was 136.71 ± 50.83 mg/L. As described in the literature, the urinary indican level was considered normal around 10–25 mg/L [10].

Correlation analysis based on Spearman's test revealed a negative correlation between LPS and occludin (rho value = -0.560, p value < 0.05), but none between indican and the other two markers.

The gut inflammation, permeability and dysbiosis marker levels in the migraine cohort were evaluated considering the migraine's features (Table 2). We observed an increase of LPS and a decrease of IgA in late migraine with respect to early onset patients, and an increase of IgA in the presence of phonophobia. We did not observe any other statistical differences for these biohumoral biomarker concentrations between patient groups (Table 2).

Discussion

This study shows that MIMIC subjects have GM profiles significantly different from those of CTRLs, and they are characterized by an increase of metabolic dysbiosis, intestinal permeability and mucosal immune activation. However, no systemic inflammation was observed.

The role of dysbiosis in migraine

Recent evidence highlighted that GM dysbiosis may contribute to migraine, although this relationship is not clearly explained. According to some researchers, there is a relationship between microbiota residents, inflammatory mediators, neuropeptides, stress hormones, and nutrient substances [3, 4]. It is well known that biochemical signals are transmitted from the gastrointestinal tract to the central nervous system (CNS) through the intestinal barrier [3, 75]. The intestinal dysbiosis could play a

(See figure on next page.)

Fig. 2 Compositional analysis of migraine patients (MIMIC) and control subjects (CTRL) gut microbiota at genus level. **A** Unsupervised multivariate analysis. Principal Component Analysis [PCA] plot showing the top 25 loadings; **B** Supervised multivariate analysis plot (Partial Least Squares Discriminant Analysis [PLS-DA]); **C** Loading variables plot (filtered for VIP > 0.85). The Root Mean Square Error (RMSE) = 0.247, R^2 value = 0.759, p-value ≤ 0.05 and Q^2 = 0.632, p value ≤ 0.05; **D** Univariate analysis (Linear Discriminant Analysis [LDA] Effect Size [LEfSe] plot. Bacterial taxa enriched in migraine patients have positive LDA scores (red), while bacterial enriched in CTRL have negative scores (green). **E** Receiver operating characteristic (ROC) analysis of the PLS-DA model. The value of AUROC = 0.969 indicates a high accuracy of the prediction model

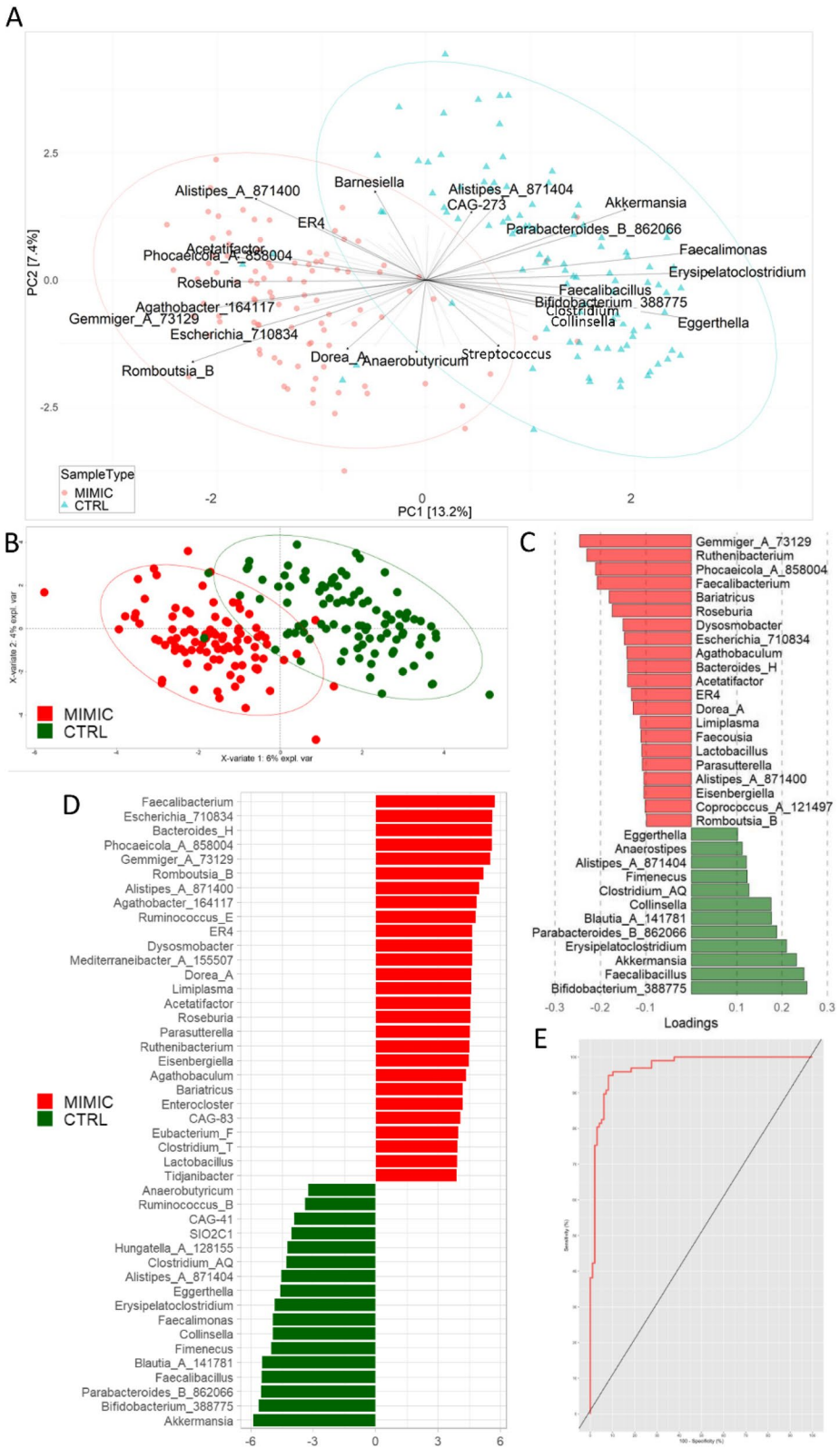

Fig. 2 (See legend on previous page.)

Fig. 3 PICRUSt2 functional prediction using the KEGG pathway Database. Predicted metabolic pathways statistically associated to migraine patients (MIMIC) and control subjects (CTRL). Red bars represent pathways increased in MIMIC; green bars represent pathways increased in CTRL. LDA, linear discriminant analysis

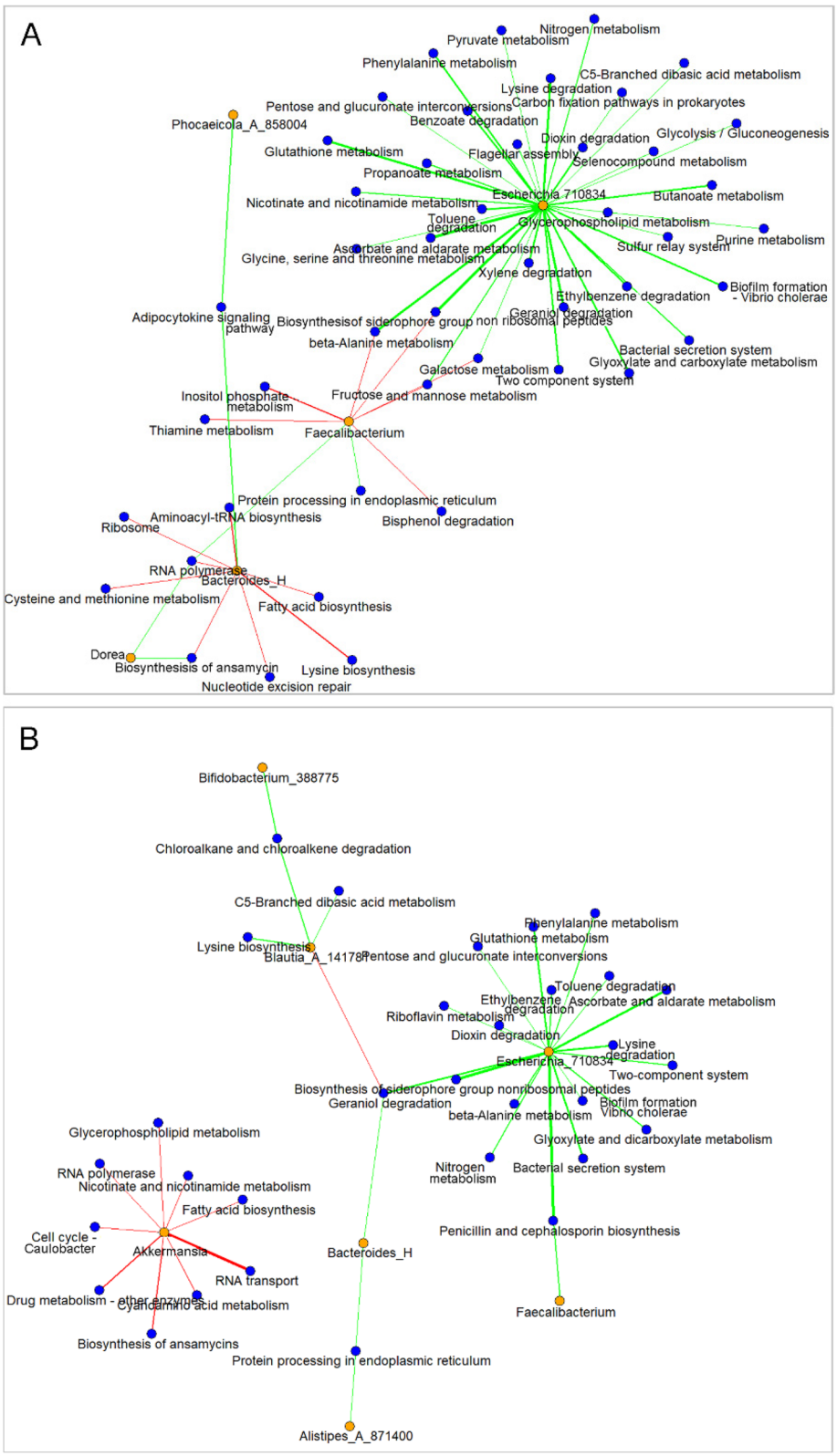

Fig. 4 Bacterial and metabolic pathways correlation network in migraine patients (MIMIC) (**A**) and in control subjects (CTRL) (**B**). Each node represents bacteria (orange circles) and metabolic pathways (blue circles). Green and red edges indicate positive and negative correlation values, respectively. Only correlations statistically significant (p-value < 0.05) are reported

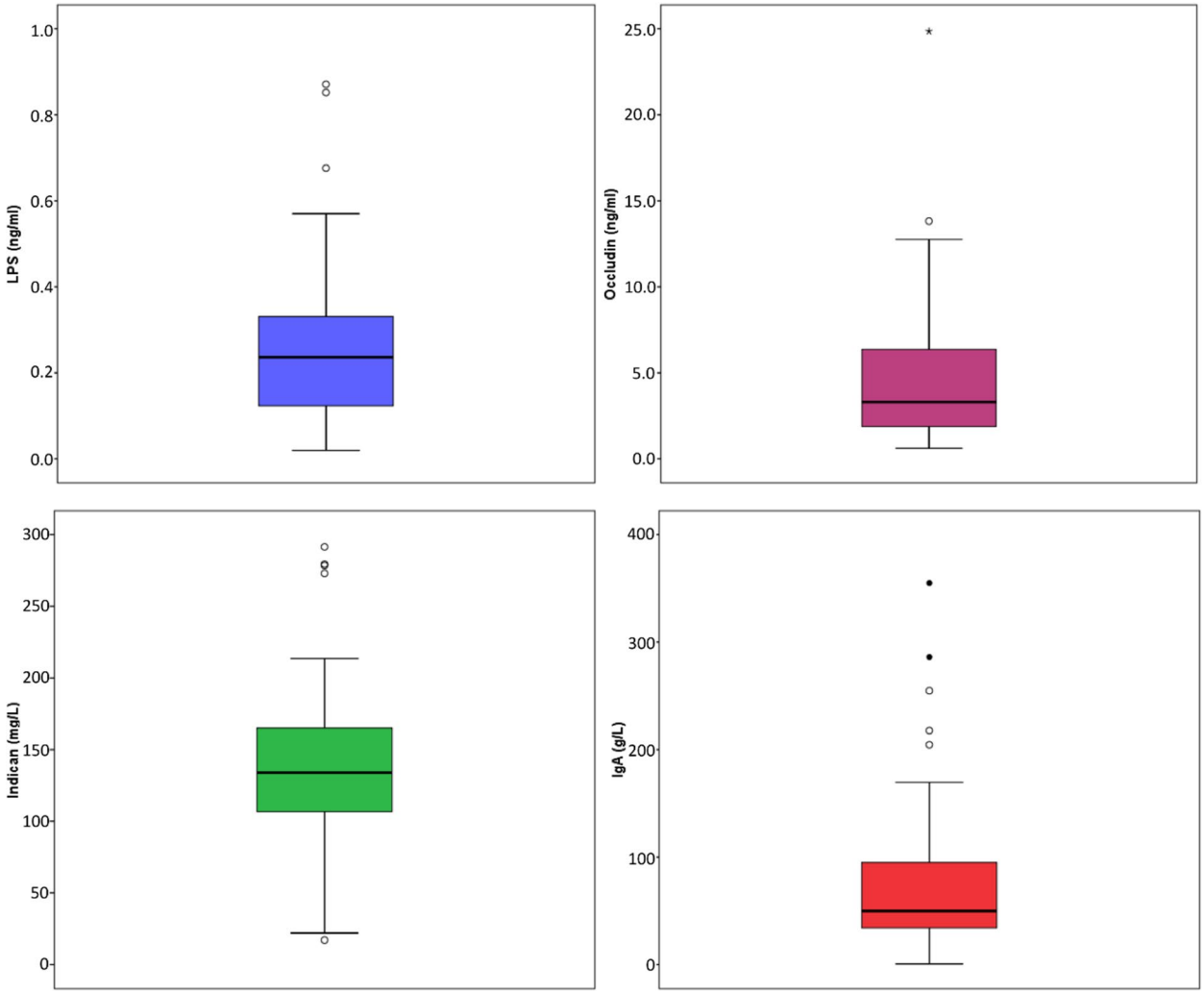

Fig. 5 Plasmatic levels of LPS, occludin, IgA and urinary level of indican for patients with migraine (MIMIC). Bar plots of LPS (ng/ml), occludin (ng/ml) and IgA (g/L) plasmatic concentrations and of urinary indican concentration (mg/L)

role in migraine attack by altering the intestinal permeability and by enhancing inflammatory processes [20, 26].

Two studies have investigated the impact of GM on pediatric migraine [7, 36]. In line with some results on adults with migraine [11], Jiang et al., [27]), both studies showed statistically significant differences for α and β-diversity between migraine patients and CTRLs groups. Unfortunately, GM profiles were retrieved from available digital datasets and the criteria for the diagnosis of migraine were not specified.

Our study showed that MIMIC subjects had higher α-diversity values of GM than CTRLs. This result is in contrast with those of the above cited studies [6, 36], in which batch effects related to geography, ethnicity and diet were not assessed, thus limiting the reproducibility of GM ecological data. Beyond its richness and diversity, the proportions of different types of bacteria in GM may have a role in migraine pathophysiology. The biochemical composition of the gut environment can be influenced by the correct balance between species.

In particular, *Dorea_A, Acetatifactor, Gemmiger, Phocaeicola, Roseburia, Alistipes_A_871400, Escherichia, ER4,* and *Rombustia* were assigned to MIMIC subjects while *Eggerthella, Erysipelatoclostridium, Alistipes_A_871404, Collinsella, Clostridium, Parabacteroides, Akkermansia and Faecalibacillus* to CTRLs.

1) *Bacteria assigned to MIMIC group*. Dorea is positively associated with intestinal permeability and an increase in INF-γ, suggesting a role in pro-inflammatory processes [32]. Acetatifactor is a producer of lithocholic acid (LCA), a secondary bile acid [70]. It is well known that bile acids have immunomodulatory properties [22]. In particular, the LCA admin-

Table 2 Occludin, LPS, indican and IgA levels in migraine cohort stratified for patients' features. *P* values were obtained by T test

Biomarkers	Categories	Occludin (ng/ml) average ± SD	*p* value	LPS (ng/ml) average ± SD	*p* value	Indican (mg/L) average ± SD	*p* value	IgA (g/L) average ± SD	*p* value
Gender	Female	4.87 ± 4.6	0.998	0.26 ± 0.19	0.764	139.87 ± 51.67	0.919	72.46 ± 76.28	0.712
	Male	4.87 ± 3.44		0.25 ± 0.13		130.81 ± 49.44		84.25 ± 66.96	
Age	< 11 years	5.49 ± 3.87	0.458	0.22 ± 0.13	0.239	128.05 ± 50.6	0.929	80.76 ± 75.46	0.977
	≥ 11 years	4.65 ± 4.34		0.27 ± 0.18		139.78 ± 50.92		75.53 ± 72.33	
Episodes frequency	low	5.29 ± 4.83	0.149	0.25 ± 0.18	0.342	137.03 ± 57.31	0.112	83.93 ± 67.2	0.806
	high	4.35 ± 3.25		0.26 ± 0.16		136.32 ± 42.08		68.43 ± 78.99	
Migraine onset	early	5.16 ± 4.56	0.478	0.21 ± 0.12	**0.003**	136.05 ± 54.25	0.539	67.85 ± 57.07	**0.031**
	late	4.44 ± 3.68		0.33 ± 0.21		137.67 ± 46.21		89.68 ± 89.68	
Phonophobia	not	6.17 ± 3.61	0.142	0.21 ± 0.15	0.161	139.29 ± 53.35	0.415	51.66 ± 32.38	**0.032**
	yes	4.4 ± 4.34		0.28 ± 0.18		135.16 ± 48.13		87.92 ± 81.307	
Photophobia	not	5.36 ± 3.56	0.555	0.23 ± 0.2	0.485	131.67 ± 45.72	0.911	71.65 ± 53.57	0.233
	yes	4.65 ± 4.42		0.27 ± 0.17		137.41 ± 50.38		81.88 ± 80.395	
Nausea/vomiting	not	5.89 ± 4.42	0.257	0.27 ± 0.17	0.779	122.98 ± 39.48	0.541	88.27 ± 75.813	0.445
	yes	4.56 ± 4.13		0.25 ± 0.17		141.84 ± 53.82		73.22 ± 71.965	
Aura	not	4.71 ± 4.2	0.229	0.26 ± 0.17	0.97	133.01 ± 47.1	0.078	81.11 ± 73.83	0.065
	yes	7.07 ± 4.17		0.26 ± 0.11		181.74 ± 74.61		25.65 ± 5.37	
Nr. of migraine characteristics	≤ 2	5.91 ± 3.86	0.115	0.18 ± 0.1	0.884	126.6 ± 54.09	0.555	73.77 ± 56	0.353
	> 2	4.51 ± 4.3		0.28 ± 0.18		139.07 ± 47.81		80.76 ± 78.87	

istration inhibited Th17 cell differentiation and promoted Treg differentiation in a mouse model [23]. A potential role of Gemmiger, as well as Eggerthella and Acetatifactor, has been suggested in patients affected by psychiatric disorders like depression, bipolar disorder, schizophrenia, and psychosis [53]. In particular, a meta-analysis on the role of GM in depression reported an increase in Eggerthella and a decrease in Acetatifactor and Gemmiger in patients [37]. Eggerthella exerts its harmful role by inducing intestinal inflammation by activating Th17 cells [2], or by the depletion of butyrate, a short fatty acid molecule with anti-inflammatory properties [67]. Mechanisms that regulate intestinal inflammation involve Eggerthella, Gemmiger, and Acetatifactor [38]. Given that in MIMIC subjects we observed an opposite trend of these bacteria compared to depression-related GM profile, it is possible that these microorganisms may act differently on inflammatory processes associated with migraine or depression. Roseburia spp. are bacteria critical for the production of butyrate, which has a neuroprotective effect, can enhance cognition, and activates the vagus nerve [51]. Roseburia was linked to a higher level of serotonin and quinolinic acid in both the brain and colon of mice [81]. Major depressive disorder patients have been reported to have a decreased abundance of Roseburia [80]. MIMIC subjects had a higher abundance of Roseburia compared to controls, which is in line with previously reported results [24]. We also observed an increase of Rombustia in MIMICsubjects. Its role in this disease could be explained by the observation that Rombustia has been correlated with an increase in serum tryptophan (Tabone et al., [69]) and that there is a link between tryptophan metabolic activities and migraine pathophysiology [36]. Phocaeicola vulgatus is a producer of 3-hydroxyphenylacetic acid [28]. It has been proposed that phenylacetic acids play a role as triggers for migraine [49, 50].

2) *Bacteria assigned to CTRL*. Alistipes had two distinct genera associated with MIMIC and CTRL groups. This observation agrees with Liu et al.'s findings (2024) which demonstrate the presence of Alistipes in both healthy subjects and migraineurs. An increased abundance of Collinsella has been reported in migraine patients with irritable bowel syndrome (IBS), compared to those with only IBS, supporting a role of this microorganism in migraine [35]. Akkermansia muciniphila is able to produce propionate, which is involved in migraine pain reduction and can reduce the frequency and intensity of migraine episodes [30]. Furthermore, Akkermansia muciniphila can increase the expression of serotonin transporters and increase the bioavailability of this molecule in the gut [78]. The potential protective role of Akkermansia against migraine is supported by these data.

Different from our results, other authors reported higher levels of *Eggerthella, Parabacteroides,* and *Erysipelatoclostridium* in children with migraine compared to CTRLs [7, 36]. Probably, different neurological alterations may be associated with different distributions of bacterial signatures in the GM.

Intestinal inflammation and permeability

We analyzed the plasmatic concentration of occludin and LPS to study intestinal permeability and inflammation in migraine patients. Tight junction proteins (e.g., occludin, zonulin, claudin) are core elements of the intestinal barrier, and their detection in the bloodstream may indicate disruption of it [76]. The intact intestinal barrier allows the passage of nutrients into the bloodstream, while restricting the leakage of LPS into the systemic circulation [9]. Low-grade inflammation has been found in different diseases, such as diabetes, obesity, inflammatory bowel diseases (IBD), chronic kidney diseases, and cardiovascular diseases [48, 73]. In the context of migraine, it is possible that LPS favours the production of cortisol which in turn acts at the level of the TVS stimulating the production of calcitonin gene-related peptide (CGRP) [65].

Adult patients suffering from chronic migraine and medication overuse headache (MOH) exhibit an elevated serum level of LPS [76]. The presence of a leaky gut and the passage of LPS into the bloodstream is demonstrated by the correlation between LPS levels and occludin levels in these patients. The same study found that patients with episodic migraine did not have an increased level of LPS, but they showed high levels of occludin when compared to healthy subjects [76]. It seems that intestinal inflammation may come up later in the course of migraine or it could be connected with the excessive use of non-steroidal anti-inflammatory drugs (NSAIDs).

This is the first study that analyze the levels of LPS, occludin, indican, and IgA in pediatric patients with episodic migraine. The relevance of this study on a pediatric cohort offers the opportunity to analyse associations between migraine and gut in an earlier phase of the disease, when the impact of environmental factors is lower than in adults. We found that the serum LPS was not increased in MIMIC subjects, which seems to confirm that intestinal inflammation associated with migraine, or vice versa, is something that occurs over time, probably when the disease becomes chronic. This hypothesis is supported by the correlation between age at onset of migraine and LPS. Despite this, all patients had high levels of occludin, which suggests that permeability increases prior to intestinal inflammation.

Is tryptophan metabolism a potential link between gut microbiota and pediatric migraine?

In the MIMIC group, tryptophan metabolism may be dysfunctional at multiple levels, as demonstrated by our study. Liu et al. had previously speculated that migraine and tryptophan metabolism were related in pediatric patients, but there were some limitations. In the study by Liu et al., the GM data were extracted from a digital database, while tryptophan metabolite levels were measured in a different migraine population [36].

Tryptophan metabolism involves three pathways: the serotonin (5-hydroxyptamine, 5-HT), kynurenine (KYN), and indole pathways [38].

The serotonin pathway is very important in the pathophysiology of migraine, which can be considered as a syndrome of chronic low serotonin levels with transient increases during attacks [14]. We observed an increase of plasmatic IgA levels in our cohort. Migraine patients have been reported to have an increased level of serum complement components and immunoglobulins, which can cause platelets aggregation and release of serotonin into the blood [40]. Serotonin is selectively absorbed by the platelets, which represent circulation reservoirs for the substance. Furthermore, a decrease in platelet 5-HT levels has been observed in patients with migraine [66]. The increased levels of immunoglobulins in migraine patients might be related to the reduced levels of 5-HT in the platelets [66].

The kynurenine pathway is the main catabolic pathway of tryptophan and, through the action of different enzymes, can lead to the production of metabolites which can have protective or harmful effects on the CNS [38].

The indole pathway is the main pathway for metabolizing tryptophan that is dominated by intestinal microbiota. The nervous system's functions are regulated by indole and its derivatives [77]. The liver transforms indole into indican and excretes it via the urine [71]. Increased production and reabsorption of indican by intestinal bacteria is indicative of bacterial overgrowth in the small intestine, malabsorption, constipation and dysbiosis [39, 41, 72, 74]. In our study, three main underlined elements are connected to the metabolism of GM and tryptophan. First, the patients' GM population showed a higher concentration of *E. coli, Bacteroides*, and *Lactobacilli* compared to CTRLs. The indole pathway's high metabolism of tryptophan is generally attributed to these bacteria [17, 36]. This finding is also consistent with an increase in indican levels in the urine of MIMIC subjects, suggesting a metabolic dysbiosis [39, 41]. Second, the patients exhibited elevated *Akkermansia,* which increases the expression of the serotonin transporter and its availability in the intestinal tract. Third, MIMIC subjects showed high

quantities of *Roseburia* that is associated with increased 5-HT level and decreased quinolinic acid and 3-hydroxykynurenine levels, in both brain and colon of mouse models [81]. Overall, our data on tryptophan metabolism suggests that the GM ecosystem in MIMIC patients may unbalance its metabolism towards indoles instead of serotonin and kynurenine pathways. Measurement of the levels of tryptophan and its metabolites in the plasma of migraneous patients is necessary for verifying this hypothesis.

Amines metabolism and gut microbiota

The abnormal levels of dopamine and norepinephrine, as well as other elusive amines, can also affect the pathophysiology of migraine attacks [13]. In our study, it was observed that the pathways responsible for the biosynthesis of phenylalanine and tyrosine decreased in migraine patients. Phenylalanine has vasoconstrictive properties and may cause headaches by altering cerebral blood flow and the release of norepinephrine from sympathetic nerve cells [33]. Additionally, phenylalanine is converted to tyrosine, which is a precursor of epinephrine, norepinephrine, and dopamine [44].

Nitric oxide metabolism and gut microbiota

Nitrate-containing compounds have been identified as common triggers of migraine [58]. In healthy volunteers and patients with migraine, the nitroglycerin (NTG) provocation model induces a headache that resembles migraine in pain characteristics and vascular manifestations [5, 58].

Under physiological conditions, the basal concentration of NO in the gut allows for control of commensal microbial populations and maintenance of the integrity of the intestinal epithelial barrier. Dysbiosis and intestinal inflammation lead to a significant increase in NO levels. High NO concentration depleted the microbiota of beneficial species and favoured potentially deleterious bacteria such as *Escherichia coli, Enterococcus faecalis,* and *Proteus mirabilis* [31].

Our investigation revealed that MIMIC subjects have an abundance of *Escherichia coli* and an increase in nitrogen metabolism pathways simultaneously. Further research is needed to confirm that there is a connection between NO metabolism mediated by GM and migraine.

Limitations

The main limitation of the present study is the absence of urinary indican, plasmatic LPS, occludin, and IgA in healthy subjects as a reference baseline. Further our study showed data that were not always comparable to those of other studies in the literature (α and β value and GM species).

This could be the effect of using different methods for data analysis and patient selection. However, beyond the richness and diversity of the GM, a role in migration may be linked to the proportion between the various species of bacteria and how these influence the biochemical composition of the intestinal environment. To accurately define the metabolic environment linked to GM, it would have been necessary to measure specific metabolites, such as tryptophan.

Conclusions

Gut dysbiosis was linked to migraine in our pediatric cohort. This evidence may lead to the identification of promising biomarkers and therapeutic targets for this disease. Furthermore, the increase in intestinal permeability and IgA levels could be a factor in the disease's trigger through the gut-brain axis. The study's strength comes from its prospective design and the involvement of pediatric specialists for headache diagnosis. Furthermore, several variables related to diet, drug intake, and other conditions that affect GM were considered as exclusion criteria. Finally, this study on pediatric patients with migraine offers the advantage of analyzing the GM in an early phase of the disease, when the role of interfering environmental factors on the GM is lower than in adults.

The present findings are a base for future advancement. The role of GM in migraine could be better defined by understanding the composition of the entire intestinal ecosystem. Metagenomics, metabolomics, and metaproteomics studies are necessary to gain a deeper understanding of metabolic pathways implicated in migraine triggering.

A better understanding of the GMB axis could lead to innovative therapeutic approaches that utilize modulation or resetting of GM. Consideration could be given to faecal transplantation in pharmacologically resistant migraine, as well as possible bacterial therapies using targeted probiotics and prebiotics.

Abbreviations

GM	Gut microbiota
LPS	Lipopolysaccharide
CTRL	Healthy subjects
TVS	Trigemino-vascular system
MGB	Microbiome-gut-brain
NO	Nitric oxide
GABA	γ-aminobutyric acid
SCFAs	Short-chain fatty acids
MIMI	Migraine-microbiota
MMD	Monthly migraine days
CQR	Conditional quantile regression
CSS	Cumulative sum scaling
PLS-DA	Partial least square discriminant analysis
RMSE	Root mean square error
AUROC	Area under the receiver operating characteristics
PCA	Principal component analysis
PICRUSt2	Phylogenetic investigation of communities by reconstruction of unobserved states

KEGG	Kyoto encyclopedia of genes and genomes
ELISA	Enzyme-linked immunosorbent assay
LDA	Linear discriminant analysis
ROC	Receiver operating characteristic
CNS	Central nervous system
LCA	Lithocholic acid
IBS	Irritable bowel syndrome
IBD	Inflammatory bowel diseases
CGRP	Calcitonin gene-related peptide
MOH	Medication overuse headache
NSAIDs	Non-steroidal anti-inflammatory drugs
5-HT	5-hydroxyptamine
KYN	Kynurenine

Supplementary Information

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Supplementary Material 1.

Authors' contributions

Conceptualization, L.P.* and F.D.; Methodology: F.T.; M.S.; S.L.M.; F.R.; F.D.; L.P.* Validation, L.P.*, F.D., L.P§. and M.V.; Writing– Original Draft Preparation, L.P.* and F.D. Writing– Review & Editing, L. P.§ and M.V; Figure and table: I.F.; M.S.; F.D.; Data setting: M.S. and L.P*.; Visualization L.P*.; M.V.; L.P.§; G.M.; F.U.; G.S.; Supervision, L.P§ and M.V. All authors reviewed the manuscript.

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Availability of data and materials

Sequence data that support the findings of this study have been deposited in NCBI BioProject database (PRJNA1120654) (https://submit.ncbi.nlm.nih.gov/subs/sra/).

Declarations

Ethics approval and consent to participate

The study was approved by the Ethical Committee of the Bambino Gesù Children's Hospital, IRCCS (protocol No. 596_OPBG_2021; protocol No. 2590_OPBG_2021; healthy subjects: protocols No. 1113_OPBG_2016 and No.2839_OPBG_2022) and was conducted in accordance with the Principles of Good Clinical Practice and the Declaration of Helsinki.

Consent for publication

Written informed consent was obtained from either parents or legal representative of the patients.

Competing interests

The authors declare no competing interests.

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