

## GASTROENTEROLOGY

**Beneficial effect of butyrate-producing Lachnospiraceae on stress-induced visceral hypersensitivity in rats**Jindong Zhang,\* Lijin Song,\* Yujing Wang,<sup>†</sup> Chang Liu,<sup>†</sup> Lu Zhang,\* Shiwei Zhu,\* Shuangjiang Liu<sup>†</sup> and Liping Duan\*\*Department of Gastroenterology, Peking University Third Hospital, and <sup>†</sup>State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China**Key words**

butyrate, intestinal barrier, microbiota, stress, visceral hypersensitivity.

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**Declaration of conflict of interest:** All authors disclosed no financial relationships relevant to this publication.**Financial support:** This work was supported by the National Natural Science Foundation of China (grant number 81670491) and Capital's Funds for Health Improvement and Research (grant number 2016-2-4093).**Abstract****Background and Aim:** Emerging evidence indicates that psychological stress is involved in the pathogenesis of irritable bowel syndrome, which is characterized by visceral hypersensitivity and may be accompanied by gut dysbiosis. However, how such stress contributes to the development of visceral hypersensitivity is incompletely understood. Here, we aimed to investigate the influence that stress-induced microbial changes exert on visceral sensitivity, as well as the possible underlying mechanisms associated with this effect.**Methods:** Male Sprague–Dawley rats underwent chronic water avoidance stress (WAS) to induce visceral hypersensitivity. Visceral sensitivity, colonic tight junction protein expression, and short-chain fatty acids of cecal contents were measured. Fecal samples were collected to characterize microbiota profiles. In a separate study, oral gavage of *Roseburia* in WAS rats was conducted to verify its potential role in the effectiveness on visceral hypersensitivity.**Results:** Repeated WAS caused visceral hypersensitivity, altered fecal microbiota composition and function, and decreased occludin expression in the colon. Stressed rats exhibited reduced representation of pathways involved in the metabolism of butyrate and reduced abundance of several operational taxonomic units associated with butyrate-producing bacteria, such as Lachnospiraceae. Consistently, supplementation with *Roseburia hominis*, a species belonging to Lachnospiraceae, significantly increased cecal butyrate content. Moreover, *Roseburia* supplementation alleviated visceral hypersensitivity and prevented the decreased expression of occludin.**Conclusions:** Reduction in the abundance of butyrate-producing Lachnospiraceae, which is beneficial for the intestinal barrier, was involved in the formation of visceral hypersensitivity. *R. hominis* is a potential probiotic for treating stress-induced visceral hypersensitivity.**Introduction**

Perturbations in gut microbiota contribute to the development of intestinal disorders, including irritable bowel syndrome (IBS). IBS patients harbor altered gut microbiota in comparison with those of healthy controls.<sup>1,2</sup> The absence or disruption of gut microbes can significantly influence visceral pain perception, leading to visceral hypersensitivity. In particular, germ-free mice exhibited increased visceral pain responses when exposed to colorectal distension (CRD) stimuli, and visceral hypersensitivity was normalized after microbial colonization.<sup>3</sup> Furthermore, rats receiving early-life antibiotic treatment also displayed visceral hypersensitivity in adulthood and altered microbiota profiles.<sup>4</sup> Moreover, supplementation with probiotics/prebiotics or non-absorbed antibiotics can reverse IBS symptoms.<sup>5,6</sup> However, the bacteria that are critical for IBS pathogenesis have not been identified, and recent results have been inconsistent.

Psychological stress is involved in the initiation, maintenance, and aggravation of IBS. Several lines of evidence have

demonstrated the impact of stress on the gut microbiota,<sup>7–10</sup> but the precise mechanisms remain incompletely understood. Interestingly, mice exposed to chronic social defeat stress exhibited reduced representation of pathways involved in the synthesis and metabolism of short-chain fatty acids (SCFAs).<sup>8</sup> SCFAs are metabolites fermented by specific anaerobic bacteria from non-digestible dietary fiber and resistant starches, and they have extensive regulatory functions in the interaction between microbes and hosts.<sup>11</sup> A few studies have shown that SCFAs are associated with IBS; however, whether they are beneficial or harmful remains controversial.<sup>12,13</sup>

In this study, to further investigate the possible role of gut microbiota in stress-induced visceral hypersensitivity, we examined the influence of chronic water avoidance stress (WAS) in rats on the intestinal barrier and bacterial community and function. We also identified stress-associated microbial species and further verified the beneficial effect of *Roseburia*, a butyrate-producing genus that was significantly reduced in WAS rats, on stress-induced visceral hypersensitivity.

## Methods

**Animals.** Male Sprague–Dawley rats (6–7 weeks old) were obtained from the Department of Laboratory Animal Science, Peking University Health Science Center, Beijing, China. All protocols were approved by the Laboratory Animal Welfare Ethics branch of the Biomedical Ethics Committee of Peking University (LA2016230). Rats were housed in a specific pathogen-free facility and maintained under a 12-h light/dark cycle (6 AM to 6 PM) at a constant temperature ( $23 \pm 2^\circ\text{C}$ ) and humidity ( $63 \pm 2\%$ ). Food and water were provided *ad libitum*. All animals were acclimatized to the facility for 7 days before the experiment began.

**Water avoidance stress treatment.** Repeated WAS procedures were conducted as described previously.<sup>14</sup> Rats were placed on a block ( $10 \times 8 \times 8$  cm) affixed to the center of the floor in a Plexiglas tank ( $45 \times 25 \times 25$  cm) filled with  $25^\circ\text{C}$  fresh water to a level 1 cm below the top of the block. Rats were kept on the block for 1 h each day (5–6 PM) for 10 consecutive days. In the sham group, rats were placed on an identical platform in containers without water for 1 h each day for 10 days. Any fecal pellets in the containers were counted at the end of each 1-h WAS or sham session. These were used to estimate the autonomic regulation of distal colonic motility as previously described.<sup>15</sup>

**Assessment of visceral sensitivity to colorectal distension.** Colorectal distension tests were performed the day after the last WAS session using a well-established and validated method.<sup>15</sup> Briefly, under light isoflurane anesthesia, a flexible balloon made of a polyethylene plastic bag (4–5 cm) was inserted into the distal colon, with its end 1 cm proximal to the anus. Then, the rats were placed in a Plexiglas cage for 20 min before the CRD test was initiated. Five series of CRDs were performed with pressures in different orders using a Distender Series IIR (G&J Electronics, Ontario, Canada) and resulting in constant pressure values of 20, 40, 60, and 80 mmHg (series 1); 40, 60, 80, and 20 mmHg (series 2); 60, 80, 20, and 40 mmHg (series 3); 80, 20, 40, and 60 mmHg (series 4); and 20, 40, 60, and 80 mmHg (series 5). Each distension lasted for a duration of 20 s, with a 4-min inter-stimulus interval. During each distention, abdominal withdrawal reflex (AWR) was scored by two blinded observers following a previously reported scale<sup>16</sup>: 0, no behavioral response to CRD; 1, brief head movement followed by immobility; 2, contraction of abdominal muscles; 3, lifting of abdomen; 4, body arching and lifting of pelvic structures.

**Microbiota 16S rRNA sequencing and analysis.** Fecal samples were collected directly from the anus by massaging the distal rectum before CRD testing, and samples were frozen at  $-80^\circ\text{C}$  until use. Microbial DNA was extracted using a QIAamp Fast Stool Mini Kit (Qiagen, Valencia, CA, USA). The V3–V4 region of the 16S rRNA gene was amplified with barcode-indexed primers (338F and 806R), and paired-end sequencing of the amplicons was performed using the Illumina MiSeq PE300 platform (Illumina, San Diego, CA, USA). The difference in the relative abundance of each taxon at different taxonomic levels

was analyzed using the linear discriminant analysis effect size method, and predictions regarding the functions of the microbiome were based on 16S rRNA-derived operational taxonomic units (OTUs) using a computational approach called phylogenetic investigation of communities using reconstruction of unobserved states.<sup>17</sup>

**Determination of cecal short-chain fatty acids.** Cecal SCFAs were determined using ultra-performance liquid chromatography coupled to tandem mass spectrometry.<sup>18</sup> Approximately 100-mg cecal content was homogenized in 1 mL 50% aqueous acetonitrile and centrifuged. For derivatization, 40- $\mu\text{L}$  supernatant was mixed with 20  $\mu\text{L}$  of 200-mM 3-nitrophenylhydrazine and 20  $\mu\text{L}$  of 120-mM *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide-HCl-6% pyridine solution. The mixture was reacted at  $40^\circ\text{C}$  for 30 min, and the solution was diluted to 800  $\mu\text{L}$  with 10% aqueous acetonitrile, of which 10  $\mu\text{L}$  was injected for measurement. An Ultimate 3000 RSLC system (Dionex Inc., Amsterdam, The Netherlands) coupled to a TSQ Quantiva Ultra triple-quadrupole mass spectrometer (Thermo Fisher, Waltham, MA, USA) was used for SCFA detection, and the final data were processed using Xcalibur 3.0.63 software (Thermo Fisher). The data are expressed as mg SCFA/g cecal content.

**Bacterial preparation.** Strain A2-183 was isolated from a healthy human fecal sample and was identified to be a representative strain of butyrate-producing *Roseburia hominis*. Strain A2-183 was routinely grown with synthetic YCFA medium. After cultivation at  $37^\circ\text{C}$  for 16 h, strain A2-183 cells were harvested by centrifugation at 5000 rpm for 5 min and were resuspended in sterile phosphate-buffered saline. Fresh bacterial cell suspension was prepared daily, and rats were administered orally at a concentration of  $2 \times 10^9$  CFU/day. In the experimental group with *R. hominis*, rats were administered with the bacterial cell suspension for four consecutive days and were subsequently subjected WAS treatment from day 5 till day 14.

**Measurement of serum corticotropin-releasing hormone levels.** Upon euthanasia, blood was withdrawn from the apex cordis and placed in a sterile microtube. After centrifugation at  $4^\circ\text{C}$  and 3000 rpm for 15 min, the supernatant was extracted and stored at  $-80^\circ\text{C}$  until analysis. A radioimmunoarray kit (HY-10175, Beijing, China) was used to determine corticotropin-releasing hormone concentrations according to the manufacturer's instructions.

**Quantitative reverse transcription-polymerase chain reaction for tight junction protein expression.** Total RNA was extracted from distal colon tissues using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Complementary DNA was synthesized using a reverse transcription kit (KR116-02, Tiangen, Beijing, China). Quantitative polymerase chain reaction (qPCR) for the typical tight junction protein occludin and  $\beta$ -actin was performed with a Real-Time PCR Detection System (QuantStudio5, Thermo Fisher Scientific) using SYBR Green detection. Primer sequences used for

qPCR were as follows: forward occludin, 5'-TCGTGATGTGC ATCGCTGTATTTCG-3'; reverse occludin, 5'-CGTAACCGTAG CCGTAACCGTAAC-3'; forward  $\beta$ -actin, 5'-GGGAAATCGTG CGTGACATT-3'; reverse  $\beta$ -actin, 5'-GCGGCAGTGGCCAT CTC-3'. The qPCR conditions were as follows: one cycle at 95°C for 3 min, followed by 40 two-temperature cycles at 95°C for 5 s and 60°C for 15 s. PCR amplifications were performed in a total volume of 20  $\mu$ L containing Talent qPCR PreMix (FP209, Tiangen, Beijing, China). Occludin transcript levels were normalized to that of  $\beta$ -actin, and relative gene expression was calculated as the fold change ( $2^{-\Delta\Delta C_t}$ ) relative to expression in the control samples.

### Assessment of tight junction protein expression.

The distal colon tissues were homogenized in RIPA extraction buffer mixed with a protease inhibitor cocktail and then centrifuged at 12 000 rpm for 10 min. The protein concentration of the supernatant was quantified by a BCA protein assay kit. Equal amounts of protein (90  $\mu$ g) were loaded onto 10% sodium dodecyl sulfate polyacrylamide gels and electrophoresed under a constant voltage of 100 V for 120 min. Proteins were transferred onto nitrocellulose membranes under a constant current of 250 mA for 100 min. The membranes were blocked in 5% (w/v) dried skim milk at 25°C for 1 h and then incubated overnight at 4°C with primary antibodies against occludin (1:10 000, ab167161, Abcam). Glyceraldehyde phosphate dehydrogenase was used as an internal reference protein. After washing three times, the membranes were incubated with antirabbit or mouse secondary antibodies for 1 h at 25°C. Finally, after repeatedly washing three times, they were scanned on an infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). Expression levels were determined as the band intensity relative to that of glyceraldehyde phosphate dehydrogenase, and then the ratio values were normalized by that of the control group.

**Statistical analysis.** Abdominal withdrawal reflex scores was analyzed using two-way ANOVA, with CRD pressure and treatment as factors followed by Least Significant Difference (LSD) post-hoc test. The data for fecal pellet output numbers, occludin expression, and SCFA content were analyzed using one-way ANOVA followed by LSD post-hoc test or unpaired Student's *t*-tests if only two groups were applied. Bacterial relative abundances were analyzed by Wilcoxon test, and the relationships between disease phenotype parameters and alterations in microbial OTUs were analyzed by Pearson's correlation test. Results are expressed as mean  $\pm$  SEM. Differences between groups were considered significant at  $P < 0.05$ . Statistical analyses were performed with SPSS version 20.0 (SPSS, Chicago, IL, USA).

## Results

**Effect of chronic water avoidance stress on visceral sensitivity and intestinal barrier.** Compared with sham controls, AWR scores were higher in WAS rats at pressures of 20 mmHg ( $1.89 \pm 0.16$  vs  $1.43 \pm 0.15$ ,  $P < 0.05$ ) and 60 mmHg ( $3.47 \pm 0.13$  vs  $3.06 \pm 0.12$ ,  $P < 0.05$ ) (Fig. 1a). Fecal pellet output numbers were significantly higher in stressed rats than in the sham group (Fig. 1b). Moreover, the relative levels of colonic occludin

mRNA ( $P < 0.05$ ) (Fig. 1c) and protein ( $P < 0.01$ ) (Fig. 1d,e) decreased significantly in stressed rats.

**Effect of chronic water avoidance stress on fecal microbiota composition and function.** Linear discriminant analysis effect size analysis showed that the relative abundances of the families Prevotellaceae and Coriobacteriaceae and the genus *Prevotella\_1* increased significantly after WAS treatment, while those of Peptococcaceae and Veillonellaceae were significantly reduced in WAS rats (Fig. 2a). Accordingly, 27 OTUs were found to be significantly altered after stress. Six OTUs were enriched following WAS and belonged to the Acidaminococcaceae ( $n = 1$ ), Coriobacteriaceae ( $n = 2$ ), Prevotellaceae ( $n = 1$ ), and Ruminococcaceae ( $n = 2$ ). Another 21 OTUs were reduced or eliminated by WAS, and most of these belonged to the Lachnospiraceae ( $n = 9$ ), Lactobacillaceae ( $n = 2$ ), Peptococcaceae ( $n = 1$ ), and Veillonellaceae ( $n = 2$ ) (Table 1).

As for the microbial function, phylogenetic investigation of communities using reconstruction of unobserved states functional analysis of the 16S rRNA data revealed that the representation of pathways involved in the metabolism of butanoate, the conjugate base of the SCFA butyrate, was significantly reduced in the WAS group (Fig. 2b).

### Correlation between altered microbiota and disease phenotype parameters.

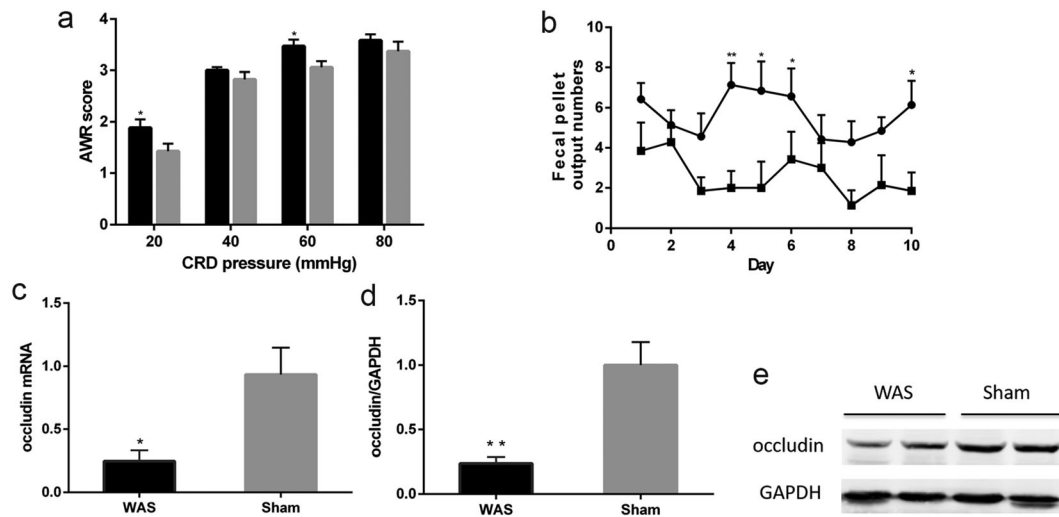
As illustrated in the correlation heatmap in Figure 2c, the abundances of OTUs that belonged to the Lachnospiraceae, Lactobacillaceae, and Veillonellaceae were negatively correlated with AWR scores. Several Lachnospiraceae, Lactobacillaceae, and Peptococcaceae-related OTUs were positively correlated with the expression of occludin. Moreover, OTU281, OTU205, and OTU309 (Lachnospiraceae) and OTU49 (Lactobacillaceae) were positively associated with the representation of butanoate metabolism functional pathways.

### Effect of *Roseburia hominis* on visceral sensitivity.

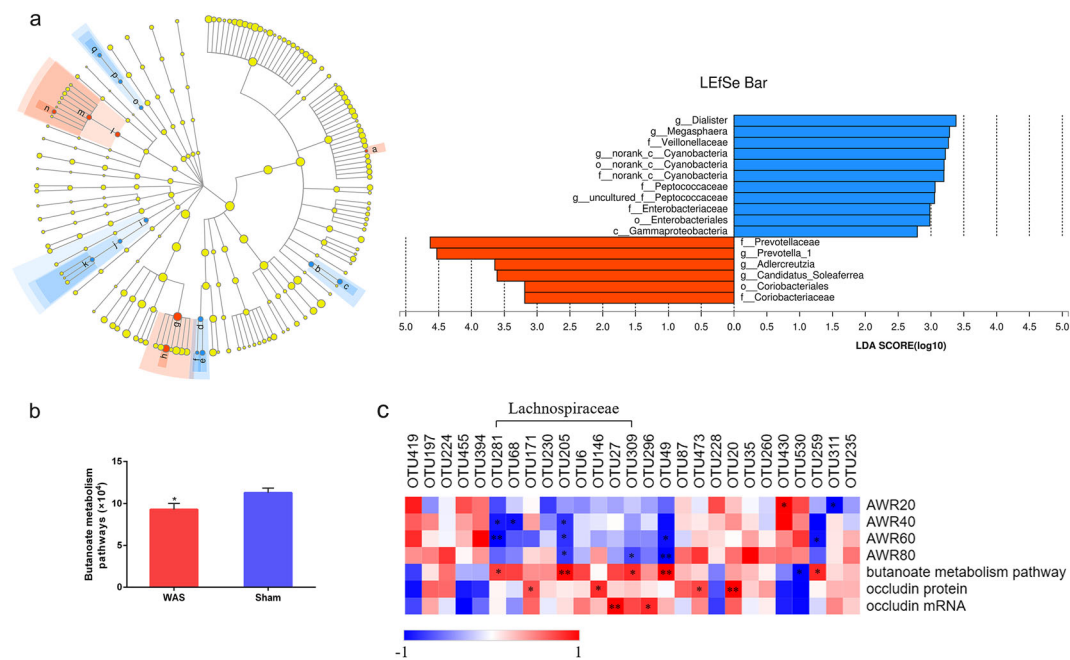
As the numbers of butanoate metabolism functional pathways and OTUs belonging to Lachnospiraceae, a major butyrate-producing family, were both significantly decreased in the stressed rats (Fig. 2b and Table 1), we examined the effect of supplementation with *R. hominis* A2-183, a representative butyrate-producing strain belonging to Lachnospiraceae, on stress-induced visceral hypersensitivity (Fig. 3a). Administration of *R. hominis* significantly attenuated the increase in AWR scores in response to CRD at 40 and 60 mmHg (both  $P < 0.05$ ) (Fig. 3b). Oral gavage of *R. hominis* also significantly decreased fecal pellet output numbers compared with those in the WAS group ( $P < 0.05$ ) (Fig. 3c).

### Effect of *Roseburia hominis* on hypothalamic-pituitary-adrenal axis and intestinal barrier.

*Roseburia hominis* prevented the WAS-induced increase in serum corticotropin-releasing hormone levels (Fig. 4b), while there was no significant alteration in the relative weight of adrenal glands after *R. hominis* administration (Fig. 4a). In addition, quantitative RT-PCR analysis showed that supplementation with *R. hominis* prevented the reduction in occludin mRNA and protein expression induced by WAS (Fig. 5a-c).



**Figure 1** Effect of chronic WAS on visceral sensitivity and intestinal tight junctions. (a) Abdominal withdrawal reflex scores in response to CRD; ■, WAS; ▒, Sham. (b) Fecal pellet output numbers found in the containers after each 1-h WAS; ●, WAS; ■, Sham. (c) Real-time quantitative RT-PCR of colonic occludin mRNA normalized to expression of β-actin. (d) Quantitative analysis of colonic occludin protein normalized to expression of GAPDH. (e) Representative image of occludin bands in western blotting experiments. \**P* < 0.05, \*\**P* < 0.01, compared with sham controls; *n* = 7 rats per group. WAS, water avoidance stress; GAPDH, glyceraldehyde phosphate dehydrogenase; CRD, colorectal distension.



**Figure 2** Effect of WAS on microbial composition and function. (a) LefSe analysis shows significantly different taxa between the two groups (left panel; ■, a: *g\_Candidatus\_Soleiferrea*; ■, b: *f\_Peptococcaceae*; ■, c: *g\_uncultured\_f\_Peptococcaceae*; ■, d: *f\_Veillonellaceae*; ■, e: *g\_Megasphaera*; ■, f: *g\_Dialister*; ■, g: *f\_Prevotellaceae*; ■, h: *g\_Prevotella\_1*; ■, i: *c\_Gammaproteobacteria*; ■, j: *o\_Enterobacteriales*; ■, k: *f\_Enterobacteriaceae*; ■, l: *o\_Coriobacteriales*; ■, m: *o\_Coriobacteriaceae*; ■, n: *g\_Adlercreutzia*; ■, o: *o\_norank\_c\_Cyanobacteria*; ■, p: *f\_norank\_c\_Cyanobacteria*; ■, q: *g\_norank\_c\_Cyanobacteria*) and bacterial taxa with significantly different abundances (LDA scores > 2) (right panel; ■, Sham; ■, WAS). Labels beginning with c\_ indicate class; o\_, order; f\_, family; g\_, genus. WAS, water avoidance stress. LDA, linear discriminant analysis; LefSe, LDA effect size. (b) Phylogenetic investigation of communities using reconstruction of unobserved states functional prediction shows significantly decreased butanoate metabolism pathways in the WAS group. \**P* < 0.05, compared with sham controls. (c) Heatmap showing correlations between altered microbial OTUs and disease phenotype parameters. Color intensity indicates the degree of correlation. Red, positive correlation; blue, negative correlation. *n* = 7 rats per group. \**P* < 0.05, \*\**P* < 0.01. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

**Table 1** Significantly different OTUs between WAS and sham control group (% , means  $\pm$  SEM)

OTU	Family	Genus	WAS	Sham	P-value
OTU419	f__Acidaminococcaceae	g__Phascolarctobacterium	0.010 $\pm$ 0.003	0.001 $\pm$ 0.001	0.014
OTU197	f__Bacteroidales_S24-7_group	g__norank_f__Bacteroidales_S24-7_group	0.133 $\pm$ 0.025	0.382 $\pm$ 0.096	0.041
OTU224	f__Christensenellaceae	g__Uncultured Christensenellaceae	0.001 $\pm$ 0.001	0.008 $\pm$ 0.002	0.029
OTU455	f__Coriobacteriaceae	g__Adlercreutzia	0.069 $\pm$ 0.030	0.016 $\pm$ 0.002	0.041
OTU394	f__Coriobacteriaceae	g__Enterorhabdus	0.008 $\pm$ 0.003	0.001 $\pm$ 0.001	0.041
OTU281	f__Lachnospiraceae	g__Lachnospiraceae_NK4A136_group	0.340 $\pm$ 0.122	1.629 $\pm$ 0.534	0.021
OTU68	f__Lachnospiraceae	g__unclassified_f__Lachnospiraceae	0.000 $\pm$ 0.000	0.254 $\pm$ 0.199	0.031
OTU171	f__Lachnospiraceae	g__uncultured_f__Lachnospiraceae	0.029 $\pm$ 0.013	0.094 $\pm$ 0.021	0.030
OTU230	f__Lachnospiraceae	g__unclassified_f__Lachnospiraceae	0.004 $\pm$ 0.002	0.032 $\pm$ 0.014	0.026
OTU205	f__Lachnospiraceae	g__Roseburia	0.001 $\pm$ 0.001	0.029 $\pm$ 0.012	0.004
OTU6	f__Lachnospiraceae	g__Lachnospiraceae_NK4A136_group	0.003 $\pm$ 0.003	0.028 $\pm$ 0.008	0.014
OTU146	f__Lachnospiraceae	g__unclassified_f__Lachnospiraceae	0.000 $\pm$ 0.000	0.026 $\pm$ 0.016	0.031
OTU27	f__Lachnospiraceae	g__unclassified_f__Lachnospiraceae	0.005 $\pm$ 0.003	0.024 $\pm$ 0.008	0.050
OTU309	f__Lachnospiraceae	g__unclassified_f__Lachnospiraceae	0.000 $\pm$ 0.000	0.005 $\pm$ 0.002	0.031
OTU296	f__Lactobacillaceae	g__Lactobacillus	0.000 $\pm$ 0.000	0.121 $\pm$ 0.075	0.031
OTU49	f__Lactobacillaceae	g__Lactobacillus	0.000 $\pm$ 0.000	0.007 $\pm$ 0.003	0.004
OTU87	f__norank_c__Cyanobacteria	g__norank_c__Cyanobacteria	0.001 $\pm$ 0.001	0.038 $\pm$ 0.023	0.010
OTU473	f__Peptococcaceae	g__uncultured_f__Peptococcaceae	0.041 $\pm$ 0.012	0.229 $\pm$ 0.075	0.025
OTU228	f__Prevotellaceae	g__Prevotella_1	9.906 $\pm$ 2.323	2.898 $\pm$ 0.742	0.041
OTU20	f__Ruminococcaceae	g__Ruminococcaceae_UCG-014	0.001 $\pm$ 0.001	0.020 $\pm$ 0.007	0.034
OTU35	f__Ruminococcaceae	g__Oscillibacter	0.005 $\pm$ 0.004	0.017 $\pm$ 0.005	0.019
OTU260	f__Ruminococcaceae	g__unclassified_f__Ruminococcaceae	0.001 $\pm$ 0.001	0.006 $\pm$ 0.002	0.048
OTU430	f__Ruminococcaceae	g__Candidatus_Soleaferrea	0.010 $\pm$ 0.003	0.004 $\pm$ 0.002	0.041
OTU530	f__Ruminococcaceae	g__Ruminiclostridium_5	0.004 $\pm$ 0.002	0.000 $\pm$ 0.000	0.031
OTU259	f__Streptococcaceae	g__Streptococcus	0.000 $\pm$ 0.000	0.013 $\pm$ 0.006	0.031
OTU311	f__Veillonellaceae	g__Megaspheara	0.000 $\pm$ 0.000	0.247 $\pm$ 0.115	0.004
OTU235	f__Veillonellaceae	g__Dialister	0.000 $\pm$ 0.000	0.021 $\pm$ 0.010	0.011

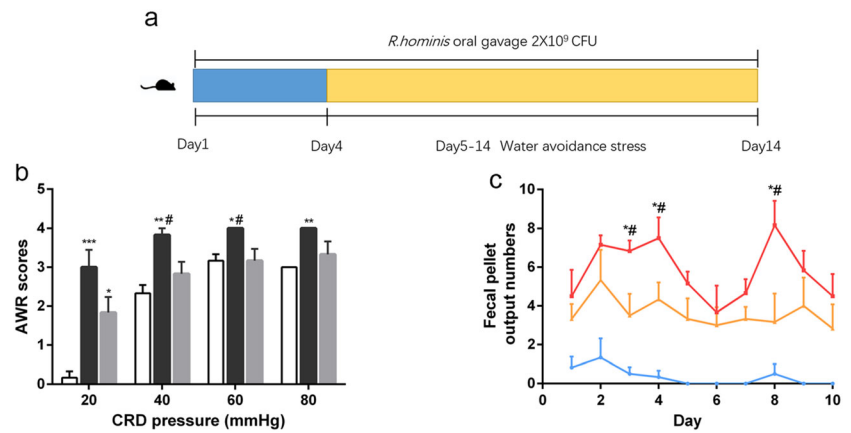
OTUs, operational taxonomic units; SEM, standard error of the mean; WAS, water avoidance stress.

**Effect of *Roseburia hominis* on short-chain fatty acids in cecal contents.** As *R. hominis* is a typical butyrate-producer, we measured SCFA concentrations in cecal contents. The results indicated that *R. hominis* administration significantly increased the cecal concentration of butyrate ( $P < 0.01$ ) (Fig. 6c). In addition, propionate concentration tended to increase ( $P = 0.053$ ) (Fig. 6b), while other SCFAs did not vary upon *R. hominis* treatment (Fig. 6a,d and Table S1).

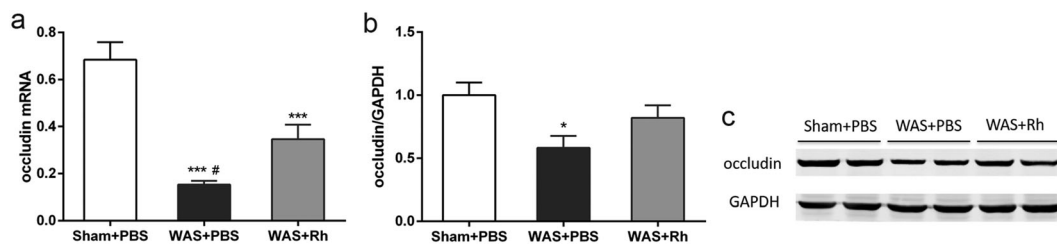
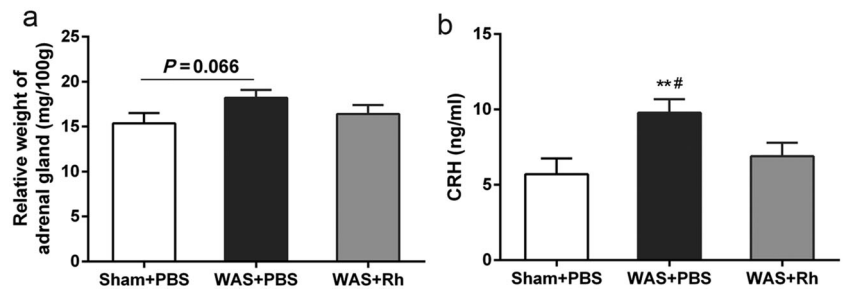
## Discussion

We found that psychological stress increased visceral hypersensitivity and decreased the expression of colonic tight junction protein occludin, accompanied by alterations to the composition and function of the gut microbiota in rats. In addition, by verifying the beneficial effect of butyrate-producing *R. hominis* on WAS rats, our results suggest that specific bacterial taxa or their

**Figure 3** Effect of *Roseburia hominis* oral gavage on visceral sensitivity. (a) Schematic diagram of the experiment. (b) Abdominal withdrawal reflex scores in response to CRD; □, Sham + PBS; ■, WAS + PBS; ▒, WAS + Rh. (c) Fecal pellet output numbers found in the containers after each 1-h WAS; —, Sham + PBS; —, WAS + PBS; —, WAS + Rh. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared with sham controls; # $P < 0.05$ , compared with WAS + Rh group.  $n = 6$  rats per group. PBS, phosphate-buffered saline. Rh, *R. hominis*; WAS, water avoidance stress; CRD, colorectal distension. [Color figure can be viewed at wileyonlinelibrary.com]



**Figure 4** Effect of *Roseburia hominis* on HPA axis activity. (a) Relative weight of adrenal gland of rats. (b) Serum corticotropin-releasing hormone level. \*\* $P < 0.01$ , compared with sham controls; # $P < 0.05$ , compared with WAS + Rh group.  $n = 6$  rats per group. HPA, hypothalamic–pituitary–adrenal; PBS, phosphate-buffered saline; Rh, *R. hominis*; WAS, water avoidance stress.



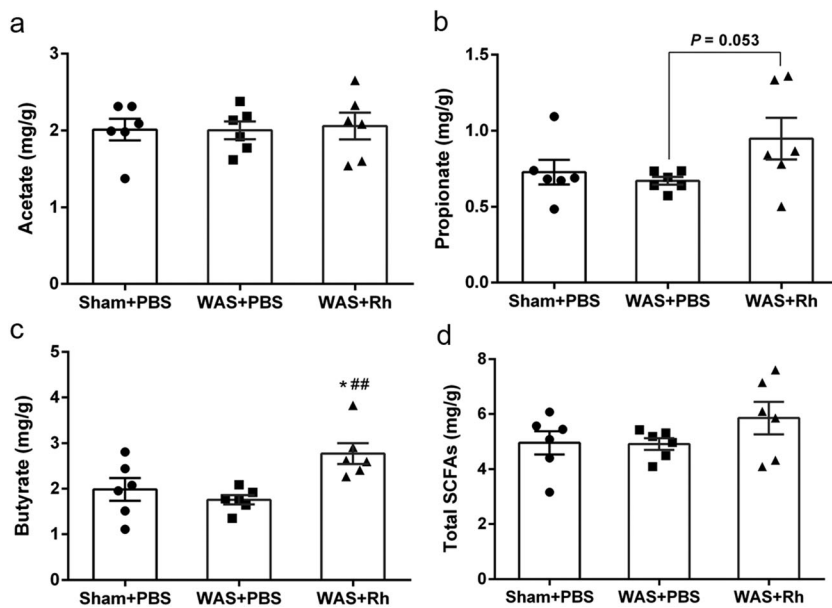
**Figure 5** Effect of *Roseburia hominis* oral gavage on intestinal barrier. (a) Real-time quantitative RT–PCR of colonic occludin mRNA normalized to expression of  $\beta$ -actin. (b) Quantitative analysis of colonic occludin protein normalized to expression of GAPDH. (c) Representative image of occludin bands in western blotting experiments. \* $P < 0.05$ , \*\*\* $P < 0.001$ , compared with sham controls; # $P < 0.05$ , compared with WAS + Rh group.  $n = 6$  rats per group. PBS, phosphate buffered saline; GAPDH, glyceraldehyde phosphate dehydrogenase; Rh, *R. hominis*; WAS, water avoidance stress.

metabolites are associated with visceral sensitivity and colonic barrier function.

Exposure to social stressors can affect the gut microbiota, manifesting as a reduction in microbial diversity and richness.<sup>7,8</sup>

As a model to mimic IBS symptoms, WAS can also lead to gut dysbiosis. At the family level, the relative abundances of Prevotellaceae and Coriobacteriaceae increased, whereas that of Peptococcaceae decreased significantly after stress treatment. Prevotellaceae is reportedly involved in several intestinal diseases. For example, Prevotellaceae is more abundant in IBS patients than in controls.<sup>19</sup> In a mouse model of chemical-induced colitis, *NLRP6*-deficient mice exhibited altered fecal microbiota, characterized by increased Prevotellaceae, which

was a key mediator of phenotype transferring from *NLRP6*-deficient mice to wild-type mice.<sup>20</sup> Similarly, the abundance of *Prevotella*, a major genus belonging to the Prevotellaceae, was also elevated in WAS rats. A previous clinical study<sup>1</sup> classified all enrolled subjects into three enterotypes, and IBS patients were assigned to *Prevotella*-dominant (25%) and *Bacteroides*-dominant (60%) types. These results support the potentially important role of Prevotellaceae and *Prevotella* in IBS morbidity. Additionally, Coriobacteriaceae is among a group of bacterial taxa found to be related to colorectal cancer,<sup>21</sup> ulcerative colitis, and Crohn's disease.<sup>22</sup> In another mouse model of stress, Coriobacteriaceae levels increased in Balb/c mice after exposure to a grid floor stimulation.<sup>23</sup> These results indicate the



**Figure 6** Effect of *Roseburia hominis* oral gavage on acetate (a), propionate (b), butyrate (c), and total SCFA concentration in cecal contents. \* $P < 0.05$ , compared with sham controls. ## $P < 0.01$ , compared with WAS group.  $n = 6$  rats per group. PBS, phosphate buffered saline; Rh, *R. hominis*; WAS, water avoidance stress; SCFAs, short-chain fatty acids.

importance of these bacterial groups to stress-induced visceral hypersensitivity, which warrants further investigations to understand in what way they interact with their hosts.

A majority of the OTUs that were reduced in the WAS group belonged to Lachnospiraceae. Members of the Lachnospiraceae are able to utilize lactate and acetate to produce butyrate via the butyryl-CoA or acetate CoA transferase pathways or the butyrate kinase pathway.<sup>24</sup> In our study, microbial functional prediction analysis showed the reduced representation of the pathways involved in butanoate metabolism in the stressed rats, and the abundances of several Lachnospiraceae-related OTUs were significantly correlated with butanoate metabolism function. Among these was *Roseburia*, one of the core genera in human gut microbiota worldwide.<sup>25</sup> *Roseburia* has been considered to be a potential indicator of intestinal health,<sup>26</sup> and links between *Roseburia* and intestinal diseases, including inflammatory bowel disease,<sup>27,28</sup> IBS,<sup>29</sup> and colon cancer,<sup>30</sup> have been reported. For example, the microbial composition in ulcerative colitis patients differs from that in healthy volunteers by showing a reduction in *R. hominis*.<sup>28</sup> Moreover, *Roseburia inulinivorans* is significantly reduced in Crohn's disease patients compared with levels in healthy individuals.<sup>31</sup> Similarly, Chassard *et al.*<sup>29</sup> reported that *Roseburia* was significantly reduced in constipation-predominant IBS patients compared with levels in healthy subjects. In our study, gavage with the chosen species, *R. hominis*, prevented the stressed rats from developing visceral hypersensitivity. This further demonstrated the possibly important role of *Roseburia* in IBS pathogenesis.

Butyrate is the preferred metabolic substrate for colonocytes and provides about 60–70% of the energy necessary for their proliferation, apoptosis, and differentiation.<sup>11</sup> Several studies have indicated the association between reduced butyrate levels and gastrointestinal diseases. Specially, patients with IBS had significantly lower serum level of butyrate, which reflects the level of butyrate in cecum and the proximal colon, following lactulose ingestion than healthy controls.<sup>32</sup> Increasing evidence demonstrate

the beneficial effect of supplementation with butyrate on IBS symptoms. One study showed that administration of microencapsulated sodium butyrate significantly reduced the frequency of abdominal pain in IBS patients.<sup>33</sup> Similarly, healthy volunteers who were given intrarectal enemas containing a physiologically relevant dose of butyrate exhibited reductions in pain perception and discomfort.<sup>34</sup> Consistent with these, our data indicated alleviation of visceral hypersensitivity and increased cecal butyrate concentration after *Roseburia* administration. It is reported that butyrate might cause a decrease in visceral pain via modulation of 5-hydroxytryptamine, leading to an increased colonic compliance. Transient receptor potential vanilloid 1 in the colonic mucosa can be activated by butyrate, which may in turn lead to 5-hydroxytryptamine release in the colonic lumens. Another mechanism by which butyrate might alleviate visceral hypersensitivity is via inhibition of histone deacetylase, thus induces microglial apoptosis and reduces inflammation-induced neurotoxicity in rat tissue.<sup>34</sup> However, the studies in this area have generated conflicting results on butyrate. For example, one previous study reported that IBS-D patients had higher n-butyrate concentration,<sup>35</sup> and other studies found that rectal instillation of butyrate to Sprague–Dawley rats resulted in a sustained decrease in their pain threshold.<sup>36,37</sup> A number of reasons have been proposed for these conflicts, including reduced absorption of butyrate due to increased motility and shorter transit times,<sup>38</sup> the amount of butyrate administered, the source of butyrate, the timing of butyrate administration, and the differences between the *in vivo* and *in vitro* environments.<sup>12</sup>

An impaired intestinal barrier may allow for pathogen invasion, and luminal commensal bacteria can also affect the barrier in various ways. Our data revealed a positive correlation between four Lachnospiraceae-related OTUs and occludin expression, and supplementation with *R. hominis* prevented the decreases in occludin mRNA and protein expression, accompanied by an increase in butyrate production in the intestinal contents. This is consistent with the finding that *R. hominis* colonization of germ-free mice can affect

intestinal barrier function genes.<sup>39</sup> These results suggest the potential role of specific butyrate-producing bacteria in preventing gut leakage through their metabolites. Moreover, it is reported that butyrate could induce MUC2 mRNA expression and MUC2 secretion in goblet-like cell,<sup>40</sup> as well as promote the assembly of occludin dependent on AMPK pathway.<sup>41</sup>

Based on these results, we speculate that stress-induced gut dysbiosis, especially a decrease in the abundance of butyrate-producing bacteria such as Lachnospiraceae, may contribute to the pathogenesis of stress-induced visceral hypersensitivity. Moreover, supplementation with butyrate-producing bacteria may be beneficial for alleviating the visceral hypersensitivity. Thus, *R. hominis* as a potential probiotic in treating stress-induced visceral hypersensitivity warrants further confirmation in the future.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Cecal short chain fatty acids difference between sham, WAS and WAS + *R. hominis* groups (means ± SEM).