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ORIGINAL RESEARCH

Increased Expression of Colonic Mucosal Melatonin in Patients with Irritable Bowel Syndrome Correlated with Gut Dysbiosis

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Abstract Dysregulation of the **gut microbiota**/gut hormone axis contributes to the pathogenesis of **irritable bowel syndrome** (IBS). **Melatonin** plays a beneficial role in gut motility and immunity. However, altered expression of local mucosal melatonin in IBS and its relationship with the gut microbiota remain unclear. Therefore, we aimed to detect the colonic melatonin levels and microbiota profiles in patients with diarrhea-predominant IBS (IBS-D) and explore their relationship in germ-free (GF) rats and BON-1 cells. Thirty-two IBS-D patients and twenty-eight healthy controls (HCs) were recruited. Fecal specimens from IBS-D patients and HCs were separately transplanted into GF rats by gavage. The levels of colon mucosal melatonin were assessed by immunohistochemical methods, and fecal microbiota communities were analyzed using 16S rDNA sequencing. The effect of **butyrate** on melatonin synthesis in BON-1 cells was evaluated by ELISA. Melatonin levels were significantly increased and negatively correlated with visceral hypersensitivity in IBS-D patients. GF rats inoculated with fecal microbiota from IBS-D patients had high colonic melatonin levels. Butyrate-producing *Clostridium* cluster XIVa species, such as *Roseburia* species and *Lachnospira* species, were positively related to colonic mucosal melatonin expression. Butyrate significantly increased melatonin secretion in BON-1 cells. Increased melatonin expression may be an adaptive protective mechanism in the development of IBS-D. Moreover, some *Clostridium* cluster XIVa species could increase melatonin expression via butyrate production. Modulation of the gut hormone/gut microbiota axis offers a promising target of interest for IBS in the future.

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Introduction

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disease that considerably affects quality of life and work productivity [1]. Gut dysmotility, visceral hypersensitivity, intestinal barrier dysfunction, immune activation, altered microbiota, and brain-gut axis dysregulation may contribute to the development of IBS [2]. Additionally, mast cells have been suggested as noteworthy players in the pathophysiology of IBS [3]. Recently, gut hormones have attracted increasing attention in IBS development and aggravation. The secretion of cholecystokinin, glucagon-like peptide 1, peptide YY, and serotonin differs between patients with diarrhea-predominant IBS (IBS-D) and healthy controls (HCs), which may contribute to the pathogenesis of IBS and the maintenance of symptoms [4].

Melatonin is widely found in nature [5,6]. In vertebrates, melatonin was first reported in the pineal gland. Subsequently, melatonin was also discovered in the retina, Harderian gland, and gastrointestinal tract (GIT). Enterochromaffin cells in the GIT mucosa have been shown to synthesize melatonin [7]. Accumulating evidence has suggested the important roles and activities of melatonin in the GIT, especially enteroprotective effects such as immunomodulatory activity, antioxidant effects, maintenance of gastric prostaglandin levels, and promotion of bicarbonate secretion [8–10]. Additionally, melatonin has also been reported to be involved in the regulation of gut motility [11,12].

Given the importance of melatonin in gastrointestinal function, it is very likely that melatonin affects the pathophysiology of IBS. Some studies found that melatonin administration could alleviate abdominal pain in IBS patients, but the mechanism remains unclear [13]. Therefore, exploration of the role of GIT melatonin in IBS is essential. However, the altered expression of local mucosal melatonin in IBS has not been evaluated to date.

Increasing evidence suggesting the effect of gut microbiota on the pathophysiology of IBS [14] and the importance of the gut microbiota/gut hormone axis in the pathogenesis of IBS has attracted increasing attention [4]. Additionally, a recent study suggested that melatonin could alleviate lipid metabolism in high fat diet-fed mice by modulating gut microbiota [15]. However, the association between mucosal melatonin and gut microbiota remains obscure.

The aims of our study were 1) to detect colonic mucosal melatonin levels and gut dysbiosis in patients with IBS-D and 2) to further assess whether altered melatonin expression correlates with gut microbiota in humans, germ-free (GF) rats, and BON-1 cells (a model of enterochromaffin cells [16]).

Results and discussion

Demographic and clinical characteristics of patients with IBS-D

32 IBS-D patients and 28 HCs were included. The demographic and clinical characteristics are presented in **Table 1**. No significant differences in age, sex, and body mass index (BMI) were found between the two groups. Anxiety and depression scores showed a higher tendency in patients with IBS-D, but the difference was not significant. The intake of protein, fat, and carbohydrate was described as a proportion of total energy consumption. No significant differences were found in the proportion of the energy intake from protein, fat, or carbohydrate between the two groups (Figure S1). 28

Table 1 Demographic and clinical features of patients with IBS-D and HCs

	IBS-D	HC	*P* value
Number of subjects	32	28	
Age (years)	32 ± 1.77	30 ± 1.56	0.471
Gender (male:female)	28:4	20:8	0.219
BMI (kg/m^2)	22.6 ± 0.62	23.1 ± 0.73	0.564
*Hospital anxiety scale	5.41 ± 0.60	3.90 ± 0.79	0.127
*Hospital depression scale	4.28 ± 0.47	2.75 ± 0.65	0.056
#Rectal distension (mmHg)			
First sensation	9.07 ± 0.60	11.14 ± 0.88	0.055
Sensation of defecation	16.50 ± 1.00	20.29 ± 1.71	0.049
Sensation of urge to defecation	24.64 ± 1.40	29.14 ± 2.35	0.089
Maximally tolerable distension	34.81 ± 1.65	39.11 ± 2.37	0.132
IBS-SSS	186.83 ± 8.42		

Note: The data are presented as mean ± SE. IBS-D, diarrhea-predominant irritable bowel syndrome; HC, healthy control; BMI, body mass index; IBS-SSS, IBS symptom severity scale. *, Number of subjects: IBS-D patients, $N = 26$; HCs, $N = 17$. #, Number of subjects: IBS-D patients, $N = 28$; HCs, $N = 17$.

IBS-D patients and 17 HCs underwent a rectal barostat test to evaluate visceral sensitivity, and the threshold for sensation of defecation in IBS-D patients was significantly lower than those in HCs (Table 1).

Increased melatonin expression was significantly negatively correlated with visceral hypersensitivity in IBS-D patients

Melatonin cell density was significantly higher in the colonic mucosa of IBS-D patients (94.88 ± 37.84/mm^2) than in that of HCs (73.61 ± 31.19/mm^2) (P = 0.0318) (**Figure 1**A–C). The arylalkylamine N-acetyltransferase (AANAT; a melatonin-synthesizing enzyme) cell density was also significantly increased in IBS-D patients (33.23 ± 24.34/mm^2) compared to HCs (24.50 ± 21.63/mm^2) (P = 0.0214) (Figure 1D–F).

To determine the role of melatonin in the GIT, it was necessary to determine whether IBS-D patients exhibited dysregulated melatonin expression (particularly mucosal melatonin expression), and this study was the first to evaluate the altered expression of mucosal melatonin in IBS patients. Melatonin synthesized in the GIT has been reported to be absorbed into the circulation and metabolized to 6-hydroxymelatonin sulfate (6-HMS) [17]. The 24-h urinary excretion of 6-HMS was also reported to be higher in the IBS-D patients, which supports our finding [18]. However, the results remain controversial. Another study reported the opposite results and found that the 24-h urinary excretion of 6-HMS was higher in the HC group [19]. Therefore, the alteration of gut melatonin may be a consequence rather than a cause according to the contradictory results regarding the melatonin metabolite 6-HMS in different studies.

To investigate the possible role of increased melatonin expression in IBS-D patients, we performed Spearman's rank correlation analyses between melatonin level and visceral hypersensitivity in IBS-D patients. The density of melatonin cells was significantly positively correlated with the thresholds of the sensation of urge to defecation and maximally tolerable distension in IBS-D patients (Figure 1G–J), which supported the idea that increased melatonin levels may be a countermeasure for the development of IBS. No correlation was found between the density of melatonin cells and other parameters,

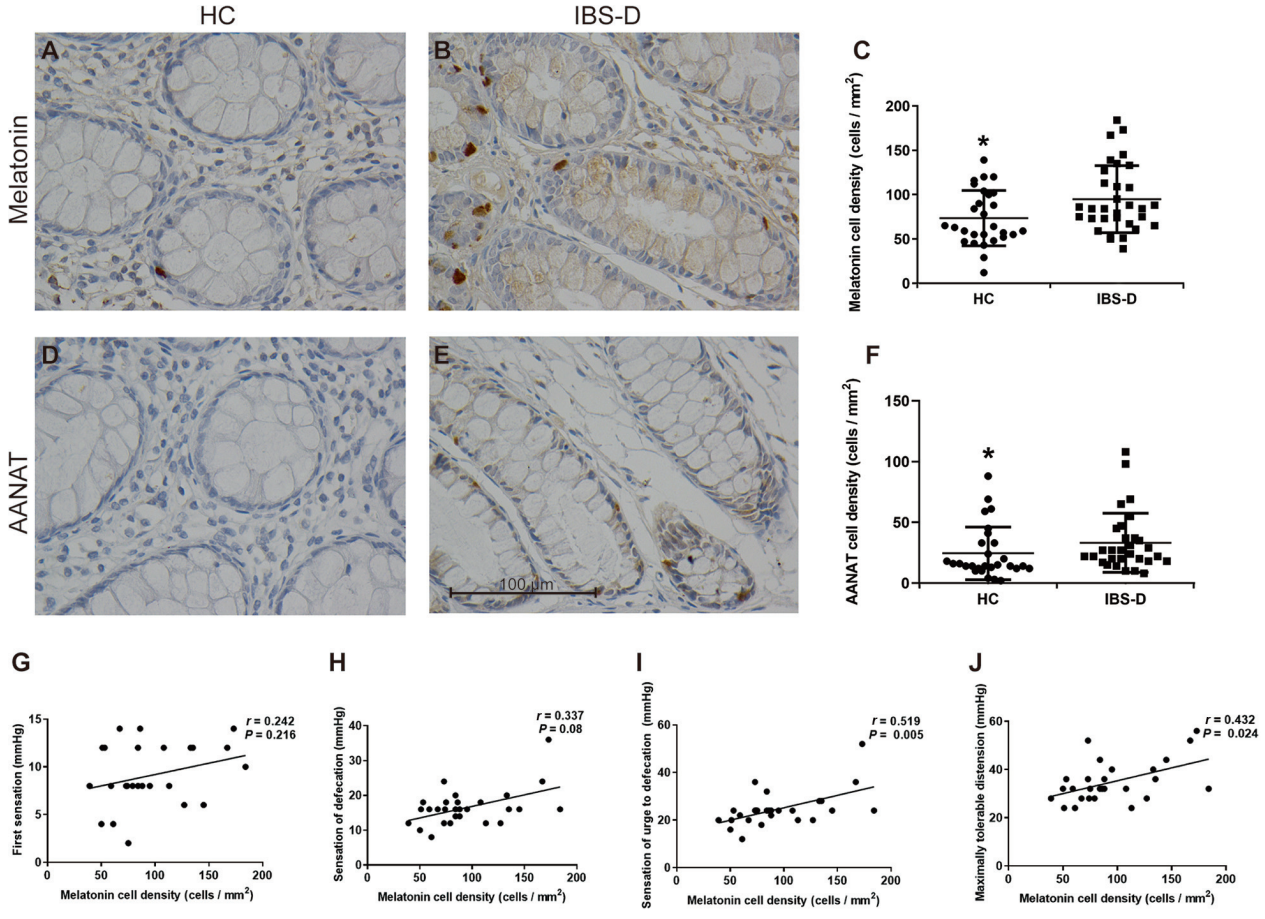

Figure 1 Increased melatonin expression was negatively associated with visceral hypersensitivity in patients with IBS-D

A. and **B.** Representative images of immunohistochemical staining of melatonin cells in the colon of HCs (A) and IBS-D patients (B) at ×400 magnification. **C.** Density of melatonin cells in the colon of HCs (N = 28) and IBS-D patients (N = 32). **D.** and **E.** Representative images of immunohistochemical staining of AANAT cells in the colon of HCs (D) and IBS-D patients (E) at ×400 magnification. **F.** Density of AANAT cells in the colon of HCs (N = 28) and IBS-D patients (N = 32). **G.**–**J.** Melatonin cell density was negatively associated with visceral hypersensitivity in IBS-D patients. The line in the scatter plots (C and F) indicates the mean value. *, 0.01 < P < 0.05. IBS-D, diarrhea-predominant irritable bowel syndrome; HC, healthy control; AANAT, arylalkylamine N-acetyltransferase.

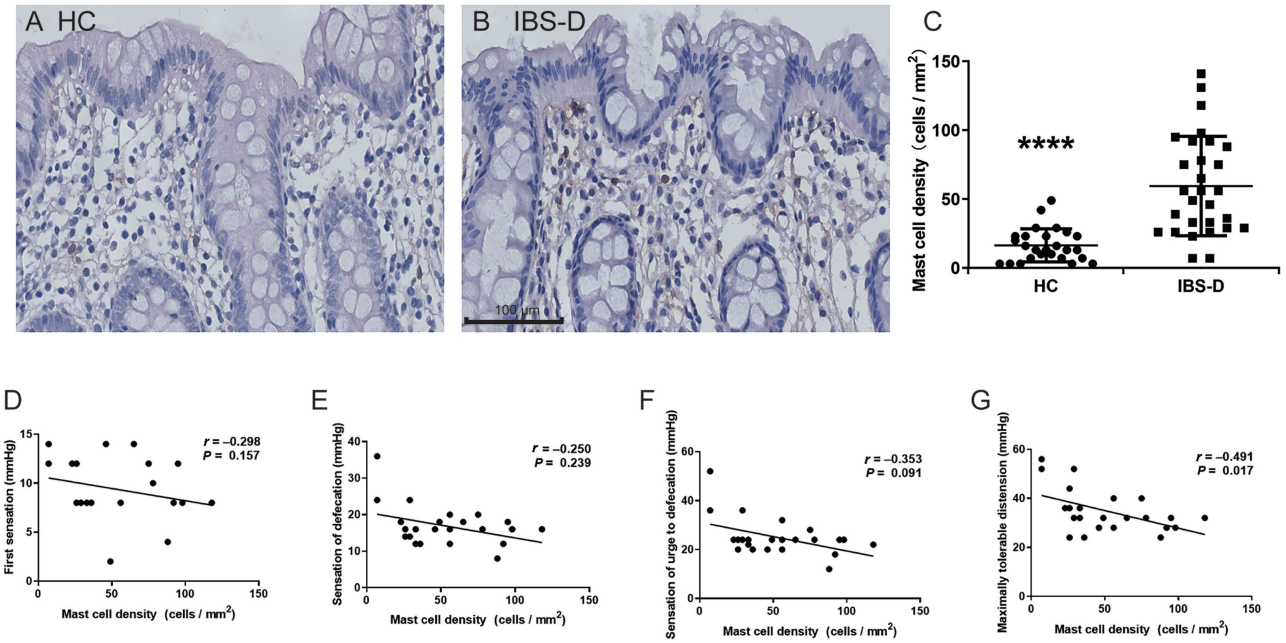

Figure 2 Increases in mast cells positively correlated with visceral hypersensitivity of IBS-D patients
A. and **B.** Representative images of immunohistochemical staining of mast cells in the colon of HCs (A) and IBS-D patients (B) at ×400 magnification. **C.** Density of mast cells in the colon of HCs ($N = 26$) and IBS-D patients ($N = 29$). **D.**–**G.** Mast cell counts were positively correlated with visceral hypersensitivity in IBS-D patients. The line in the scatter plot (C) indicates the mean value. ****, $P < 0.0001$.

including psychological features and IBS symptom severity scale (IBS-SSS) scores (Table S1).

Low-grade inflammation has been confirmed in the colonic mucosa of IBS and mast cells are considered to play an essential role in mucosal inflammation [20]. In the present study, the mast cell count was significantly higher in the colonic mucosa of IBS-D patients (59.48 ± 36.09/mm^2) than in that of HCs (16.42 ± 12.01/mm^2) ($P = 0.000$) (**Figure 2**A–C). Furthermore, the mast cell count was significantly negatively associated with the threshold of maximally tolerable distension in patients with IBS-D (Figure 2D–G). Numerous studies have suggested an increased mast cell count in the colons of patients with IBS [21]. In addition, the increased mast cell count and the activation of mast cells in the colons of patients with IBS may play an important role in the pathophysiology of IBS, especially in visceral hypersensitivity [3,22]. All of these findings are in line with our results.

Moreover, melatonin has been confirmed to inhibit the activation and proliferation of mast cells by *in vitro* and *in vivo* studies [23–25]. In our study, no significant differences were detected between the densities of melatonin cells and mast cells ($r = 0.111$, $P = 0.567$). Interestingly, when the mast cell counts were considered between one standard deviation above the mean density of the HCs (28.43/mm^2) and nine standard deviations below the mean density of the HCs (124.51/mm^2), the density of melatonin cells was significantly negatively correlated with the mast cell count in patients with IBS-D ($r = -0.455$, $P = 0.038$). In addition, accumulating evidence has demonstrated the protective effects of melatonin on the GIT, including immune enhancement, antioxidant activity, regulation of disturbed GI motility, visceral sensitivity reduction, and anxiolytic and antidepressant activity [8]. Above all, the elevated level of colonic mucosal melatonin in IBS-D patients may be based on an adaptive mechanism to modulate the dysfunction of the GIT.

Transplantation of IBS-D patient fecal microbiota significantly elevated melatonin expression in GF rats

To further explore the correlations between gut dysbiosis and melatonin observed in patients with altered expression, we performed fecal microbiota transplantation (FMT) in male GF rats. We chose male GF rats for two reasons. First, estrogen in female rats has been confirmed to be involved in the regulation of motor and sensory functions in the GIT and may confound the results [26]. Second, the gut microbiota composition may be influenced by estrogen and altered during the physiological cycle [27,28]. The density of melatonin cells in the GI group (GF rats receiving fecal specimens from one IBS-D patient) (57.83 ± 13.78/mm^2) was significantly increased compared with the GH group (GF rats receiving fecal specimens from one HC) (25 ± 10.46/mm^2) and the GN group (GF rats without FMT) (20.2 ± 6.26/mm^2) (**Figure 3**). The increased level of mucosal melatonin expression indicates that abnormal gut microbiota results in abnormal melatonin secretion in the colon via direct or indirect mechanisms.

The GI rats also exhibited visceral hypersensitivity. Abdominal withdrawal reflex (AWR) scores were significantly higher in GI rats than in GH rats, regardless of the pressures applied (from 20 to 80 mmHg) (Figure S2), which is in accordance with a previous study in which fecal microbiota from a single IBS patient was transferred to GF rats [29]. In the colonic mucosa specimens, the density of mast cells showed a

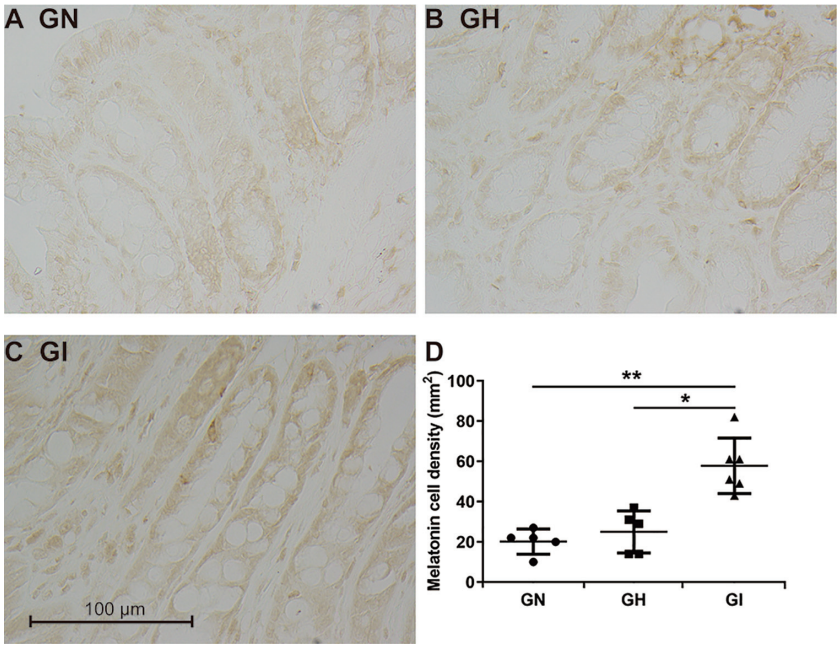

Figure 3 Melatonin expression in FMT rats

A.–C. Representive images of immunohistochemical staining of melatonin in the colon of the GN group (A), GH group (B), and GI group (C) at ×400 magnification. **D.** Density of melatonin cells in the GN, GH, and GI groups. The line in the scatter plot indicates the mean value. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$. FMT, fecal microbiota transplantation; GN, germ-free rats without FMT; GH, germ-free rats receiving FMT from one HC; GI, germ-free rats receiving FMT from one IBS-D patient.

higher tendency in GI rats (118.2 ± 64.02/mm^2) than in GH rats (93.20 ± 32.90/mm^2), but this difference was not significant (Figure S3).

Gut dysbiosis was detected in IBS-D patients

Fecal samples from 22 IBS-D patients and 18 HCs were collected for microbial analysis. The fecal microbiota richness was significantly increased in IBS-D patients compared with that in HCs based on the abundance-based coverage estimator (ACE; 246.78 ± 84.07 *vs*. 197.62 ± 48.88, $P = 0.035$) and the Chao1 estimator (Chao; 243.16 ± 71.85 *vs*. 198.36 ± 50.89, $P = 0.032$) richness indices. No significant differences in the Shannon (3.27 ± 0.60 *vs*. 3.08 ± 0.43, $P = 0.33$) and Simpson (0.10 ± 0.089 *vs*. 0.11 ± 0.058, $P = 0.60$) diversity indices were found between the two groups (Figure S4; Table S2). There were 636 operational taxonomic units (OTUs) in IBS-D patients and 524 OTUs in HCs. In total, 463 OTUs coexisted in the two groups. The microbial community in the two groups could not be separated by principal coordinate analysis (PCoA) (Figure S5), which may be due to the heterogeneity within each group and the relatively small sample size. However, the partial least squares-discriminant analysis (PLS-DA) revealed separation in microbial composition between IBS-D patients and HCs, which was significantly different based on the cross-validation ANOVA (CV-ANOVA) ($P = 0.000321$) (**Figure 4**A). PLS-DA yielded a model with five components with a cumulated index ($Q^2 = 0.492$) that measures the overall goodness of fit of the extremely high model explaining 98.1% (R^2Y) of the variability between the HC and IBS-D groups. An analysis of similarity (ANOSIM) ($R = 0.0659$, $P = 0.041$, permutation number = 999) was also performed. A tendency of increase in Firmicutes and decrease in Bacteroidetes was found in the IBS-D group in this study based on the community abundance bar plot (Figure 4B), which is in accordance with previous studies [30,31]. Moreover, the alteration in abundance of Firmicutes and Bacteroidetes in patients with IBS has been shown in many studies, but the results were controversial [32,33]. The reason for this discrepancy may be due to sample size, different DNA extraction methods, and the various dietary habits of the recruited subjects [34]. The abundance of *Prevotella_9* was significantly increased in the IBS-D group at both the genus and species levels based on the Wilcoxon rank-sum test (Figure 4C and D). *Prevotella* enrichment has been reported as a risk factor in IBS-D patients [35]. The enrichment and reduction in the observed bacterial communities in IBS-D patients are displayed in a Manhattan plot (Figure 4E). The percentage of community abundance at the genus level in the two groups is shown in Table S3.

Additionally, no significant difference was found in the amount of fecal short-chain fatty acids (SCFAs) between the two groups (Figure S6), which may be due to the following reasons. First, the content of fecal butyrate depends on production by bacterial fermentation and absorption in the colon. Second, the relatively small sample size may be a factor. The multifactorial determinants of fecal SCFAs may contribute to the inconsistent results.

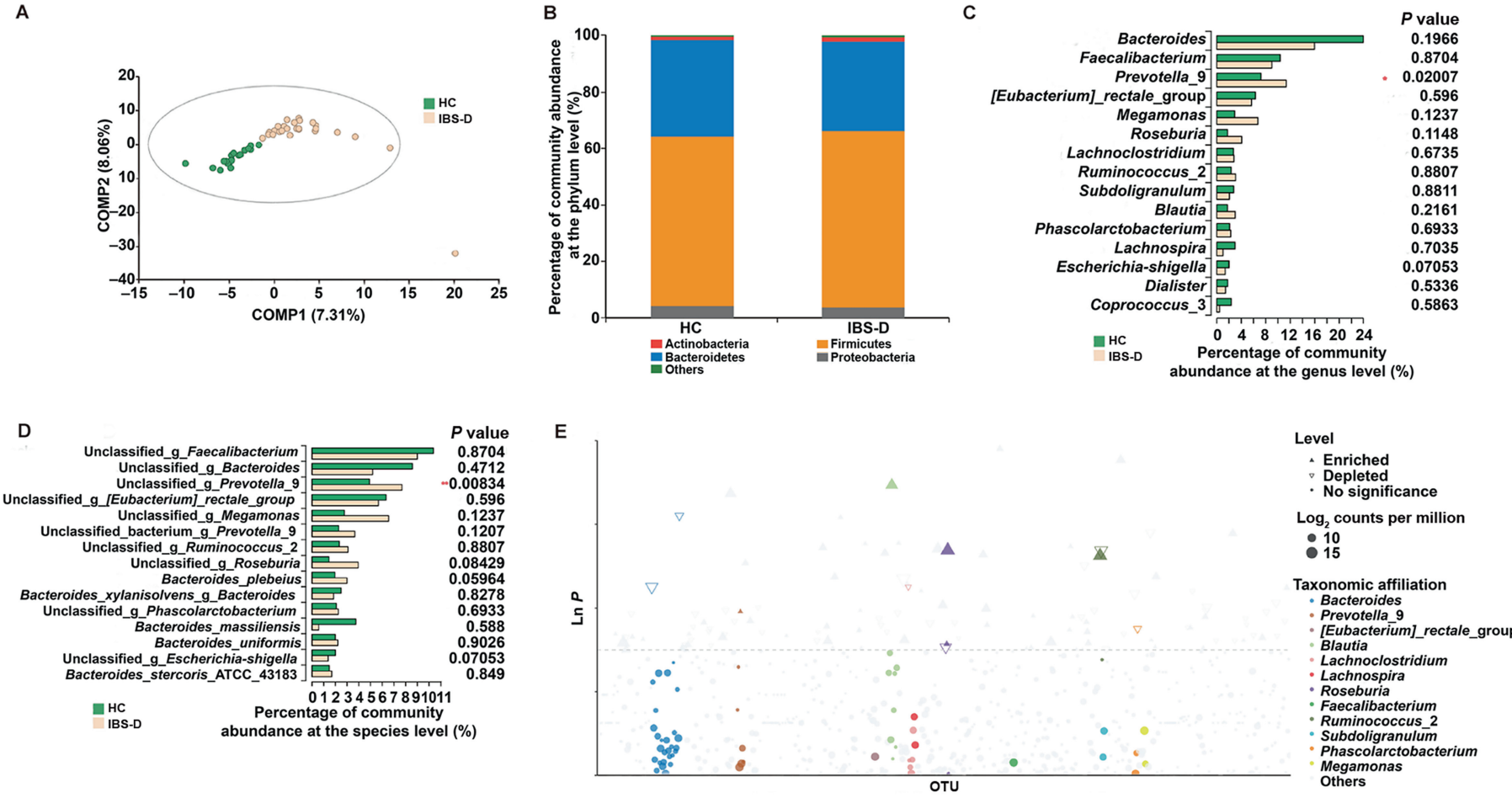

Figure 4 Gut dysbiosis in IBS-D patients

A. PLS-DA revealed separation in microbial composition between patients with IBS-D and HCs. **B.** The percentage of community abundance in IBS-D patients and HCs at the phylum level. **C.** and **D.** The percentage of community abundance in IBS-D patients and HCs at the genus (C) and species (D) levels. Wilcoxon rank-sum test. *, $0.01 < P < 0.05$. **E.** Manhattan plot showing enriched and reduced OTUs in IBS-D patients. OTUs that are significantly enriched are depicted as full triangles. The dashed line indicates the threshold of significance ($P = 0.05$). The color and size of each dot represents the different taxonomic affiliation of the OTUs and relative abundance, respectively (at the genus level). PLS-DA, partial least squares-discriminant analysis; OTU, operational taxonomic unit.

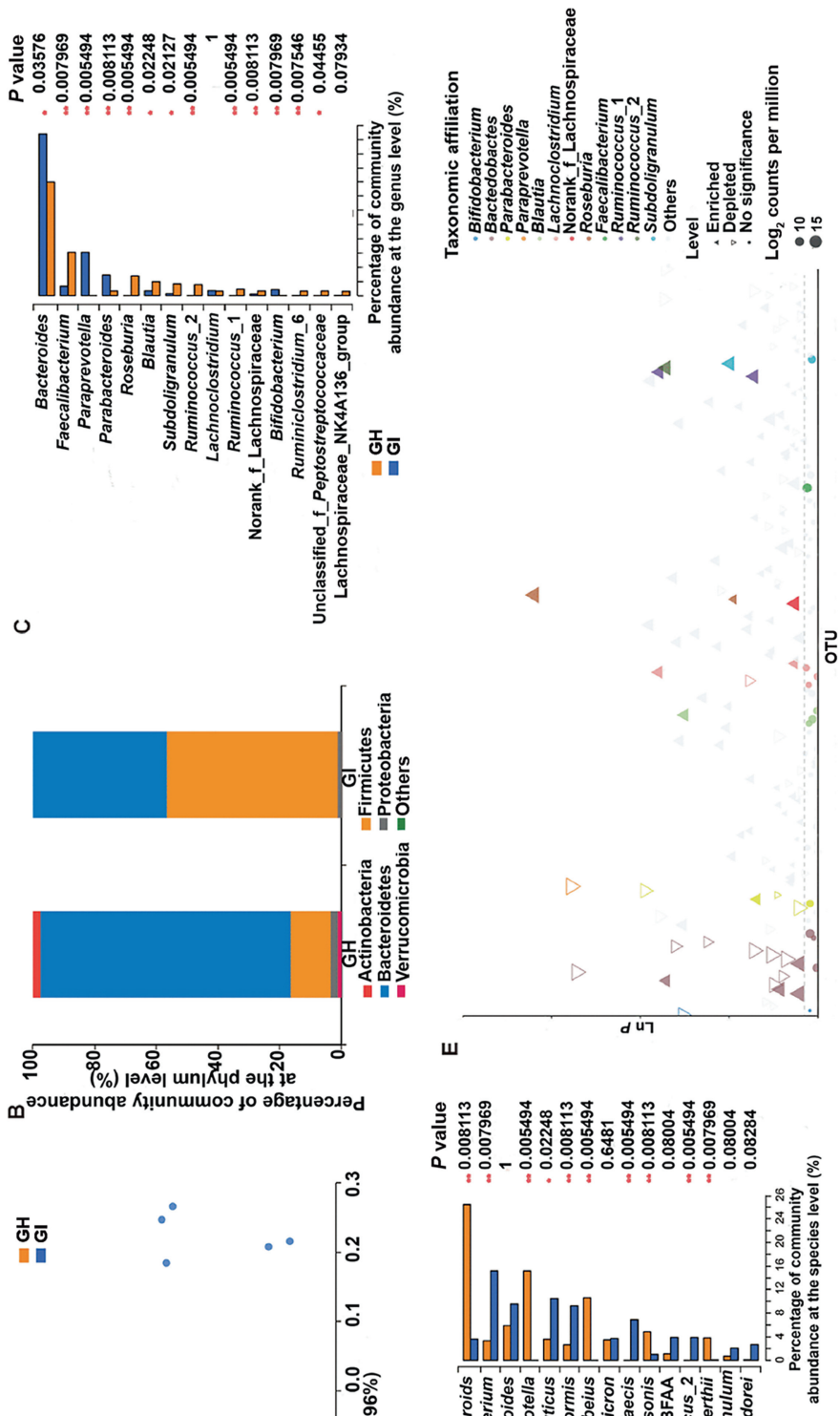

Figure 5 Significant differences in gut microbiota between the GI and GH groups

A. PCoA indicated significant difference in microbial composition between the GI group and GH group. **B.** The percentage of community abundance in the GI and GH groups at the phylum level. **C.** and **D.** The percentage of community abundance in the GI and GH groups at the genus (C) and species (D) levels. Wilcoxon rank-sum test. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$. **E.** Manhattan plot showing enriched and reduced OTUs in the GI group. OTUs that are significantly enriched are depicted as full triangles. The dashed line indicates the threshold of significance ($P = 0.05$). The color and size of each dot represent the different taxonomic affiliation of the OTUs and relative abundance, respectively (at the genus level). PCoA, Principal coordinate analysis.

Figure 6 Relationship between melatonin and gut microbiota in humans and FMT rats

A. Spearman correlation heatmap showing the relationships of human colonic mucosal melatonin expression, AANAT expression, and butyrate level with gut microbiota at the species level. **B.** Spearman correlation heatmap showing the relationship of FMT rat colonic mucosal melatonin expression with gut microbiota at the species level. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$. MT, melatonin.

Significant difference in gut microbiota between the GI and GH groups

The fecal microbiota richness in the GI group was remarkably higher than that in the GH group based on the ACE (137.24 ± 4.65 *vs.* 94.26 ± 8.59, $P < 0.000$), Shannon (3.17 ± 0.12 *vs.* 2.91 ± 0.21, $P = 0.028$), and Chao (137.61 ± 5.03 *vs.* 94.06 ± 9.27, $P < 0.000$) richness indices. No significant difference was detected in the Simpson (0.077 ± 0.012 *vs.* 0.095 ± 0.043, $P = 0.35$) diversity index between the two groups (Figure S7; Table S4). There were 153 OTUs in the GI group and 113 OTUs in the GH group. In total, 91 OTUs appeared simultaneously in the two groups. A significant difference in microbial composition was found between the two groups by PCoA

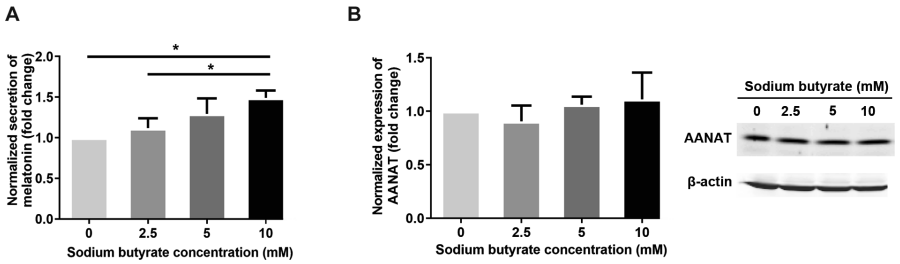

Figure 7 Promotion of butyrate on melatonin and AANAT expression in BON-1 cells
A. Relative quantification of melatonin in sodium butyrate-treated BON-1 cells by ELISA. **B.** Relative quantification of AANAT in sodium butyrate-treated BON-1 cells by Western blot analysis. Data are presented as mean ± SEM. *, $0.01 < P < 0.05$.

(**Figure 5**A). The abundance of Firmicutes was significantly enriched, and the abundance of Bacteroidetes was significantly reduced in the GI group based on a community abundance bar plot (Figure 5B).

The abundances of *Faecalibacterium*, *Roseburia*, and *Ruminococcus* were significantly enriched, and the abundance of *Bacteroides* was significantly reduced in the GI group based on the Wilcoxon rank-sum test at the genus and species levels (Figure 5 C and D). The enrichment and reduction in the observed bacterial communities in the GI group are displayed in a Manhattan plot (Figure 5E). The percentage of community abundance at the genus level in FMT rats is shown in Table S5.

The amounts of fecal formate, butyrate, valerate, and isovalerate were remarkably increased, and the amount of fecal propionate was remarkably reduced in the GI group (Figure S8).

Some butyrate-producing *Clostridium* cluster XIVa bacteria species were positively associated with colonic mucosal melatonin expression

As shown in the heatmap of Spearman's rank correlation coefficients in humans, *Roseburia* was significantly positively correlated with melatonin and AANAT expression. *Lachnospira* and *Parasutterella* were significantly positively associated with AANAT expression (**Figure 6**A). Additionally, *Roseburia* and *Lachnospira* were significantly positively related to fecal butyrate levels. The genus *Roseburia*, which belongs to *Clostridium* cluster XIVa, consists of *R. intestinalis*, *R. hominis*, *R. inulinivorans*, *R. faecis*, and *R. cecicola* [36]. The ability of *Roseburia* species to penetrate the mucus layer enables them to interact with gut epithelial cell surfaces and exert physiological effects [37]. As SCFA producers, *Lachnospira* species also belong to *Clostridium* cluster XIVa [38,39]. On the one hand, *Clostridium* cluster XIVa species are known to be butyrate-producing bacteria and play a potential role in the regulation of gut motility and innate immunity (mainly anti-inflammatory effects) [36,40]. Butyrate has been confirmed to stimulate the expression of colonic serotonin (a precursor of melatonin) in both *in vivo* and *in vitro* studies [41,42]. Moreover, butyrate has been reported to promote melatonin expression in an intestinal epithelial cell line (Caco-2 cells) [43], indicating that *Clostridium* cluster XIVa species may promote melatonin expression via butyrate. On the other hand, melatonin facilitates goblet proliferation and MUC2 expression [44]. As mucin-adherent bacteria, *Clostridium* cluster XIVa species compose 60% of the mucosal microbiota, and stimulating mucus production may lead to a higher abundance of mucin-adherent bacteria [37]. Therefore, we postulate that melatonin could increase the abundance of *Roseburia* and *Lachnospira* via the promotion of mucus secretion, in turn stimulating melatonin expression and constituting a positive feedback loop.

In view of the correlation analyses in FMT rats, most of the bacteria that positively correlated with melatonin expression belong to *Clostridium* cluster XIVa such as *Roseburia*, *Lachnospiraceae*, *Blautia*, and *Ruminiclostridium* species (Figure 6B), which is consistent with the results in humans. Furthermore, the changes in the abundance of butyrate-producing *Clostridium* cluster XIVa species (such as *Roseburia*) in IBS patients were not consistent among different studies [45,46] indicating that these changes may also be a consequence rather than a cause of the disease.

Sodium butyrate promotes melatonin secretion in BON-1 cells

To confirm the effect of butyrate, one of the main metabolites of butyrate-producing *Clostridium* cluster XIVa bacteria species, we used sodium butyrate to treat BON-1 cells and detected the melatonin concentration and AANAT expression in the treated cells using ELISA and Western blot analysis, respectively. After treatment with sodium butyrate for 24 h, the melatonin level was significantly increased in a dose-dependent manner in BON-1 cells (**Figure 7**A). The 10 mM sodium butyrate-treated group showed a significant increase in melatonin secretion compared with the untreated control group and the 2.5 mM sodium butyrate-treated group. However, only a tendency of increased AANAT expression was observed when BON-1 cells were treated with 5 mM and 10 mM sodium butyrate (Figure 7B). Therefore, butyrate could increase melatonin expression in a dose-dependent manner according to our research, which was consistent with a previous study [43]. Moreover, many studies have suggested that butyrate can promote tight junction-mediated barrier integrity and exert anti-inflammatory functions. Considering the protective effects of melatonin, the butyrate-melatonin link may be a protective mechanism of butyrate in IBS.

There were several limitations in this study. First, this study involved only IBS-D patients, and additional studies are wanted to evaluate the alteration of melatonin in patients with other IBS subtypes. Second, cause and effect inference cannot

be made based on our research. Further studies are needed to explore the potential mechanisms underlying the association between gut microbiota and mucosal melatonin.

In conclusion, our study is the first to demonstrate that colonic mucosa melatonin expression is increased in IBS-D patients. Increased melatonin expression may be an adaptive protective mechanism to reduce visceral hypersensitivity. Notably, the study assessed the relationships between gut microbiota and melatonin expression and suggested that different microbiota structures may contribute to different colonic mucosal melatonin levels. Moreover, some butyrate-producing *Clostridium* cluster XIVa species, such as *Roseburia* and *Lachnospira*, could promote melatonin expression via butyrate. These preliminary findings provide some clues for the involvement of the dysregulated gut hormone/gut microbiota axis in the elusive pathophysiology of IBS and for the search for new targeted approaches in the treatment of IBS.

Materials and methods

Subjects

Patients with IBS-D (aged 18–65 years) were enrolled from the gastroenterology clinic of Peking University Third Hospital between March 2015 and January 2016 following the Rome III criteria [47]. Healthy volunteers without any symptoms (aged 18–65 years) were recruited by an Internet advertisement. The inclusion criteria were as follows: 1) normal colonoscopy and 2) no abnormalities in routine blood or stool exams or in liver or kidney function. The exclusion criteria were as follows: 1) organic gastrointestinal or systemic diseases, such as inflammatory bowel diseases or diabetes mellitus; 2) current infectious diseases of the respiratory, digestive, or urinary system; 3) a history of abdominal surgery; 4) history of antibiotic or probiotic use during the previous 4 weeks; 5) continuous laxative or antidiarrheal use for more than 3 days; 6) lactating or pregnant women; and 7) mental illness. Symptom severity was measured with the IBS-SSS. The severity of anxiety and depression symptoms was evaluated with the hospital anxiety and depression scale (HADS). Eating habits were investigated with a 3-day dietary record sheet. Fecal samples were collected for sequencing on the Illumina MiSeq platform. Visceral sensitivity was assessed by rectal barostat, as previously described in detail [48]; sensory thresholds for first sensation, sensation of defecation, sensation of urge to defecation, and maximally tolerable distension were recorded as the lowest distention pressures (mmHg). All participants underwent colonoscopy. Sigmoid colonic mucosal biopsies were obtained during colonoscopy.

Immunohistochemistry

Sections were cut with a Leica microtome at a thickness of 4 μm. The sections were deparaffinized by following routine procedures. Rabbit anti-melatonin (1:800; catalog No. abx100179, Abbexa, Cambridge, UK), rabbit anti-AANAT (1:800; catalog No. orb5712, Biorbyt, Wuhan, China), and anti-mast cell tryptase (1:3000; catalog No. ab2378, Abcam, Cambridge, UK) primary antibodies were used to detect melatonin, AANAT, and mast cells, respectively. A horseradish peroxidase/3,3′-diaminobenzidine detection system (Catalog Nos. PV-6000 and PV-6001, Zhongshan Golden Bridge, Beijing, China) was used for visualization.

For the quantification of melatonin and AANAT expression, ten fields were randomly selected at a magnification of ×400 from each slide under a Leica DM 2500 microscope (LEICA Microsystems, Germany), captured using a Nikon DS-Vi1 camera (Nikon Instruments, China), and exported by the Nikon NIS-Elements imaging software. The number of immunoreactive cells was counted in every field and the area of every field was calculated using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., USA). To quantify mast cells, the NanoZoomer-SQ digital slide scanner (Hamamatsu Photonics, Japan) was used for whole slide scanning, and three randomly selected fields at a magnification of ×400 were exported by NDP view2 viewing software (Hamamatsu Photonics, Japan). The density of immunoreactive cells was expressed as the average number of cells per square millimeter of mucosal epithelium.

DNA extraction of fecal microbiota

Microbial DNA was extracted from fecal samples with an E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA). Two primers [338F (5′-ACTCCTACGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT)] were used to amplify the hypervariable regions (V3 to V4) of the bacterial 16S ribosomal RNA gene. PCR was performed with the following program: 3 min of denaturation at 95 °C; 27 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; and a final extension at 72 °C for 10 min. Purified amplicons were pooled in equimolar amounts, and paired-end sequencing (2 × 300) was performed on the Illumina MiSeq platform (Illumina, San Diego, CA).

Processing of sequencing data

Trimmomatic was chosen to demultiplex and quality-filter raw FASTQ files and raw FASTQ files were merged by fast length adjustment of short reads (FLASH). OTU clustering was performed by UPRISE (version 7.0 http://drive5.com/uparse/). UCHIME was used to identify and remove chimeric sequences. The taxonomy of each 16S rRNA gene sequence analysis was performed using the RDP Classifier algorithm against the Silva (SSU128) 16S rRNA database with confidence threshold of 70%.

Quantification of fecal SCFAs

Ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) was used to determine the levels of SCFAs (formate, acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate acetate) in feces as previously described [49].

Animal experiments

Male GF Sprague-Dawley (SD) rats were obtained from the Department of Laboratory Animal Science at Peking University Health Science Center, China. GF rats were divided into three groups and housed in separated sterilized isolators. Different groups of GF rats were maintained in different sterile

bubbles, and every GF rat was housed in a separate cage to avoid cage effects. A total of 16 GF rats (6 in the GI group, 5 in the GH group, and 5 in the GN group) were used in this study. At six weeks of age, GF rats in different groups were subjected to FMT [obtained from one IBS-D patient (GI group) and one healthy volunteer (GH group)] separately by gavage. Rats in the same group received fecal microbiota from the same sample, and transplantation was performed only once. Two weeks after FMT, fecal samples were collected from the rats for further microbiota analyses. Colorectal distension (CRD) tests were conducted 24 days after FMT as described previously [48]. Then, GF rats were sacrificed with anesthesia. Colon tissue was fixed in 10% buffered formalin for immunohistochemistry.

Cell culture

BON-1 cells were purchased from Shanghai Shun Ran Biotechnology Co., Ltd., China and cultured in DMEM (Catalog No. SH30022.01, HyClone, UT) supplemented with 10% FBS (Catalog No. 900–108, Gemini, CA) and 1% penicillin/streptomycin (Catalog No. 15140122, Gibco, MA) at 37 °C in a humidified atmosphere of 5% CO_2. Cells were subcultured every 2 days.

Melatonin measurements in BON-1 cells

BON-1 cells were seeded in 60-mm dishes, allowed to attach and treated with sodium butyrate (Catalog No. 303410, Sigma-Aldrich) at concentrations ranging from 0 mM to 10 mM for 24 h. Then, supernatants were harvested and melatonin levels were determined with an ELISA kit (Catalog No. E-EL-H2O16c, Elabscience, Wuhan, China). Readings were normalized to total protein content, which was detected using a BCA assay. The data compiled from different treatment groups were expressed as melatonin concentrations normalized to the untreated control group.

Western blot analysis

wProteins from BON-1 cells were extracted with RIPA extraction buffer mixed with a protease inhibitor cocktail. Equal amounts of protein (30 μg) were electrophoresed in 12% sodium dodecyl sulfate polyacrylamide gels and transferred onto nitrocellulose membranes under a constant current of 250 mA for 90 min. The membranes were incubated with rabbit anti-AANAT antibody (1:1000; catalog No. ab3505, Abcam) overnight at 4 °C. β-Actin was chosen as the internal reference protein. The primary antibodies were detected using anti-rabbit or mouse secondary antibodies and were visualized with an infrared imaging system (LI-COR Biosciences, Lincoln). The band intensity relative to that of β-actin was calculated, and the results were expressed as fold change from control values.

Statistical analysis

The mean number of melatonin and AANAT immunoreactive cells per square millimeter of mucosal epithelium was compared using the nonparametric Mann-Whitney test (between IBS-D patients and HCs) and Kruskal-Wallis nonparametric test with posttest (among different GF rats). The differences in age and BMI were analyzed by independent samples *t*-test. The χ^2 test or Fisher's exact test was used to detect sex differences between IBS-D patients and HCs. Correlations between melatonin and clinical/experimental parameters were explored using Spearman's rank correlation analysis. The statistical analyses were performed using SPSS software version 24.0 (SPSS Inc., Chicago, IL). Alpha diversity based on the OTU table was analyzed with the ACE, Chao, and the Shannon and Simpson diversity indices in Mothur (version v.1.30.1). β-Diversity was calculated using UniFrac distance, and PCoA was performed in R (version 3.3.1) to visualize the difference in community composition among different groups. PLS-DA and CV-ANOVA were performed using SIMCA (version 14.1.0). The associations among the expression levels of melatonin, AANAT, SCFAs (acetate, propionate, butyrate) and bacterial taxa were visualized and identified by heatmaps of Spearman's rank correlation coefficients using the pheatmap package in R (version 3.3.1). One-way ANOVA with post hoc testing was conducted using SPSS to determine differences in melatonin secretion according to ELISA among different treatment groups.

Ethical statement

The study was approved by the Ethics Committee of Peking University Health Science Center (RB00001052–14091). Written informed consent was obtained from all participants. For the animal experiments, all protocols were approved by the Laboratory Animal Welfare Ethics branch of the Biomedical Ethics Committee of Peking University (LA2016230).

Data availability

The raw sequence data have been deposited in the Genome Sequence Archive [50] in the National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences / China National Center for Bioinformation (GSA: CRA001604), and are publicly accessible at http://bigd.big.ac.cn/gsa.

CRediT author statement

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Competing interests

The authors have declared no competing interests.

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Supplementary material

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