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Early-stage Hyperglycemia-induced Gut Microbial Changes Are Partially Associated with Mechanical Allodynia in *db/db* Mice

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Abstract: Recent studies have demonstrated that pain is partially regulated by the gut microbiota. However, the association of gut microbiota with painful diabetic neuropathy, a common complication of diabetes, remains unclear. Herein, we investigated whether the gut microbiota is associated with mechanical allodynia during the early stage of hyperglycemia in 7-week-old *db/db* mice. The *db/db* mice were intraperitoneally injected with metformin for 2 weeks. Using the von Frey test and gut microbiota analyses, we investigated the association of gut microbial changes with mechanical allodynia in 7-week-old mice. In *db/db* mice, colonic microbial community profiles were altered, and both unweighted and weighted UniFrac distances were reduced. Colonic genus-level abundances of *Alloprevotella* and *Prevotellaceae_UCG-001* were positively correlated with mechanical allodynia in *db/db* mice, while the abundance of *Odoribacter* was negatively correlated. Intraperitoneal injection of metformin for 2 weeks alleviated mechanical allodynia in *db/db* mice but did not achieve an anti-diabetic effect. Metformin altered colonic microbial communities and increased weighted UniFrac distance in *db/db* mice, although its analgesic effect was not associated with specific bacteria. Additionally, alteration of small intestinal microbial community profiles and reduction in weighted UniFrac distance were observed in *db/db* mice, which were not affected by metformin. These results provide potential evidence of the association of the gut microbiota with mechanical allodynia during early-stage of hyperglycemia.

Keywords: Gut Microbiota, Painful Diabetic Neuropathy, Mechanical Allodynia, Metformin

1. Introduction

Pain is a common and complex problem with multiple causes. Emerging evidence indicates that the gut microbiota is one of the factors associated with pain, such as visceral, inflammatory, and neuropathic pain [1, 2]. Germ-free (GF) or antibiotic-treated mice exhibit visceral hypersensitivity due to upregulation of toll-like receptors, cytokines, and altered ion channels, which can be attenuated by microbiota transplantation [3, 4]. Probiotic therapy ameliorates gut microbiota-associated visceral hypersensitivity by attenuating the elevation of myeloperoxidase activity and substance P expression [5]. In contrast, inflammatory and neuropathic pain are alleviated in GF or antibiotic-treated mice. The upregulation of pro-inflammatory cytokines in GF mice reduces hypersensitivity in inflammatory animal model via systematic administration of carrageenan and lipopolysaccharides [6, 7]. Additionally, abnormal composition of the gut microbiota is associated with depression in an animal pain model with nerve injury [8]. Collectively, these findings imply that the gut microbiota

plays important and diverse roles in pathological pain.

Painful diabetic neuropathy (PDN) is the most common early complication in patients with diabetes; 30-50% of patients who have diabetic neuropathy develop PDN during their lifetime [9, 10]. The Aδ and C fibers that transduce mechanical and thermal nociception, are damaged early and associated with the development of PDN [11]. Thus, hypersensitivity to mechanical stimulation is a common symptom of patients with diabetes [12], and mechanical allodynia occurs at an early stage of diabetic neuropathy in patients and animal models [13, 14]. A previous study identified an impairment of adenosine monophosphate-activated protein kinase (AMPK) activation during the early stage of hyperglycemia in *db/db* mice, an animal model that mimicked type 2 diabetes mellitus (T2DM), which can cause transient receptor potential A1 (TRPA1) channel to accumulate in the membrane of dorsal root ganglion (DRG) neurons and concomitantly induce mechanical allodynia [13]. Intraperitoneal injection of metformin, an AMPK activator, for 2 weeks restores abnormal TRPA1 expression in DRG neurons and attenuates mechanical allodynia in *db/db* mice without anti-hyperglycemic and anti-obesity effects [13]. Systematic administration of metformin can alter the gut microbiota, which is associated with its anti-diabetic effect [15, 16]. However, whether early PDN is associated with the gut microbiota and whether metformin inhibits mechanical allodynia via the gut microbiota remain unclear. In this study, we investigated the association between gut microbiota and mechanical allodynia, as well as the effect of injection of metformin for 2 weeks on the gut microbiota in 7-week-old *db/db* mice.

2. Methods

2.1. Animals

Sixteen and eight 4-week-old male BKS.Cg-+Leprdb/+Leprdb/Jcl (*db/db*) and BKS.Cg-m+/m+/Jcl (*m/m*) mice, respectively, were purchased from Nanjing Junke Biological. Mice were raised at the Institute of Psychology of the Chinese Academy of Sciences, under 23±1°C and 50–60% relative humidity. The mice were adaptively fed for 7 days to perform the behavior study. Starting from 5 weeks age, eight *db/db* mice were daily injected with metformin (250 mg/2 ml/kg, i.p.). Saline was used as a vehicle for another eight *db/db* and *m/m* mice. Body weight and non-fasting glucose levels in tail-tip blood were measured in 7-week-old mice. All procedures involving the care and use of animals were approved by the Institutional Review Board of the Institute of Psychology, Chinese Academy of Sciences (A20002).

2.2. Von Frey Test

The von Frey test was used to evaluate mechanical allodynia in 7-week-old mice. Three hours after intraperitoneal injection, mice were placed in a plastic cage with a metal mesh floor (10 mm × 10 mm) for a 1 h adaptation. Testing was initiated with 1 g filament intensity, and von Frey filaments were applied to the mid-plantar surface for up to 3–5 s with a 3-min interval. Cut-offs of 0.16 and 4 g filament intensities were selected as the low and high testing limits, respectively. All behavioral tests were performed within 1 h. The 50% withdrawal threshold was assessed using an up-down paradigm.

2.3. Gut Microbiota Analysis Via 16S rDNA Sequencing

The 16S rDNA from stool samples of the small intestine and colon were sequenced by Allwegene Technology (Beijing, China). Genomic DNA was extracted using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and used as the template for amplifying the V3-V4 regions of 16S rDNA. Amplicon libraries were sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA). After removing barcodes and chimeras, cleaned tags were clustered into group with 97% similarity using vsearch (version 2.7.1 https://github.com/torognes/vsearch). These operational taxonomic units (OTUs) were analyzed based on classification using the Ramer-Douglas-Peucker (RDP) classifier algorithm and compared against the SILVA database (release128/132; http://www.arb-silva.de). The confidence threshold was set at 0.7. Comparative abundance and correlation analyses were performed at the genus level.

2.4. Statistical Analysis

All results are expressed as mean±SEM. One-way analysis of variance (ANOVA) followed by the Bonferroni test was used for data with normal distribution and equal variance. Non-parametric data were analyzed with the Wilcoxon rank-sum and Kruskal Wallis test.

Linear discriminant analysis Effect Size (LEfSe) was used to identify biomarkers that differed significantly in abundance between groups. First, ANOVA test detected species with significant between-group differences in abundance (threshold=0.05). Second, among significantly different species, paired Wilcoxon rank-sum test was used for analysis of between-group variation (threshold=0.05). Finally, linear discriminant analysis (LDA) downscaled and assessed the effect of species with significant differences (threshold=3).

Relationships between bacterial relative abundance and mechanical allodynia were assigned using Pearson's correlation. Significance was set at $p<0.05$.

3. Results

3.1. Two-week Intraperitoneal Injection of Metformin Attenuated Mechanical Allodynia in db/db Mice

We observed mechanical allodynia in 7-week-old *db/db* mice (Figure 1a). Two weeks of intraperitoneal metformin injection attenuated mechanical allodynia (Figure 1a). In addition, increased body weight and blood glucose levels in *db/db* mice were not ameliorated by metformin (Figure 1b, c), suggesting that short-term treatment with metformin acted as an analgesic without anti-diabetic effect. Thus, we used metformin to distinguish between mechanical allodynia- and diabetes-induced changes in gut microbiota.

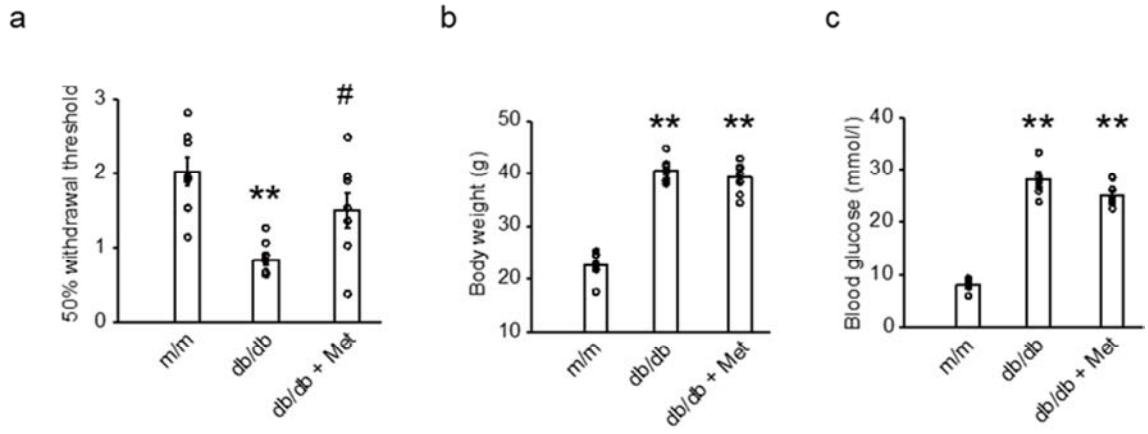

Figure 1. *Changes to mechanical allodynia, body weight and blood glucose in 7-week-old mice.*

(a) Analyses of von-Frey test in *m/m*, *db/db*, and metformin-treated *db/db* (db/db + Met) mice. (b) Body weight per group. (c) Non-fasting blood glucose per group. Met, metformin. ** p<0.01, vs. *m/m* mice; # p<0.05, vs. *db/db* mice (one-way ANOVA and Bonferroni test). n=8 per group.

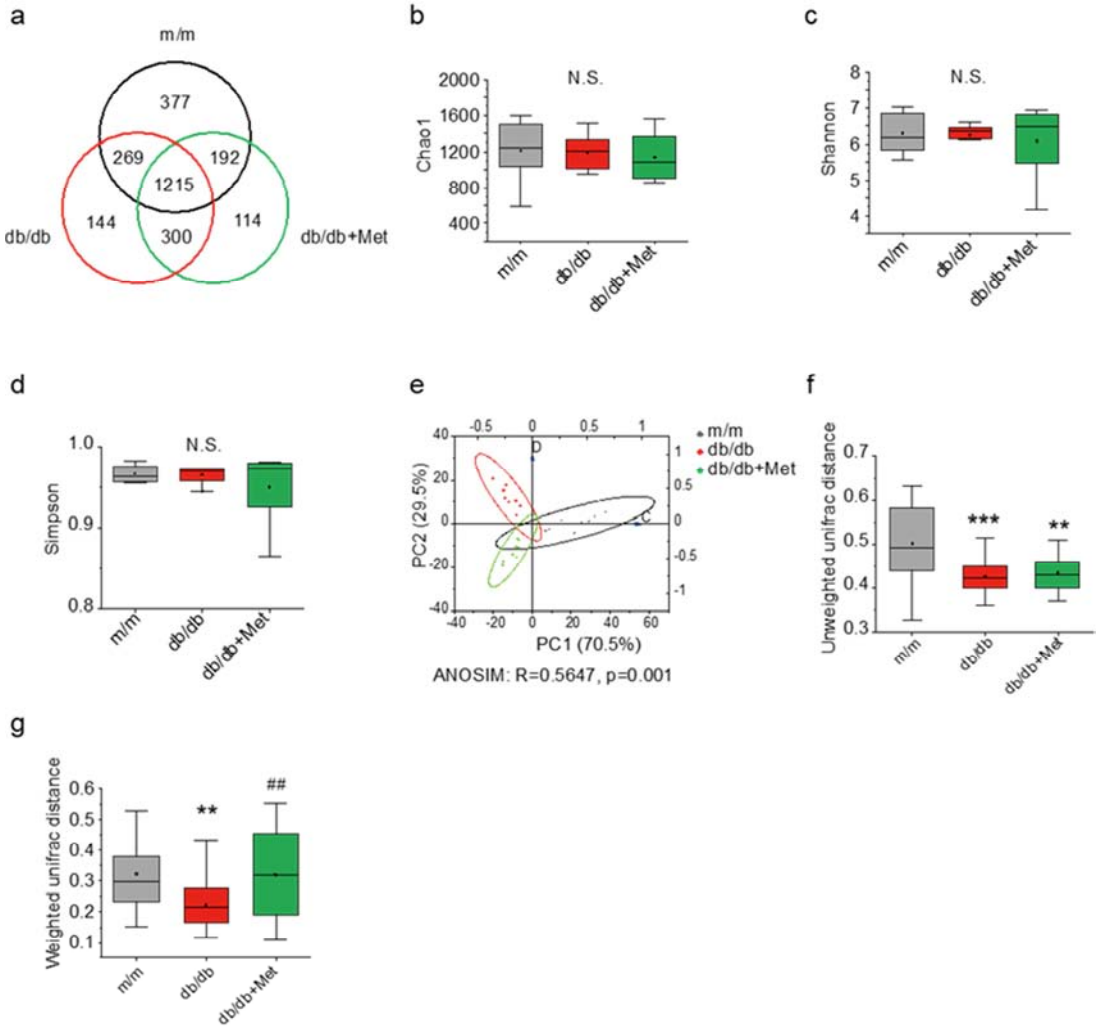

Figure 2. *Colon microbiota diversity.*

(a) Venn diagrams. (b-d) Alpha diversity was evaluated using Chao1 (b), Shannon (c), and Simpson (d) indices. (e) Partial least squares discriminant analysis (PLS-DA); ANOSIM: R=0.5647, p=0.001. Un-weighted (f) and the weighted UniFrac distance (g) analysis. Met, metformin. N. S., no significant difference in three groups. *** p<0.001, ** p<0.01, vs *m/m* mice, ## p<0.01, vs *db/db* mice (one-way ANOVA with Bonferroni test for b; Kruskal Wallis test used for c, d, f, and g). n=8 per group.

3.2. Gut Microbiota Diversity Was Altered in db/db Mice

We investigated gut microbiota in the colon and small intestine of *db/db* mice. In the colon, 1215 OTUs were shared across the three experimental groups, while 377, 144, and 114 OTUs were unique to *m/m*, *db/db*, or metformin-treated *db/db* mice, respectively (Figure 2a). Neither the richness (Chao1) nor diversity (Shannon and Simpson indices) of colonic microbiota changed in any group (Figure 2b-d). Partial least squares discriminant analysis (PLS-DA) identified significantly different microbial community profiles in the colon across the three groups (ANOSIM (analysis of similarities): R=0.5647, p=0.001) (Figure 2e). UniFrac distance was used to further discern between-group dissimilarities, with unweighted UniFrac examining evolutionary distances and weighted UniFrac accounting for phylogenetic relatedness and relative abundance. Both unweighted and weighted UniFrac distance were reduced in *db/db* mice (Figure 2f, g), while metformin mitigated the reduction in weighted but not unweighted UniFrac distance (Figure 2g). These results suggested that colonic microbial community abundance changed during the early stage of disease development in *db/db* mice, which possibly affected the diabetic mechanical allodynia.

The small intestine had fewer OTUs (Figure 3a) than the colon (Figure 2a). There were 179 shared OTUs, while there were 63, 50, and 14 unique OTUs among *m/m*, *db/db*, and metformin-treated *db/db* mice, respectively (Figure 3a). The microbiota richness and diversity did not change in the small intestine (Figure 3b-d). The PLS-DA plot combined with ANOSIM test (R=0.2294, p=0.02) showed that the three mouse groups differed in small intestinal microbial communities (Figure 3e). In contrast to the colon, the small intestine of *db/db* mice showed a decrease in weight but not unweighted UniFrac distance, and neither was affected by metformin (Figure 3f, g). These results suggested that metformin-induced alleviation of mechanical allodynia was not associated with small intestinal microbial profiles in *db/db* mice, although their community composition and abundance changed in diabetic mice.

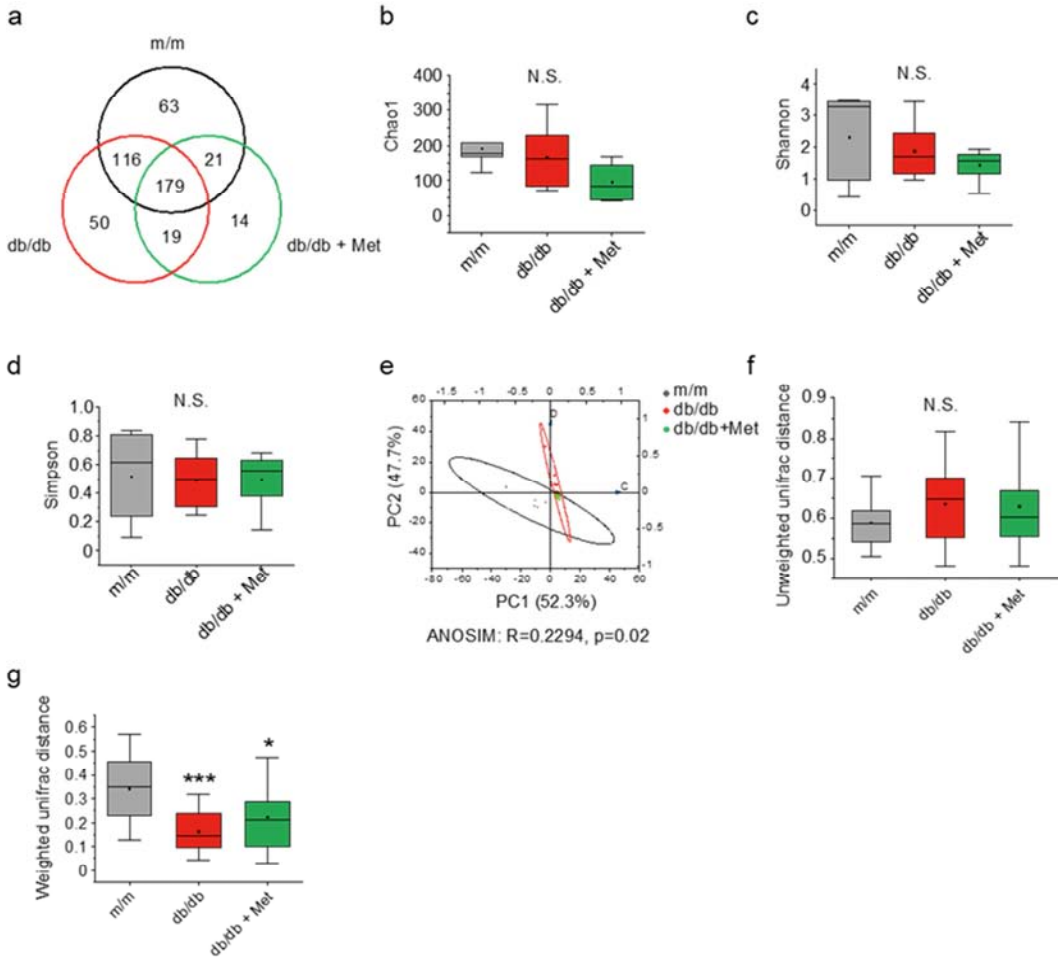

Figure 3. *Small intestine microbiota diversity.*

(a) Venn Diagrams. (b-d) Alpha diversity was evaluated using Chao1 (b), Shannon (c), and Simpson (d) indices. (e) Partial least squares discriminant analysis (PLS-DA); ANOSIM: R=0.2294, p=0.02. Unweighted (f) and the weighted UniFrac distance (g) analysis. Met, metformin. N. S, no significant difference in three groups. *** p<0.001, ** p<0.01, vs *m/m* mice (one-way ANOVA with Bonferroni used for b, d, and g; Kruskal Wallis test used for c and f). n=5~8 per group.

3.3. Alteration of Gut Microbial Communities in db/db Mice with or Without Metformin

We used LEfSe to determine biomarkers of colonic and small intestinal microbial communities in *m/m*, *db/db*, and metformin-treated *db/db* mice. The composition of colonic microbiota differed in each group (LDA score>3, $p<0.05$) (Figure 4a). Eighteen, nine, and eight microbial species were enriched in the colons of *m/m*, *db/db*, and metformin-treated *db/db* mice, respectively. The dominant species were *p-Proteobacteria* (*f-Desulfovibrionaceae*, o-*Desulfovibrionales*, and *c-Deltaprobacteria*) and *p-Actinobacteria* (*f-Coriobacteriaceae*, *o-Coriobacteriales*, and *c-Coriobacteria*) in *m/m* mice, whereas *p-Bacteroidetes* (*o-Bacteroidales*, *c-Bacteroidia*, *f-Bacteroidales-S24-7-group*, and *f-Prevotellaceae*) was dominant in *db/db* mice. Metformin injection shifted abundance to *p-Firmicutes* (*s-Lactobacillus-gasseri*) and *p-Cyanobacteria* (*o-Gastranaerophilales* and *c-Melainabacteria*) in *db/db* mice (Figure 4a).

The most abundant small intestinal microbial species in *m/m* were *p-Proteobacteria* (*f-Brucellaceae*, *o-Rhizobiales*, *f-Mitochondria*, *o-Rickettsiales*, *c-Alphaproteobacteria*, and *o-Pseudomonadales*). In *db/db* mice, *g-Erysipelatoclostridium* and *f-Closteidiales-vadinBB60-group* were enriched. Metformin injection caused a shift to *p-Firmicutes* (*f-Lactobacilaceae*, *g-Lactobacillus*, and *s-Lactobacillus-gasseri*) (Figure 4b).

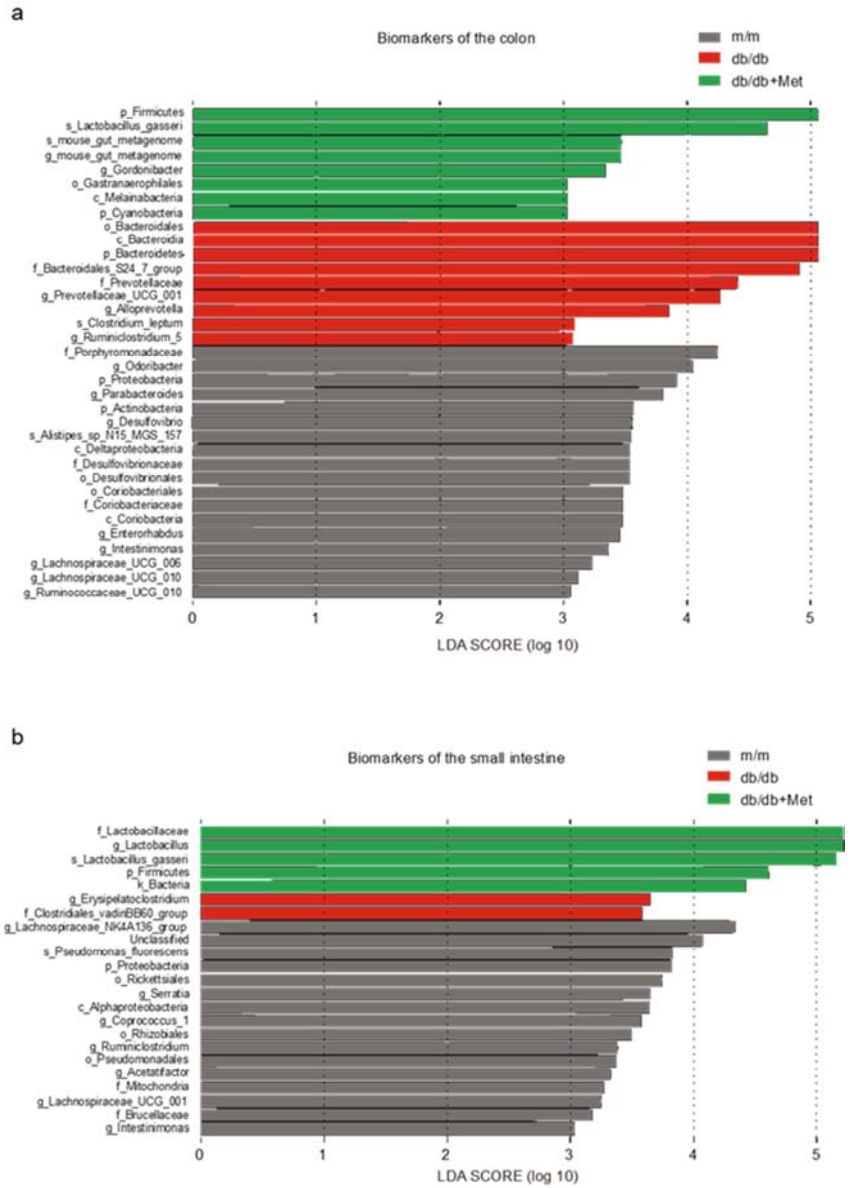

Figure 4. *Linear discriminant analysis Effect Size (LEfSe) of colon and small intestine.*

Colon (a) and small intestine (b) microbiota in *m/m*, *db/db*, and metformin-treated *db/db* mice. Only categories with LDA values>3 are shown. The letters p, c, o, f, and g represent phylum, class, order, family, and genus.

We then analyzed the species relative abundance on genus level and found that the colon (Table 1) and small intestine (Table 2) differed in the composition and abundance of dominant bacterial species. In the colon, bacteria such as *Bacteroides*, *Lachnospiraceae_NK4A136_group*, and *Lactobacillus* occupied a large proportion of the microbial community (Table 1). Additionally, the three groups of mice differed significantly in *Alloprevotella*, *Prevotellaceae-UCG-001*, and *Odoribacter* abundance. *Alloprevotella* and *Prevotellaceae-UCG-001* were increased, while *Odoribacter* was decreased in *db/db* mice. Metformin injection restored *Alloprevotella* and *Prevotellaceae-UCG-001* abundance in *db/db* mice, while the abundance of *Odoribacter* remained unchanged (Figure 5a). *Streptococcus*, *Lachnospiraceae_NK4A136_group*, *Lactobacillus*, and *Candidatus_Arthromitus* accounted for majority of the small intestine communities; there was no significant change in bacteria detected in each group of the small intestine (Table 2).

Table 1. *Relative abundance of colonic microbiota in each group (Mean ± SEM).*

Bacteria	m/m	db/db	db/db + Met*
Tyzzerella	0.008±0.005	0.007±0.005	0.006±0.004
Helicobacter	0.011±0.009	0.060±0.005	0.004±0.002
Parabacteroides	0.014±0.013	0.006±0.007	0.002±0.001
Lachnoclostridium	0.016±0.011	0.009±0.004	0.006±0.003
Anaerotruncus	0.013±0.008	0.008±0.006	0.010±0.008
Roseburia	0.019±0.018	0.010±0.008	0.018±0.012
Alistipes	0.021±0.010	0.014±0.012	0.013±0.013
Rikenellaceae_RC9_gut_group	0.035±0.022	0.021±0.008	0.017±0.013
Bacteroides	0.089±0.067	0.048±0.037	0.040±0.030
Lachnospiraceae_NK4A136_group	0.079±0.113	0.082±0.554	0.130±0.101
Lactobacillus	0.104±0.102	0.057±0.034	0.191±0.223
Ruminiclostridium	0.008±0.007	0.005±0.005	0.004±0.003
Ruminococcaceae_UCG-014	0.005±0.003	0.008±0.011	0.006±0.002
Ruminiclostridium_9	0.006±0.004	0.007±0.004	0.006±0.003
Eubacterium_xylanophilum_group	0.006±0.007	0.012±0.014	0.005±0.006
Lachnospiraceae_UCG-001	0.003±0.004	0.013±0.013	0.020±0.024

* Met, metformin

Table 2. *Relative abundance of small intestinal microbiota in each group (Mean ± SEM).*

Bacteria	m/m	db/db	db/db + Met*
Rikenellaceae_RC9_gut_group	<0.001	0.001±0.002	0
Oscillibacter	0.001±0.003	<0.001	<0.001
Bacteroides	<0.001	0.001±0.003	0
Mucispirillum	0.002±0.005	<0.001	0
Lachnospiraceae_UCG-006	0.001±0.001	<0.001	<0.001
Candidatus_Saccharimonas	<0.001	0.001±0.001	<0.001
Eubacterium_xylanophilum_group	0.002±0.003	<0.001	<0.001
Lachnospiraceae_UCG-001	0.002±0.003	<0.001	<0.001
Ruminiclostridium	0.003±0.006	<0.001	<0.001
Anaerotruncus	0.002±0.004	<0.001	<0.001
Desulfovibrio	0.008±0.013	0.001±0.002	<0.001
Lachnoclostridium	0.006±0.009	0.002±0.003	0.001±0.003
Helicobacter	0.001±0.003	0.007±0.021	<0.001
Roseburia	0.012±0.017	<0.001	<0.001
Enterorhabdus	0.015±0.026	0.005±0.006	0.001±0.002
Streptococcus	0.028±0.028	0.001±0.001	0.001±0.001
Lachnospiraceae_NK4A136_group	0.035±0.056	0.007±0.012	<0.001
Lactobacillus	0.245±0.300	0.233±0.168	0.595±0.342
Candidatus_Arthromitus	0.541±0.399	0.671±0.175	0.386±0.357

* Met, metformin

3.4. Mechanical Allodynia Is Associated with Specific Colonic Microbiota

To explore whether specific bacteria are associated with mechanical allodynia in *db/db* mice, we performed correlation analyses using Pearson's correlation analysis. We found that the abundances of *Prevotellaceae-UCG-001*, *Alloprevotella*, and *Odoribacter* were highly correlated with the 50% withdrawal threshold in *m/m* and *db/db* mice (Figure 5b-d). The 50% withdrawal threshold was positively correlated with *Odoribacter* (p=0.014) and negatively correlated with *Prevotellaceae-UCG-001* (p=0.027) and *Alloprevotella* (p=0.042). However, variation in microbiota was not correlated with the analgesic effect of metformin on

mechanical allodynia in *db/db* mice (*Prevotelaceae-UCG-001*, p=0.334; *Alloprevotella*, p=0.117; *Odoribicter*, p=0.322) (Figure 5e-g).

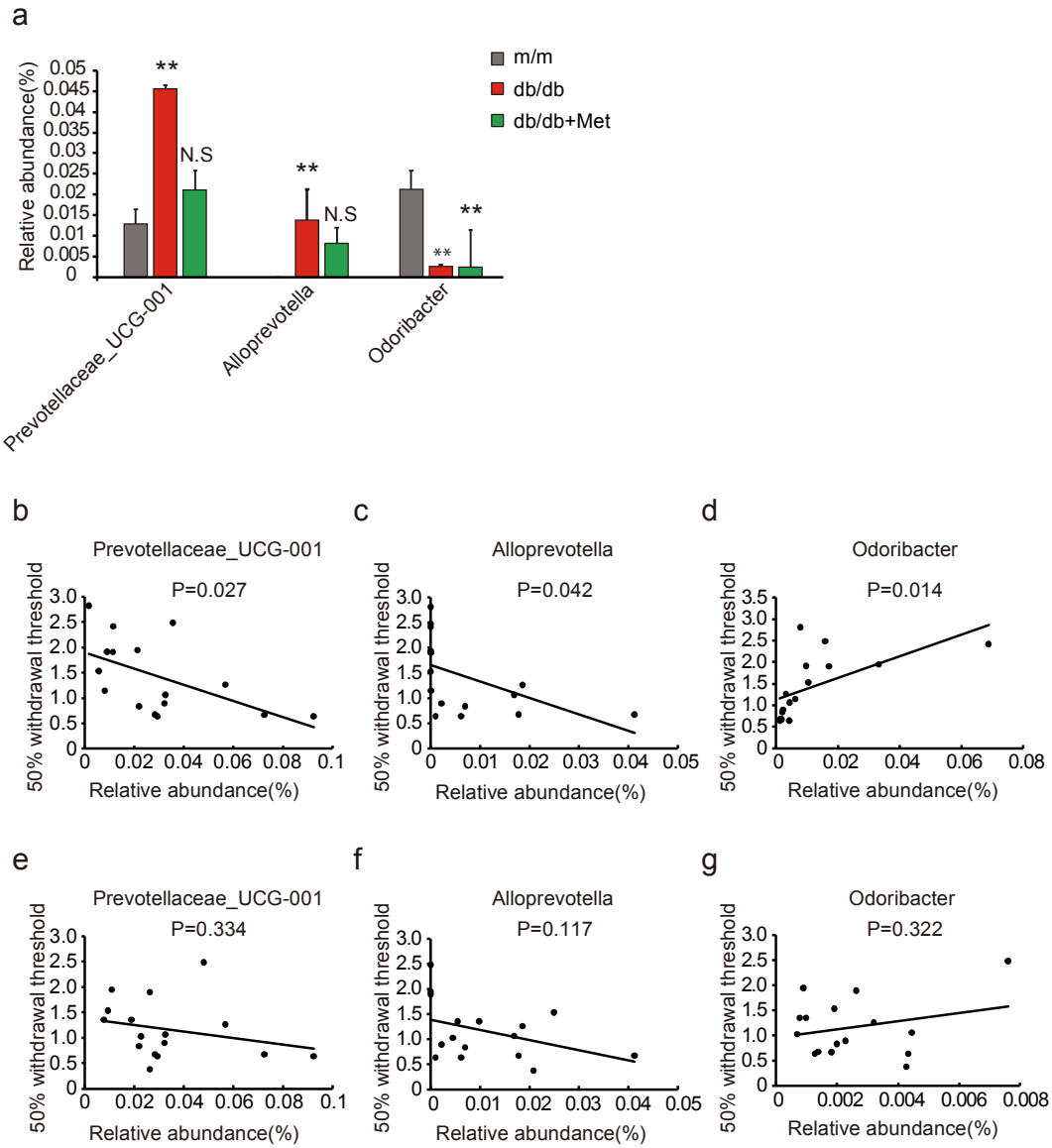

Figure 5. *Correlations between altered colonic bacterial genera and mechanical allodynia.*

(a) Comparison of bacterial relative abundance in colon. Met, metformin. ** p<0.01 and N. S, no significant, vs *m/m* mice (Kruskal Wallis test). n=8 in each group. (b-d): Correlations of *Prevotellaceae_UCG-001* (b), *Alloprevotella* (c), and *Odoribacter* (d) with mechanical allodynia in *m/m* and *db/db* mice. (e-g): Correlations of *Prevotellaceae_UCG-001* (e), *Alloprevotella* (f), and *Odoribacter* (g) with mechanical allodynia in *db/db* mice with or without metformin.

4. Discussion

In this study, we observed increased body weight, blood glucose levels and mechanical allodynia in 7-week-old *db/db* mice. Two weeks of intraperitoneal injection of metformin ameliorated diabetic mechanical allodynia, but not obesity and blood glucose. This study is the first to identify three bacterial genera that were correlated with the mechanical allodynia of *db/db* mice in the early stage of hyperglycemia. Metformin improved microbiota diversity, but its effect on mechanical allodynia was not associated with these three bacteria in *db/db* mice.

Both diabetes mellitus and the effect of anti-diabetic medicine (e.g., metformin) are associated with the regulation of gut microbiota, [16-19] making it difficult to distinguish whether gut microbial changes are caused by diabetes or its complications. Our previous study showed that metformin injection for 2 weeks attenuates diabetic mechanical allodynia without an anti-diabetic effect, enabling us to determine the effect of gut microbial changes on PDN. In the current study, we demonstrated that changes in gut microbiota abundance, but not gut microbial evolutionary relationships, are associated with mechanical allodynia. Furthermore, this association exists in the colon but not small intestine.

In present study, bacteria associated with PDN (*Prevotellaceae_UCG-001*, *Alloprevotella* and *Odoribacter*) were commonly involved in metabolic regulation [19-22], and there is little direct evidence on the association between pain and these bacteria. Short-chain fatty acids (SCFAs) are important metabolic products of these bacteria [23-25], which also increase in animal model with nerve injury and contribute to neuropathic pain [26, 27], suggesting that increased abundance of *Prevotellaceae_UCG-001* and *Alloprevotella* might be associated with diabetic mechanical allodynia through their metabolic products. On the other hand, the roles of SCFAs in pain regulation are selective [27]. Thus, negative association between *Odoribacter* abundance and mechanical allodynia in *db/db* mice may be caused by distinct dominant SCFAs. Our results suggested that multiple pathways are involved in diabetic mechanical allodynia during early-stage hyperglycemia, including direct regulation of neuronal excitability through impairing AMPK activity [13, 28] and indirect regulation through the gut microbiota.

We also investigated gut microbiota characteristics during early-stage hyperglycemia in *db/db* mice. In contrast to reduced microbial community abundance in 7-week-old *db/db* mice, the alpha diversity analyses demonstrated that neither microbiota richness nor uniformity changed in the colon and small intestine of any group which is consistent with some clinical reports that diabetes is not associated with alpha diversity indices (Chao 1, Shannon and Simpson) [17]. In diabetic animal models with long-term hyperglycemia, abundances of the bacterial genera *Helicobacter* and *Alistipes* are increased [29, 30], while those of *Tyzzerella*, *Lachnoclostridium*, *Anaerotruncus*, *Roseburia*, *Bacteroides*, *Parabacteroides*, and *Lactobacillus* are decreased [31-35]. We did not observe any change in the colonic abundance of these bacteria during early-stage hyperglycemia (7-week-old), although body weight and blood glucose levels increased from 5 weeks age [13]. This outcome indicates that these bacteria are more associated with diabetes progression than with onset. Our results indicated that *Prevotellaceae_UCG-001* and *Alloprevotella* abundances were increased in 7-week-old *db/db* mice, which is consistent with previous studies that *Prevotellaceae_UCG-001* increase the risk of T2DM [19] and are correlated with fasting blood glucose levels [22], while *Alloprevotella* is associated with obesity [21, 36]. However, *Prevotellaceae_UCG-001* abundance is decreased in diabetic mice (12-week-old) with a long-term hyperglycemia [25]. The available data suggest that abundance patterns of some colonic bacteria might vary as the disease develops. Therefore, previous results may reflect bacterial abundance in a long-term disorder, whereas our results represented abundance during the early stage of hyperglycemia and obesity. Further research is needed to understand whether the various changes in bacterial abundance indicate a special stage of diabetes.

The effect of metformin on the gut microbiota has been widely studied in both patients and animal models. Metformin increases the abundance of colonic bacteria (e.g., *Prevotellaceae_UCG-001*, *Roseburia*, *Lactobacillus* and *Rikenellaceae_RC9_gut_group*) in diabetic and some digestive tract diseases while decreasing *Helicobacter* abundance [29, 32, 35, 37]. Additionally, metformin increases *Lactobacillus* abundance and decreases *Bacteroides* abundance in the small intestine [38, 39]. However, these studies examined long-term metformin treatment, and the effect of short-term metformin treatment on the gut microbiota was unclear. Our results showed that 2 weeks of metformin treatment can affect the gut microbiota in *db/db* mice, mainly by increasing microbial community abundance. In contrast to that with long-term treatment, only *Alloprevotella* and *Prevotellaceae-UCG-001* were restored in the colon after 2 weeks, suggesting that these two bacteria are more susceptible to metformin.

5. Conclusions and Perspectives

In summary, our results showed that the abundance of gut microbial communities changed in 7-week-old *db/db* mice. Bacteria associated with metabolic disorders are correlated with pain in *db/db* mice. We found a limited effect on gut microbiota after 2 weeks of intraperitoneal metformin injection, and its analgesic effect on diabetic mechanical allodynia in *db/db* mice was not associated with specific colonic bacteria. Based on the results of this study, further clinical exploration can be carried out to analyze the correlation between the gut microbiota of early diabetic patients and their pain symptoms, to explore the link between changes in the commensal bacteria of patients and changes in abnormal sensation, and to identify bacteria in the gut microbiota that are closely associated with diabetes as markers of lesions.

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