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ORIGINAL RESEARCH

Differences in Gut Microbiota Composition Depending on the Site of Pain in Patients with Chronic Pain

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Background: There are many factors associated with chronic pain, including changes in the nervous and musculoskeletal systems and so on. Recently, it has become clear that the gut microbiota (GM) influences these factors, and there are many reports of GM dysbiosis in patients with chronic pain. However, the relationship between pain and GM remains unclear. Our previous study reported that defecation status, which reflects GM composition, was associated with pain intensity and that this relationship was different for each pain site. Our study investigated the association between pain site and the GM composition of feces in chronic pain patients.

Methods: The subjects were 136 patients with chronic pain and 125 healthy controls. Patients were classified into four groups, whole body (WB) pain, lower back and lower extremity (LL) pain, headache, and upper back and upper extremity pain, based on the site of pain, and we investigated differences in GM taxonomy groups compared with healthy subject.

Results: Chronic pain patients had a lower alpha diversity (effect size=0.16, p=0.02). But each pain site group did not differ in alpha diversity. WB pain patients showed higher Eggerthellaceae (LDA=3.09, p<0.01) and lower Halomonas (LDA =−2.72, p<0.01). LL pain patients had increased Fusobacterium and Sellimonas (LDA=4.09,3.03 p<0.01, 0.01) but reduced Halomonas (LDA=−2.59, p<0.01), and other key taxa.

Conclusion: WB and LL patients may have GM compositions different from healthy controls, but larger studies are needed to confirm this.

Keywords: gut microbiota, chronic pain, whole body pain, low back pain

Introduction

Chronic pain is a common problem that substantially impairs physical and psychological health and economic well-being. The prevalence of chronic pain is high, with 39% reported in the Japanese study population[1] and 35~51% in the U.K.[2] In addition, chronic pain of moderate to severe intensity occurs in 19% of adult Europeans,[3] and a study of adults in the US showed that 20.5% of them reported pain on most days or every day.[4]

There are many potential causes and risks of chronic pain, which are generally known to include changes in the nervous system, musculoskeletal degeneration, immune system disorders, physical attributes, and psychologic states.[5–7] Recently, a number of studies have reported that the gut microbiota (GM), which may influence these various factors, is associated with chronic pain. Specifically, signaling molecules derived from the gut microbiota, including metabolites, neurotransmitters, and neuromodulators, play a critical role in the pathogenesis of chronic pain. These molecules exert their effects by interacting with specific receptors and modulating both peripheral and central sensitization. In the peripheral nervous system, gut microbiota-derived mediators serve as key regulators of primary nociceptive neuron excitability, contributing to the induction of peripheral sensitization. In the central nervous system, these mediators

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influence neuroinflammatory processes by activating blood-brain barrier cells, microglia, and infiltrating immune cells. Additionally, they are involved in the modulation of both the induction and maintenance of central sensitization.[8]

For example, the GM composition of fibromyalgia (FM) and low back pain (LBP) patients differed from healthy controls in the abundance of certain taxa, and these changes correlated with pain symptoms.[9,10] The mechanisms implicating GM in FM symptoms include the involvement of short chain fatty acids, bile acids and neurotransmitters.[10] A systematic review of osteoarthritis (OA) and GM has shown a slight relationship between pain and inflammatory symptoms and GM, and the involvement of inflammatory microbiomes such as Clostridium genus and Streptococcus family.[11] A study on the relationship between migraine and GM in sterile mice suggests that GM dysbiosis contributes to chronicity of migraine-like pain.[12] Thus, research has shown a relationship between chronic pain in all parts of the body, not just visceral pain. However, due to differences between humans and animals, differences between races, and differences in methods, evidence has not yet been established for each of these diseases. It is noteworthy that the pain mechanisms involved in GM affect both local and systemic pain, and the possibility that GM may have the same effect across disease boundaries cannot be ruled out. Therefore, we believe it is necessary to confirm the relationship between pain symptoms and GM in the same race and by the same method, without distinguishing between diseases.

We previously investigated the relationship between defecation status, which is reported to reflect the GM composition, and pain severity in chronic pain patients at different pain sites. As a result, we showed that the defecation status differed with the pain site, suggesting a possible relationship between constipation symptoms and pain intensity, especially in patients with whole-body pain and lower back and lower extremity pain.[13] In addition, constipated patients have been known to exhibit a GM composition that differs from that of healthy controls.[14] These suggests that patients with whole-body pain and low back and lower extremity pain may have specific GM composition, but this was not investigated. Therefore, the purpose of this study was to compare the composition of GM in feces of chronic pain patients with that of healthy controls in terms of GM abundance and diversity by pain site, based on the hypothesis that GM composition is related to the pain site of chronic pain patients.

Method

Participants

After receiving approval from the IRB (Aichi Medical University reference number: 12–067), a cross-sectional survey was administered to a total of 178 chronic pain patients suffering from chronic pain who visited the pain center of Aichi Medical University Hospital for the management of their chronic pain between April 2019 and June 2022. Informed consent was obtained from all participants in this study and all procedures complied with the principles of the Declaration of Helsinki. For participants under the age of 18, informed consent was obtained from a parent or legal guardian in accordance with ethical guidelines.

Eligibility Criteria

The inclusion criteria were patients who experienced a pain intensity of ≥ 3 on the numerical rating scale (NRS: 0 indicates “no pain” and 10 “the greatest pain possible”) for at least 3 months. The exclusion criteria were acute inflammatory findings related to pain, digestive disease other than irritable bowel syndrome (IBS) that may be causing constipation and diarrhea, stoma in situ, nutritional disorders, being on a restricted diet, diabetes, metabolic disorders, neurological disease such as spinal cord injury and autonomic disturbance, or cognitive disease.

Controls (125 healthy subjects) were matched 2 to 1 on the basis of subjects’ demographic features such as age and gender of the patient’s group for each group, and sample data were provided by Mykinso gut microbiota testing service (Cykinso, Inc. Tokyo, Japan). Informed consent for the use of these data for the study was obtained when the subjects submitted their fecal samples, and all procedures were performed in accordance with the principles of the Declaration of Helsinki.

Demographic Features

We measured demographics (age, gender, body mass index), pain intensity, pain site, psychological status and drug use in all patients.

Pain Intensity and Site

Pain intensity was measured using NRS for the average pain intensity over the past week. The pain site was self-reported by the patients, and if the patient was experiencing pain in more than one site, they were asked to report all of the pain sites. They were allocated into four groups according to the pain region (ie, whole-body: WB, low back and/or lower extremities: LL, neck and/or upper back and/or upper extremities: NUU, head). In our previous study, we reported that, of these categories, NUU pain and LL pain were associated with psychological disturbances and LL pain had a poorer prognosis.[15] We also examined the relationship between pain location and defecation status in chronic pain patients and reported that constipation symptoms correlated with pain symptoms in patients with WB pain and LL pain.[14]

The WB group (eg, fibromyalgia, rheumatoid arthritis, and primary widespread pain, etc.) included patients with pain spread across a multiple of four categories. Patients experiencing pain sites outside of any of these four categories (eg, orofacial pain, pelvic pain, vulvodynia) were excluded.

Psychological Status

Questionnaires included Hospital Anxiety and Depression Scale (HADS) and Pain Catastrophizing Scale. These psychological assessments were performed on the same day as the assessment of pain intensity and site.

The HADS is a four-point, 14-item self-assessment scale to measure psychological distress, and has two factors: anxiety and depression. The Japanese version of the HADS has been validated among Japanese cancer survivors.[16]

Pain Catastrophizing Scale has been considered a negative "mental set" that feels more threatening than necessary when experiencing actual or anticipated pain. The PCS consists of 13 items, and the participants responded to each item from 0 ("not at all") to 4 ("all the time"), with higher scores (0–52 points) indicating a greater degree of catastrophic thinking.[17] This scale is well known for its reliability and validity in the Japanese version.[18]

Drug Use

Drugs can affect not only pain symptoms, but also the gut environment. Therefore, we investigated the use of drugs primarily used to treat pain: nonsteroidal anti-inflammatory drugs, acetaminophen, steroids, opioids, pregabalin, anti-depressants, antiepileptic drugs, antipsychotic drugs, and muscle relaxants.

Composition of the Gut Microbiota

The analysis was performed according to the method of Watanabe S (2020)[19] using the following procedure.

Fecal Sampling, DNA Extraction, and Sequencing

GM analysis was performed using Mykinso Pro, a gut microbiome testing service (Cykinso, Inc. Tokyo, Japan). Fecal samples were collected using Mykinso fecal collection kits containing guanidine thiocyanate solution (Cykinso, Inc. Tokyo, Japan) and stored at 4°C. The patients were instructed to collect fecal samples at the time of defecation closest to the date of the pain and psychological status assessment. DNA extraction from the fecal samples was performed using an automated DNA extraction machine (GENE PREP STAR PI-1200A, Kurabo Industries Ltd, Osaka, Japan) according to the manufacturer's protocol. The V1–V2 region of the 16S rRNA gene was amplified using forward primer (16S_27Fmod: TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG AGR GTT TGA TYM TGG CTC AG) and reverse primer (16S_338R: GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTG CTG CCT CCC GTA GGA GT) with KAPA HiFi Hot Start Ready Mix (Roche). To sequence 16S amplicons by Illumina MiSeq platform, dual index adapters were attached using the Nextera XT Index kit. Each library was diluted to 5 ng/μL, and equal volumes of the libraries were mixed to 4 nM. The DNA concentration of the mixed libraries was quantified by qPCR with KAPA SYBR FAST qPCR Master mix (KK4601, KAPA Biosystems) using primer 1 (AAT GAT ACG GCG ACC ACC) and primer 2 (CAA GCA GAA GAC GGC ATA CGA). The library preparations were carried out according to 16S library preparation protocol of Illumina (Illumina, San Diego, CA, USA). Libraries were sequenced using the MiSeq Reagent Kit v2 (500 Cycles), 250 bp paired end.

Taxonomy Assignment Based on 16S rRNA Gene Sequences

The paired end reads of the partial 16S rRNA gene sequences were analyzed using QIIME 2 (version 2020.8).[20] The steps for data processing and assignment based on the QIIME 2 pipeline were as follows: (1) DADA2 for joining paired-

end reads, filtering, and denoising; (2) assigning taxonomic information to each ASV using naive Bayes classifier in QIIME 2 classifier with the 16S gene of V1-V2 region data of SILVA[21] (version 138) to determine the identity and composition of the bacterial genera.

Diversity Index

Alpha diversity and beta diversity were evaluated as indicators of GM diversity. Alpha diversity was assessed with the Shannon index based on 97% sequence identity. These values represent the richness or diversity of a sample, with higher values indicating more diversity. Beta diversity represents the difference in diversity between two samples. ie the difference in structure of the GM. The further the unifrac distance, the more different the composition between the two samples.[22] In this study, weighted and unweighted unilac distances were used to measure and compare distances between groups.[22]

Statistical Analysis

All data manipulation, analyses, and graph creation were conducted using qiime2 (version 2020.08)[19] or SPSS ver. 26 (IBM, New York, USA).

The Kruskal Wallis test and Pearson's chi-square test were used for intergroup comparisons of pain site on patient characteristics. The results were summarized by frequency (%) for categorical variables and mean (standard deviation) or median (interquartile range: IQR) for continuous variables. These differences were evaluated using Fisher's exact test for categorical variables and *t*-test for continuous variables.

The α diversity was evaluated at the ASV level using the Shannon index, and significant differences were evaluated using Kruskal Wallis test from the q2-diversity plugin in QIIME2. β diversity was used to evaluate differences in the community composition between samples using the unweighted UniFrac distance method. Differences in Bray-Curtis distances between groups were assessed using a non-parametric permutation-based multivariate analysis of variation (PERMANOVA) test with 999 permutations[23] from the q2-diversity plugin in QIIME2.

To assess cross-sectional differences in the relative abundances (RA) of phylum-, genus-, and species-level taxa, we performed differential abundance analyses using an Analysis of Composition of Microbiomes (ANCOM)[24] from the q2-composition plugin in QIIME 2. To identify differences among substrates for specific microbial taxa, the linear discriminant analysis effect size method (LEfSe)[25] was used.

Results

Participants Characteristics

One hundred and seventy-eight chronic pain patients were screened for this study. Sixteen patients who did not submit stool samples were excluded, and samples were obtained from 162 patients. Furthermore, 26 patients (2 vulvodynia, 4 orofacial pain, 1 pelvic pain, 5 unknown pain site, and 14 data unavailable) were excluded, leaving 136 patients for analysis. Of the 136 patients, 45 had WB pain, 41 LL pain, 30 headache, and 20 NUU pain (Figure 1). The healthy control data included 68 for the total participants group, 19 for the WB group, 17 for the LL group, 14 for the headache group, and 7 for the NUU group. The characteristics of the subjects in each pain site group are shown in Table 1. Although the headache group was significantly younger than the other groups (adj. $p<0.05$), there were no other clear differences between pain sites in the patient groups (Table 1). For medications, steroid use differed between pain sites, and only patients with WB pain were using steroids (Table 2). Details of pain site in the LL and NUU groups are displayed in Table 3. In the LL group, over half of the patients had simple back pain, and only 4 patients had pain in both the lower back and lower extremities. In addition, knee osteoarthritis was present in three patients. In the NUU group, only 3 patients had simple neck pain, with most patients having pain from the upper back to the neck. The headache group included only tension type and migraines and did not include cluster headaches.

Alpha and Beta Diversity

Alpha Diversity

Shannon alpha-diversity index was significantly lower in chronic pain patients than in healthy controls at the total participants. There were no significant differences in sample diversity between the patients and the control subjects at

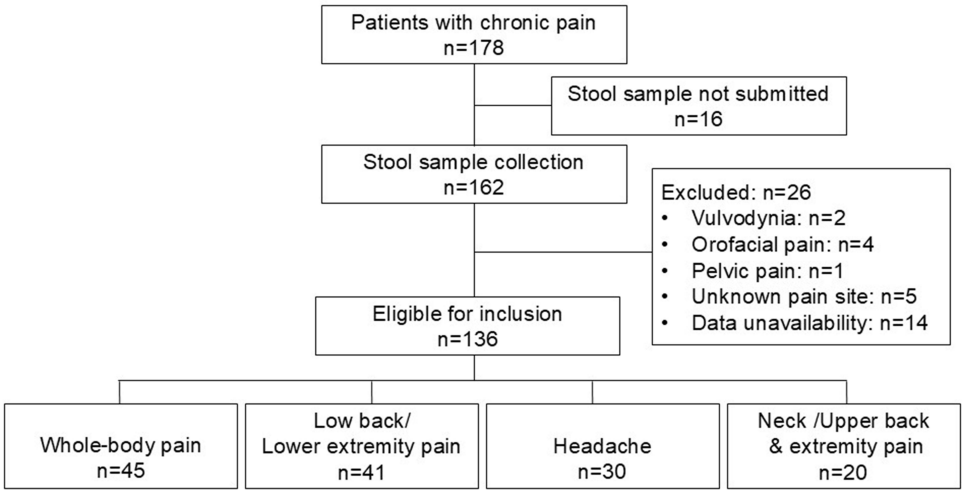

Figure 1 Flow chart of participants through the study.

pain site groups (Table 4). Furthermore, there were no significant differences in comparisons among the pain sites (Figure 2, adj. p=0.596).

Beta Diversity

In the total participants and the low back and lower extremity pain group, significant differences were found in the Bray-Curtis dissimilarity for control-control (C-C), control-patient (C-Pt or C-LL), and patient-patient (Pt-Pt or LL-LL) pairs (unweighted: p=0.001, 0.002, weighted: p=0.033, 0.021, Figure 3A and B). However, each effect size was very small (unweighted: 0.03, <0.01, weighted: <0.01, <0.01). In the headache group, only unweighted unifrac distance showed

Table 1 Characteristics of Participants

Pain Site	Total Participants		Whole-body		Low Back / Lower Extremity		Head		Neck /Upper Back & Extremity	
	Patient	Control	Patient	Control	Patient	Control	Patient	Control	Patient	Control
Number (male)	136	68	45 (5)	19 (1)	41 (19)	17 (7)	30 (9)	14 (3)	20 (7)	7 (2)
BMI (kg/m^2)* <18.5	15	8	4	3	2	2	6	3	3	2
18.5- <25.0	93	43	32	14	30	12	18	10	13	4
25.0- <30.0	20	13	7	2	5	1	5	1	3	1
30.0 and over	8	4	2	0	4	2	1	0	1	0
Age (year) * <19	7	6	1	0	0	0	5	2	1	0
20–29	9	2	1	1	1	0	5	1	2	0
30–39	14	4	4	1	4	1	4	2	2	1
40–49	38	16	11	7	11	8	11	7	5	3
50–59	43	31	23	9	12	5	4	2	4	1
60–69	16	6	4	1	8	2	0	0	4	2
70 and over	9	3	1	0	5	1	1	0	2	0
Pain intensity (NRS)#	5 (3–10)	-	6 (3–10)	-	5 (3–9)	-	5.5 (3–10)	-	4 (3–8)	-
HADS-Anxiety#	8 (0–20)	-	9 (0–20)	-	7 (1–16)	-	7.5 (1–19)	-	8 (1–17)	-
HADS-Depression#	8 (0–17)	-	8 (0–16)	-	7 (0–16)	-	8 (0–15)	-	8 (0–17)	-
PCS#	29 (0–51)	-	32 (10–51)	-	25 (0–45)	-	30 (11–44)	-	26 (4–49)	-

Note: *value: number, #, value: median (range).
Abbreviations: BMI, body mass index; NRS, numerical rating scale; HADS, hospital anxiety and depression scale; PCS, pain catastrophizing scale.

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Table 2 Comparison of Medications Used in Patient Groups

	Whole-body (n=45)	Low back / Lower Extremity (n=41)	Head (n=30)	Neck /Upper back and extremity (n=20)	adj. P value
Medication					
NSAIDs	6 (13.3)	12 (29.3)	9 (30.0)	4 (20)	0.237
Acetaminophen	3 (6.6)	6 (14.6)	0 (0)	0 (0)	0.050
Steroid	4 (8.8)	0 (0)	0 (0)	0 (0)	0.040
Opioid	3 (6.6)	7 (17.0)	0 (0)	1 (5.0)	0.058
Pregabalin	6 (13.3)	6 (14.6)	0 (0)	1 (5.0)	0.132
Antidepressant	5 (11.1)	2 (4.9)	6 (20.0)	1 (5.0)	0.172
Antiepileptic	4 (8.8)	0 (0)	3 (10.0)	1 (5.0)	0.236
Antipsychotic	1 (2.2)	0 (0)	2 (6.7)	0 (0)	0.246
Muscle relaxant	4 (8.8)	5 (12.2)	4 (13.3)	2 (10.0)	0.930

Note: Value: number (%).

Table 3 Detail of Pain Site in Low Back/ Lower Extremity and Neck/Upper Back & Extremity

Low Back /LOWER Extremity (n=41)		Neck /Upper Back & Extremity (n=20)	
Pain site	n	Pain site	n
Low back	29	Neck	3
Low back and lower extremity	4	Upper back	1
Lower extremity	8	Upper extremity	3
Lower extremity details		Neck & Upper back	8
Thigh	1	Neck & Upper extremity	3
Leg	3	Upper back and extremity	0
Knee	3	Neck & upper back and upper extremity	2
Ankle	1		
Foot	0		

Table 4 Alpha-Shannon Entropy

Pain Site		Alpha Shannon	Effect Size	P-value
Total participants	Patients (n=136)	6.03 (0.90)	0.16	0.020
	Controls (n=68)	6.38 (0.78)		
Whole-body	Patients (n=45)	6.03 (0.90)	0.03	0.797
	Controls (n=19)	6.08 (0.74)		
Low back /Lower extremity	Patients (n=41)	6.05 (0.68)	0.15	0.270
	Controls (n=17)	6.20 (0.59)		
Head	Patients (n=30)	5.88 (0.64)	0.11	0.450
	Controls (n=14)	6.08 (0.77)		
Neck /Upper back and extremity	Patients (n=20)	6.12 (0.74)	0.02	0.911
	Controls (n=7)	6.05 (0.83)		

Note: Value: median (IQR).

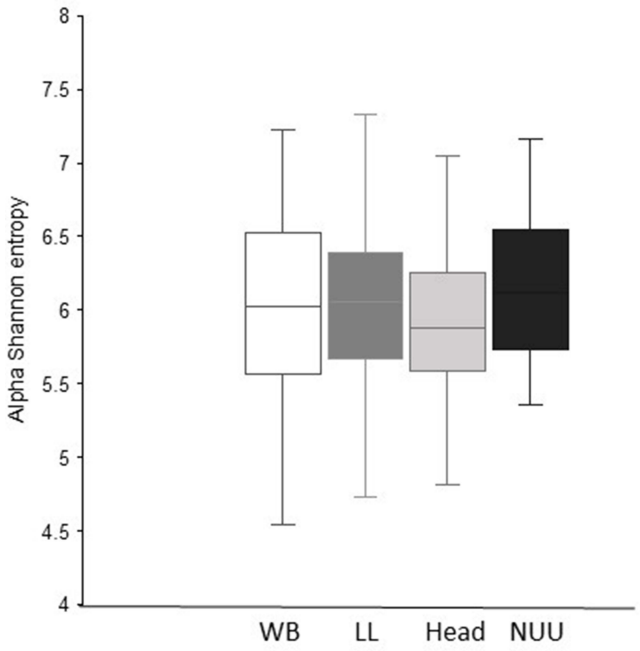

Figure 2 Comparison of alpha diversity indices for each pain site.
Abbreviations: WB, whole-body pain; LL, low back and lower extremity pain; NUU, neck; upper back and upper extremity pain.

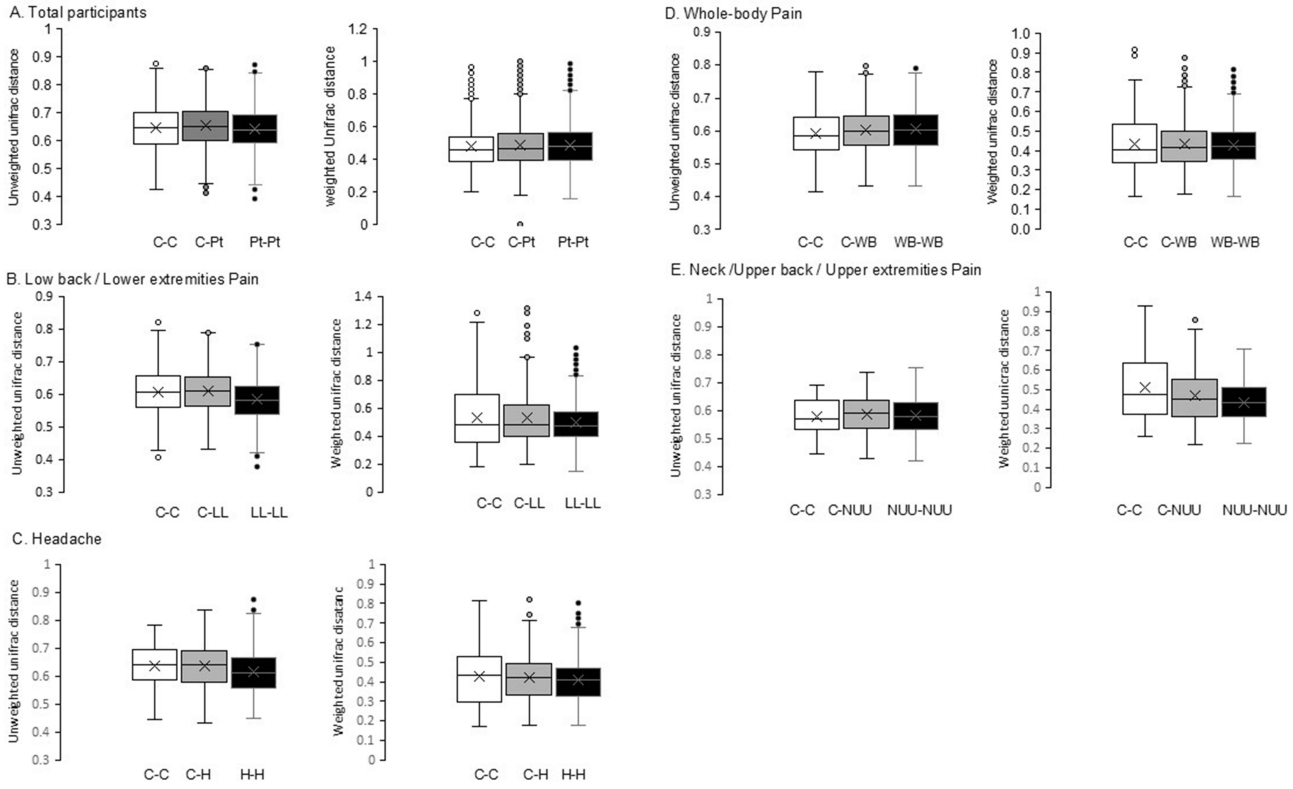

Figure 3 (**A**–**E**) Beta diversity: unifrac distance.
Abbreviations: C, control; Pt, patient; WB, whole-body pain; LL, low back and lower extremity pain; H, headache; NUU, neck; upper back and upper extremity pain.

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a significant difference, but the effect size was very small (effect size <0.01, p=0.022, Figure 3C). There were no significant differences in unifrac distance in the WB group or NUU group (Figure 3D and E).

Compositional Differences in the GM

We performed 16SrRNA sequencing on the stool samples and evaluated high dimensional class comparison via LEfSe on the composition of GM between the patients and controls in each pain site group. GM composition of total chronic pain patients did not differ significantly from that of healthy controls (Figure 4). The patients with WB pain had a significantly higher abundance of *Eggerthellaceae* family (linear discriminant analysis (LDA) score=3.093, p=0.008) but less abundant in *Halomonas* genus (LDA score=−2.716, p<0.001) and *Lachnospira* genus (LDA score=−3.164, p=0.024) than those of the control group (Figure 5). In patients with LL pain, *Fusobacterium* family (LDA score=4.087, p=0.006) and *Sellimonas* genus (LDA score=3.029, p=0.013) were significantly more abundant than in controls. On the other hand, *Halomonas* genus (LDA score=−2.592, p<0.001), *Romboutsia* genus (LDA score=−2.424, p=0.022), *Subdoligranulum* genus (LDA score=−3.520, p=0.016), *Faecalibacterium* genus (LDA score=−4.198, p=0.008), *ErysipelotrichaceaeUCG_003* genus (LDA score=−3.865, p<0.001), and *Ruminococcaceae* family (LDA score=−4.348, p=0.012) were significantly less abundant in the patient group compared to controls (Figure 6). Headache patients showed more *Actinomycetaceae* family (LDA score=2.812, p=0.045) and *Eggerthellaceae* family (LDA score=2.999, p=0.025), and less *Romboutsia* genus (LDA score=−3.060, p=0.001) and *Turicibacter* genus (LDA score=−2.942, p=0.007) (Figure 7). Patients with NUU pain showed more *Oscillibacter* genus (LDA score=3.201, p=0.015), *Flavonifractor* genus (LDA score=2.917, p=0.020), *Ruminococcus_torquesgroup* genus (LDA score=4.005,

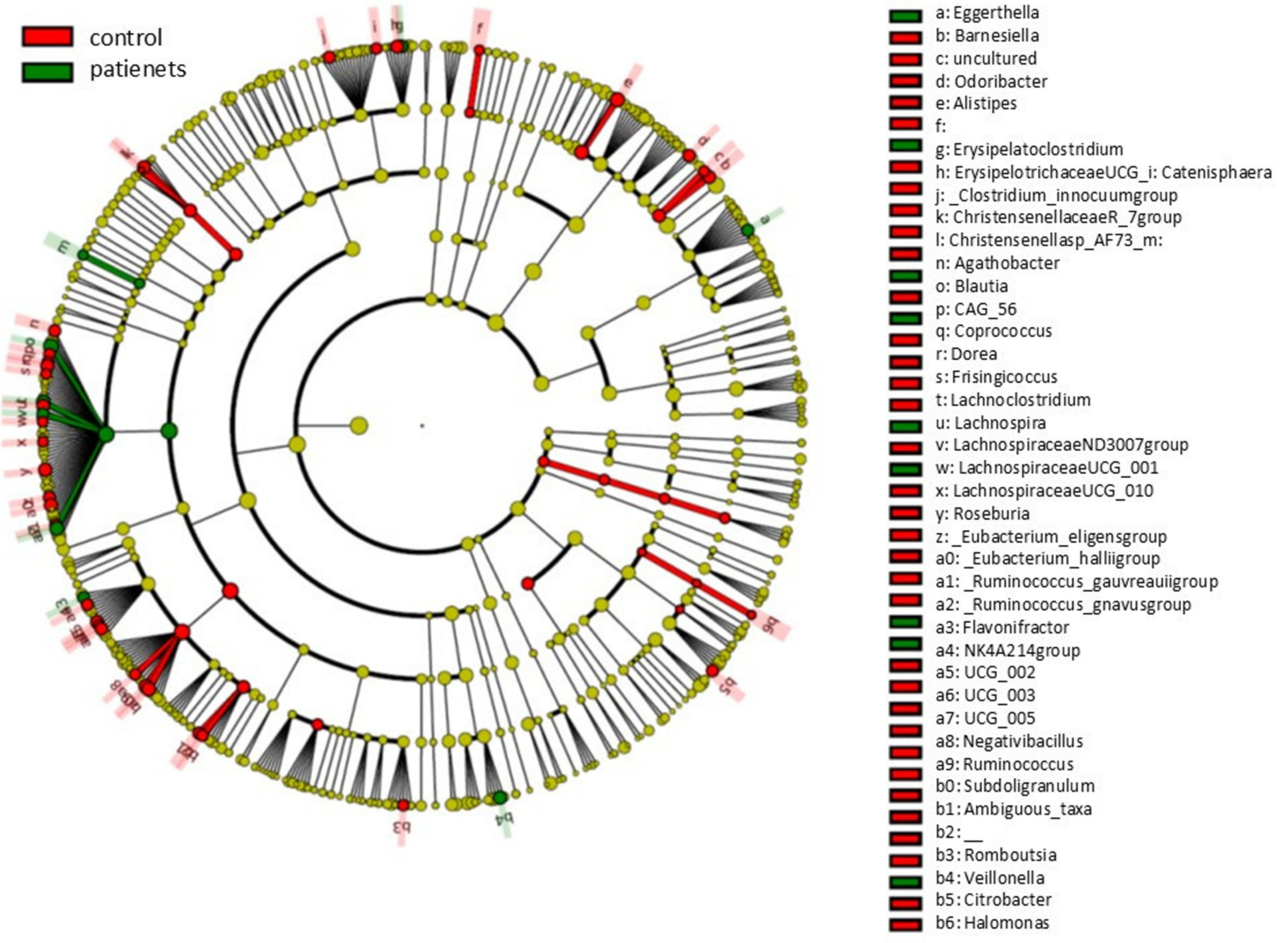

Figure 4 Compositional differences in the gut microbiome –all patients—.

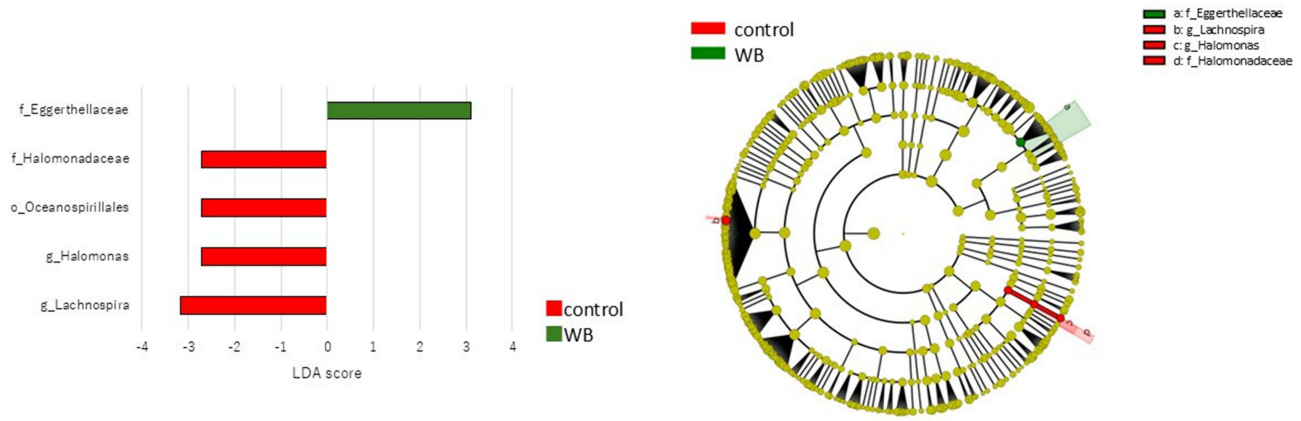

Figure 5 Compositional differences in the gut microbiome –Whole-body pain-.
Abbreviations: WB, whole-body pain; LDA, linear discriminant analysis; g, genus; f, family; o, order.

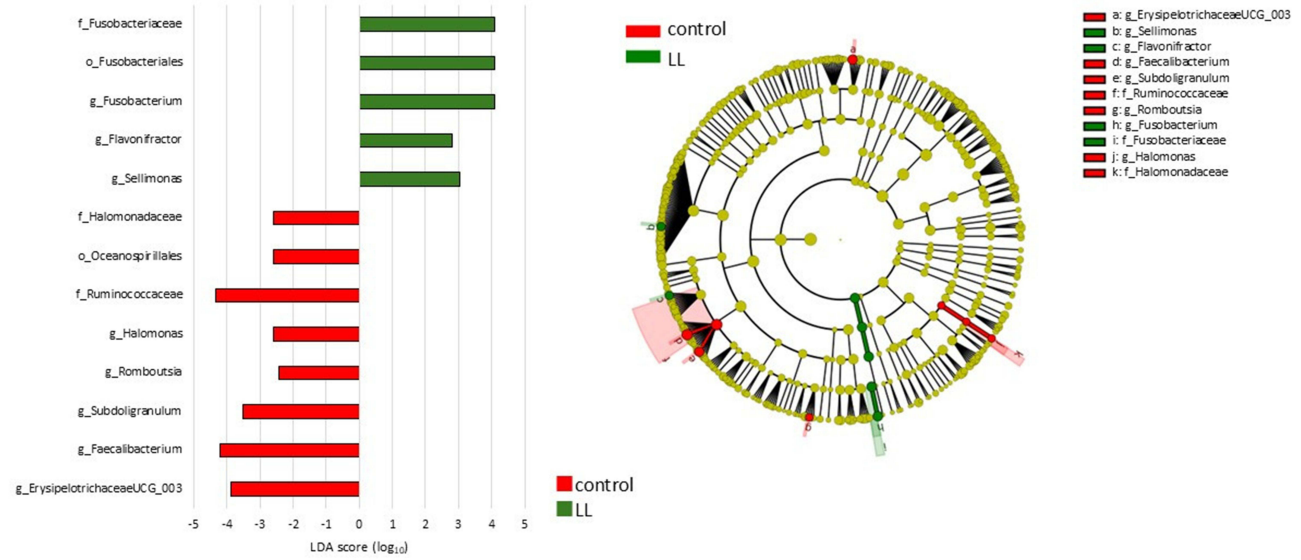

Figure 6 Compositional differences in the gut microbiome -Low back, Lower extremity pain.
Abbreviations: LL, low back/ lower extremities pain; LDA, linear discriminant analysis; g, genus; f, family; o, order.

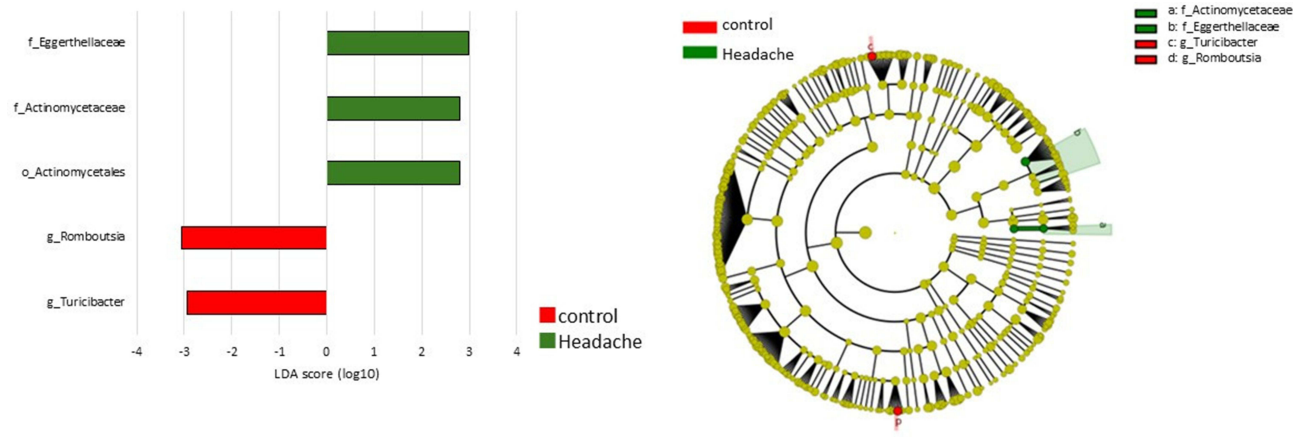

Figure 7 Compositional differences in the gut microbiome –Headache.
Abbreviations: LDA, linear discriminant analysis; g, genus; f, family; o, order.

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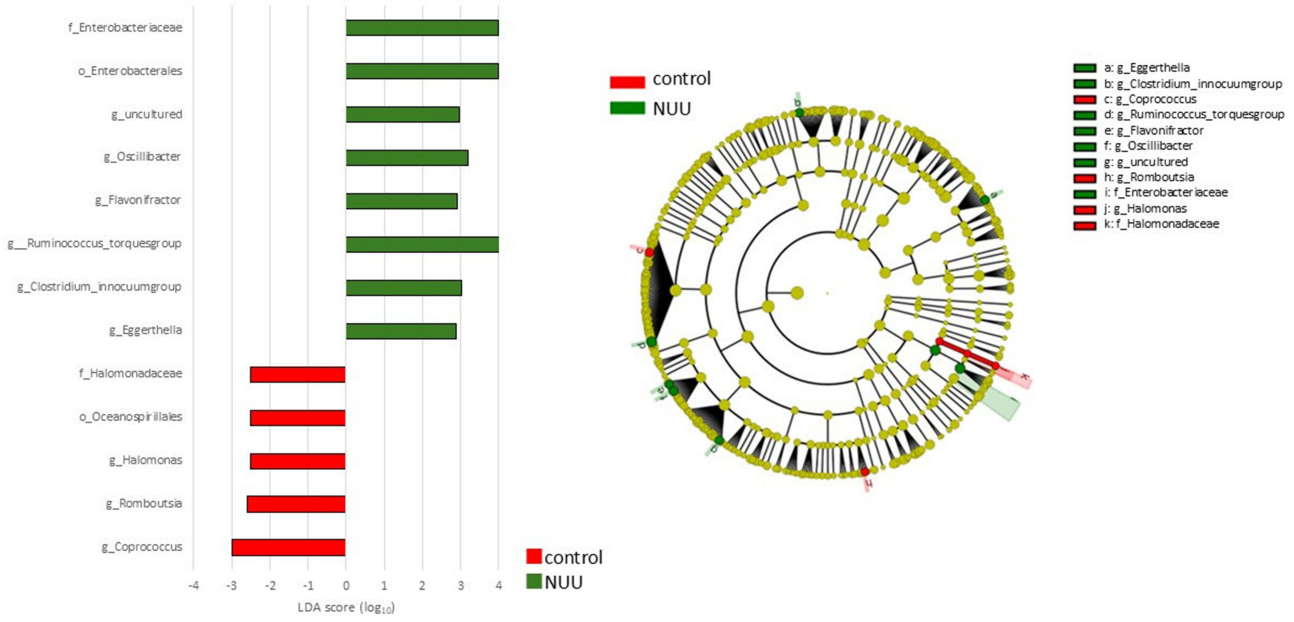

Figure 8 Compositional differences in the gut microbiome –Neck, Upper back and Upper extremity pain.
Abbreviations: NUU, neck, upper back and upper extremity pain; LDA, linear discriminant analysis; g, genus; f, family; o, order.

p=0.007), *Clostridium_innocuum group* genus (LDA score=3.015, p=0.009), and *Eggerthella* genus (LDA score=2.891, p=0.020) compared to the control group, and *Halomonas* genus (LDA score=−2.512, p<0.001) was significantly less abundant as shown in patients with WB pain and LL pain. In addition, *Romboutsia* genus (LDA score=−2.585, p=0.015) and *Coprococcus* genus (LDA score=−2.989, p=0.012) were less abundant in the patients' groups compared to the controls (Figure 8).

Furthermore, there was not a clear relationship between GM diversity and composition and pain intensity, anxiety, depression, or pain catastrophizing in all groups.

Discussion

According to our previous study investigating the relationship between defecation status and pain-related symptoms in patients with chronic pain, patients with low back and lower extremity pain tended to have more severe pain with more severe constipation.[13] Since constipation has been reported to be associated with GM composition,[14] the aim of this study was to investigate whether there is a specific GM composition in the feces depending on the pain site in patients with chronic pain. Chronic pain patients had slightly lower alpha diversity than healthy controls. Decreased GM diversity has been reported in several diseases such as psychiatric disorders,[26] rheumatoid arthritis,[27] and IBS,[28] and similar results were observed in patients with chronic pain. On the other hand, each pain site group did not differ in alpha diversity, but the GM composition may differ by pain site.

The LL group was characterized by an abundance of *Fusobacterium*, which is a gram-negative bacterium and part of the normal human microbiota. Its surface is covered with lipopolysaccharides (LPS), which interacts with the immune system and may be involved in a variety of processes in health conditions and pathologies.[29] For example, some research has suggested that *Fusobacterium* nucleatum is associated with the initiation and metastasis of colorectal cancer.[30] Although the mechanism is not clear, it has been thought that infiltration of epithelial and endothelial cells might be involved.[31,32] In addition to gastrointestinal diseases, relationships with cardiovascular diseases, rheumatoid arthritis, respiratory infections, and Alzheimer's disease have also been reported.[33] To date, there have been few studies on the relationship between chronic pain and the *Fusobacterium* family, although one study reported on patients with burning mouth syndrome, in which the *Fusobacterium* family was found to be more abundant in healthy controls than in patients, in contrast to the results of our study.[34] Rheumatoid arthritis, a painful chronic disease, was not reported to be related to

pain, but a high prevalence of *Fusobacterium* family was detected in the synovial fluid.[35] Thus, there is little known about the relationship between chronic pain and the *Fusobacterium* family.

However, some researchers have reported an association between an increase in LPS-releasing gram-negative bacteria and systemic inflammation.[36] Parenteral administration of low doses of LPS to humans increases the plasma inflammatory cytokines IL-6 and TNF-a, the anti-inflammatory cytokines IL-10 and IL-1, and cortisol and norepinephrine levels in saliva and plasma. These changes are accompanied by depressed mood and increased anxiety.[37] Furthermore, decreased sensitivity thresholds to visceral pain have been reported.[33]

On the other hand, *Ruminococcaceae*, one family of the major butyrate-producing bacteria, showed less in patients with LL pain. Butyrate can affect the gut-brain axis by potentiating cholinergic neurons via epigenetic mechanisms.[33] Butyrate is also included in short-chain fatty acids (SCFA) along with acetic acid and propionic acid, and reduction of SCFA is known to affect a variety of diseases, including diabetes, obesity, autoimmune diseases, cancer, and depression.[38] These may suggest that a decrease in *Ruminococcaceae* family may be associated with chronic pain in the low back and lower extremity. Due to the small sample size of our present study, we should be cautious about confirming the relationship between chronic low back and lower extremity pain and *Fusobacterium, Ruminococcaceae* family, and therefore, we postulate that further research is needed, such as investigating the LBP and lower extremity pain separately.

In addition, to the best of our knowledge, the relationship between lower extremity pain and GM has not been reported, except for the reports on knee osteoarthritis (OA). A systematic review of the relationship between OA and GM showed an association between diet, GM dysbiosis, and radiological severity and self-reported symptoms of OA.[39] Others reported that probiotic consumption may serve as a novel treatment option in the clinical management of knee OA, improving treatment outcome likely through reducing serum hs-CRP levels.[40] However, only 3 of the 41 low back and lower extremity pain cases had knee pain in our present study. Therefore, it is highly unlikely that the relationship between knee OA and GM influenced the present results.

The relationship between LBP and GM has been reported in a few studies, with anaerobic bacteria reported to influence disc degeneration and disc herniation.[41,42] On the other hand, there is very limited evidence to support altered GM composition in patients with chronic LBP other than discogenic LBP. One study comparing the GM composition of overweight chronic LBP patients with controls has reported a higher abundance of the genera Adlercreutzia, Roseburia, and Uncl. Christensenellacae in patients compared to controls.[43] In addition, these overweight participants with LBP showed a high diversity of GM.[43] The obesity rate in the LL group of this study was very low, which may have influenced the findings. Consequently, our results did not support these previous reports. Nonetheless, as with our study, these reports were based on small sample sizes, highlighting the need for further investigation.

As seen in other pain sites except headache, the patients had fewer *Halomonas* genus than healthy controls. There have been very few reports on *Halomonas* genus in humans, and although some have shown a relationship with gastric cancer[44] and necrotizing enterocolitis,[45] it has not been discussed as a major contributing bacterium. Although there are no reports on the relationship between chronic pain and *Halomonas* genus so far, the results of our present study suggest that musculoskeletal chronic pain in particular might have some relationship to *Halomonas* genus.

Our previous study showed that pain intensity in patients with WB pain as well as LL pain was related to constipation,[13] but this study did not clarify the GM composition specific to WB pain. This may have been influenced by variability in patient characteristics and symptoms, as WB pain includes a number of different diseases.

Headache, and NUU pain require further investigation because of the small sample size. However, the patients with headache showed a tendency to have a different GM composition from the patients with other pain sites. Since pain-related symptoms and influential factors were different for headache and musculoskeletal pain, GM involvement in the headache patients may be different from other pain sites. However, the headache group had a higher proportion of patients who were younger than the other pain sites. It is known that GM composition is influenced by age. In addition, Migraine and tension-type headaches are very common, and both are distinguished by differences in duration and pain intensity, whether the pain site is unilateral or bilateral, and whether the symptoms are aggravated or not by physical activity. However, because the mechanism of both is unknown, it is difficult to make a clear diagnosis, and they are often

mistaken for each other.[46] Therefore, we did not distinguish between migraine and tension-type headache in this study, but distinguishing between the two may lead to new findings.

There were several limitations to this study. Firstly, our results only showed that the patients with chronic pain have different GM compositions compared with healthy controls depending on the pain site, and the effect of the extracted GM on pain was not known. It was also unclear why chronic pain patients have different GM compositions depending on the pain site. Secondly, the sample size for each group was small as the subjects were separated into 4 groups according to the pain site. Thirdly, there was a higher frequency of females in all groups, indicating a gender bias in our study. It has been reported that there are gender differences in GM and that there is an interaction between gut microbiota and sex hormones.[47] Therefore, it was not clear whether the results of this study would also be applicable to men or not. Finally, WB pain and LL pain had higher rates of steroid and opioid use. These drugs may have an impact on the gut environment, and we were not able to consider the involvement of the drugs used in the present results.

Conclusion

We investigated whether there was a pain site specific to GM composition in patients with chronic pain. Patients with low back and lower extremity pain had a different GM composition from healthy individuals and characteristically showed an abundance of the *Fusobacterium* family. In addition, *Halomonas* genus was found to be less abundant in pain sites other than headache. We have reported that pain symptoms differ by pain site in chronic pain patients, with lower back and lower extremity pain having a particularly poor prognosis, and that constipation symptoms are associated with pain symptoms. These results may suggest that such differences in pain symptoms by pain site may be influenced by differences in GM. The relationship between GM may also be different for headache and musculoskeletal pain, but when considering the results, the small sample size should be kept in mind as a limitation of our study.

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Disclosure

The authors report no conflicts of interest in this work.

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