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# Microbiota alterations are related to migraine food triggers and inflammatory markers in chronic migraine patients with medication overuse headache

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## Abstract

**Objective** Chronic migraine (CM) patients with medication overuse headache (MOH) were recently shown to be associated with leaky gut and inflammation. We aimed to investigate gut microbiota profiles of CM patients with MOH, and their correlations with inflammatory serum parameters, migraine food triggers, and comorbid anxiety and depression.

**Materials and methods** The study included women participants (32 CM patients with NSAID overuse headache, and 16 healthy non-headache sufferers). Migraine duration, monthly migraine headache days, presence of irritable bowel syndrome symptoms, and HADS-D and HADS-A scores were recorded. Serum samples were collected to measure circulating LPS, HMGB1, HIF-1 $\alpha$ , and IL-6. The gut microbiota profiles of the patients were evaluated using fecal samples.

**Results** Serum LPS, HMGB1, HIF-1 $\alpha$ , and IL-6 levels were significantly higher in the CM + MOH group compared to the healthy controls. HADS-A and HADS-D scores were considerably higher in the CM + MOH group compared to the healthy controls. In the microbiota analysis, alpha and beta diversities were similar between the two groups. The class Clostridia, the order Eubacteriales, and the genus Ruminococcus were less abundant in the CM + NSAID overuse headache group compared to the control group. At the genus level *Desulfovibrio*, *Gemmiger*, and *Dialister* and at the species level, *Clostridium fessum*, *Blautia luti*, *Dorea longicatena*, *Eubacterium coprostanoligenes*, and *Gemmiger formicilis* were more abundant in the CM + NSAID overuse headache group compared to the control group. *Desulfovibrio*, *Gemmiger*, *Dialister*, *Ethanoligenens harbinense*, *Eubacterium coprostanoligenes*, *Dorea longicatena*, and *Thermoclostridium stercorarium* showed positive correlations and Clostridia bacteria showed negative correlations with migraine food triggers. Positive correlations were found between LPS and Hapalosiphonaceae, HMGB1 and Melghirimyces, HIF-1 $\alpha$  and *Rouxella* and *Blautia luti*, IL-6 and Melghirimyces and Ruminococcus.

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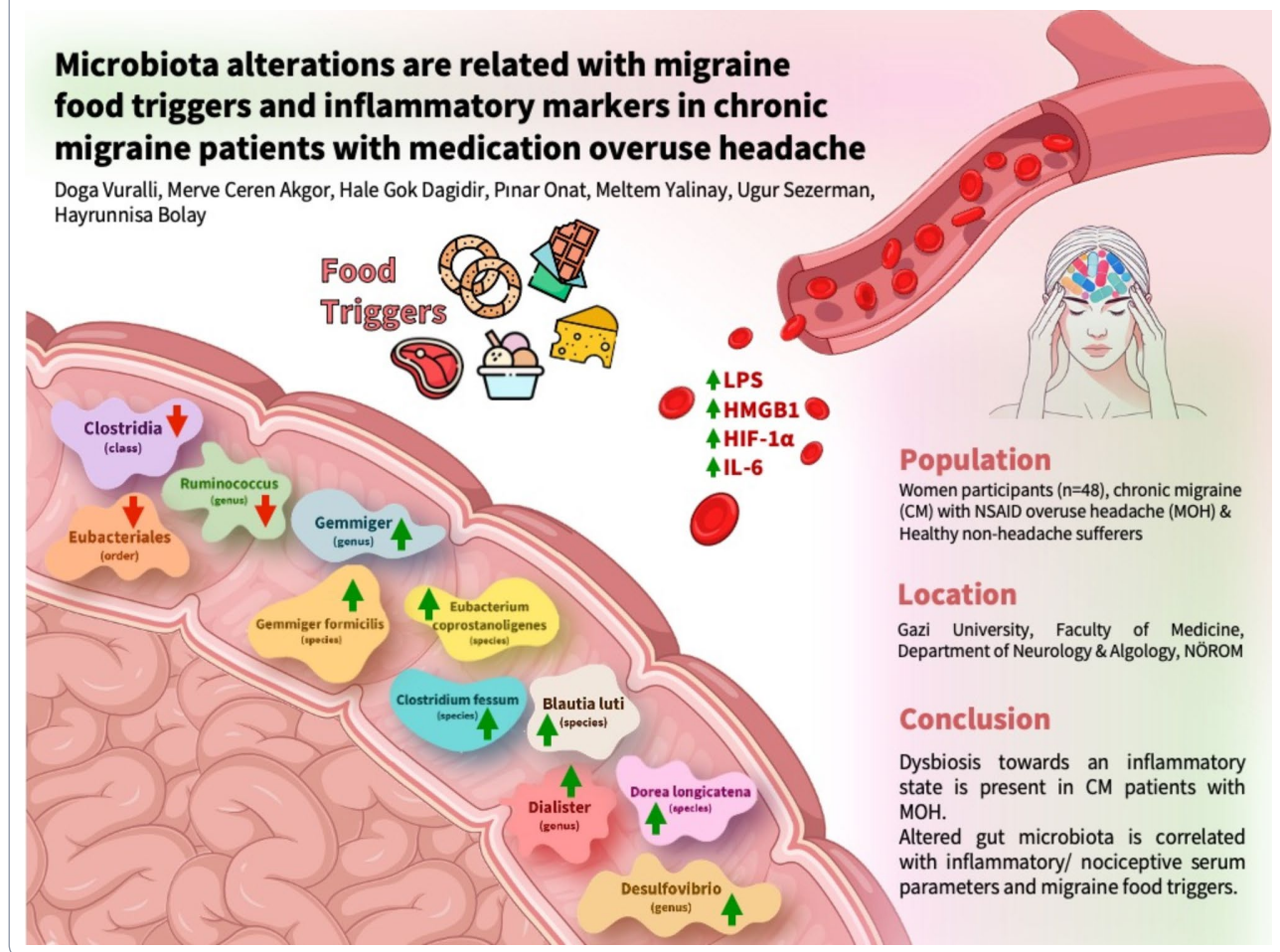


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**Conclusion** In CM patients with MOH, we have revealed the presence of dysbiosis towards an inflammatory state, and positive correlations were shown between altered gut microbiota and inflammatory serum parameters and migraine food triggers.

**Keywords** Medication overuse headache, Chronic migraine, Microbiota, Lipopolysaccharide, Leaky gut, HMGB1, HIF-1 $\alpha$ , Inflammation

### Graphical abstract



### Introduction

Gut microbiota has a pivotal role in the regulation of human physiology and the bidirectional pathways of the gut-brain axis are involved in many neurological diseases and their role in migraine has gained attention in recent years. Factors such as the microbiota composition and inflammatory mediators influence the gut-brain axis. Altered microbiota profiles are suggested to play a crucial role in cardiovascular, neurological, and psychiatric diseases [1]. It has been shown that the microbiota profiles of migraine patients were different compared to the healthy controls [2]. Chronic migraine (CM) and medication overuse headache (MOH) are associated with increased gut permeability [3] and altered gut microbiota composition may underlie the intestinal

hyperpermeability and inflammation seen in this headache disorder.

MOH is recognized as a secondary headache frequently seen in CM patients according to the International Classification of Headache Disorders 3rd edition (ICHD-3), even though some clinicians see MOH as a complication of primary headache disorders including CM [4]. Non-steroidal anti-inflammatory drugs (NSAIDs) are the most overused analgesic agents [5] that play a role in the development of MOH in migraine patients. Recently, it was shown that CM patients with MOH were associated with the increased passage of lipopolysaccharide (LPS), bacterial endotoxin, into the bloodstream and consequent release of pro-inflammatory and/or nociceptive molecules such as high mobility group box-1 protein

(HMGB1), calcitonin gene-related peptide (CGRP), hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and interleukin 6 (IL-6) levels suggesting that leaky gut may be involved in MOH pathophysiology [3].

Microbiota profiles of CM patients with MOH have not been investigated before. We aimed to investigate microbiota profiles of CM patients with MOH and the association between microbiota profiles with migraine food triggers and factors associated with leaky gut and inflammation such as LPS, HMGB1, HIF-1 $\alpha$ , and IL-6 levels. This study aims to provide information about the possible link between CM and NSAID overuse headache, altered microbiota profiles, leaky gut, and migraine food triggers.

## Methods

Female CM patients with MOH (NSAID overuse headache) were consecutively recruited from Gazi University Faculty of Medicine, Department of Neurology and Algology, Headache outpatient clinic between July 2023 and December 2023. Only women participants were enrolled in the study. CM patients with MOH were included in the study if, (1) the age was between 18 and 65 years of age, (2) they had a definite diagnosis of chronic migraine (1.3) according to ICHD-3, (4) they had a diagnosis of NSAID overuse headache (8.2.3.2) according to ICHD-3, (5) they did not use any migraine prophylactic medications, (6) they agreed to give a blood sample for enzyme-linked immunosorbent assay (ELISA) analysis, (6) they agreed to bring fecal sample and (7) they had normal white blood cell count (WBC), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels. The inclusion criteria for healthy controls were; (1) age between 18 and 65 years of age, (2) no history of headaches, (3) agreeing to bring fecal samples and (4) having normal WBC, ESR, and CRP levels. Exclusion criteria for the participants were; (1) history of any other chronic or neurological disease or having any laboratory findings suggesting a chronic systemic disease, (2) abnormally hard or loose stool state types [6] or failure to bring the fecal sample, (3) any history of food intolerance or food allergies, (3) chronic daily use of any medications, (4) any infections within 7 days before and after the blood sample or fecal sample collection, (5) history of alcohol or drug abuse (6) smoking and (7) history of any major psychiatric disease. Two headache experts (DV and HB) included the patients in the study after a detailed clinical assessment. The patients were called a month later to question whether they received a diagnosis of any chronic disease within one month after the blood sample and fecal sample collection.

The number of migraine headache days and migraine duration were recorded for CM patients with MOH. Hospital Anxiety and Depression Scale Anxiety

(HADS-A) and Depression subscales (HADS-D) scores were recorded for both groups. A detailed migraine food trigger questionnaire was filled out [5] and the irritable bowel syndrome (IBS) symptoms were assessed in both groups according to the Rome IV Criteria [7].

Serum LPS, HMGB1, HIF-1 $\alpha$ , and IL-6 levels were assessed using enzyme-linked immunosorbent assay (ELISA). The blood samples collected from both groups were centrifuged (20 min 1000\* $g$  at 2–8 °C) to obtain the serum samples and they were stored at –80 °C until further analysis. The local ethics committee approved the study and it complies with the Declaration of Helsinki.

## Enzyme-linked Immuno Sorbent Assay (ELISA)

Serum LPS, HMGB1, HIF-1 $\alpha$ , and IL-6 levels were measured with high-sensitivity ELISA kits. ELISA kits were obtained from Elabscience Biotechnology Inc., Houston, TX, USA [IL-6 (E-EL-H6156), HMGB1 (E-EL-H1554), HIF-1  $\alpha$  (E-EL-H6066)] and CUSABIO Inc., Wuhan, China [Human LPS (CSB-E0994h)]. The coefficients of variation were <10%.

All of the reagents and serum samples were brought to room temperature before the analysis. Dilution of the reference standards provided in each kit was made according to the instructions of the manufacturer. Analytical-grade deionized water was added to the concentrated wash buffers to obtain the wash buffers. The kits were used following the assay procedure. Washing processes were performed using the Combiwash Human ELISA plate washer. A stop solution was added to terminate the enzyme-substrate reaction and a rapid color change to yellow was detected. The optical density of each well at 450 nm was measured using Chromate microplate reader.

## Microbiota analysis

### Sample processing and sequencing

After obtaining the samples, bacterial DNA isolation was performed with the ZymoBIOMICS DNA Miniprep kit. The obtained DNAs were checked by measuring both the precise DNA amount with Qubit 2.0 and the purity with NanodropONEc. In the cases where the purity of the DNA was not found sufficient, cleaning was performed with the magnetic bead method. Since amplicon creation for 16 S rRNA-targeted amplicon sequencing was carried out by the polymerase chain reaction (PCR) method, DNA purity was very important. DNAs additionally purified with magnetic beads were amplified with 27 F-1453R universal 16 S primers to create an amplicon library. After measurements of the amplified PCR products were made with Qubit 2.0, they were purified again with the magnetic bead method to remove impurities caused by PCR. The DNA library of the purified samples was prepared with the 16S024 kit provided by Oxford Nanopore

Technologies. Barcoded samples for adapter and multiplexing were purified again with magnetic beads, then combined with sequencing buffer and sequenced with MinION FLO-MIN106D flowcells. Sequencing was performed to obtain at least 30,000 sequence readings from each sample. Unlike the 16 S (V3-V4 regions) sequencing performed with Illumina MiSeq technology, since all regions for 16 S were sequenced, the average quality score (phred score) according to the Lambda phage loaded with the barcoding kit for long multiple runs rather than the high number of sequence reads is Q17. After sequencing, the read values were optimized and debugged according to the reference of the 3.6 kb Lambda phage included in the kit, and ONT FAST5 signal Hiles were converted into fastq Hiles. Consensus sequences were created with the obtained Fastq Hiles and annotated according to the NCBI 16 S database. Taxonomy was created at the species or genus level based on 95% accuracy in taxonomy creation.

#### Statistical analysis

Data analysis was performed using IBM SPSS statistical software version 22.0 (USA) and python 3.11.4. The normal distribution of data was investigated by the Shapiro-Wilk normality test. Continuous variables were indicated as mean±standard deviation and categorical variables were given as frequency and percentage values. The analysis of normally distributed continuous variables was performed with a student t-test. The analysis of non-normally distributed continuous variables was performed with the Mann-Whitney's U test.  $p < 0.05$  was considered statistically significant.

#### Explorative analyses

In order to explore the dataset using visualizations and in terms of bacterial diversity, firstly relative abundance plots were created using the plot functionality of the pandas package.

Each group's mean/median diversity was then compared using either Mann Whitney U test or t-test (based on the results of the Shapiro-Wilk normality test, if both groups were normally distributed, t-test was used, if not, Mann Whitney U was used) using python package scipy, and its related functions. For alpha diversities, Shannon index, Simpson index, and Observed OTU metrics were used. Differences in alpha diversities were visualized using boxplots made using the seaborn package. To explore the beta diversities, PCoA was applied using the skbio package. As the distance metric, Bray-Curtis was chosen. To show the features (bacteria) most involved in the first two components, a loadings plot was created along with the scatter plot of the original PCoA, both done with the seaborn package.

#### Comparative analyses

Comparative analyses were applied with comparative statistical testing, applying either Mann Whitney U or t-test to group comparisons depending on the normality of the groups. The results were then visualized with seaborn boxplots. Bacteria that were seen in significantly different levels in compared groups were then selected ( $p$  value  $< 0.05$ ) to be visualized in a plot showing both the log fold changes (logFCs) and the  $p$ -values. For this, a violin plot was created with the seaborn package.

#### Correlation analyses

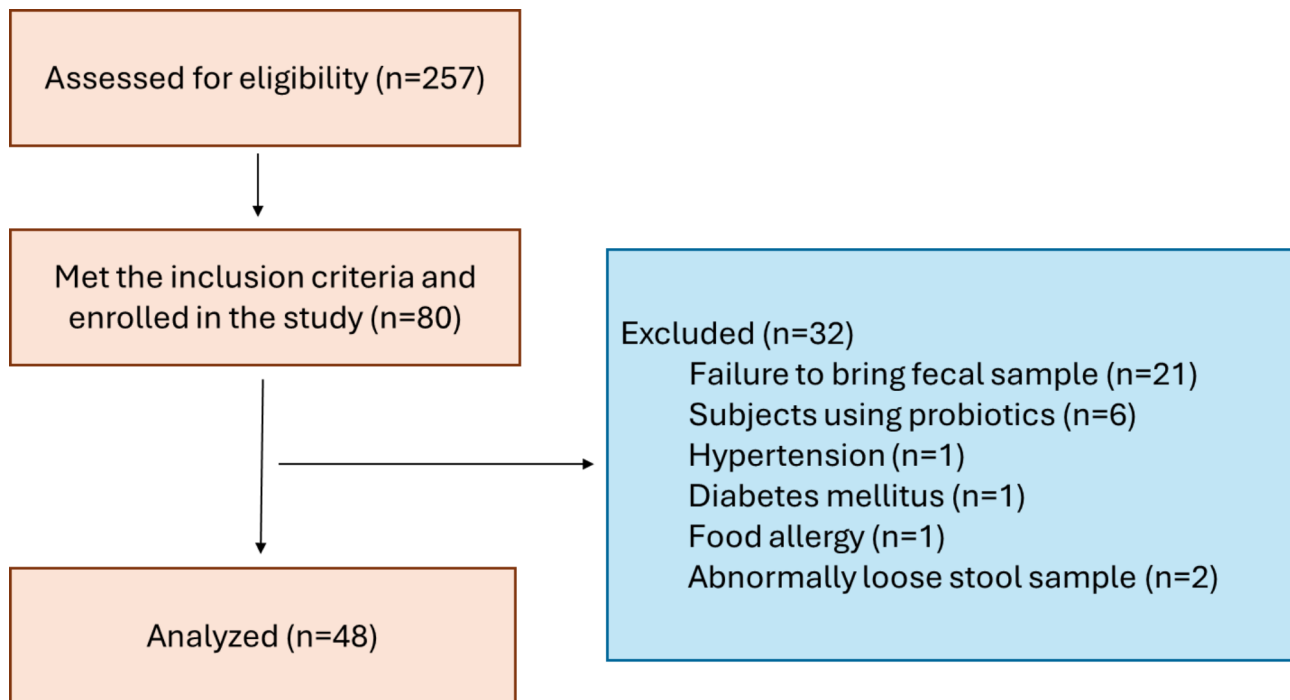
Correlation analyses were applied on either continuous and categorical data (when correlating bacterial percentage values and migraine food trigger categories), or continuous and continuous data (when correlating bacterial percentage values and inflammatory serum marker levels). For correlating continuous and categorical data, Point Biserial Correlation (scipy package) was used, and for correlating continuous and continuous groups, Spearman correlation (scipy package) was used, as most groups contain many zeros and not normally distributed. Each correlation analysis was visualized using seaborn heatmaps.

In correlation analyses including bacterial value data, only the bacteria that were seen significantly different in the related group comparisons were used, to limit the size of the graphs and to filter the output information.

#### Results

Flow-chart of the study is given in Fig. 1. Thirty-two CM+MOH patients and 16 healthy controls were included in the study. The mean age was  $41.4 \pm 8.5$  years in the chronic migraine and MOH group and  $41.0 \pm 12.1$  years in the healthy controls ( $p = 0.89$ ). In the chronic migraine and MOH group, migraine duration was  $244.1 \pm 78.8$  months, and mean monthly migraine headache days was  $22.2 \pm 7.4$  days.

Serum LPS, HMGB1, HIF-1 $\alpha$  and IL-6 levels were significantly higher in the CM+MOH patients compared to the healthy controls ( $p = 0.002$ ,  $p = 0.023$ ,  $p = 0.009$  and  $p = 0.042$  respectively) (Fig. 2). HADS-A scores and HADS-D scores were significantly higher in the CM+MOH patients compared to the healthy controls ( $p < 0.0001$  and  $p < 0.0001$  respectively) (Fig. 2). IBS symptoms were defined by 20 CM+MOH patients (62.5%) and 5 healthy controls (31.3%). IBS symptoms were higher in CM+MOH patients compared to the healthy controls ( $p < 0.0001$ ). Twenty-five CM patients with MOH (78.1%) were fast eaters with a chewing time of each bite less than 20 s while 14 healthy controls (87.5%) were fast eaters. The percentage of fast eaters was similar between the two groups ( $p = 0.43$ ).



**Fig. 1** The flowchart of the study

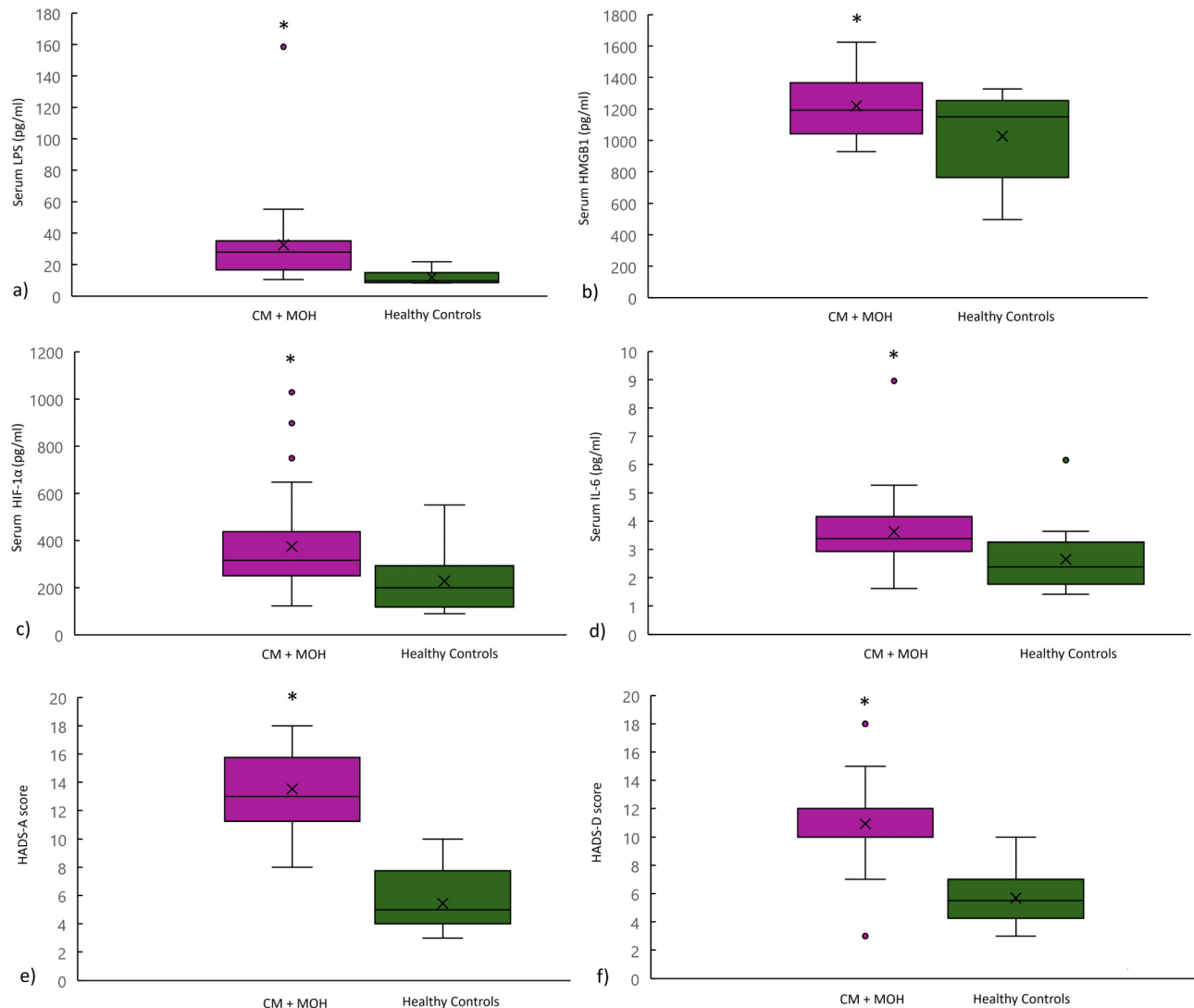
Alpha diversity represents the richness and evenness of microbial community. Alpha diversities evaluated by Shannon and Simpson indices and Observed OTU richness were similar between CM and control groups ( $p=0.45$ ,  $p=0.75$  and  $p=0.88$  respectively) (Fig. 3). Beta diversity shows the dissimilarity of community composition between the groups. PCoA analysis with Bray-Curtis dissimilarity index was performed to evaluate overall diversity of gut microbiota between CM patients with MOH and healthy non-headache sufferers. PCoA with Bray-Curtis dissimilarity index for beta diversity at the genus level revealed no significant difference between the groups ( $p=0.128$ ) (Fig. 3).

The microbiota analysis was performed at the class, order, family, genus, and species levels (Fig. 4). At the class level, Clostridia was less abundant in the CM+NSAID overuse headache group compared to the control group ( $p=0.035$ ). At the order level, Eubacteriales was less abundant in the CM+NSAID overuse headache group compared to the control group ( $p=0.035$ ). At the genus level, Ruminococcus was less abundant and Desulfovibrio, Gemmiger and Dialister were more abundant in the CM+NSAID overuse headache group compared to the healthy controls ( $p=0.034$ ,  $p=0.045$ ,  $p=0.034$  and  $p=0.027$  respectively). At the species level, Clostridium fessum, Blautia luti, Dorea longicatena, Eubacterium coprostanoligenes, and Gemmiger formicilis showed relative positive predominancy in the CM+NSAID overuse headache group compared to the control group ( $p=0.002$ ,  $p=0.016$ ,  $p=0.027$  and  $p=0.024$  respectively).

Clostridium fessum was also significantly abundant in fast eater CM+MOH patients compared to fast eater healthy controls ( $p=0.002$ ). LogFC of significantly different bacteria between chronic migraine patients and healthy controls is given in Fig. 5a. LogFC of significantly different bacteria between subjects with and without irritable bowel syndrome is given in Fig. 5b. LogFC of significantly different bacteria between subjects with a chewing time of less or more than 20 s for each bite is given in Fig. 5c.

#### Correlation analysis

The correlations between migraine food triggers and altered gut bacteria in the chronic migraine group were analyzed (Fig. 6). The relative abundance of Desulfovibrio was positively correlated with peanut butter ( $r=0.88$ ,  $p=0.015$ ), grapefruit ( $r=0.59$ ,  $p<0.001$ ), soya sauce ( $r=0.45$ ,  $p=0.01$ ), coffee cream/milk powder ( $r=0.38$ ,  $p=0.03$ ) and ice cream ( $r=0.38$ ,  $p=0.03$ ). Increase in Gemmiger was positively associated with ice cream ( $r=0.37$ ,  $p=0.035$ ). The genus Dialister was positively correlated with frozen meat and chicken products ( $r=0.58$ ,  $p<0.001$ ), hamburger/pizza ( $r=0.53$ ,  $p=0.002$ ), red meat/minced meat ( $r=0.52$ ,  $p=0.002$ ), aged cheese ( $r=0.51$ ,  $p=0.003$ ), ketchup ( $r=0.49$ ,  $p=0.005$ ), coffee cream/milk powder ( $r=0.47$ ,  $p=0.006$ ), cream filled pastries ( $r=0.47$ ,  $p=0.006$ ), pickles ( $r=0.47$ ,  $p=0.007$ ), sweet sauces ( $r=0.43$ ,  $p=0.01$ ), soya sauce ( $r=0.41$ ,  $p=0.02$ ), vinegar ( $r=0.40$ ,  $p=0.02$ ) and chocolate/cacao ( $r=0.38$ ,  $p=0.03$ ). Ethanoligenens harbinense

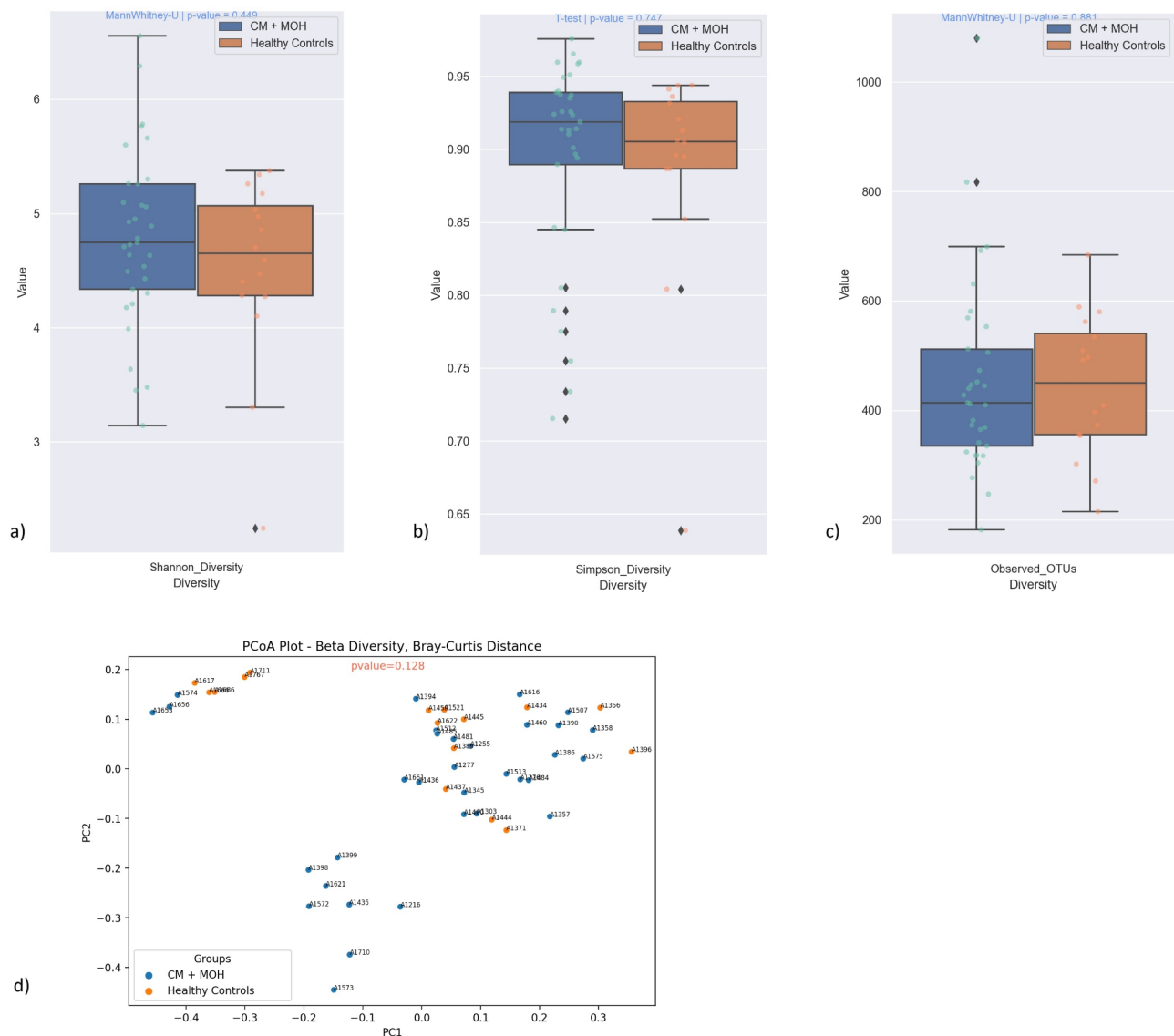


**Fig. 2** Serum (a) LPS, (b) HMGB1, (c) HIF-1α and (d) IL-6 levels were significantly higher in CM+MOH patients compared to healthy controls ( $p=0.002$ ,  $p=0.023$ ,  $p=0.009$  and  $p=0.042$  respectively). CM+MOH patients had higher (e) HADS-A and (f) HADS-D scores compared to healthy controls ( $p < 0.0001$  and  $p < 0.0001$  respectively). Data are shown as whisker-box plots (whisker: full range; line: median; cross: mean)

was positively correlated with chocolate/cacao ( $r=0.38$ ,  $p=0.03$ ), canned tuna fish ( $r=0.56$ ,  $p < 0.001$ ), ready-to-eat canned foods ( $r=0.56$ ,  $p < 0.001$ ), cream filled pastries ( $r=0.36$ ,  $p=0.04$ ) and excessive carbohydrate consumption in a single meal ( $r=0.43$ ,  $p=0.01$ ). Eubacterium coprostanoligenes was positively associated with peanut ( $r=0.74$ ,  $p < 0.001$ ), onion/garlic ( $r=0.51$ ,  $p=0.003$ ), pastries/simit (Turkish bagel) ( $r=0.37$ ,  $p=0.035$ ), orange juice ( $r=0.47$ ,  $p=0.006$ ), cream filled pastries ( $r=0.36$ ,  $p=0.04$ ), cream cheese ( $r=0.75$ ,  $p < 0.001$ ), ready-to-use sauces (such as pesto sauce) ( $r=0.38$ ,  $p=0.03$ ) and ready-to-eat canned foods ( $r=0.63$ ,  $p < 0.001$ ). Dorea longicatena was positively correlated with yoghurt ( $r=0.45$ ,  $p=0.009$ ), soudjouk/sausage ( $r=0.64$ ,  $p < 0.001$ ), salami/pastrami ( $r=0.48$ ,  $p < 0.001$ ), green pepper/green beans ( $r=0.45$ ,  $p=0.009$ ). Thermoclostridium stercorarium

was positively correlated with peanut ( $r=0.41$ ,  $p < 0.001$ ) and cream cheese ( $r=0.43$ ,  $p < 0.001$ ). Clostridia bacteria was negatively correlated with chocolate/cacao ( $r = -0.4$ ,  $p=0.02$ ), pickles ( $r = -0.49$ ,  $p=0.004$ ), butter ( $r = -0.46$ ,  $p=0.008$ ), chicken ( $r = -0.46$ ,  $p=0.008$ ), milk ( $r = -0.6$ ,  $p < 0.001$ ), pastrami/salami ( $r = -0.44$ ,  $p=0.01$ ), mushroom ( $r = -0.46$ ,  $p=0.008$ ), red meat/minced meat ( $r = -0.45$ ,  $p=0.01$ ), ketchup ( $r = -0.44$ ,  $p=0.01$ ), frozen meat and chicken products ( $r = -0.53$ ,  $p=0.002$ ) and chickpea and beans ( $r = -0.46$ ,  $p=0.008$ ).

LPS was positively correlated with Hapalosiphonaceae ( $r=0.37$ ,  $p=0.01$ ), HMGB1 was positively associated with Melghirimyces ( $r=0.39$ ,  $p=0.002$ ), HIF1-α was positively correlated with Rouxeilla ( $r=0.40$ ,  $p=0.005$ ) in both groups and Blautia luti ( $r=0.30$ ,  $p=0.048$ ) in CM+MOH group (Fig. 7), IL-6 was positively correlated

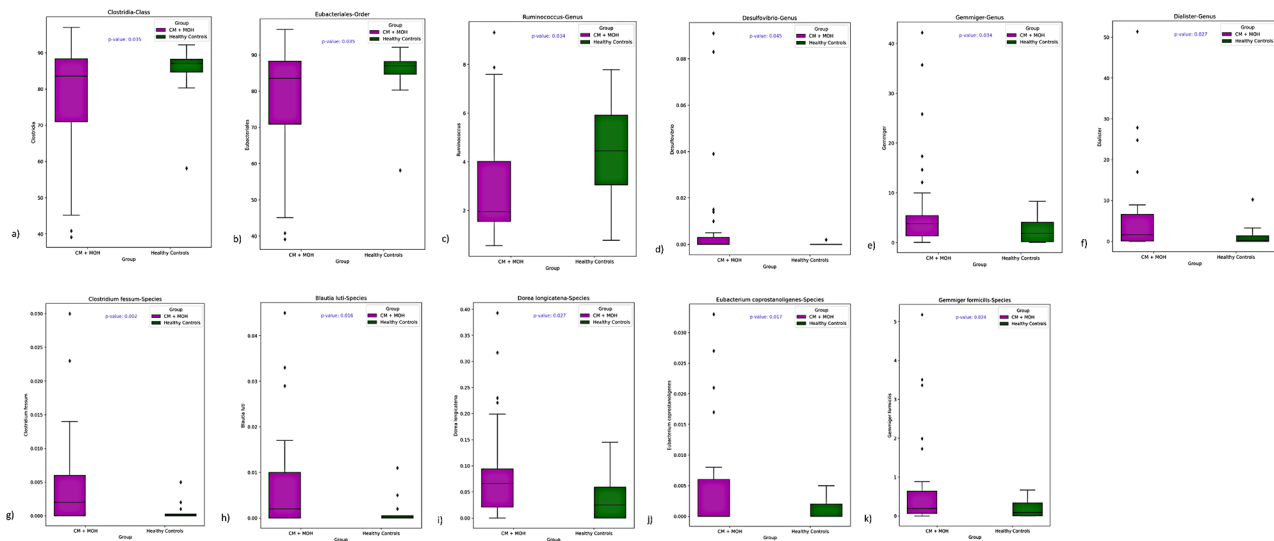


**Fig. 3** Alpha diversities measured by Shannon and Simpson indices and Observed OTU richness were comparable between CM+MOH and control groups ( $p=0.45$ ,  $p=0.75$  and  $p=0.88$  respectively). PCoA with Bray-Curtis dissimilarity index for beta diversity at the genus level showed no significant difference between CM+MOH and healthy control groups ( $p=0.128$ )

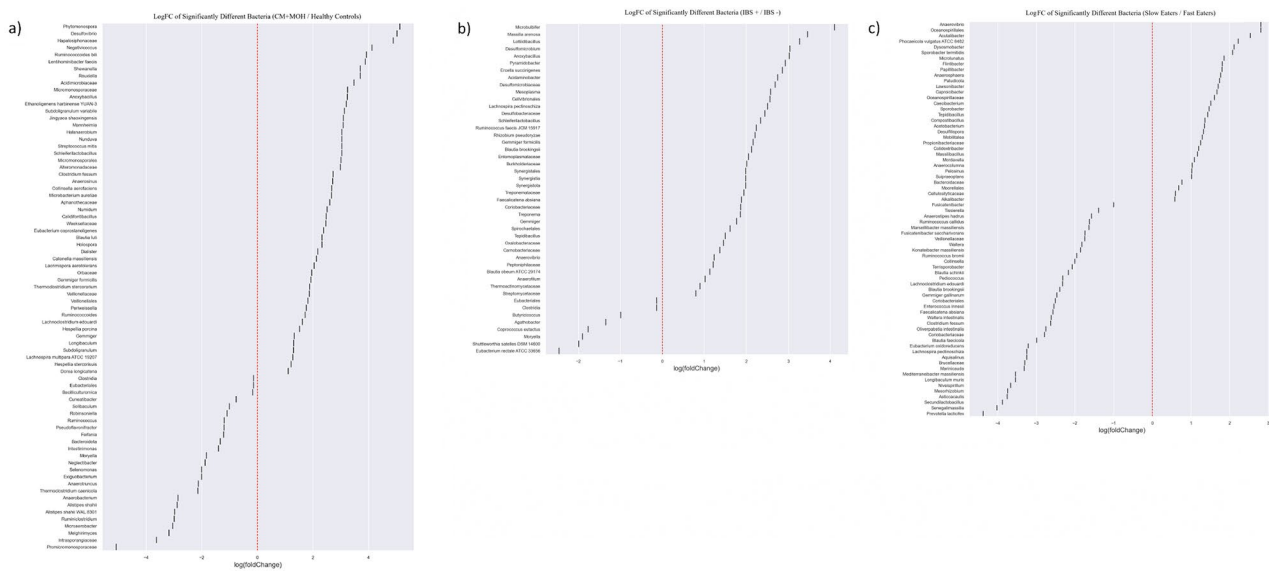
with *Melghirimyces* ( $r=0.35$ ,  $p=0.048$ ) and *Ruminococcus* ( $r=0.60$ ,  $p<0.001$ ) in CM+MOH patients (Fig. 7) while negatively correlated with *Subdoligranulum variabile* ( $r=-0.48$ ,  $p=0.006$ ) and the genus *Subdoligranulum* ( $r=-0.45$ ,  $p=0.009$ ) in CM+MOH patients. HAD anxiety scores were positively correlated with an increase in *Subdoligranulum variabile* ( $r=0.41$ ,  $p=0.001$ ) and, *Desulfovibrio* ( $r=0.45$ ,  $p=0.002$ ), *Anoxybacillus* ( $r=0.48$ ,  $p=0.002$ ) and *Aphanothecaceae* ( $r=0.36$ ,  $p=0.01$ ) in both groups and HAD depression scores were positively associated with *Moryella* ( $r=0.38$ ,  $p=0.03$ ) in CM+MOH patients and negatively associated with *Neglectibacter* ( $r=-0.44$ ,  $p=0.002$ ) in both groups (Fig. 7).

## Discussion

We showed the presence of dysbiosis in the gut microbiota of CM patients with MOH towards an inflammatory state as evidenced by the relative abundance of *Desulfovibrio*, *Dialister*, *Dorea longicatena*, and *Blautia luti* and relative depletion of *Ruminococcus*. Inflammatory serum parameters such as LPS, HMGB1, IL-6, and HIF1- $\alpha$  levels were positively correlated with these certain bacteria in the altered gut microbiota of CM patients. Additionally, alterations in microbiota composition were correlated with migraine food triggers. The microbiota changes were also linked to anxiety and depression scores, and the presence of irritable bowel syndrome symptoms.



**Fig. 4** (a) The class Clostridia ( $p=0.035$ ), (b) the order Eubacteriales ( $p=0.035$ ) and (c) the genus Ruminococcus ( $p=0.034$ ) were less abundant in the CM + MOH group compared to the control group. The genera (d) Desulfovibrio, (e) Gemmiger and (f) Dialister were predominant in the CM + MOH group compared to the healthy controls ( $p=0.045$ ,  $p=0.034$  and  $p=0.027$  respectively). At the species level, (g) Clostridium fissum, (h) Blautia luti, (i) Dorea longicatena, (j) Eubacterium coprostanoligenes, and (k) Gemmiger formicilis were more abundant in the CM + MOH group compared to the control group ( $p=0.002$ ,  $p=0.016$ ,  $p=0.027$  and  $p=0.024$  respectively)

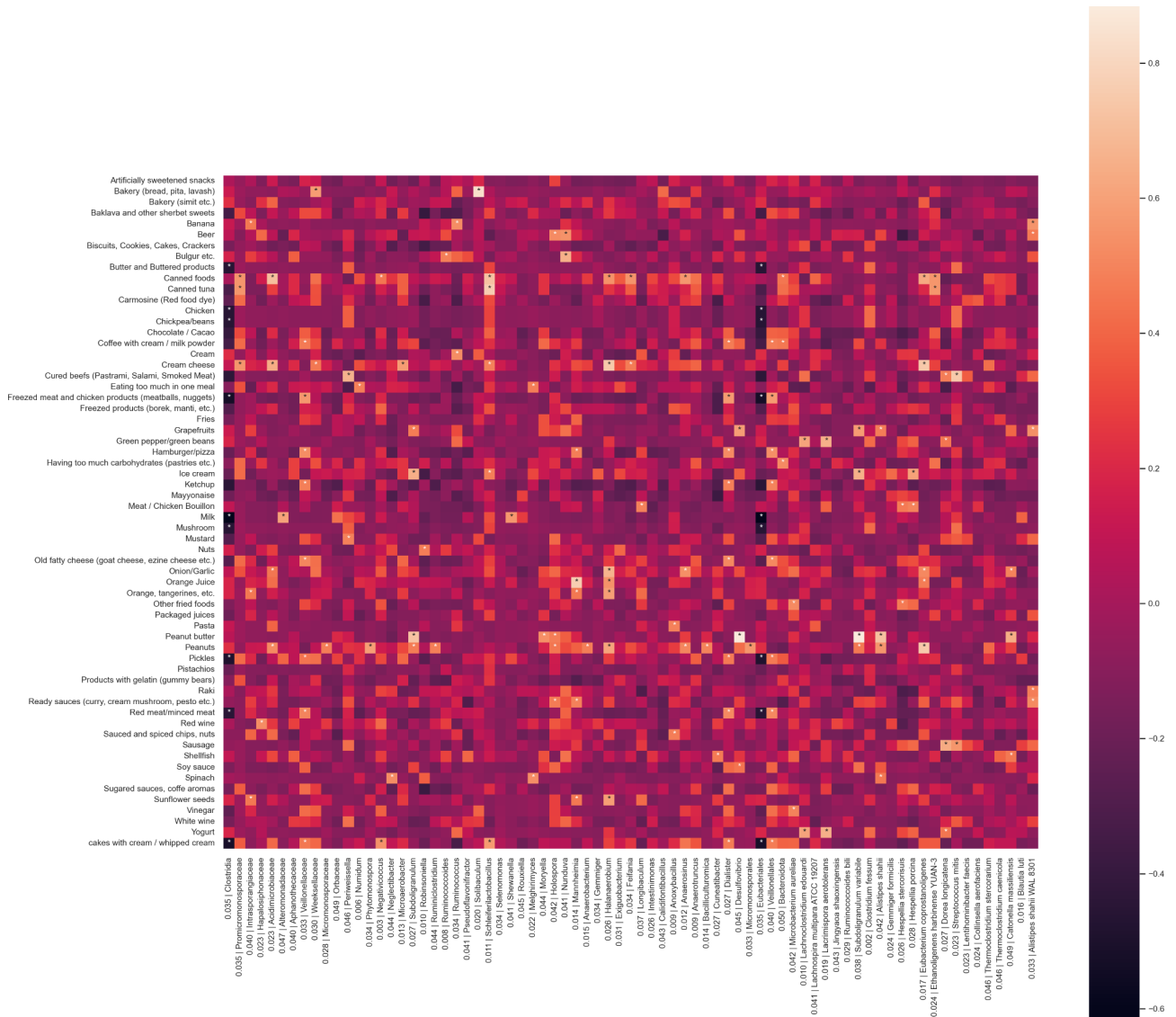


**Fig. 5** (a) Log Fold Change (LogFC) calculated as Chronic migraine / Healthy Controls. Positive logFC indicates bacteria that were significantly more abundant in chronic migraine group and negative logFC indicates bacteria that were significantly more abundant in healthy controls. (b) LogFC of significantly different bacteria between subjects with and without IBS. Positive logFC indicates bacteria that were significantly more abundant in subjects with IBS and negative logFC indicates bacteria that were significantly more abundant in subjects without IBS. (c) LogFC of significantly different bacteria between subjects with a chewing time of less or more than 20 s for each bite. Positive logFC indicates bacteria that were more abundant in subjects with a chewing time of more than 20 s and negative logFC indicates bacteria that were more abundant in subjects with a chewing time of less than 20 s. The fold change of all bacteria that showed significant differences between the two groups in statistical tests with a  $p$ -value less than 0.05 is shown in this figure

Studies have addressed strong correlations between gut microbiota alterations and commonly used medications such as NSAIDs [8]. Intestinal injury induced by NSAIDs is also shown to be associated with alterations in gut microbiota and dysbiosis. Several animal studies have assessed the role of NSAIDs on gut microbiota, however,

there are only few human studies that have investigated the effect of NSAIDs on gut bacteria. The use of NSAIDs, both in preclinical and clinical studies had no clear effect on alpha diversity [9–14]. In our study, there was no difference regarding the alpha diversity between CM patients with NSAID overuse headache and healthy



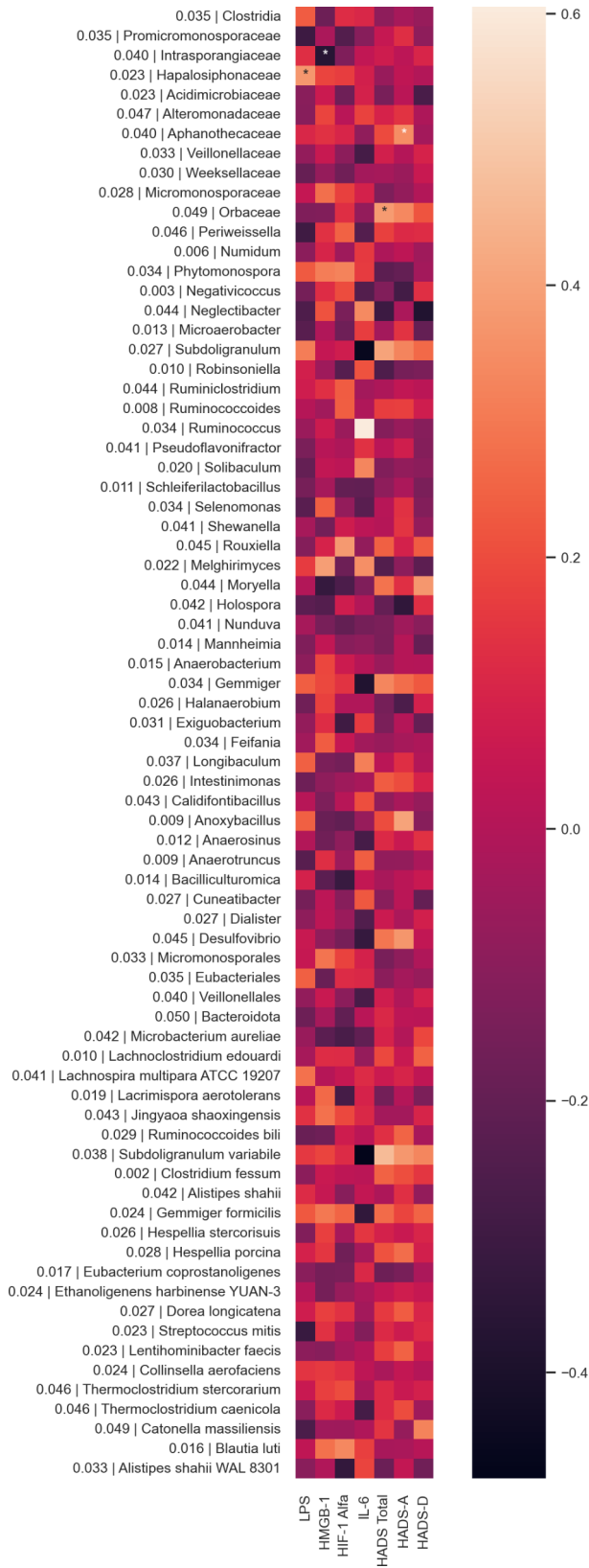


**Fig. 6** Heatmaps showing correlations between migraine food triggers and altered gut microbiota

controls. Previously Edogawa et al. [9] showed that indomethacin decreased the abundance of Ruminococcus in women and similarly, in this study, CM patients with NSAID overuse headache had less abundance of Ruminococcus compared to healthy non-headache sufferers. In a study conducted by Rogers and Aronoff in their community residents, the bacteria family Desulfovibrionaceae was shown to be more common in NSAID users than in the controls [11]. In the present study, the genus Desulfovibrio was more abundant in the CM+NSAID overuse group than in the healthy controls. In a recent study that investigated the composition of gut microbiota in pediatric migraine patients, similar to our results, Gemmiger was more abundant in migraine patients [15]. Previously, Gemmiger was also suggested to have role in depression [16]. The genus Ruminococcus was less abundant in CM

patients with MOH. Migraine is known to be associated with certain comorbidities such as depression, irritable bowel syndrome and inflammatory bowel disease. Ruminococcus depletion has been observed in major depressive disorder, irritable bowel syndrome and inflammatory bowel disease patients [17–19].

Blautia luti was another bacteria species that was more abundant in the CM patients with NSAID overuse headache and it was also positively correlated with HIF1- $\alpha$  levels. In previous studies, Blautia luti was significantly increased in inflammatory bowel disease patients compared to the control group [20]. Blautia luti was also reported to be higher in abundance in depression patients compared to healthy controls [21]. However, there are controversial results regarding Blautia luti species. Decreased incidence of Blautia luti was shown in gut



**Fig. 7** Heatmaps showing correlations between LPS, HMGB1, HIF-1α, IL-6, HADS-A, HADS-D scores and altered gut microbiota

microbiota of children with obesity and the decrease was more pronounced in the gut microbiota of obese children who also had insulin resistance suggesting that depletion of *Blautia luti* could be associated with metabolic inflammation [22].

*Dorea longicatena* was more abundant in CM patients with MOH compared to healthy non-headache sufferers in our study. *Dorea longicatena* abundance was reported to be associated with being overweight and obesity [23] and insulin resistance after gastric bypass [24]. *Dorea longicatena* also metabolizes sialic acid found at the terminal ends of mucins and release of sialic acid is associated with mucin degradation that increases gut permeability [25]. IL-6 levels were negatively correlated with *Subdoligranulum* in this study. Similarly, in a previous study, *subdoligranulum* was also shown to be negatively correlated with IL-6 levels in overweight and obese individuals [26].

*Desulfovibrio* (DSV) bacteria are sulfate-reducing and anaerobic bacteria that are associated with many diseases such as irritable bowel syndrome, inflammatory bowel diseases, Parkinson's disease, bacteremia, autism, liver and chronic kidney diseases, autoimmune diseases and metabolic syndrome [27]. *Desulfovibrio desulfuricans* has been reported to have inflammatory effects through LPS [28–30]. *Desulfovibrio fairfieldensis* have outer membrane vesicles that are associated with increased inflammation and tight junction dysfunction in the intestinal barrier [31]. DSV abundance also has a role in impaired cognition and memory deficits [32]. DSV abundance was also shown to be correlated with anxiety and depression in ulcerative colitis patients [33]. Similarly in our study, in CM patients with MOH, the abundance of DSV bacteria was positively correlated with HAD anxiety scores.

In a previous study that investigated alterations in gut microbiota in pediatric migraine patients, *Dialister* was relatively abundant in female migraine patients compared to female healthy controls whereas, *Desulfovibrio* was more abundant in the migraine patients compared to healthy controls in the male group [34]. A positive correlation between the abundance of the genus *Dialister* and Ankylosing Spondylitis Disease Activity Score was shown in patients with spondyloarthritis and it was suggested as a marker of inflammation [35]. *Desulfovibrio* and *Dialister* were also more abundant in the CM patients with NSAID overuse headache compared to the healthy controls in our study.

Alterations in microbiota composition may contribute to the role of certain foods as migraine triggers. Probable mechanisms underlying the correlation between certain bacteria alterations and significantly related migraine food triggers are given in Table 1. *Clostridia*, *Dialister*, DSV, *Gemmiger*, *Ethanoligenens harbinense*,

**Table 1** The correlation between certain bacteria alterations and significantly related migraine food triggers in this study and the probable underlying mechanisms

Altered bacteria	Migraine food triggers	Explanation	The probable mechanism
Clostridia (class) ↓	Chocolate/cacao, pickles, butter, chicken, milk, pastrami, sausage, mushroom, red meat / minced meat, ketchup and frozen meat and chicken products	Clostridia has been shown to produce indole acetic acid through tryptophan metabolism [38]. Chocolate/cacao, red meat and red meat products, milk and milk products and poultry are rich in tryptophan [36, 37]. A decrease in the class clostridia could result in excess tryptophan.	Tryptophan metabolism and serotonergic mechanisms
Desulfovibrio (genus) ↑	Peanut butter, grapefruit, soya sauce, clotted cream, custard, ice cream and excessive carbohydrate consumption in a single meal	Desulfovibrio is a sulfate-reducing bacteria and sulfate and sulfite are derived from dietary sources especially food preservatives [27]. Sulfites trigger headache via histamine release.	Histaminergic mechanisms
Gemmiger (genus) ↑	Ice cream	Gemmiger is a butyrate producing bacteria and butyrate has an influence on serotonin release from intestinal cells [45]. Milk products are rich in tryptophan [36–38].	Tryptophan metabolism and serotonergic mechanisms
Dialister (genus) ↑	Frozen meat and chicken products, hamburger, pizza, coffee cream and milk powder, ketchup, cream filled pastries, red meat/minced meat, pickles, aged cheese, chocolate/cacao, sweet sauces, soya sauce and vinegar	Chocolate/cacao, red meat and red meat products, milk and milk products and poultry are rich in tryptophan [36–38]. Dietary tryptophan significantly increased the relative abundance of Dialister in the caecum microbiota of weaned piglets [40].	Tryptophan metabolism and serotonergic mechanisms
Ethanoligenens harbinense (species) ↑	Chocolate/cacao, canned tuna fish, cream filled pastries, ready-to-eat canned foods and excessive eating during one meal	Ethanoligenens harbinense is capable of ethanol-type fermentation and converts sugars into cellular energy and produces ethanol and carbon dioxide [47].	Ethanol-type fermentation
Eubacterium coprostanoligenes (species) ↑	Peanut, pastries, simit (Turkish bagel), cream filled pastries, cream cheese, ready-to-use sauces (such as pesto sauce), ready-to-eat canned foods, onion/garlic, orange juice	Eubacterium coprostanoligenes ferments carbohydrates into acetic, formic and succinic acids [51, 52]. Succinate was shown to be correlated with colonic inflammation [53].	Colonic inflammation and decreased gut barrier function and increased intestinal permeability
Dorea longicatena (species) ↑	Yoghurt, soudjouk, salami, pastrami, bell pepper and beans	Dorea longicatena is involved in glucose fermentation and produces hydrogen and carbon dioxide [50]. Dorea longicatena metabolizes sialic acid found in mucins and release of sialic acid is associated with mucin degradation which increases gut permeability [25].	Decreased gut barrier function and increased intestinal permeability
Thermoclostridium stercorarium (species) ↑	Peanut and cream cheese	Thermoclostridium stercorarium is a thermophilic bacteria that degrades polysaccharides and produces acetate, ethanol, carbon dioxide and hydrogen [48].	Ethanol mediated activation of nuclear factor- $\kappa$ B signaling pathway, decreased barrier integrity and increased intestinal permeability

Dorea longicatena, Thermoclostridium stercorarium, and Eubacterium coprostanoligenes that are involved in tryptophan metabolism, sulfate reduction, histaminergic mechanisms, ethanol-type fermentation, polysaccharide degradation were shown to be associated with migraine food triggers. Clostridia bacteria was negatively correlated with chocolate/cacao, pickles, butter, chicken, pastrami/salami, red meat/minced meat, frozen meat, and chicken products. Chocolate/cacao, red meat and red meat products, milk and milk products, and poultry are rich in tryptophan [36–38]. Clostridia has been shown to produce indole acetic acid through tryptophan metabolism [34]. Tryptophan metabolism could be one of the mechanisms underlying the association between food triggers and microbiota. Tryptophan is a precursor for serotonin, a neurotransmitter involved in migraine

pathophysiology and high brain serotonin levels were shown in migraine patients between the attacks [39]. The genus Dialister was positively correlated with frozen meat and chicken products, red meat/minced meat, aged cheese, and chocolate/cacao in our study. These migraine food triggers are rich in tryptophan. Dialister in the caecum microbiota of weaned piglets was shown to be significantly increased by dietary tryptophan [40]. The genus Dialister was also shown to be increased with 4-week interventions with a vegan diet and whole grain barley [41, 42]. DSV is a sulfate-reducing bacteria and hydrogen sulfide is a by-product of DSV which may be toxic to the host in high concentrations. Sulfate and sulfite are derived from dietary sources especially food preservatives [27]. Sulfites are suggested to provoke headache via the release of histamine [43, 44]. Gemmiger

is a butyrate-producing bacteria and butyrate influences serotonin release from intestinal cells [45]. The abundance of Gemmiger was positively associated with ice cream. Milk products contain tryptophan [36–38]. Ultra-processed food consumption (>5 servings per day) was also shown to be associated with an increase in the genus Gemmiger [46]. *Ethanoligenens harbinense* is capable of ethanol-type fermentation and converts sugars into cellular energy and produces ethanol and carbon dioxide (CO<sup>2</sup>) [47]. The abundance of *thermoclostridium stercorarium* was correlated with peanut and cream cheese. *Thermoclostridium stercorarium* is a thermophilic bacteria that degrades polysaccharides and produces acetate, ethanol, CO<sup>2</sup>, and hydrogen [48]. Ethanol-producing bacteria is associated with ethanol-mediated activation of the nuclear factor- $\kappa$ B signaling pathway which changes the gut barrier integrity and increases systemic endotoxemia [49]. *Dorea longicatena* is involved in glucose fermentation and produces hydrogen and CO<sup>2</sup> [50]. *Dorea longicatena* also metabolizes sialic acid found in mucins and the release of sialic acid is associated with mucin degradation which increases gut permeability and thus results in mild systemic inflammation. *Eubacterium coprostanoligenes* is a cholesterol-reducing bacteria that converts cholesterol to coprostanol and ferments carbohydrates into acetic, formic, and succinic acids [51, 52]. Succinate was shown to be correlated with colonic inflammation [53]. Monosodium glutamate (MSG) is also accused of triggering migraine attacks and can be found in canned and frozen foods, ketchup, and sauces [54]. MSG consumption was shown to induce changes in microbiota and metabolic dysbiosis in a systematic review [55]. Onions contain sulfites and sulfites trigger headaches via histamine release [43, 44]. Migraine patients are more susceptible to histamine-induced headache compared to controls [56]. Histamine infusion was shown to provoke a delayed migraine attack in migraine patients [57]. Elevated plasma histamine levels were reported both during and between the attacks in migraine patients [57]. Peanuts, citrus fruits, pickled and fermented foods contain tyramine and excess dietary tyramine may interfere with gastrointestinal homeostasis and is suggested to be involved in intestinal inflammation. Dietary tyramine and histamine were shown to have synergistic cytotoxic effects on human intestinal cell culture [58]. Tyramine was also suggested to play a role in dietary migraine [59, 60].

The tryptophan-kynurenine (Trp-Kyn) pathway was suggested to be one of the main pathways in the microbiota-gut-brain axis [61]. The gut bacterial strains have synthetic enzymes that may produce tryptophan metabolites and tryptophan is the precursor of serotonin [62, 63]. The germ-free mice were reported to have higher TRP plasma levels [64] and higher serotonin turnover [65].

The probiotic supplementations were shown to reduce 5-hydroxyindoleacetic acid and kynurenine in the frontal cortex [66]. Inflammation is associated with tryptophan metabolism [67]. Tryptophan is metabolized mainly by the indole pathway by the intestinal microbiota. Indican, a metabolite of indole, was found to be high in the urine of pediatric migraine patients [15]. The production of indican by intestinal bacteria increases when there is bacterial overgrowth and dysbiosis [15].

Mainly dietary patterns influence gut microbiota, however, other factors such as age, sex, lifestyle, exercise, genetics, geographic location, cultural traditions, antibacterial drugs, psychological situation, and stress also play a role in this dynamic ecosystem [68, 69]. The same factors may also influence migraine headache frequency in patients with migraine. The association between microbiota and migraine food triggers and serum inflammatory parameters could be influenced by these confounding factors.

The disruption in intestinal barrier integrity results in the leakage of LPS into the circulation and triggers a low-grade systemic inflammatory response. LPS triggers the release of HMGB1, a potent proinflammatory molecule of innate immunity that mediates trigeminal nociception [70, 71, 72]. LPS and HMGB1 act through toll-like receptor-4 (TLR-4) and amplify the immune response including IL-6 [70, 73, 74]. Proinflammatory cytokines such as IL-6 and IL-1 $\beta$  released upon LPS leak, are also nociceptive in the trigeminovascular system and play a role in the development of headache [3, 70, 75].

Increased systemic pro-inflammatory cytokine levels such as TNF- $\alpha$  and IL-6 have been shown in migraine patients [76, 77]. Increased production of interferon-gamma and granulocyte/macrophage colony-stimulating factor was shown in dietary migraine patients after the induction of a migraine headache attack with the dietary trigger [78]. Although elevated systemic inflammatory markers were demonstrated in migraine patients, the underlying cause of the systemic inflammation is unknown. We recently showed increased serum LPS, LPS binding protein, HIF-1 $\alpha$ , VE-cadherin, and IL-6 levels in chronic migraine patients with NSAID overuse headache [3] suggesting leaky gut and LPS escape into the circulation as a source of inflammation. In this study, we have shown correlations between altered microbiota and systemic inflammatory parameters further supporting the role of dysbiosis and consequent leaky gut in CM patients with NSAID headache. Moreover, a link between microbiota changes and migraine food triggers is revealed. Alterations in microbiota composition were also correlated with migraine food triggers shedding light on the mechanisms that may contribute to certain foods' role as migraine triggers. The dysbiosis in gut microbiota was shown previously in migraine patients [79]. The intestinal

dysbiosis was suggested to play a role in migraine by increased intestinal permeability and consequent inflammatory processes [80]. Therapeutic strategies targeting LPS and HMGB1 should be investigated in migraine patients.

Gut dysbiosis reduces the barrier integrity and increases gut permeability and dysregulates immune responses resulting in inflammation and oxidative stress [81]. LPS in the systemic circulation is evidence of intestinal hyperpermeability. Short-chain fatty acids are produced by gut bacteria and play an important role in the gut barrier integrity [82]. SCFAs have neuroprotective and anti-inflammatory effects on the central nervous system [83]. SCFAs exhibit anti-inflammatory effects by regulating the expression of proinflammatory cytokines such as tumor necrosis factor- $\alpha$  and IL-6 [84]. Dysbiosis was shown to impair the production of SCFAs [85]. Colon dysbiosis was shown to be associated with migraine-like pain via TNF- $\alpha$  up-regulation in the trigeminal nociceptive system in mice [86].

A ketogenic diet regulates intestinal microbiota and has anti-inflammatory effects [87] that may have positive effects on metabolic dysbiosis seen in chronic migraine with NSAID overuse headache. Moreover, ketosis was also shown to prevent neurogenic inflammation in mouse models [88]. Inulin supplementation was shown to increase total antioxidant capacity and decrease high sensitivity-CRP levels and oxidative stress index levels in migraine patients [89]. Zonulin, a tight junction protein, was also shown to be decreased after inulin supplementation but it was not significant compared to the control group [89]. The decrease in inflammatory parameters was suggested to be due to improved gut integrity and reduced LPS leakage into the bloodstream [89]. A low glycemic diet also was shown to modify inflammatory responses [90]. TNF- $\alpha$  receptors II and CRP levels were significantly decreased with a low glycemic diet [91]. High omega-3 with low omega-6 diet resulted in a higher headache improvement compared to low omega-6 diet in chronic migraine patients [92]. In a randomized controlled trial (RCT) in episodic migraine patients, omega-3 and nano-curcumin supplementation reduced the expression of TNF- $\alpha$  mRNA and serum TNF- $\alpha$  level [93]. The effects of elimination diets on migraine frequency were studied in two RCTs [94, 95]. In these studies, personalized migraine food triggers were eliminated and a beneficial effect on migraine headache frequency was shown in one of them [94]. Histaminergic mechanisms are also involved in migraine food triggers. Histamine-free diet was also shown to decrease headache attacks and analgesic consumption in chronic headache patients [96]. More studies are required to evaluate the effects of dietary interventions on microbiota, migraine food triggers, and inflammatory parameters in migraine.

There are some limitations of the study. Our sample size was small due to strict inclusion criteria for both CM patients with MOH and healthy controls. We only included female chronic migraine patients with NSAID overuse headache. We conducted the study in this specific group of patients since we have provided recent evidence of leaky gut and enhanced inflammation in female CM patients with NSAID overuse headache [3]. However, the inclusion of such a specific group of patients was also a strength of our study to define the correlations between microbiota and inflammatory markers and migraine food triggers. Another limitation of the study was that we had not studied an extensive inflammatory marker panel. We only evaluated inflammatory markers that were significantly different in CM patients with NSAID overuse headache in our previous study [3]. A more detailed inflammatory marker panel may reveal more specific mechanisms of gut dysbiosis. We have not assessed the microbiota profile of episodic migraine patients and compared the altered microbiota in episodic migraine and chronic migraine patients. This comparison remains to be established. The changes in microbiota and inflammatory markers after the preventive treatment of migraine remain to be evaluated in longitudinal studies. Both CM and MOH are more prevalent in women and NSAIDs are the most overused analgesics worldwide. Therefore, we only included female subjects and patients overusing NSAIDs in our study. However, the latter limits the generalizability of our results to all migraine patients. The microbiota changes should be investigated in patients using other analgesic medications such as triptans. The correlations revealed in our study do not imply a causal relationship, experimental studies are required to show causality. The strength of our study is that we evaluated the correlations between microbiota, inflammatory markers, and migraine food triggers for the first time in a specific group of female CM patients with NSAID overuse headache without using any migraine prophylactic treatments. We involved subjects without any other chronic disease and chronic use of other medications to exclude the influence of other drugs and diseases on gut microbiota.

## Conclusion

In the gut microbiota of CM patients with MOH, we have demonstrated the presence of dysbiosis towards an inflammatory state, and inflammatory serum parameters such as LPS, HMGB1, IL-6, and HIF1- $\alpha$  levels were shown to be positively correlated with certain bacteria in the gut microbiota. The associations between migraine food triggers and altered gut bacteria in CM patients with MOH were significant for some bacteria, even though the exact mechanisms underlying these associations are not known. Few studies investigated the complex link

between migraine and microbiota and this is the first study that evaluated the correlation between migraine food triggers, microbiota changes, and inflammatory parameters in chronic migraine with NSAID overuse headache. Further studies are required to enlighten the role of microbiota in migraine and migraine food triggers and to identify promising biomarkers and their correlations with clinical features that will enlighten new pathways for therapeutic targets. Metabolites of probable pathways underlying the link between microbiota and migraine food triggers, especially the tryptophan pathway, should be investigated in both serum and urine in further studies to verify our findings. The role of dietary interventions, prebiotics, and probiotics on these pathways remains to be investigated.

#### Abbreviations

CM	Chronic migraine
MOH	Medication overuse headache
NSAIDs	Non-steroidal anti-inflammatory drugs
LPS	Lipopolysaccharide
HMGB1	High mobility group box-1 protein
CGRP	Calcitonin gene-related peptide
HIF-1 $\alpha$	Hypoxia-inducible factor-1 $\alpha$
IL-6	Interleukin 6
ICHD-3	International Classification of Headache Disorders 3rd edition
ELISA	Enzyme-linked immunosorbent assay
WBC	White blood cell count
ESR	Erythrocyte sedimentation rate
CRP	C-reactive protein
HADS-A	Hospital Anxiety and Depression Scale Anxiety subscale
HADS-D	Hospital Anxiety and Depression Scale Depression subscale
IBS	Irritable bowel syndrome
PCR	Polymerase chain reaction
logFC	Log fold change
DSV	Desulfovibrio
CO <sup>2</sup>	Carbon dioxide
Trp	Tryptophan
Kyn	Kynurenine
SCFAs	Short-chain fatty acids
RCT	Randomized controlled trial

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#### Author contributions

DV and HB designed the study. DV, MCA, HGD, and HB collected the data. DV, MCA, PO, MY, US, and HB analyzed the data. DV and HB wrote the manuscript. All authors reviewed, contributed, and edited the final draft. All authors approved the final version.

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#### Data availability

No datasets were generated or analysed during the current study.

#### Declarations

##### Ethics approval and consent to participate

The study was approved by the Clinical Research Ethics Committee of Gazi University Faculty of Medicine. Written informed consent was obtained from all participants.

#### Consent for publication

Not applicable.

#### Competing interests

HB is an Associate Editor of the Journal of Headache and Pain. The rest of the authors declare no competing interests.

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