TITLE PAGE

Title: Psychological stress exacerbates NSAID-induced small bowel injury by inducing changes in intestinal microbiota and permeability via glucocorticoid receptor signaling.

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Abstract

[Background]: Nonsteroidal anti-inflammatory drugs (NSAIDs) are popular painkillers, however, there are serious side effects not only in the upper gastrointestinal tract, but also in the small intestine. It is well known that psychological stress may exacerbate various gastrointestinal diseases. The aim of this study was to determine whether psychological stress exacerbate NSAID enteropathy and to determine the possible underlying mechanisms. [Methods] Experiment1: Mice were exposed to water avoidance stress (WAS) or sham stress for 1h per day for 8 consecutive days, then enteropathy was induced by indomethacin (IND). Experiment2: The mice were transplanted with cecal contents from stress(-) or (+) mice after the administration of antibiotics and induction of NSAID enteropthy without WAS. Experiment3: Mifepristone, a glucocorticoid receptor antagonist, was injected before WAS for 8 days. Small intestinal injury, mRNA expression of TNFα, intestinal permeability, and microbial community were assessed. [Results] Psychological stress exacerbated NSAID

enteropathy and increased the intestinal permeability. Psychological stress induced the ileal microbial changes, which were characterized by increase in the total bacterial number and the proportion of gram-negative bacteria. The increased susceptibility to NSAIDs and intestinal permeability by WAS was transferable via cecal microbiota transplantation. Increased permeability and aggravation of NSAID enteropathy caused by WAS were blocked by the administration of mifepristone. [Conclusions]: This study found the relevance of NSAID enteropathy and psychological stress, and showed the possibility of using microbial approach to elucidate the pathophysiology of NSAID enteropathy. It also found the impact of stress on the microbiota and mucosal barrier in gastrointestinal diseases.

KEY WORDS

NSAID enteropathy, HPA-axis, fecal microbiota transplantation, small intestinal permeability, water avoidance stress

ABREVIATIONS

NSAIDs	Nonsteroidal anti-inflammatory drugs
HPA-axis	Hypothalamic-pituitary-adrenal axis
MGB-axis	Microbiota-gut-brain axis
WAS	Water avoidance stress
DMSO	Dimethyl sulfoxide
RT-PCR	Reverse transcription polymerase chain reaction
FITC-dextran	Fluorescein isothiocyanate dextran
T-RFLP	Terminal Restriction Fragment Length Polymorphism
PBS	Pphosphate buffered saline
FMT	Fecal microbiota transplantation
TLR	Toll-like receptor
GPB	Gram-positive bacteria
GNB	Gram-negative bacteria
NLRP6	NOD-like receptor family pyrin domain containing 6
CRH	Corticotropin-releasing hormone

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most popular painkillers in the world. NSAIDs are prescribed to a wide of variety patients, regardless of whether the pain is acute or chronic, the pain region, and the type of disease. NSAIDs are easy to use for both patients and medical doctors, making the amount of NSAID prescription to increase in recent years [1]. The number of patients using NSAIDs, whether for single or regular use, will increase more in the aging society. However, NSAID use is recently reported to have serious complications in not only the upper gastrointestinal tract, but also the small intestine and colon, especially among elderly patients and patients on long-term NSAID use [2]. The clinical manifestations of NSAID-induced small intestinal injury (NSAID enteropathy) are iron-deficiency anemia, hypoalbuminemia, and malabsorption, based on bleeding ulcers. Autopsy and recent advances in endoscopy, which includes video capsule endoscopy and double balloon enteroscopy (DBE), revealed that subclinical and unrecognized NSAID enteropathy

were more frequent than conventionally supposed [3, 4]. Subclinical mucosal breaks were reported in 50% of NSAID-users in the study using DBE [3], while the patients who presented clinical manifestations were limited to a similar part of NSAID-users. We hypothesized that certain factors increased the activity of small intestinal injury via changing in intestinal permeability and intestinal microbiota, which are the established pathophysiologies of NSAID enteropathy [5, 6], then leading to the uncovering of clinical manifestations.

It has been established that there is close contact between the gastrointestinal tract and the brain to maintain vital homeostasis [7]. It is suggested that the communication routes between the gastrointestinal tract and the brain are the hypothalamic-pituitary-adrenal axis (HPA-axis) and the autonomic nervous system. Psychological stress was reported to change the phenotypes of various gastrointestinal diseases, which includes inflammatory bowel disease and irritable bowel syndrome. Recently, the intestinal microbiota has been recognized as an important modulator of the mucosal immune

system – a concept known as the microbiota-gut-brain axis (MGB-axis) [8]. However, the effect of psychological stress on NSAID enteropathy is unknown. The aim of this study were to determine whether psychological stress affects the intestinal lesions induced by NSAIDs and to elucidate the role of intestinal microbiota on psychological stress-induced modification of activity in NSAID enteropathy using murine experimental model. Three experiments were conducted in this study: the observation of stress effect on NSAID enteropathy, the transferring susceptibility of psychological effect on NSAID enteropathy by microbial transplantation, and the observation of the effect of glucocorticoid receptor antagonist on NSAID enteropathy and small intestinal permeability.

METHODS

Ethics and Animals

Animal Research Committee of National Defense Medical College (NDMC) in Japan (No.14085) approved the experimental protocol. Male C57BL/6J mice (Clea, Tokyo,

Japan) were housed in plastic cages and maintained under a 12-hour light and dark cycle with standard laboratory chow (SLC, Tokyo, Japan). The care and use of laboratory animals were in accordance with the guidelines of the animal facility in NDMC.

Psychological stress

The mice were divided into 4 groups at random: stress(-)IND(-), stress(-)IND(+), stress(+)IND(-), and stress(+)IND(+). The mice of stress(+) group were exposed to water avoidance stress (WAS) treatment as previously reported [9]. The mouse was placed on a circular platform (4cm diameter) located at the center of a standard plastic cage (410 × 250 × 200 mm), which was filled with tap water (20℃) to 1 cm below the surface of the platform. The mice of the stress group were subjected to WAS for 1 h/day, which was repeated for 8 consecutive days, while the sham stress groups were placed in a similar cage without water for the same period.

Induction of small intestinal injury

Indomethacin (IND, Wako Pure Chemical Industries, Ltd, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO, 20 ng/mL) to 1 mg/mL concentration [10]. To induce small intestinal injury in mice of IND(+) group, 10 mg/kg IND was administered subcutaneously at a single time [11] 24 h after the last stress loading. The mice of the sham injection group were administered the same concentration of DMSO solution. The mice were euthanized 24 hours after IND administration.

Evaluation of intestinal injury and histology

Evans blue (1%; Wako Pure Chemical Industries, Ltd) was injected to the mice intravenously 30 minutes before euthanasia. The small intestine was removed after euthanasia and then the lumen was opened along the anti-mesenteric attachment. The blue-stained depression areas were measured and summed per small intestine using a grid sheet [12]. Ileum tissue samples, which were acquired 5cm from the terminal ileum toward the oral side, were fixed in 10% buffered formalin.

The tissues were embedded in paraffin and stained with hematoxylin and eosin stain. Histological damage score was determined as described in a previous study [13]. The degree of the tissue damage was graded from 0 to 5 according to the following criteria: 0 for normal structure; 1 if there is a development of subepithelial space at the apex of the villus; 2 if there is an extension of the subepithelial space with moderate lifting of the epithelial layer; 3 if there are massive epithelial spaces with a few shortened villi and the presence of cells in the lumen; 4 if there are denuded villi with lamina propria and dilated capillaries with cells in the lumen; and, 5 if there is a collapse of the lamina propria with bleeding and ulcer.

Quantitative RT-PCR for intestinal mRNA expression

The degree of mRNA expression of TNFα in the intestinal mucosa was measured as reported in a previous study [14]. The samples were collected from the distal ileum, avoiding the location of deep ulcer to eliminate bias [12]. The total mRNA was extracted using the RNeasy Mini Isolation Kit (Qiagen,

Valencia, CA, USA). TaqMan reverse transcription polymerase chain reaction (RT-PCR) was performed in triplicate for each sample using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The primer and probes used in this study were purchased from Applied Biosystems: TNFα (Mm00443258). GAPDH was used as the housekeeping gene.

Measurement of intestinal permeability

Intestinal permeability was measured in mice that were exposed to 8 days of WAS and sham stress as reported previously [15]. 4000 Daltons of fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich, St. Louis, MO) was dissolved in distilled water to 15mM under shading condition. The mice were given 600 mg/kg body weight of FITC-dextran via oral route 6 hours after the fasting condition. One hour after administration, the blood was collected from the postcaval veins of mice. The plasma was obtained from the supernatant of the blood that was centrifuged at 4℃, 12000 g, for 3 min. The

FITC-dextran concentration of the plasma was analyzed with a fluorescence spectrophotometer (Gemini EM Microplate Reader, Molecular Devices, CA, USA) at an excitation wavelength of 485nm and an emission wavelength of 535 nm.

Microbial DNA extraction from intestinal feces and PCR amplification

The bacterial DNA content of distal ileum was extracted using the bead-phenol method as described in a previous study [16]. Briefly, the bacterial bodies from the samples were crushed using glass beads in the extraction buffer. The intensive crush was then held to separate the precipitated proteins and cell walls in the buffer containing phenol. After isopropanol precipitation, the DNA was extracted, washed using 70% ethanol, extracted into the TE buffer [(10 mM Tris-HCl, 1 mM EDTA (pH8.0)].

PCR of 16SrRNA gene and T-RFLP analysis of microbial communities

Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis was performed using the protocols described by Hayashi et al. [17] and Sakamoto et al. [18]. The primers used for the PCR amplification of 16SrRNA gene sequences were 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), labeled at the 5' end with 6-FAM (6-carboxyfluorescein), and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The amplification was performed using Takara Ex Taq HS Hot Start Version (Takara Bio, Sigma, Japan) programed at 95℃ for 3 min, 30 cycles of 95℃ for 30 sec, 50℃ for 30sec, and 72℃ for 90 sec, followed by 72℃ for 10 min. After amplification, the PCR product was digested with restriction enzymes, Hha I and Msp I . The length of fluorescent labelling terminal restriction fragment (T-RF) from the DNA end was analyzed using DNA sequencer (ABI PRISM 310 Genetic analyzer, Applied Biosystems).

RT-PCR analysis of microbial communities

Absolute quantification was conducted for phylum levels using representative bacteria for standard DNA in each phylum as

follows: *Firmicutes* for *Blautia coccoides*, *Bacteroidetes* for *Bacteroides fragilis*, *Bifidobacterium* for *Bifidobacterium breve*, *Atopobium cluster* for *Collinsella aerofacens*, *γ-Proteobacteria* for *Escherichia coli*, and all bacteria for *Blautia coccoides*. The DNA was extracted from the bacterial body by measuring the number under the microscope for proper dilution. The intercalation assay was performed using SYBR Green I as the fluorescent dye, Thermal Cycler Dice TP800 (Takara) as the detection system, and SYBR Premix Ex Taq Ⅱ Tli RNaseH Plus (Takara) as the detection reagent. The primer sequences and PCR conditions were described in Table1 [16, 19, 20]. *Actinobacteria* was calculated by summing up *Bifidobacterium* and *Atopobium cluster*.

Effect of microbiota transplantation on small intestinal injury

The administration of antibiotics and microbial contents were conducted, following the protocol described in previous studies [21,22]. The antibiotic cocktail (1g/L ampicillin, 500mg/L vancomycin, 1g/L neomycin sulfate, 1g/L metronidazole) mixed

in drinking water was administered orally to the mice ad libitum for 28 days. This combination was shown to deplete all detectable intestinal microbiota [23]. The cecal microbial contents of donor mice (stress mice and sham stress mice) were harvested and rapidly stirred in phosphate buffered saline (PBS, 1.5mL/0.2mL of cecal volume). Extraction liquid (0.2 mL) of the cecal contents was administered to mice of the corresponding group orally via gastric gavage tube once a day for 5 successive days. Twenty-four hours after the last cecal content administration, 10 mg/kg IND was administered subcutaneously at a single time. After which, intestinal injury was compared between the mice that received cecal contents from sham stress mice and those that received cecal contents from stress mice. In some mice, intestinal permeability and microbial community were investigated.

Effect of glucocorticoid receptor antagonist (mifepristone) on NSAID enteropahty of WAS treated mice

The effect of mifepristone (RU-486), a glucocorticoid receptor

antagonist, on stress-induced changes in small intestinal permeability and microbial community was studied. The mice were divided into 2 groups at random: RU-486(-) and RU-486(+). 0.2 mL mifepristone liquid, containing 20mg/kg mifepristone, was prepared, and the same volume of vehicle was also prepared for sham injection [24]. Mifepristone or vehicle was injected subcutaneously 30 minutes before each stress session. After 8 consecutive days, 10 mg/kg IND was administered subcutaneously at a single time. Then, IND-induced intestinal injury was assessed and compared between RU-486(-) stress(+) group and RU-486(+) stress(+) group. In some mice, intestinal permeability and microbial community were investigated.

Statistics

All results were expressed as mean ± standard deviation (SD). Statistical analyses were performed using Mann-Whitney U test or Kruskal-Wallis test via JMP 11 software. The differences in P values less than 0.05 were considered as statistically significant.

RESULTS

Psychological stress exacerbated NSAID-induced enteropathy

The intestinal ulcer area and histopathological damage scores are shown in Figure1A and 1B. The stress(+) IND(-) mice group showed a slight but significant increase in intestinal damage score compared to stress(-) IND(-) mice. The stress(-) IND(+) mice showed, the ulcer area was 21.4 ± 3.88 mm^2 and the damage score was 2.5 ± 0.5. The stress(+) IND(+) mice showed significantly exacerbated intestinal injury, the ulcer area was 25.9 ± 3.42 mm^2 and the damage score was 3.1 ± 0.5 compared to stress(-) IND(+) mice. The representative pictures of samples in each group are shown in Figure1C: Normal structure in stress(-) IND(-) group; Almost normal structure of mucosal villi with small subepithelial space in spots in stress(+) IND(-) group: Existence of mild subepithelial space with mild inflammatory cells proliferation in stress(-) IND(+) group: Exposure of lamina propria to lumen with high-grade subepithlial space and inflammatory cells in

stress(+) IND(+) group.

Psychological stress increased the degree of TNFα mRNA expression in damaged intestinal tissue induced by IND treatment

The degree of TNFα mRNA expression in the small intestine are shown in Figure 1D. IND treatment significantly increased the expression of TNFα. The addition of psychological stress on IND treatment significantly enhanced the degree of expression of TNFα mRNA. The relative expression were 1.06±0.28 in stress(-) IND(-) group, 1.24±0.31 in stress(+) IND(-) group, 2.55 ± 1.58 in stress(-) IND(+) group, and 5.56 ± 5.36 in stress(+) IND(+) group.

Intestinal permeability was increased by psychological stress

The plasma FITC-dextran concentrations after stress and sham stress treatment are shown in Figure 1E. Stress treatment alone significantly increased in the plasma FITC-dextran concentration, indicating that stress treatment

increased the intestinal epithelial permeability. In the IND treated mice, stress treatment further increased the plasma FITC-dextran concentration significantly. Plasma FITC-dextran concentration were 4.98±1.15 μg/mL in the stress(-) IND(-) group, 8.28±3.06 μg/mL in the stress(+) IND(-) group, 13.81±10.18 μg/mL in the stress(-) IND(+) group, and 30.60±22.09 μg/mL in the stress(+) IND(+) group.

Stress-induced alteration of gut microbiota analyzed by T-RFLP method

The results of T-RFLP analysis of the distal ileum contents after a series of stress treatment are shown in Figure 2A. After digestion with Hha I , T-RFs at bp 200-206, 208-212, 223-224, 245-248, and 396-399 were detected in the stress(-) samples. The microbial community shifted and detected bp 88-89, 232-234, 245-248, and especially 359-361 in the stress(+) group. After digestion with Msp I , the microbial community also shifted. T-RFs at bp 113-120, 175-177, and 181-189 were detected in stress(-) samples, while bp 138-149, 181-189, and

554-559 were detected in stress(+) samples. Bp 113-120 was increased in stress(+) samples compared to stress(-) samples. The respective results of the three samples in Figure5 and all the samples within same group showed similar pattern in microbial community assessed via T-RFLP method.

Altered proportion and number of microbial count induced by WAS in phylum level analysis

The number of intestinal microbial community was quantified in the phylum level by quantitative RT-PCR. The results of the effect of stress were shown in Figure 2B. The number of bacteria of all four phyla is increased in the stress(+) mice. The ratio comparing the bacterial count of the stress(+) group to that of the stress(-) group were increased 11 times for *Firmicutes*, 146 times for *Bacteroidetes*, 91 times for *Actinobacteria*, and 4 times for *γ-Proteobacteria*. The number of *Bacteroidetes* and *Actinobacteria* phylum were notably increased. Figure 2B shows the ratio of the 4 phyla. In stress(-) mice, the proportion of microbiota consisted mainly of

Firmicutes. In stress(+) mice, the ratio of *Bacteroidetes* increased, while the ratio of *Firmicutes* decreased.

Psychological stress-induced exacerbation of NSAIDs enteropathy is transferable via gut microbiota transplantation

We observed that stress induced changes in the gut microbiota. In order to clarify that the stress-induced exacerbation of NSAID enteropathy was caused by changes in the gut microbiota, we investigated whether stress-induced exacerbation was transferrable by fecal microbiota transplantation (FMT). The comparison of IND-induced intestinal injury after FMT between stress and sham stress mice is shown in Figure 3A and 3B. The mice treated with cecal contents from stress(+) mice were more susceptible to NSAID enteropathy than those treated with cecal contents from stress(-) based on macroscopic and histological assessment. The ulcer area after FMT of the stress(+) mice group were significantly larger than that of the stress(-) mice group, 10.1 $\pm$1.50 mm^2 vs. 6.5 $\pm$ 0.53 mm^2, respectively. The intestinal

damage score after FMT of the stress(+) mice group were significantly more severe than that of the stress(-) mice group, 2.78±0.46 vs. 1.44±0.73, respectively. The comparison of the degree of expression of TNFα mRNA and the plasma FITC-dextran concentrations after FMT between stress and sham stress mice is shown in Figure 3C and 3D. The mice treated with cecal contents from stress(+) mice group showed a significantly enhanced degree of expression of TNFα mRNA compared to those treated with cecal contents from stress(-) group, 2.59 ± 1.86 vs. 7.43 ± 4.01 in relative expression, respectively. In NSAID-treated mice, the stress(+) group also showed a significantly enhanced degree of TNFα expression. The mice also administered with cecal contents from stress(+) mice showed higher plasma FITC-dextran condition compared to those administered with cecal contents from stress(-) mice, 15.32±1.02 μg/mL vs. 37.67±18.00 μg/mL, respectively. The success of transfer of intestinal permeability via FMT from stress(+) mice suggests that stress-induced changes in microbiota is responsible for stress-induced increase in

intestinal permeability.

Gut microbiota community analysis of FMT treated mice

The results of quantification of intestinal microbial community in the phylum level of mice administered with cecal contents from stress(-) or stress(+) mice are shown in Figure 4. The number of total bacteria per gram of feces was equivalent to the number of bacteria of mice that did not receive antibiotics or FMT treatment (Figure 2B). In the phylum level, *Actinobacteria* was not detected after qRT-PCR in both groups. The number of *Firmicutes* was nearly the same between the FMT from stress(-) group and FMT from stress(+) group. Other phyla were higher in the FMT from stress(+) groups than the FMT from stress(-) groups - 5.56 times in *Bacteroidetes* and 10.48 times in *γ Proteobacteria*. The proportion of the 4 phyla in the ileal contents of mice that received FMT from stress(-) mice showed 97.5% of *Firmicutes*, 0.2% of *γ Proteobacteria*, 2.3% of *Bacteroidetes*, and 0% of *Actinobacteria*. In contrast, the proportion of the 4 phyla in the ileal contents of mice that

received FMT from stress(+) mice showed 84.5% of *Firmicutes*, 6.0% of *γ Proteobacteria*, 9.5% of *Bacteroidetes*, and 0% of *Actinobacteria*.

Effect of RU-486 (mifepristone) on NSAID enteropahty of WAS treated mice

The comparison of IND-induced intestinal injury and the plasma FITC concentration after RU-486 treatment and WAS treatment are shown in Figure 5. The mice were administered with vehicle or 20 mg/kg RU-486 subcutaneously before they were given sham stress treatment or WAS treatment for 8 consecutive days. The mice of RU-486(-) stress(+) group showed significantly higher IND-induced intestinal injury and plasma FITC-dextran concentration compared to RU-486(-) stress(-) group, 26.1 ± 1.05 mm^2 vs. 20.7 ± 4.27 mm^2 and 13.5 ± 4.30 μg/mL vs. 5.4 ± 2.01 μg/mL, respectively, as shown in Figure 3. The intestinal ulcer area and the plasma FITC-dextran concentration of the mice of RU-486(+) stress(+) group was 22.9 ± 3.06 mm^2 and 6.6 ± 2.51 μg/mL, which is

significantly lower compared to that of RU-486(-) stress(+) group, indicating that the increase in IND-induced intestinal injury and intestinal permeability by WAS treatment was dependent on glucocorticoid signaling.

Effect of RU-486 on changes in gut microbiota by WAS treatment.

The results of the quantification of intestinal microbial community among mice administered with RU-486 and WAS treatments are shown in Figure 6. In mice of the RU-486(-) stress(-) and RU-486(-) stress(+) groups, the number of total bacteria, *Firmicutes*, *Bacteroidetes,* *Actinobacteria*, and *γ Proteobacteria* were equivalent to the former experiment as shown in Figure 2B - that is the number of all 4 phyla tend to be higher in the stress(+) groups. The increase in the number of total bacteria in RU-486(+) stress(+) group as well as in all the investigated phyla in RU-486(-) stress(+) group were cancelled, indicating that the changes in microbiota by WAS treatment was dependent on glucocorticoid signaling.

DISCUSSION

This is the first report that investigate the relevance of psychological stress to NSAID-induced small intestinal injury. This study showed four points. 1) Psychological stress exacerbated the indomethacin-induced enteropathy of mice accompanied with increased permeability. 2) Psychological stress-induced ileal microbial changes characterized by increase in the total bacterial number and increase in the proportion of *Bacteroidetes* phylum and *Actinobacteria* phylum. 3) The increase in the disease activity by NSAIDs and intestinal permeability by psychological stress were transferable via cecal microbiota transplantation. 4) Increased permeability and microbial change caused by psychological stress were blocked by the administration of glucocorticoid receptor antagonist.

Previous reports assessed the intestinal permeability using various stress models, such as WAS, communication box-induced stress, and restraint stress. They assessed the

intestinal permeability in different organs such as duodenum, ileum, and colon. Psychological stress generally increased the gut permeability, regardless of the type of stress or location. The mechanism of increased permeability by stress remains unclear, but the involvement of CRH [9], mast cells [25], the functional change of cannabinoid type 1 receptor [26, 27], and the modification and redistribution of tight junction proteins [28] have already been reported. In this study, we clearly showed that the FMT from mice that received stress has increased the intestinal permeability, suggesting that the change in microbiota is responsible for increased intestinal permeability. There are reports showing that microbiota and probiotics are associated with the intestinal barrier function in *vitro* and in *vivo*, such that the murine colon barrier dysfunction by chronic WAS was improved by probiotic treatment [29]. The possible mechanisms for this include short chain fatty acid, tight junction protein, and pattern recognition receptor. Butyrate is a typical short chain acid, which plays a key role in the intestinal epithelial function produced by

microbiota [30, 31]. Decreased butyrate production was reported to increase the bacterial translocation in the cell model [32]. Tight junction protein is also an important factor for paracellular transfer. The formation of a tight junction complex consisted of occludin, Zo-1, and claudin protein families. There are reports concerning the changes in the expression and redistribution of tight junction proteins by pathogens, endotoxin, antibiotics and probiotics [33-39]. In this study, significant difference in the degree of mRNA expressions of occludin mRNA in the intestinal mucosa was not observed between in stress(-) group and in stress(+) group, while stress treatment tended to decrease in the degree of mRNA expression of Zo-1 ($p=0.07$). Toll-like receptor 2 (TLR2), a representative pattern recognition receptor like TLR4, is stimulated by the recognition of the peptidoglycan derived from Gram-positive bacteria (GPB). The TLR2 pathway activates protein kinase C, which affects the transepithelial electrical resistance [40].

In this study we showed that RU-486 blocked the WAS-induced aggravation of NSAID enteropathy. In addition,

increased intestinal permeability by stress was significantly inhibited by RU-486 injection, which suggests that glucocorticoid stimulation plays an important role in the increase in permeability by stress. This result was consistent with previous studies that showed glucocorticoid-related increase intestinal permeability [24, 41, 42]. In addition, we showed that the changes in microflora by WAS treatment was cancelled by RU-486 treatment, suggesting that stress-induced dysbiosis was dependent on glucocorticoid signaling.

We observed quantitative and qualitative changes in the microbiota. The total bacterial count was increased in the stress(+) group. All of the 4 analyzed phyla also showed an increase in absolute number. However, in terms of the proportion of these phyla, *Firmicutes* (GPB) decreased, while γ *Proteobacteria* and *Bacteroidetes*, which are Gram-negative bacteria (GNB), both increased - that is, the proportion of GNB was increased by psychological stress. Previous studies showed the importance of GNB to the pathophysiology of NSAID enteropathy [43-45]. In fact, the therapy that targeted a

decrease in GNB with ampicillin and aztoreonam or kanamycin improved the NSAID-induced small bowel injury, while the one that targeted a decrease in GPB with vancomycin had no preventive effect [46]. Thus, we suppose that the balance between GPB and GNB is important for gut homeostasis and inclined condition of microbiota rich in GNB by stress might be vulnerable to NSAID iletitis. However, in the current study, we could not exclude the possibility that the stress treatment has induced the increase of specific harmful bacteria or the decrease of a specific beneficial bacteria, which lead to the increase in vulnerability for NSAID ileitis.

In this study, we did not clarify how stress changed the microbiota. Stress-induced microbial shift has been reported in previous studies [15, 47]. WAS-induced microbial shift in rats was mainly analyzed by a sequencer [47]. In mice, stress changed the *Lactobacillaceae* and unclassified *Clostridiales* content of the small intestine, while decreasing the *Lacnospiraceae* and unclassified *Bacteroidetes* content of the small intestinal content [15]. Some mechanisms of microbial

change caused by stress were proposed previously. It was reported that a decrease in IL-18 followed by a decrease in NOD-like receptor family pyrin domain containing 6 (NLRP6) inflammasome have caused the microbial change [15]. Recently, it was reported that the increase in serum corticotropin-releasing hormone (CRH) decreased NLRP-6 expression, leading to the suppression of innate immunity and host defense against pathogens [48]. When taken together, it is possible that stress has changed the microbiota through HPA-NLRP6 axis, although we did not clarify relationship between psychological stress and NLRP6 expression.

This study found out the possibility of using a microbial approach in elucidating the pathophysiology of NSAID enteropathy. The study also found the impact of stress on the microbiota and mucosal barrier in gastrointestinal diseases.

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FIGURE LEGENDS

Table1.

Primer sequences for phylum specific 16SrRNA gene and PCR programs

Figure1.

The results of NSAID enteropathy after psychological stress or sham stress. (A) Macroscopic intestinal ulcer calculated from blue stained depression area after Evans blue injection. (B) Intestinal damage score based on the pathological assessment in hematoxylin and eosin stain. (C) Pathological images of intestinal injury. (D) Inflammatory cytokine TNFα mRNA expression of intestinal tissues. (E) Plasma FITC-dextran concentration. N=10 each group, mean ± SD. ☆ $p<0.05$ vs. stress(-) IND(-) group, § $p<0.05$ vs. stress(+) IND(-) group , ⋆ $p<0.05$ vs. stress(-) IND(+) group.

Figure2.

The results of microbial community analysis of NSAID enteropathy after psychological stress or sham stress. (A) T-RFLP analysis digested by Hha I and Msp I . (B)

Quantification and the proportion of four phyla by using quantitative PCR. N=5 each group.

Figure3.

The results of NSAID enteropathy after fecal microbiota transplantation from stress(+) or stress(-) mice . (A) Macroscopic intestinal ulcer calculated from blue stained depression area after Evans blue injection. (B) Intestinal damage score based on the pathological assessment in hematoxylin and eosin stain. (C) Inflammatory cytokine TNFα mRNA expression of intestinal tissues. (D) Plasma FITC-dextran concentration. N=10 each group, mean ± SD. ★ $p<0.05$ vs. FMT from stress(-) mice group.

Figure4.

The results of microbial community analysis of NSAID enteropathy after fecal microbiota transplantation from stress(+) or stress(-) mice. N=5 each group, mean ± SD.

Figure5.

The results of IND-induced intestinal injury and plasma

FITC-dextran concentration of the mice that administered RU-486 or sham injection after exposure of psychological stress or sham stress. N=11 each group, mean ± SD. ★ $p<0.05$ vs. RU-486(-)stress(-) group and RU-486(+)stress(+) group, § not significant vs. RU-486(-) stress(-) group

Figure 6.

Quantitative PCR for microbial community in phylum levels of the mice that administered RU-486 or sham injection after exposure of psychological stress or sham stress. N=4, each group, mean ± SD.

Table1

Target group	Primer	Sequence(5'→3')	Approximate size(bp) of product	PCR programs	cycle
Firmicutes	Firm934F	GGAGYATGTGGTTTAATTCGAAGCA	126	95℃(15s)→60℃(60s)	40
	Firm1060R	AGCTGACGACAACCATGCAC			
Bacteroidetes	Bact934F	GGARCATGTGGTTTAATTCGATGAT	126	95℃(15s)→60℃(60s)	40
	Bact1060R	AGCTGACGACAACCATGCAG			
Bifidobacterium	g-Bifid-F	CTCCTGGAAACGGGTGG	550	95℃(5s)→55℃(10s)→72℃(60s)	40
	g-Bifid-R	GGTGTTCTTCCCGATATCTACA			
Atopobium cluster	c-Atopo-F	GGGTTGAGAGACCGACC	190	95℃(5s)→55℃(10s)→72℃(60s)	40
	c-Atopo-R	CGGRGCTTCTTCTGCAGG			
γ-Proteobacteria	1080γF	TCGTCAGCTCGTGTYGTGA	122	95℃(15s)→56℃(15s)→72℃(20s)	40
	γ1202R	CGTAAGGGCCATGATG			
All bacteria	Eub338F	ACTCCTACGGGAGGCAGCAG	180	95℃(15s)→60℃(60s)	40
	Eub518R	ATTACCGCGGCTGCTGG			

Figure1

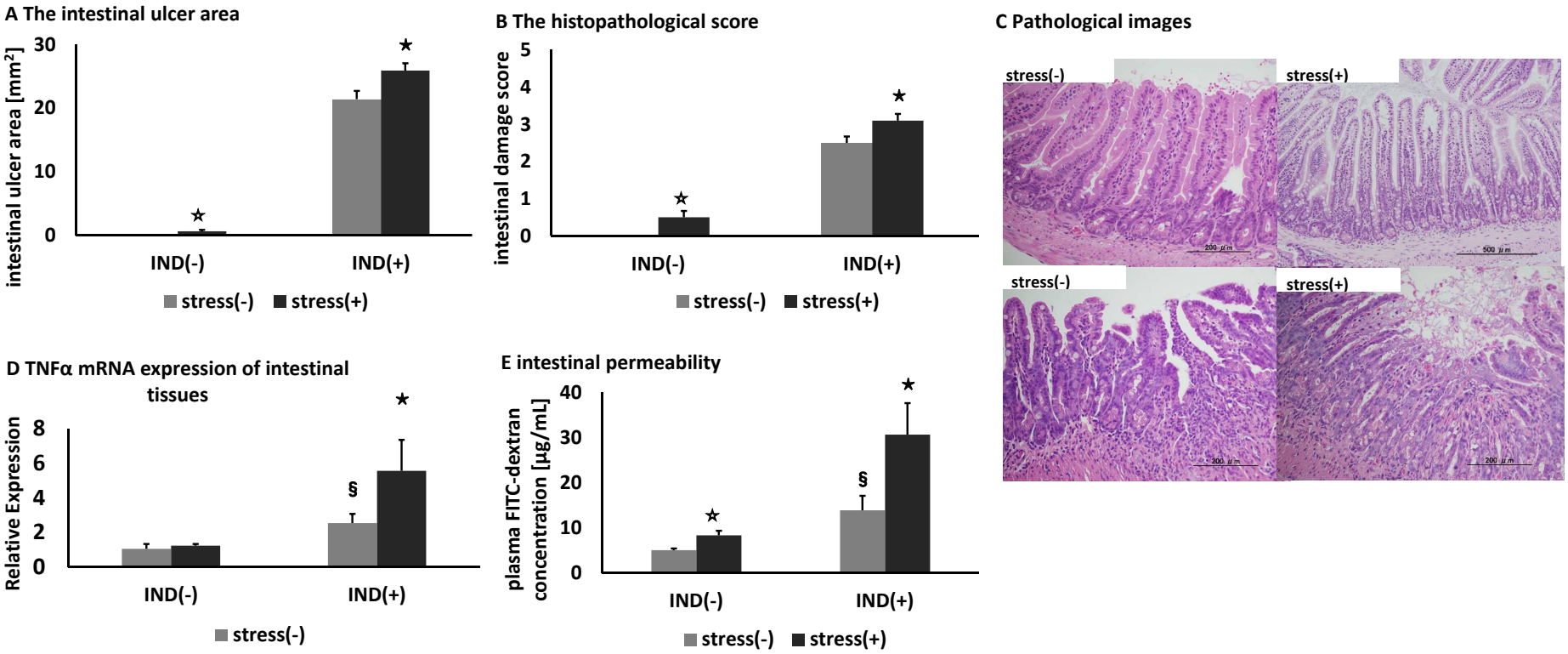

A The intestinal ulcer area
intestinal ulcer area [mm²]
30
20
10
0
IND(-)
IND(+)
stress(-)
stress(+)
B The histopathological score
intestinal damage score
5
4
3
2
1
0
IND(-)
IND(+)
stress(-)
stress(+)
C Pathological images
stress(-)
stress(+)
stress(-)
stress(+)
D TNFα mRNA expression of intestinal tissues
Relative Expression
8
6
4
2
0
§
IND(-)
IND(+)
stress(-)
E intestinal permeability
plasma FITC-dextran concentration [μg/mL]
40
30
20
10
0
§
IND(-)
IND(+)
stress(-)
stress(+)

Figure2

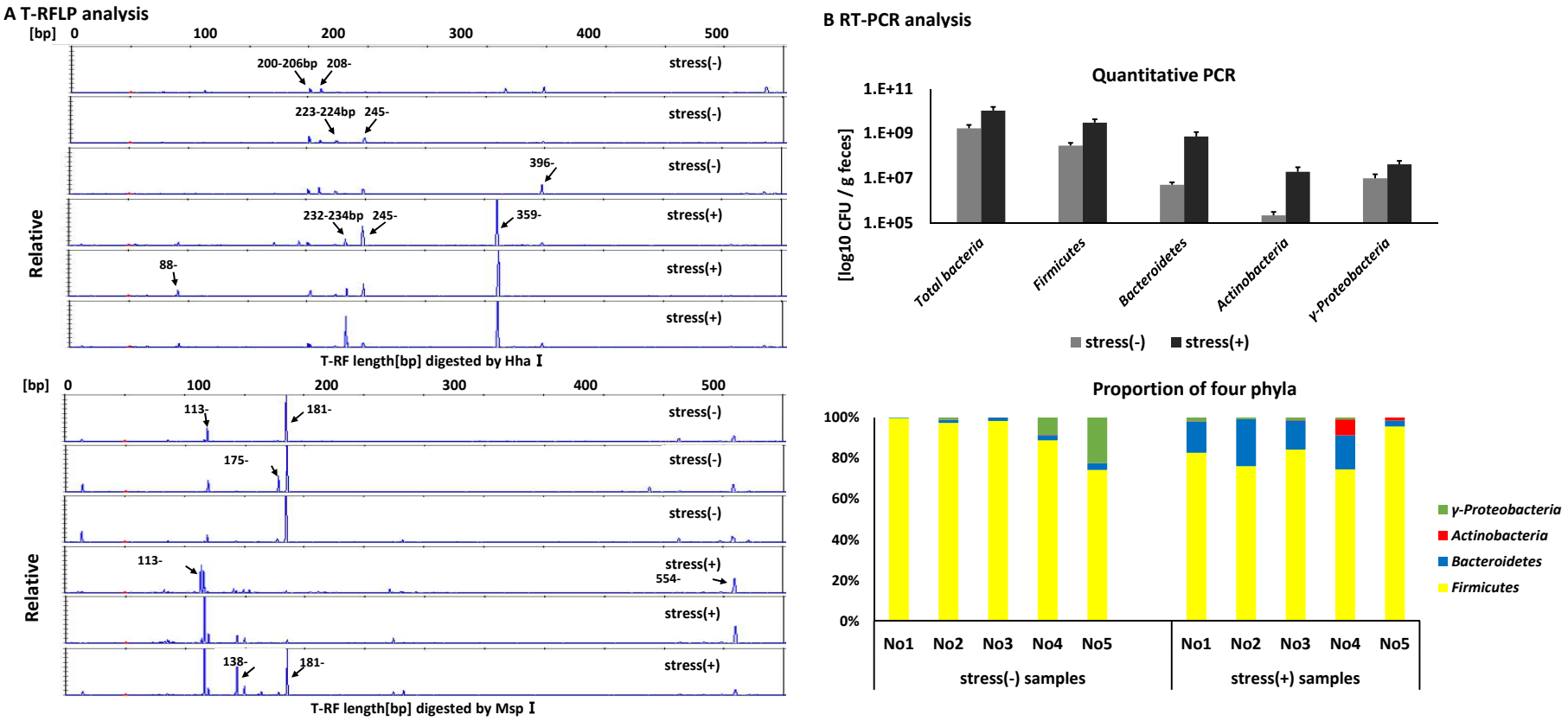

A T-RFLP analysis
[bp] 0 100 200 300 400 500
200-206bp 208-
223-224bp 245-
396-
232-234bp 245-
359-
88-
stress(-)
stress(+)
Relative
T-RF length[bp] digested by Hha I
113-
181-
175-
554-
138-
T-RF length[bp] digested by Msp I
B RT-PCR analysis
Quantitative PCR
[log10 CFU / g feces]
1.E+11
1.E+09
1.E+07
1.E+05
Total bacteria
Firmicutes
Bacteroidetes
Actinobacteria
γ-Proteobacteria
Proportion of four phyla
100%
80%
60%
40%
20%
0%
No1 No2 No3 No4 No5
stress(-) samples
stress(+) samples
γ-Proteobacteria
Actinobacteria
Bacteroidetes
Firmicutes

Figure3

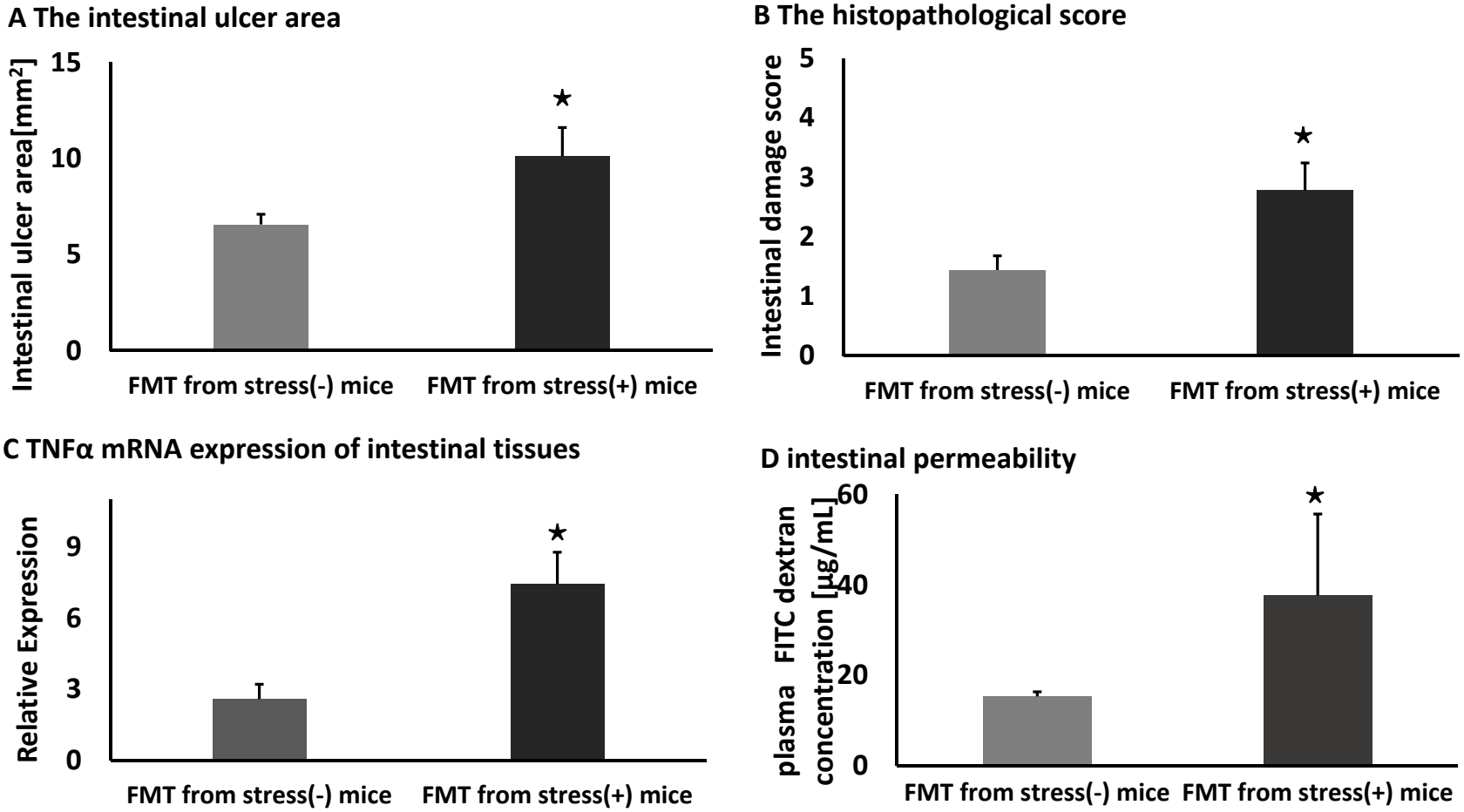

A The intestinal ulcer area
Intestinal ulcer area[mm²]
15
10
5
0
★
FMT from stress(-) mice
FMT from stress(+) mice
B The histopathological score
Intestinal damage score
5
4
3
2
1
0
★
FMT from stress(-) mice
FMT from stress(+) mice
C TNFα mRNA expression of intestinal tissues
Relative Expression
9
6
3
0
★
FMT from stress(-) mice
FMT from stress(+) mice
D intestinal permeability
plasma FITC dextran concentration [μg/mL]
60
40
20
0
★
FMT from stress(-) mice
FMT from stress(+) mice

Figure4

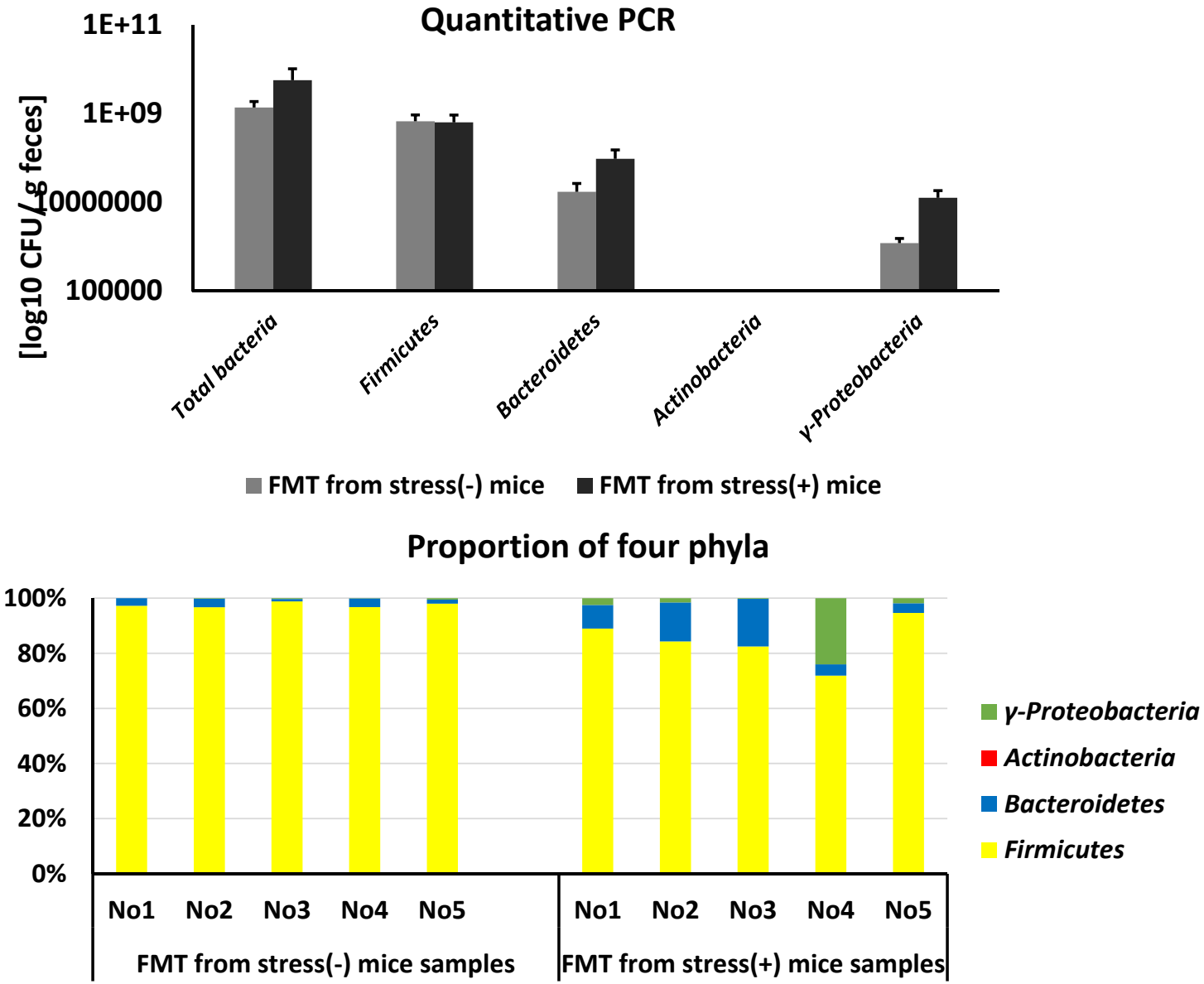

Quantitative PCR
1E+11
1E+09
10000000
100000
[log10 CFU/g feces]
Total bacteria
Firmicutes
Bacteroidetes
Actinobacteria
γ-Proteobacteria
FMT from stress(-) mice
FMT from stress(+) mice
Proportion of four phyla
100%
80%
60%
40%
20%
0%
No1
No2
No3
No4
No5
No1
No2
No3
No4
No5
FMT from stress(-) mice samples
FMT from stress(+) mice samples
γ-Proteobacteria
Actinobacteria
Bacteroidetes
Firmicutes

Figure5

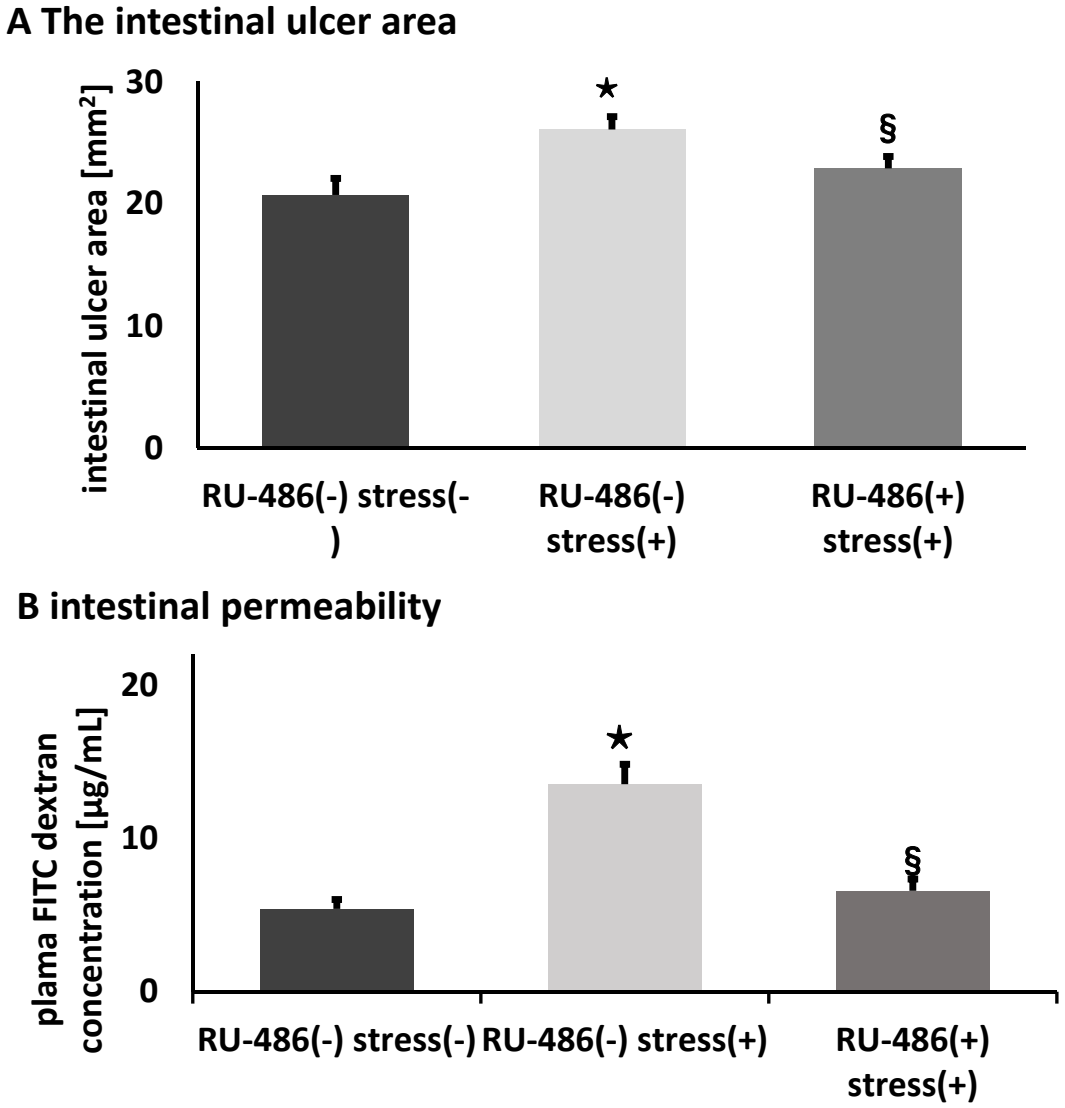
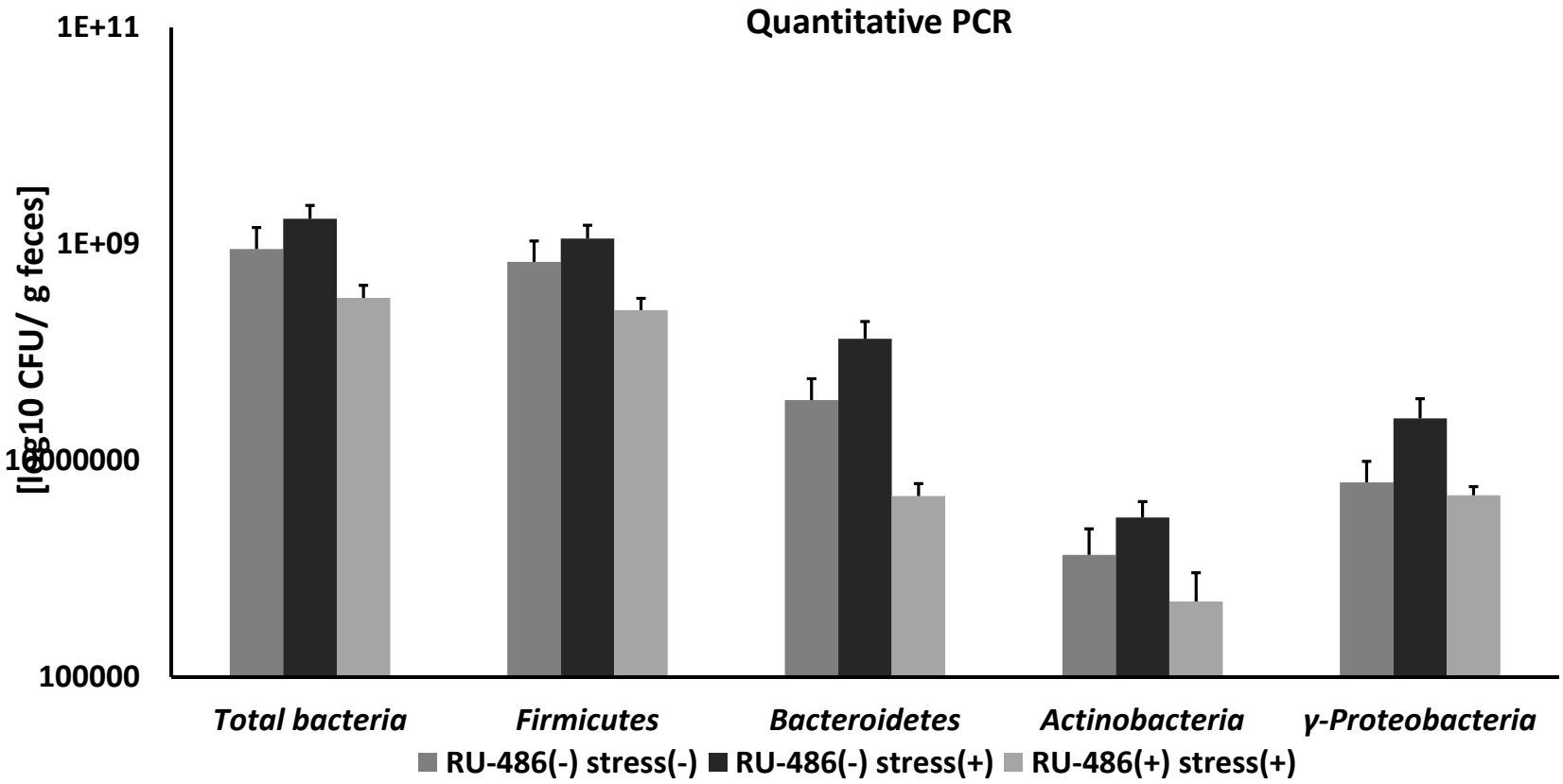

A The intestinal ulcer area
intestinal ulcer area [mm²]
30
20
10
0
★
§
RU-486(-) stress(-)
RU-486(-) stress(+)
RU-486(+) stress(+)
B intestinal permeability
plama FITC dextran concentration [μg/mL]
20
10
0
★
§
RU-486(-) stress(-)
RU-486(-) stress(+)
RU-486(+) stress(+)

Figure6


Quantitative PCR
1E+11
1E+09
10000000
100000
[log10 CFU/ g feces]
Total bacteria
Firmicutes
Bacteroidetes
Actinobacteria
γ-Proteobacteria
RU-486(-) stress(-)
RU-486(-) stress(+)
RU-486(+) stress(+)