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Overcrowding Stress in Livestock Production Alters Gut Microbiota Composition and nNOS Expression in nNOS-HiBiT Knock-in Mouse Model

Abstract

Overcrowding stress in livestock farming is a significant concern for animal health and livestock products such as meats, milk, and eggs. It affects gut health by altering microbiota and regulating neuronal nitric oxide synthase (nNOS). This study aimed to investigate the effects of overcrowding stress on the gut microbiota composition and nNOS expression. We generated an nNOS-HiBiT knock-in mouse model using the HiBiT system, a highly sensitive tool for accurately quantifying gene expression. Overcrowding stress was induced by housing twenty mice per cage (MPC20) and compared with a control group of two mice per cage (MPC2). Overcrowding stress increases nNOS levels in the hypothalamus and ileum and serum corticosterone levels. Gut microbial composition differed between the control and overcrowding stress-induced groups in the ileum, cecum, and colon. Specifically, *Bifidobacterium* and *Akkermansia* decreased in all three regions of MPC20, whereas *Helicobacter* in the ileum and colon and *Parasuterella* in the cecum increased in MPC20. Notably, *Bifidobacterium* consistently decreased when nNOS and corticosterone expression were used as covariates under overcrowding stress. These regional variations reflect the differential impact of overcrowding stress on the intestinal tract, indicating complex interactions through nNOS expression within the brain-gut-microbiome axis. Importantly, the addition of probiotic feed, particularly those containing *Bifidobacterium*, may counteract these decreases, leading to enhanced gut health and improved quality of livestock food products. This study enhances our understanding of the correlation between overcrowding stress and the gut microbiota, providing valuable data for improving the management environment in livestock farming.

Keywords: overcrowding stress, gut microbiota, neuronal nitric oxide synthase (nNOS), HiBiT system, Bifidobacterium

Introduction

Stress resulting from density is a significant concern livestock farming. Despite ongoing regulations regarding density, there are persistent cases of noncompliance with the standards for the appropriate number of livestock per area. Overcrowding is often linked to increased farm profitability (De Vries et al., 2016), however, the stress it induces not only leads to various diseases but also causes changes in gut microbiome, which can compromise the quality of livestock products (Peixoto et al., 2021). For example, overcrowding stress has been shown to decrease macrophage activity and increase Salmonella Enteritidis invasion in broiler chickens (Gomes et al., 2014). Similarly, overcrowding in dairy cows has been linked to reduced milk production and deteriorating health (Jensen et al., 2019). Additionally, it elevates stress hormones such as dehydroepiandrosterone (DHEA) and cortisol (Fustini et al., 2017).

Stress stimulates the brain and impacts the intestines, either directly or indirectly. The hypothalamic-pituitary-adrenal (HPA) axis is activated when stressed, such as in cases of overcrowding. This activation releases cortisol and other glucocorticoid stress hormones, generating oxygen and nitrogen radicals (Chen et al., 2015). Nitric oxide is synthesized from L-arginine by nitric oxide synthase (NOS), and neuronal NOS (nNOS) is known to colocalizes with the HPA axis (Mogor et al., 2021). nNOS is transcriptionally controlled by glucocorticoids and plays a significant role in stress response (Chen et al., 2015). Chronic stress promotes increased nNOS expression in the brain (Zhou et al., 2011). Furthermore, nNOS has recently been shown to modulate the sensitivity to social stress through cyclin-dependent kinase 5 (Yin et al., 2021). Stress-induced activation of the HPA axis also regulates the physiological functions of the digestive system, such as smooth muscle relaxation in the gastrointestinal tract, blood flow regulation, and alterations in neurotransmitter secretion (Mogor et al., 2021).

The gut microbiota is linked to stress response and the HPA axis (Warren et al., 2024). A germ-free mouse model study showed that stress activated a hyperactive HPA axis, including

elevated adrenocorticotropic hormone (ACTH) and corticosterone levels, and normal nervous system function was restored after fecal microbiota transplantation from healthy mice (Cenit et al., 2017). Additionally, numerous studies on the consumption of probiotic supplements have shown improvements in stress and emotional responses through changes in the gut microbiota (Allen et al., 2016; Boehme et al., 2023; Culpepper et al., 2016; Wang et al., 2019; Zhang et al., 2020). Importantly, the addition of probiotic feed containing Bifidobacterium may counteract the adverse effects of overcrowding stress, potentially enhancing gut health and improving the quality of livestock food products. However, the intricate interaction between the stress response and gut microbiota remains largely unexplored.

This research utilized the nNOS-HiBiT knock-in mouse model designed by Han et al. (2021). The HiBiT system detects luciferase intensity, allowing for the accurate quantification of nNOS expression with high sensitivity. In previous work, nNOS was identified as a biomarker of stress-activated bowel motility in a stress-induced neonatal maternal separation (NMS) mouse model using nNOS-HiBiT knock-in mice (Han et al., 2021). We employed nNOS-HiBiT knock-in mice to investigate the effects of crowding stress on the gut microbiota, aiming to provide insights into the brain-gut-microbiome axis by focusing on the differential expression of nNOS and the resulting microbial alterations in the ileum, cecum, and colon. The primary objective of this study is to elucidate the relationship between overcrowding stress and gut microbiota changes, thereby informing strategies to enhance livestock health and productivity.

Materials and Methods

Animals

Thirty-six male C57BL/6J mice (6 weeks old), each harboring a 33-base pair HiBiT sequence knock-in in the nNOS gene were used. Genetically modified mice were generated as described previously (Han et al., 2021).

Mice were genotyped by snipping their tails. A piece shorter than 2 mm was quickly cut from the tail tip using sharp surgical scissors, and hemostasis was achieved by applying slight pressure to the incision site. Genotyping was performed using a MightyAmp™ Genotyping Kit (Takara Bio Inc., Seoul, South Korea) according to the manufacturer's protocol. In brief, the mouse tail was incubated in extraction buffer with Proteinase K for 5 min at 60 ℃, heated at 98 ℃ for 2 min, and then centrifuged. The resulting supernatant was used as the template for PCR. Amplification of the nNOS-HiBiT gene was conducted using C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, USA) under condition 98 ℃ for 2 min; 40 cycles of 98 ℃ for 10 sec, 60 ℃ for 15 sec, 68 ℃ for 1 min/kb. The PCR solution containing 5X Loading Dye was analyzed by electrophoresis.

The animal experiment in this study were approved by Institution Animal Care and Use Committee (IACUC) of Seoul National University (Approval No. SNU-200803-1).

Treatments and sample collection

All the mice were kept in a specific pathogen-free (SPF) animal room at a temperature of 23±1 ℃ and humidity of 55±5%. The lights were turned on and off in a light-dark cycle with 12-hour intervals.

The mice were randomly divided into three groups as follows: the MPC2 group (n=6; two mice per cage), the MPC10 group (n=10; ten mice per cage), and the MPC20 group (n=20; twenty mice per cage) with cages measuring 391 mm × 199 mm × 160 mm (Sealsafe Plus GM500, Tecniplast, West Chester, USA). MPC2 was regarded as the control group, while MPC10 and MPC20 were considered as the stress-induced groups. Stress was induced for two weeks, during which time the cage bedding was changed for MPC2 but not for MPC10 and MPC20. All the mice had *ad libitum* access to a standard laboratory chow diet and water.

The mice were euthanized at the end of the 14-day experiment in accordance with the ethical guidelines. For corticosterone quantification, blood was collected via cardiac puncture into BD Microtainer® Blood Collection Tube and centrifuged at 10,000 rpm for 3 min to obtain serum. The hypothalamus, ileum, cecum, and colon samples were collected for nNOS quantification. Additionally, a portion of intestinal tissue from MPC2 and MPC20 was used for 16s rRNA sequencing. All samples were immediately frozen at -80 ℃ and stored until further analysis.

Quantification of nNOS

The total protein in each hypothalamus, ileum, cecum, and colon sample was quantified using a BCA Protein Assay Kit (Takara Bio, Inc., Seoul, South Korea). Proteins were extracted from the samples using RIPA Lysis and Extraction buffer (Thermo Scientific™, Waltham, USA) and homogenized with a bead homogenizer (BeadBug™ 6, Bechmark Scientific, Inc, Sayreville, USA). The 10-fold diluted samples were mixed with 1 ml of a working solution composed of BCA reagents A and B, and the absorbance was measured at 562 nm in triplicate using a spectrophotometer (Spectrostar Nano, BMG Labtech, Ortenberg, Germany).

The amount of HiBiT-tagged nNOS proteins was quantified using the Nano-Glo® HiBiT Lytic Detection System (Promega, Madison, USA). The homogenized samples were incubated with the Nano-Glo® HiBiT Lytic Reagent in a 1:1 ratio for 10 min. HiBiT binds tightly to LgBiT protein derived from the reagent solution and generates luminescence, measured in triplicate using a GloMax® 96 Microplate Luminometer (Promega, Madison, USA).

Quantification of serum corticosterone

Serum corticosterone concentration was determined using a Mouse/Rat Corticosterone ELISA Kit (Arigo Biolaboratories, Hsinchu, Taiwan). Briefly, 10 μL of serum was added in duplicate into the antibody-coated microplate and incubated for 2 h at room temperature (RT) with 100 μL

of Incubation Buffer and 50 μL of HRP-conjugated Corticosterone. After four times of washing step, 200 μL of TMB substrate was added and incubated for 30 min at RT in the dark. The absorbance was measured at 450 nm.

DNA extraction, 16S rRNA library preparation, and sequencing analysis

Each 1 mg of mouse ileum, cecum, and colon sample was placed in 1 ml of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Waltham, USA) for 10 min and heated to 95 ℃ for 5 min using a heating block. Lysis was achieved by performing two cycles of bead-beating at maximum speed for 90 sec using BeadBug™ 6 (Bechmark Scientific, Inc, Sayreville, USA). According to the manufacturer's instructions, DNA extraction was continued using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Irvine, USA). After estimating quality and quantity, extracted DNA was diluted to a 5 ng/μL concentration and stored at -20 ℃ until further processing.

Library preparation was conducted using a two-step PCR protocol according to Illumina's 16S Metagenomic Sequencing Library Preparation Guide. Amplicon PCR was performed as follows for amplification of bacterial 16S rRNA gene with V3-V4 universal primers: initial denaturation 94 ℃ for 3 min; 25 cycles of 95 ℃ for 30 sec, 55 ℃ for 30 sec, 72 ℃ for 30 sec; and a final extension at 72 ℃ for 3 min. The resulting product was purified using AMPure XP beads (Beckman Coulter, Brea, USA). Index PCR was performed as follows using 16s rRNA V3-V4 amplicons and Nextera® XT Index primers (Illumina, San Diego, USA): initial denaturation 95 ℃ for 3 min; 8 cycles of 95 ℃ for 30 sec, 55 ℃ for 30 sec, 72 ℃ for 30 sec; and a final extension at 72 ℃ for 5 min. Indexed libraries were cleaned using AMPure XP beads. The concentration of libraries was quantified using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen, Waltham, USA). Equal amounts of libraries were pooled and sequenced using the Illumina MiSeq system (Macrogen, Seoul, South Korea).

Illumina adapters and primers were trimmed using the Trimmomatic software (version 0.38) (Bolger et al., 2014). Paired-end reads were analyzed on the Nephele platform (version 2.31.2) (Weber et al., 2017), employing the DADA2 pipeline (version 1.28) (Callahan et al., 2016) with default parameters. This pipeline facilitates sequence denoising, chimera removal, and amplicon sequence variants (ASVs) assignment. Taxonomic classification was performed using the RDP method against the SILVA (version 138.1) database (Quast et al., 2012). Samples with low depths were excluded from the subsequent analyses.

Microbial composition and diversity were analyzed using MicrobiomeAnalyst 2.0 (Lu et al., 2023). Taxonomic differences between microbial communities in the groups were explored using heat tree analysis, visualizing those with a Wilcoxon p-value ≤ 0.05. Alpha diversity was estimated using ecological indices (Chao1, Shannon, and Fisher's alpha) and assessed using the Mann-Whitney U test. Beta diversity was evaluated using Principal Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity, and statistical significance among the groups was determined using permutational multivariate analysis of variance (PERMANOVA). Dendrograms were constructed from feature-level calculations using unweighted UniFrac distances. The correlation between nNOS and corticosterone was analyzed using Pearson's correlation coefficients, and between nNOS and microbial taxa using Spearman's correlation coefficients. A multiple linear regression analysis was conducted using MaAsLin2 (Mallick et al., 2021), with nNOS and corticosterone included as covariates. P-values were adjusted using the False Discovery Rate (FDR), and those less than 0.05 were considered statistically significant.

Quantification and statistical analysis

Statistical analyses were conducted using GraphPad Prism software. Data are presented as mean ± standard deviation (SD). Continuous variables between the MPC2 and MPC20 groups were compared using unpaired t-tests or Mann-Whitney U tests, whereas differences among

three or more groups were assessed using one-way analysis of variance (ANOVA) with Tukey's post hoc test for multiple comparisons. Statistical significance was set at $p < 0.05$. significant.

Results

Overcrowding stress affects nNOS and corticosterone expression

Mice carrying a HiBiT knock-in sequence within nNOS were generated and confirmed by genotyping (Fig. S1), and were subsequently included in the experiment. The nNOS-HiBiT knock-in mice were randomly divided into three groups: two mice per cage (MPC2), ten mice per cage (MPC10), and twenty mice per cage (MPC20). nNOS expression in the hypothalamus exhibited an elevation with increasing density and was significantly increased in MPC20 compared to MPC2 (Fig. 1A). Serum corticosterone levels were also significantly higher in the MPC20 mice than in the MPC2 mice (Fig. 1B). Overexpression of hypothalamic nNOS and corticosterone indicated that overcrowding condition in MPC20 induced considerable stress. Additionally, variations were observed in the expression of nNOS in the ileum, cecum, and colon (Fig. 1C). In the ileum, nNOS levels were significantly higher in MPC20 than in MPC2. There was no significant difference in the nNOS levels in the cecum, but a decreasing trend was observed in the colon ($p=0.06$).

Overcrowding stress leads to alteration in gut microbial abundance

Microbial community profiling was performed on the MPC2 and MPC20. MPC20 exhibited higher alpha diversity, as estimated by the Chao1, Shannon, and Fisher's alpha indices, compared to MPC2 (Fig. 2A-C). The colon of MPC20 mice showed a significant increase in all alpha diversity indices. The alpha diversity of gut microbiota has been reported to either increase or decrease in stressful situations (Ma et al., 2023). In the beta diversity analysis, MPC2 and MPC20 clustered differently in the ileum, cecum, and colon, with PERMANOVA confirming

significant differences (Fig. 2D-2F). Clustering based on phylogenetic diversity revealed a clear distinction between the two groups in the dendrogram (Fig. 2G-2I). Overcrowding stress induces considerable changes in the gut microbial community profile.

Regarding the microbial composition at the phylum level, MPC20 showed a higher relative abundance (RA) of Proteobacteria than MPC2 in the ileum, cecum, and colon. Camphylobacterota showed higher RAs in the ileum and colon of MPC20 than MPC2. In contrast, the MPC20 RAs of Actinobacteriota and Verrucomicrobiota were lower than those in MPC2 (Fig. 3A). Genus-level heat tree analysis revealed significantly lower RAs in MPC20 for *Bifidobacterium* (Actinobacteriota), *Akkermansia* (Verrucomicrobiota), *Ligilactobacillus*, *Faecalibaculum* (Bacillota). In contrast, levels of *Helicobacter* (Campylobacterota), *Parasutterella*, *Muribaculum* (Proteobacteria), *Oscillibacter*, *Anaerotruncus*, and *Roseburia* (Bacillota) RAs were significantly elevated in the MPC20 group (Fig. 3B and Fig. S2). Regardless of the intestinal region, the RAs of *Bifidobacterium* and *Akkermansia* were significantly lower in MPC20 (Fig. 3C-3D). Meanwhile, *Helicobacter* populations significantly increased in the ileum and colon, and *Parasutterella* abundance significantly increased in the cecum of MPC20 (Fig. 3E-3F).

Overcrowding stress reduces Bifidobacterium regardless of intestinal region

Different correlation patterns between nNOS and corticosterone were observed depending on the intestinal region: the ileum, cecum, or colon (Fig. 4A-4C). A tendency for a positive correlation was observed in the ileum ($p=0.055$) (Fig. 4A). However, no significant correlation was found between nNOS and corticosterone levels in the cecum or colon (Fig. 4B-4C). This result was presumed to be due to the significantly increased expression of nNOS in the ileum, as shown in Fig. 1C, which was not observed in the cecum or colon.

The correlation between nNOS and microbial taxa at the species level in the ileum, cecum, and colon was analyzed (Fig. 4D-4F). Distinct correlation patterns were observed in each intestinal region. Specifically, *A2*, *Helicobacter*, and *Enterorhabdus* showed a positive correlation in the ileum but a negative correlation in the cecum and colon. *Oscillibacter* and *Parasutterella* were positively correlated in the ileum and cecum yet negatively correlated in the colon. *Turibacter* exhibited a negative correlation in the ileum but a positive correlation in the cecum and colon.

Furthermore, we used a multiple linear regression model to elucidate the association between the covariates (nNOS and corticosterone) and microbiota composition (Table 1). Remarkably, *Bifidobacterium* abundance decreased significantly across all three intestinal regions. This indicates that *Bifidobacterium* is vulnerable to overcrowding stress. Furthermore, the *faecalibaculum* and *prevotellaceae_NK3B31_*groups showed a significant decrease in the cecum and colon. *Odoribacter* and *Bacteroides* were decreased in the cecum and colon, respectively.

Discussion

Stress causes an increase in nNOS expression mainly in the hypothalamus (Chen et al., 2015; Mogor et al., 2021; Shirakawa et al., 2004), but it also significantly affects nNOS expression in the intestine (Schneider et al., 2023). The enteric nervous system functions independently of the central nervous system. Because of this functional autonomy, enteric nerves have the plasticity to respond flexibly depending on the situation (Yunker and J. Galligan, 1998). Therefore, distinct responses to the exact circumstances may occur depending on the region in the gastrointestinal tract.

Motor activity in the small intestine is reduced in response to psychological stress (Kellow et al., 1992; Mönnikes et al., 2001). Stress and mechanical stimulation caused by fasting or feeding increase nNOS expression in the small intestine (Ito et al., 2017). Additionally, nNOS

immunohistochemistry of the small intestine showed a significant increase in nNOS-containing cells in patients with intestinal pseudo-obstruction, who had symptoms of intestinal obstruction but no physical blockage (Wang et al., 2000). We also found that chronic stress induced by overcrowding increased nNOS expression in the ileum.

The cecum contains a large proportion of vagally innervated myenteric ganglia (Berthoud et al., 1990). About 65-66% of the vagus nerve is found in the cecum, whereas the ileum accounts for 35% and the distal colon for 16% (Berthoud et al., 1990). In an experiment on nNOS expression following neurectomy, splanchnic ganglionectomy, which removes the intestinal ganglion, or 6-OH-dopamine treatment, which mainly suppresses the sympathetic nervous system by chemically destroying specific nerves, increased intestinal nNOS expression (Nakao et al., 1998). However, vagotomy, which involves removal of the vagus nerve, had no significant effect on nNOS mRNA expression (Nakao et al., 1998). Therefore, high vagal nerve distribution is presumed to be responsible for the unaffected nNOS levels in the cecum.

Chronic psychological stress is closely associated with colonic inflammation (Duffy et al., 1991; Garrett et al., 1991; Levenstein et al., 2000). When colitis develops, the physical length of the colon decreases, and peristalsis decreases as the colon shortens (Delaroque et al., 2021; Reber et al., 2006). In the colon of a dextran sulfate sodium-induced colitis mouse model, the number of nNOS-immunopositive cells, NOS activity, and nNOS synthesis decreased (Mizuta et al., 2000). Recently, it has been demonstrated that stress directly affects the enteric nervous system, lowering the number of nNOS-expressing neurons in the colon (Schneider et al., 2023).

We extrapolated that overcrowding stress affects the nervous system and intestinal motility, altering gut microbiota composition. To the best of our knowledge, little is known about the impact of overcrowding stress on the gut microbiota. According to Delaroque et al. (Delaroque et al., 2021), overcrowding stress in mice results in hyperglycemia, moderate chronic inflammation, and changes in the gut microbiota. We found that *Bifidobacterium* (Chen et al., 2021; Tojo et al.,

2014), a representative probiotic, and *Akkermansia* (Ding et al., 2021; Zhai et al., 2019), a next-generation probiotic, significantly decreased in response to overcrowded stress. Conversely, *Helicobacter* (Guo et al., 2009; Li et al., 1998; Maltz et al., 2019), which is associated with gastrointestinal inflammation, and *Parasutterella* (Everard et al., 2011; Henneke et al., 2022), which is associated with the onset of obesity and diabetes, were significantly increased. These results suggest that overcrowding stress has an unfavorable impact on the composition of the gut microbial community.

Akkermansia has a protective effect against obesity, diabetes, colitis (Wang et al., 2020; Zhai et al., 2019), and neurological diseases such as amyotrophic lateral sclerosis (Blacher et al., 2019), Alzheimer's disease (Ou et al., 2020), and autism (Wang et al., 2011). Psychological stress diminishes the presence of intestinal *Akkermansia* (McGaughey et al., 2019). More specifically, *Akkermansia muciniphila* alleviates depression caused by chronic stress by modulating the gut microbiota and serum metabolomics (Cheng et al., 2021; Ding et al., 2021). *Helicobacter* has primarily been studied as the cause of upper digestive tract diseases; however, it has also been linked to dyspepsia and visceral hypersensitivity in the lower digestive tract (Budzyński and Kłopocka, 2014; Gerards et al., 2001; Li et al., 1998; Su et al., 2000). Psychological stress promotes gastric colonization by *Helicobacter pylori* and contributes to developing peptic ulcers (Guo et al., 2009). However, there is ongoing controversy over the potential health effects of *Helicobacter* colonization of the lower digestive tract due to chronic stress. *Parasutterella* has been associated with the development of inflammatory bowel disease (Chen et al., 2018), obesity (Everard et al., 2011; Henneke et al., 2022), and diabetes (Henneke et al., 2022). This bacterium suppresses energy metabolism and short-chain fatty acid synthesis in intestinal microorganisms (Luo et al., 2018). Furthermore, a strong correlation has been detected between high *Parasutterella* abundance and low intracranial volume (Hu et al., 2024). Thus, it is

assumed that *Parasutterella* is connected to the brain-gut-microbiome axis; however, its significance in stress mechanisms requires further research.

Meanwhile, *Bifidobacterium* was decreased in all three intestinal regions (ileum, cecum, and colon) when the correlation with changes in the gut microbiota was analyzed using nNOS and corticosterone as covariates. Previous studies have confirmed that *Bifidobacterium* abundance is reduced in various digestive diseases and under conditions of chronic stress (Chen et al., 2021; Yang et al., 2017). Animal studies have demonstrated that *Bifidobacterium* supplementation alleviates stress and enhances immune function. *Bifidobacterium* was below the detection limit in social defeat stress-induced mice but was significantly present in the stress-resilient group (Yang et al., 2017). Oral administration of *Bifidobacterium* LAC-B improves resilience to chronic social defeat stress (Yang et al., 2017). In another mouse experiment that induced social defeat stress, the oral administration of *Bifidobacterium longum* R0175 increased anti-inflammatory responses and stress resistance (Partrick et al., 2021).

In studies involving livestock such as broilers, pigs, beef cattle, and dairy cows, *Bifidobacterium* supplementation has been shown to boost immunity, improve the quality of livestock products, and increase overall productivity. In broilers, the addition of *Bifidobacterium* has been linked to improved gut health and reduced disease incidence, thereby enhancing growth performance (Estrada et al., 2001; Kabir et al., 2004). Similarly, in pigs, *Bifidobacterium* supplementation enhances immune response and growth rates, resulting in higher-quality meat (Méndez-Palacios et al., 2018; Pang et al., 2022). For beef cattle, the inclusion of *Bifidobacterium* in their diet has been associated with improved feed efficiency and weight gain, contributing to enhanced meat quality (Alawneh et al., 2020; Uyeno et al., 2015). Additionally, in dairy cows, *Bifidobacterium* supplementation has been shown to improve milk yield and quality, while also promoting overall health (Krishnan et al., 2020; Nalla et al., 2022). These finding collectively highlight the potential of *Bifidobacterium* as a beneficial probiotic for

improving livestock health and productivity, ultimately leading to enhanced quality of livestock products.

Overall, overcrowding stress induced ileum nNOS and serum corticosterone levels, altering the gut microbiota composition. Specifically, overcrowding stress led to a decrease in *Bifidobacterium* and *Akkermansia*, along with an increase in *Helicobacter* and *Parasutterella*. Notably, *Bifidobacterium* was found to be especially vulnerable to overcrowding stress, as it consistently declined, regardless of the intestinal region. This study supports previous findings indicating that *Bifidobacterium* plays a significant role in regulating the brain-gut-microbiome axis. However, further studies are needed, as our research is not sufficient to fully explain the complex interaction between NO mechanisms and the gut microbiome. For example, NO produced by *Bacillus* is known to have an anti-aging effect (Ayala et al., 2017). Therefore, research that considers NO produced by gut microbiota separately from the host's NO mechanism is also required.

In conclusion, we have demonstrated that overcrowding stress can have negative effects on the intestinal health of animals. The findings of this study hold significant implications for livestock management practices. By understanding the effects of overcrowding stress on gut health and the microbiota, producers can implement strategies, such as the inclusion of probiotics like *Bifidobacterium* in feed, to mitigate stress-related issues. This approach not only enhances animal welfare but also improves the quality and safety of livestock products, ultimately contributing to greater economic sustainability in the livestock industry.

References

Alawneh JI, Barreto MO, Moore RJ, Soust M, Al-Harbi H, James AS, Krishnan D, Olchowy TWJ. 2020. Systematic review of an intervention: The use of probiotics to improve health and productivity of calves. Preventive Veterinary Medicine 183:105147.

Allen AP, Hutch W, Borre YE, Kennedy PJ, Temko A, Boylan G, Murphy E, Cryan JF, Dinan TG, Clarke G. 2016. Bifidobacterium longum 1714 as a translational psychobiotic: Modulation of stress, electrophysiology and neurocognition in healthy volunteers. Transl Psychiatry 6:e939-e939.

Ayala FR, Bauman C, Cogliati S, Leñini C, Bartolini M, Grau R. 2017. Microbial flora, probiotics, bacillus subtilis and the search for a long and healthy human longevity. Microb Cell 4:133-136.

Berthoud H-R, Jedrzejewska A, Powley TL. 1990. Simultaneous labeling of vagal innervation of the gut and afferent projections from the visceral forebrain with dil injected into the dorsal vagal complex in the rat. J Comp Neurol 301:65-79.

Blacher E, Bashiardes S, Shapiro H, Rothschild D, Mor U, Dori-Bachash M, Kleimeyer C, Moresi C, Harnik Y, Zur M, Zabari M, Brik RB-Z, Kviatcovsky D, Zmora N, Cohen Y, Bar N, Levi I, Amar N, Mehlman T, Brandis A, Biton I, Kuperman Y, Tsoory M, Alfahel L, Harmelin A, Schwartz M, Israelson A, Arike L, Johansson MEV, Hansson GC, Gotkine M, Segal E, Elinav E. 2019. Potential roles of gut microbiome and metabolites in modulating als in mice. Nature 572:474-480.

Boehme M, Rémond-Derbez N, Lerond C, Lavalle L, Keddani S, Steinmann M, Rytz A, Dalile B, Verbeke K, Van Oudenhove L, Steiner P, Berger B, Vicario M, Bergonzelli G, Colombo Mottaz S, Hudry J. 2023. Bifidobacterium longum subsp. Longum reduces perceived psychological stress in healthy adults: An exploratory clinical trial. Nutrients 15:3122.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: A flexible trimmer for illumina sequence data. Bioinformatics 30:2114-2120.

Budzyński J, Kłopocka M. 2014. Brain-gut axis in the pathogenesis of helicobacter pylori infection. World J Gastroenterol 20:5212-5225.

Callahan BJ, Mcmurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. Dada2: High-resolution sample inference from illumina amplicon data. Nat Methods 13:581-583.

Cenit MC, Sanz Y, Codoñer-Franch P. 2017. Influence of gut microbiota on neuropsychiatric disorders. World J Gastroenterol 23:5486-5498.

Chen H-JC, Spiers JG, Sernia C, Lavidis NA. 2015. Response of the nitrergic system to activation of the neuroendocrine stress axis. Front Neurosci 9:3.

Chen J, Chen X, Ho CL. 2021. Recent development of probiotic bifidobacteria for treating human diseases. Front Bioeng Biotechnol 9:770248.

Chen Y-J, Wu H, Wu S-D, Lu N, Wang Y-T, Liu H-N, Dong L, Liu T-T, Shen X-Z. 2018. Parasutterella, in association with irritable bowel syndrome and intestinal chronic inflammation. Gastroenterology 33:1844-1852.

Cheng R, Xu W, Wang J, Tang Z, Zhang M. 2021. The outer membrane protein amuc_1100 of akkermansia muciniphila alleviates the depression-like behavior of depressed mice induced by chronic stress. Biochem Biophys Res Commun 566:170-176.

Culpepper T, Christman M, Nieves Jr C, Specht G, Rowe C, Spaiser S, Ford A, Dahl W, Girard S, Langkamp-Henken B. 2016. Bifidobacterium bifidum r0071 decreases stress-associated diarrhoea-related symptoms and self-reported stress: A secondary analysis of a randomised trial. Benef Microbes 7:327-336.

De Vries A, Dechassa H, Hogeveen H. 2016. Economic evaluation of stall stocking density of lactating dairy cows. J Dairy Sci 99:3848-3857.

Delaroque C, Chervy M, Gewirtz AT, Chassaing B. 2021. Social overcrowding impacts gut microbiota, promoting stress, inflammation, and dysglycemia. Gut Microbes 13:2000275.

Ding Y, Bu F, Chen T, Shi G, Yuan X, Feng Z, Duan Z, Wang R, Zhang S, Wang Q, Zhou J, Chen Y. 2021. A next-generation probiotic: Akkermansia muciniphila ameliorates chronic stress–induced depressive-like behavior in mice by regulating gut microbiota and metabolites. Appl Microbiol Biotechnol 105:8411-8426.

Duffy LC, Zielezny MA, Marshall JR, Byers TE, Weiser MM, Phillips JF, Calkins BM, Ogra PL, Graham S. 1991. Relevance of major stress events as an indicator of disease activity prevalence in inflammatory bowel disease. Behav Med 17:101-110.

Estrada A, Wilkie D, Drew M. 2001. Administration of bifidobacterium bifidum to chicken broilers reduces the number of carcass condemnations for cellulitis at the abattoir. J Appl Poult Res 10:329-334.

Everard A, Lazarevic V, Derrien M, Girard M, Muccioli GG, Neyrinck AM, Possemiers S, Van Holle A, François P, De Vos WM, Delzenne NM, Schrenzel J, Cani PD. 2011. Responses of gut microbiota and glucose and lipid metabolism to prebiotics in genetic obese and diet-induced leptin-resistant mice. Diabetes 60:2775-

2786.
Fustini M, Galeati G, Gabai G, Mammi LE, Bucci D, Baratta M, Accorsi PA, Formigoni A. 2017. Overstocking dairy cows during the dry period affects dehydroepiandrosterone and cortisol secretion. J Dairy Sci 100:620-628.
Garrett VD, Brantley PJ, Jones GN, Mcknight GT. 1991. The relation between daily stress and crohn's disease. J Behav Med 14:87-96.
Gerards C, Leodolter A, Glasbrenner B, Malfertheiner P. 2001. H. Pylori infection and visceral hypersensitivity in patients with irritable bowel syndrome. Dig Dis 19:170-173.
Gomes AVS, Quinteiro-Filho WM, Ribeiro A, Ferraz-De-Paula V, Pinheiro ML, Baskeville E, Akamine AT, Astolfi-Ferreira CS, Ferreira AJP, Palermo-Neto J. 2014. Overcrowding stress decreases macrophage activity and increases salmonella enteritidis invasion in broiler chickens. Avian Pathol 43:82-90.
Guo G, Jia K-R, Shi Y, Liu X-F, Liu K-Y, Qi W, Guo Y, Zhang W-J, Wang T, Xiao B. 2009. Psychological stress enhances the colonization of the stomach by helicobacter pylori in the balb/c mouse. Stress 12:478-485.
Han JP, Lee JH, Lee GS, Koo OJ, Yeom SC. 2021. Positive correlation between nnos and stress-activated bowel motility is confirmed by in vivo hibit system. Cells 10:1028.
Henneke L, Schlicht K, Andreani NA, Hollstein T, Demetrowitsch T, Knappe C, Hartmann K, Jensen-Kroll J, Rohmann N, Pohlschneider D, Geisler C, Schulte DM, Settgast U, Türk K, Zimmermann J, Kaleta C, Baines JF, Shearer J, Shah S, Shen-Tu G, Schwarz K, Franke A, Schreiber S, Laudes M. 2022. A dietary carbohydrate – gut parasutterella – human fatty acid biosynthesis metabolic axis in obesity and type 2 diabetes. Gut Microbes 14:2057778.
Hu X, Fang Z, Wang F, Mei Z, Huang X, Lin Y, Lin Z. 2024. A causal relationship between gut microbiota and subcortical brain structures contributes to the microbiota–gut–brain axis: A mendelian randomization study. Cereb Cortex 34:bhae056.
Ito J, Uchida H, Machida N, Ohtake K, Saito Y, Kobayashi J. 2017. Inducible and neuronal nitric oxide synthases exert contrasting effects during rat intestinal recovery following fasting. Exp Biol Med 242:762-772.
Jensen KC, Frömke C, Schneider B, Do Duc P, Gundling F, Birnstiel K, Schönherr F, Scheu T, Kaiser-Wichern A, Woudstra S, Seyboldt C, Hoedemaker M, Campe A. 2019. Case-control study on factors associated with a decreased milk yield and a depressed health status of dairy herds in northern germany. BMC Veterinary Research 15:442.
Kabir S, Rahman M, Rahman M, Rahman M, Ahmed S. 2004. The dynamics of probiotics on growth performance and immune response in broilers. Int J Poult Sci 3:361–364.
Kellow JE, Langeluddecke PM, Eckersley GM, Jones MP, Tennant CC. 1992. Effects of acute psychologic stress on small-intestinal motility in health and the irritable bowel syndrome. Scand J Gastroenterol 27:53-58.
Krishnan D, Al-Harbi H, Gibson J, Olchowy T, Alawneh J. 2020. On the use of probiotics to improve dairy cattle health and productivity. Microbiology Australia 41:86-90.
Levenstein S, Prantera C, Varvo V, Scribano ML, Andreoli A, Luzi C, Arcà M, Berto E, Milite G, Marcheggiano A. 2000. Stress and exacerbation in ulcerative colitis: A prospective study of patients enrolled in remission. Am J Gastroenterol 95:1213-1220.
Li X, Fox JG, Whary MT, Yan L, Shames B, Zhao Z. 1998. Scid/ncr mice naturally infected with helicobacter hepaticus develop progressive hepatitis, proliferative typhlitis, and colitis. Infect Immun 66:5477-5484.
Lu Y, Zhou G, Ewald J, Pang Z, Shiri T, Xia J. 2023. Microbiomeanalyst 2.0: Comprehensive statistical, functional and integrative analysis of microbiome data. Nucleic Acids Res 51:W310-W318.
Luo L, Hu M, Li Y, Chen Y, Zhang S, Chen J, Wang Y, Lu B, Xie Z, Liao Q. 2018. Association between metabolic profile and microbiomic changes in rats with functional dyspepsia. RSC Adv 8:20166-20181.
Ma L, Yan Y, Webb RJ, Li Y, Mehrabani S, Xin B, Sun X, Wang Y, Mazidi M. 2023. Psychological stress and gut microbiota composition: A systematic review of human studies. Neuropsychobiology 82:247-262.
Mallick H, Rahnavard A, Mciver LJ, Ma S, Zhang Y, Nguyen LH, Tickle TL, Weingart G, Ren B, Schwager EH, Chatterjee S, Thompson KN, Wilkinson JE, Subramanian A, Lu Y, Waldron L, Paulson JN, Franzosa EA, Bravo HC, Huttenhower C. 2021. Multivariable association discovery in population-scale meta-omics studies. PLoS Comp Biol 17:e1009442.
Maltz RM, Keirsey J, Kim SC, Mackos AR, Gharaibeh RZ, Moore CC, Xu J, Somogyi A, Bailey MT. 2019. Social stress affects colonic inflammation, the gut microbiome, and short-chain fatty acid levels and receptors. Gastroenterology 68:533-540.
Mcgaughey KD, Yilmaz-Swenson T, Elsayed NM, Cruz DA, Rodriguiz RM, Kritzer MD, Peterchev AV, Roach J, Wetsel WC, Williamson DE. 2019. Relative abundance of akkermansia spp. And other bacterial phylotypes correlates with anxiety- and depressive-like behavior following social defeat in mice. Sci Rep 9:3281.
Méndez-Palacios N, Méndez-Mendoza M, Vázquez-Flores F, Castro-Colombres JG, Ramírez-Bribiesca JE. 2018. Productive and economic parameters of pigs supplemented from weaning to finishing with prebiotic and

probiotic feed additives. Anim Sci J 89:994-1001.
Mizuta Y, Isomoto H, Takahashi T. 2000. Impaired nitrergic innervation in rat colitis induced by dextran sulfate sodium. Gastroenterology 118:714-723.
Mogor F, Kovács T, Lohinai Z, Dora D. 2021. The enteric nervous system and the microenvironment of the gut: The translational aspects of the microbiome-gut-brain axis. Appl Sci 11:12000.
Mönnikes H, Tebbe JJ, Hildebrandt M, Arck P, Osmanoglou E, Rose M, Klapp B, Wiedenmann B, Heymann-Mönnikes I. 2001. Role of stress in functional gastrointestinal disorders : Evidence for stress-induced alterations in gastrointestinal motility and sensitivity. Dig Dis 19:201-211.
Nakao K, Takahashi T, Utsunomiya J, Owyang C. 1998. Extrinsic neural control of nitric oxide synthase expression in the myenteric plexus of rat jejunum. J Physiol 507:549-560.
Nalla K, Manda NK, Dhillon HS, Kanade SR, Rokana N, Hess M, Puniya AK. 2022. Impact of probiotics on dairy production efficiency. Front Microbiol 13.
Ou Z, Deng L, Lu Z, Wu F, Liu W, Huang D, Peng Y. 2020. Protective effects of akkermansia muciniphila on cognitive deficits and amyloid pathology in a mouse model of alzheimer's disease. Nutri Diabetes 10:12.
Pang J, Liu Y, Kang L, Ye H, Zang J, Wang J, Han D. 2022. Bifidobacterium animalis promotes the growth of weaning piglets by improving intestinal development, enhancing antioxidant capacity, and modulating gut microbiota. Food Microbiol 88:e01296-01222.
Partrick KA, Rosenhauer AM, Auger J, Arnold AR, Ronczkowski NM, Jackson LM, Lord MN, Abdulla SM, Chassaing B, Huhman KL. 2021. Ingestion of probiotic (lactobacillus helveticus and bifidobacterium longum) alters intestinal microbial structure and behavioral expression following social defeat stress. Sci Rep 11:3763.
Peixoto RS, Harkins DM, Nelson KE. 2021. Advances in microbiome research for animal health. Annual Review of Annimal Biosciences 9:289-311.
Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2012. The silva ribosomal rna gene database project: Improved data processing and web-based tools. Nucleic Acids Res 41:D590-D596.
Reber SO, Obermeier F, Straub HR, Falk W, Neumann ID. 2006. Chronic intermittent psychosocial stress (social defeat/overcrowding) in mice increases the severity of an acute dss-induced colitis and impairs regeneration. Endocrinology 147:4968-4976.
Schneider KM, Blank N, Alvarez Y, Thum K, Lundgren P, Litichevskiy L, Sleeman M, Bahnsen K, Kim J, Kardo S. 2023. The enteric nervous system relays psychological stress to intestinal inflammation. Cell 186:2823-2838.
Shirakawa T, Mitome M, Kikuiri T, Nakamura W, Oshima S, Hasegawa T, Shindoh M, Oguchi H. 2004. Immobilization induces acute nitric oxide production in the rat hypothalamus: A role of ionotropic glutamate receptors in the paraventricular nucleus. Endocrinology 145:3603-3607.
Su Y-C, Wang W-M, Wang S-Y, Lu S-N, Chen L-T, Wu D-C, Chen C-Y, Jan C-M, Horowitz M. 2000. The association between helicobacter pylori infection and functional dyspepsia in patients with irritable bowel syndrome. Am J Gastroenterol 95:1900-1905.
Tojo R, Suárez A, Clemente MG, De Los Reyes-Gavilán CG, Margolles A, Gueimonde M, Ruas-Madiedo P. 2014. Intestinal microbiota in health and disease: Role of bifidobacteria in gut homeostasis. World J Gastroenterol 20:15163-15176.
Uyeno Y, Shigemori S, Shimosato T. 2015. Effect of probiotics/prebiotics on cattle health and productivity. Microbes Environ 30:126-132.
Wang H, Braun C, Murphy EF, Enck P, Acg. 2019. Bifidobacterium longum 1714™ strain modulates brain activity of healthy volunteers during social stress. Am J Gasteroenterol 114:1152-1162.
Wang L, Christophersen CT, Sorich MJ, Gerber JP, Angley MT, Conlon MA. 2011. Low relative abundances of the mucolytic bacterium akkermansia muciniphila and bifidobacterium spp. In feces of children with autism. Appl Environ Microbiol 77:6718-6721.
Wang L, Tang L, Feng Y, Zhao S, Han M, Zhang C, Yuan G, Zhu J, Cao S, Wu Q. 2020. A purified membrane protein from akkermansia muciniphila or the pasteurised bacterium blunts colitis associated tumourigenesis by modulation of cd8+ t cells in mice. Gut 69:1988-1997.
Wang Z-Q, Watanabe Y, Toki A, Kohno S, Hasegawa S, Hamazaki M. 2000. Involvement of endogenous nitric oxide and c-kit—expressing cells in chronic intestinal pseudo-obstruction. J Pediatr Surg 35:539-544.
Warren A, Nyavor Y, Beguelin A, Frame LA. 2024. Dangers of the chronic stress response in the context of the microbiota-gut-immune-brain axis and mental health: A narrative review. Front Immunol 15:1365871.
Weber N, Liou D, Dommer J, Macmenamin P, Quiñones M, Misner I, Oler AJ, Wan J, Kim L, Coakley Mccarthy M, Ezeji S, Noble K, Hurt DE. 2017. Nephele: A cloud platform for simplified, standardized and reproducible microbiome data analysis. Bioinformatics 34:1411-1413.
Yang C, Fujita Y, Ren Q, Ma M, Dong C, Hashimoto K. 2017. Bifidobacterium in the gut microbiota confer

resilience to chronic social defeat stress in mice. Sci Rep 7:45942.
Yin C-Y, Huang S-Y, Gao L, Lin Y-H, Chang L, Wu H-Y, Zhu D-Y, Luo C-X. 2021. Neuronal nitric oxide synthase in nucleus accumbens specifically mediates susceptibility to social defeat stress through cyclin-dependent kinase 5. J Neurosci 41:2523-2539.
Yunker AMR, J. Galligan J. 1998. Extrinsic denervation increases myenteric nitric oxide synthase-containing neurons and inhibitory neuromuscular transmission in guinea pig. J Auton Nerv Syst 71:148-158.
Zhai Q, Feng S, Arjan N, Chen W. 2019. A next generation probiotic, akkermansia muciniphila. Crit Rev Food Sci Nutr 59:3227-3236.
Zhang N, Zhang Y, Li M, Wang W, Liu Z, Xi C, Huang X, Liu J, Huang J, Tian D, Mu J, Liao X, Zhai S. 2020. Efficacy of probiotics on stress in healthy volunteers: A systematic review and meta-analysis based on randomized controlled trials. Brain Behav 10:e01699.
Zhou Q-G, Zhu L-J, Chen C, Wu H-Y, Luo C-X, Chang L, Zhu D-Y. 2011. Hippocampal neuronal nitric oxide synthase mediates the stress-related depressive behaviors of glucocorticoids by downregulating glucocorticoid receptor. J Neurosci 31:7579-7590.

Tables and Figures

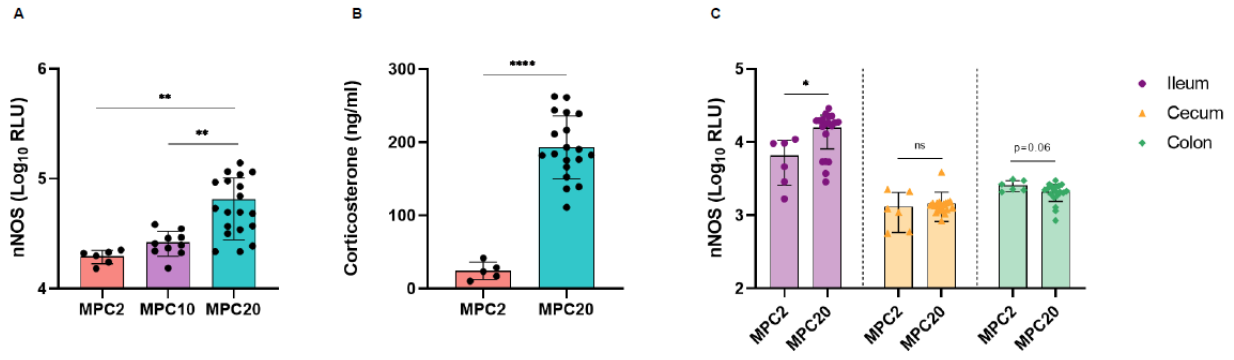

Fig. 1. Comparison of nNOS and corticosterone levels in mice groups induced by overcrowding stress.

(A) Hypothalamic nNOS levels in MPC2, MPC10, and MPC20. (B) Serum corticosterone levels in MPC2 and MPC20 mice. (C) The nNOS levels in the ileum, cecum, and colon of MPC2 and MPC20. MPC2, 2 mice per cage; MPC10, 10 mice per cage; MPC20, 20 mice per cage. Data are presented as mean ± SD. Statistical significance was determined using an unpaired t-test or one-way ANOVA. Significant differences are indicated by **, $p<0.005$; ****, $p<0.0001$; ns, not significant.

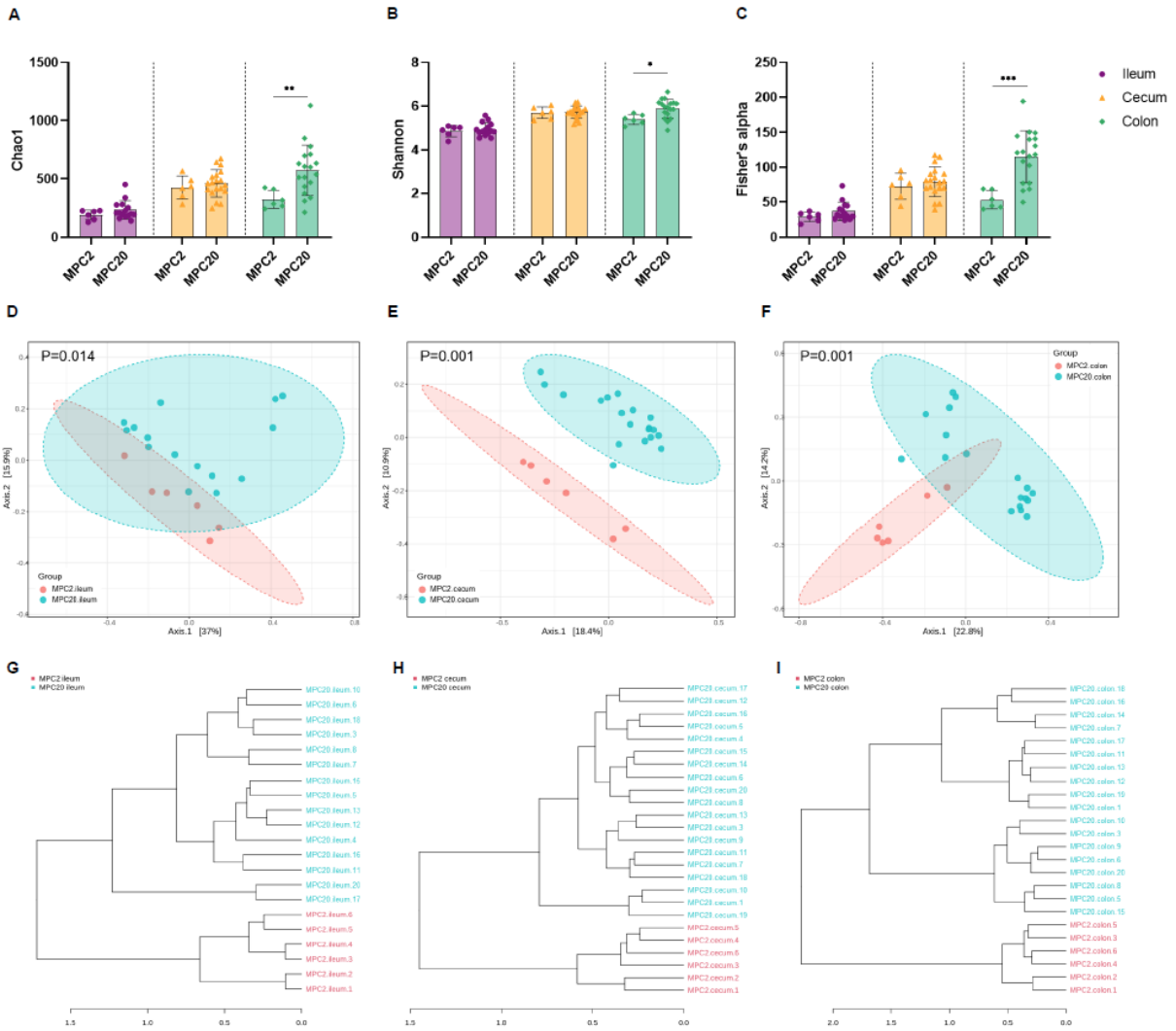

Fig. 2. Microbial community profiles of MPC2 and MPC20.

(A-C) Alpha diversity indices: (A) Chao1, (B) Shannon, and (C) Fisher's alpha indices for the intestinal regions of MPC2 and MPC20. Significant differences are indicated by *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$. (D-F) PCoA plots of beta diversity based on– the Bray-Curtis distance: (D) ileum, (E) cecum, and (F) colon between MPC2 and MPC20. P-values were assessed using PERMANOVA. (G-I) Dendrograms constructed using Unweighted UniFrac Distance: (G) ileum, (H) cecum, and (I) colon, showing clustering of MPC2 and MPC20.

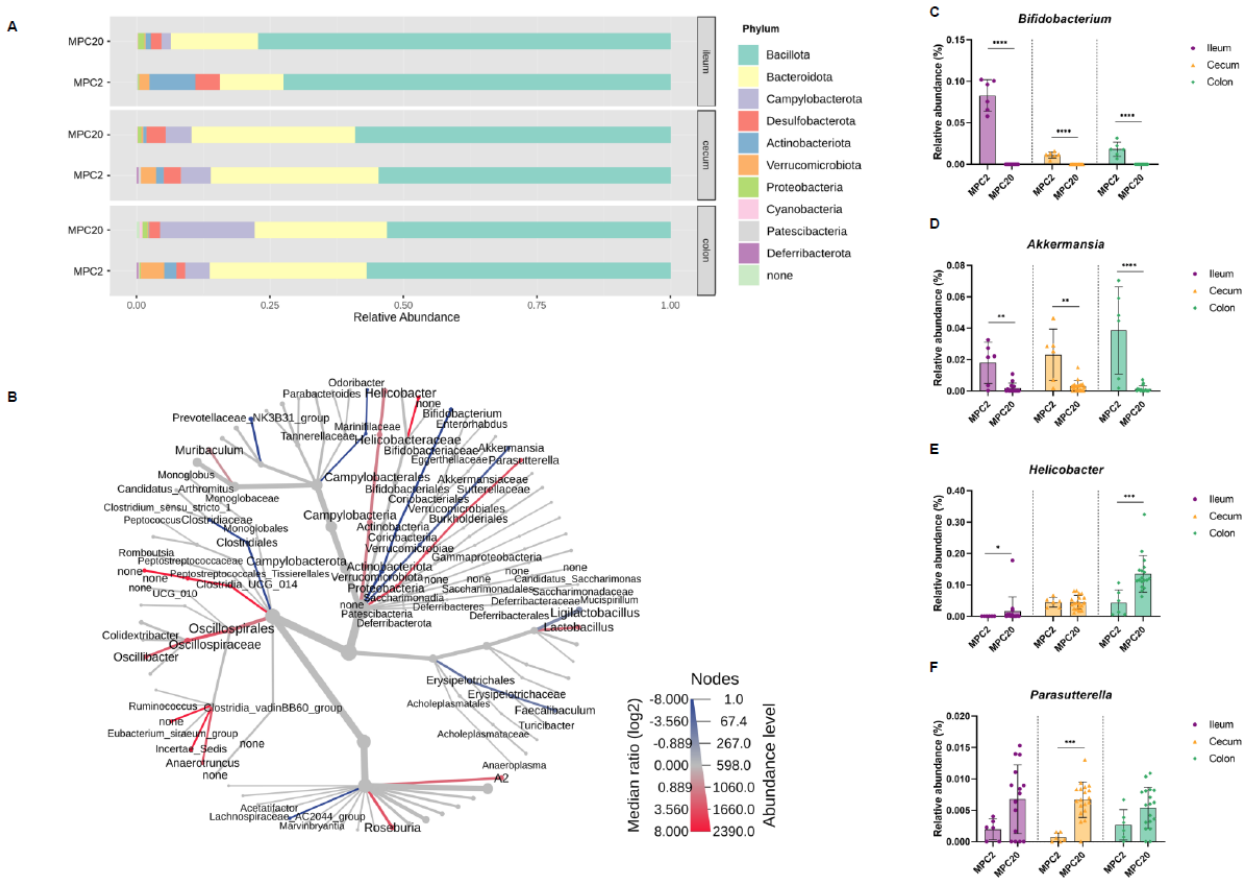

Fig. 3. Comparison of microbial composition between MPC2 and MPC20.

(A) Microbial composition in the intestinal regions of MPC2 and MPC20 at the phylum level.

(B) Heat tree analysis of microbial composition at the genus level, comparing MPC20 to MPC2. Only genera with a Wilcoxon p-value ≤ 0.05 are shown. (C-F) Comparison of the relative abundance of genera in the intestinal regions of MPC2 and MPC20: (C) *Bifidobacterium*, (D) *Akkermansia*, (E) *Helicobacter*, and (F) *Parasutterella*. Data are presented as mean ± SD. Statistical analysis was performed using the Mann-Whitney U test. Significant differences are indicated by *, p<0.05; **, p<0.005; ****, p<0.0001.

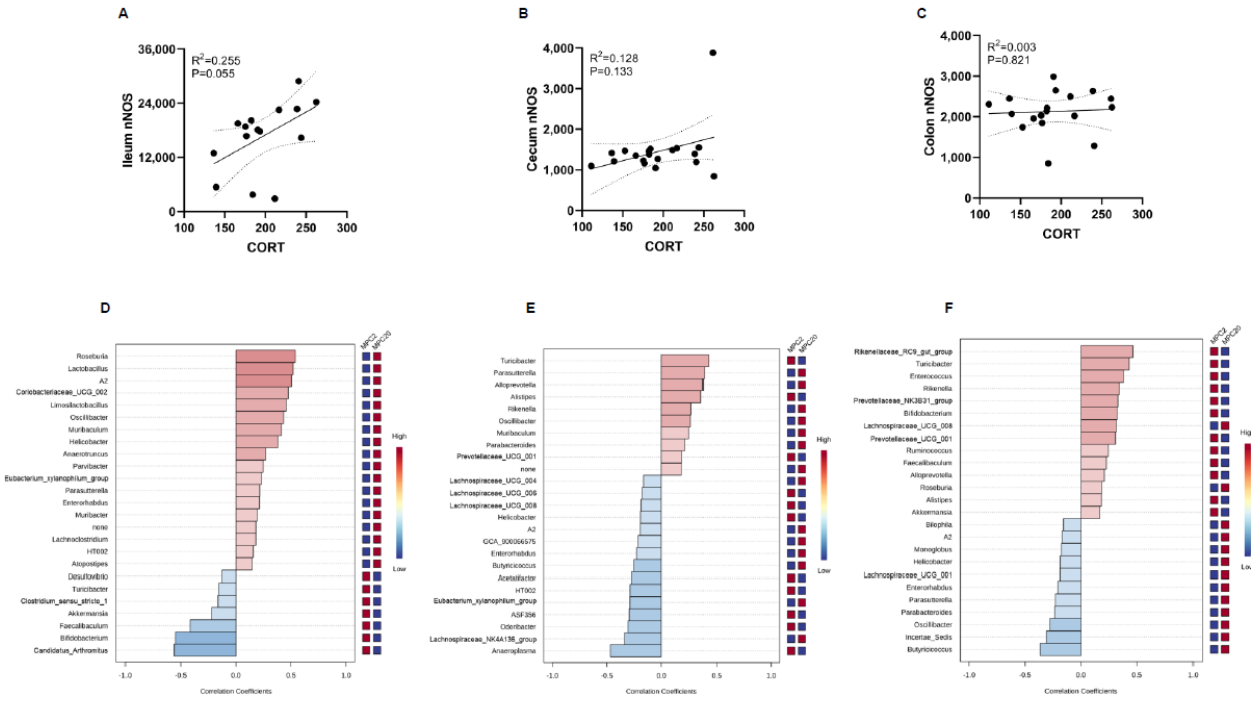

Fig. 4. Correlation analysis of nNOS, corticosterone, and microbial taxa across the intestinal regions.

(A-C) Correlation between nNOS and corticosterone (CORT) in the intestinal region: (A) ileum, (B) cecum, and (C) colon. The Pearson's correlation coefficient was used. (D-F) Correlations between nNOS and microbial taxa at the species level in the intestinal region: (D) ileum, (E) cecum, and (F) colon. Only the top 25 correlations with nNOS, analyzed using Spearman's correlation, are displayed. Blue bars represent negative correlations, and red bars represent positive correlations. The deeper the color, the stronger the correlation.

Table 1. Microbial relative abundance comparison in MPC2 and MPC20 adjusted for nNOS and corticosterone.

	Effect size[1] (Log_2FC)	Standard error	P-value	FDR[2]
Ileum				
Bifidobacterium	-1230	250	0.0001	0.0127
Cecum				
Prevotellaceae_NK3B31_group	-680	75.9	<0.0001	<0.0001
Odoribacter	-148	35.1	0.0004	0.0117
Faecalibaculum	-546	146	0.0013	0.0261
Bifidobacterium	-295	85.2	0.0023	0.0382
Colon				
Faecalibaculum	-865	124	<0.0001	0.0001
Prevotellaceae_NK3B31_group	-386	63.7	<0.0001	0.0004
Bifidobacterium	-389	89.7	0.0004	0.0112
Bacteroides	-770	183	0.0003	0.0112

[1] Multiple linear regression analysis was conducted using MaAsLin2 with nNOS and corticosterone as covariates. The effect size indicates the change in microbial relative abundance of MPC20 compared to that of MPC2.

[2] False Discovery Rate (FDR). Results with a statistically significant FDR ($p<0.05$) are shown in the table.

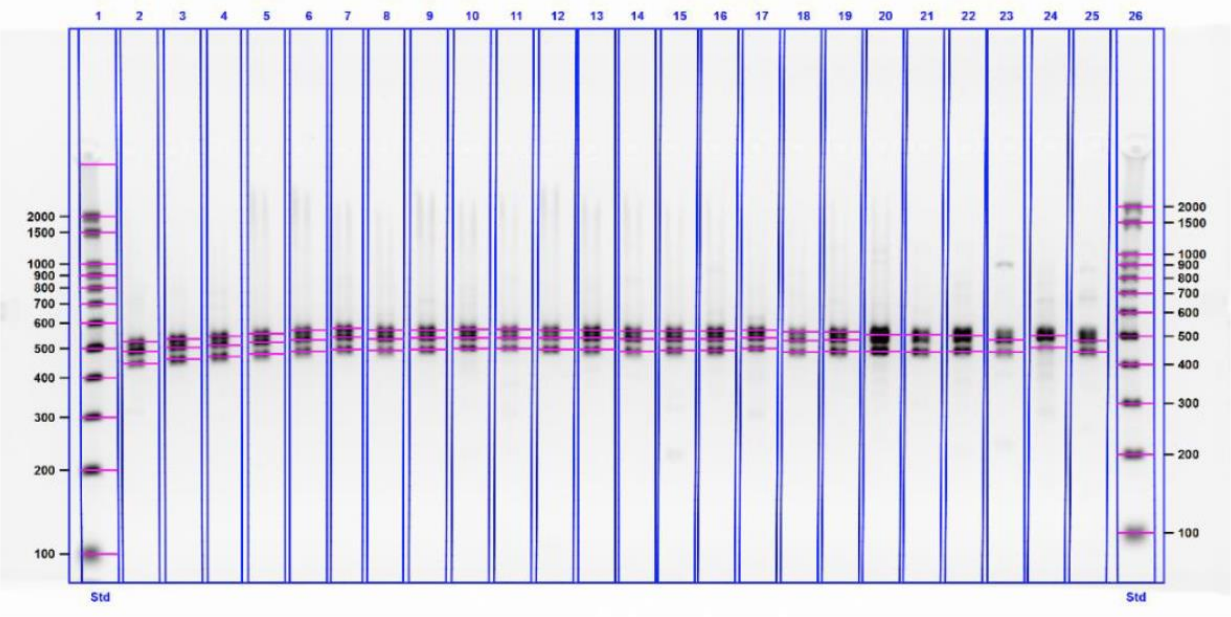

Fig. S1. The results of PCR genotyping for the nNOS-HiBiT gene are displayed on agarose gel electrophoresis. Std, standard DNA ladder.

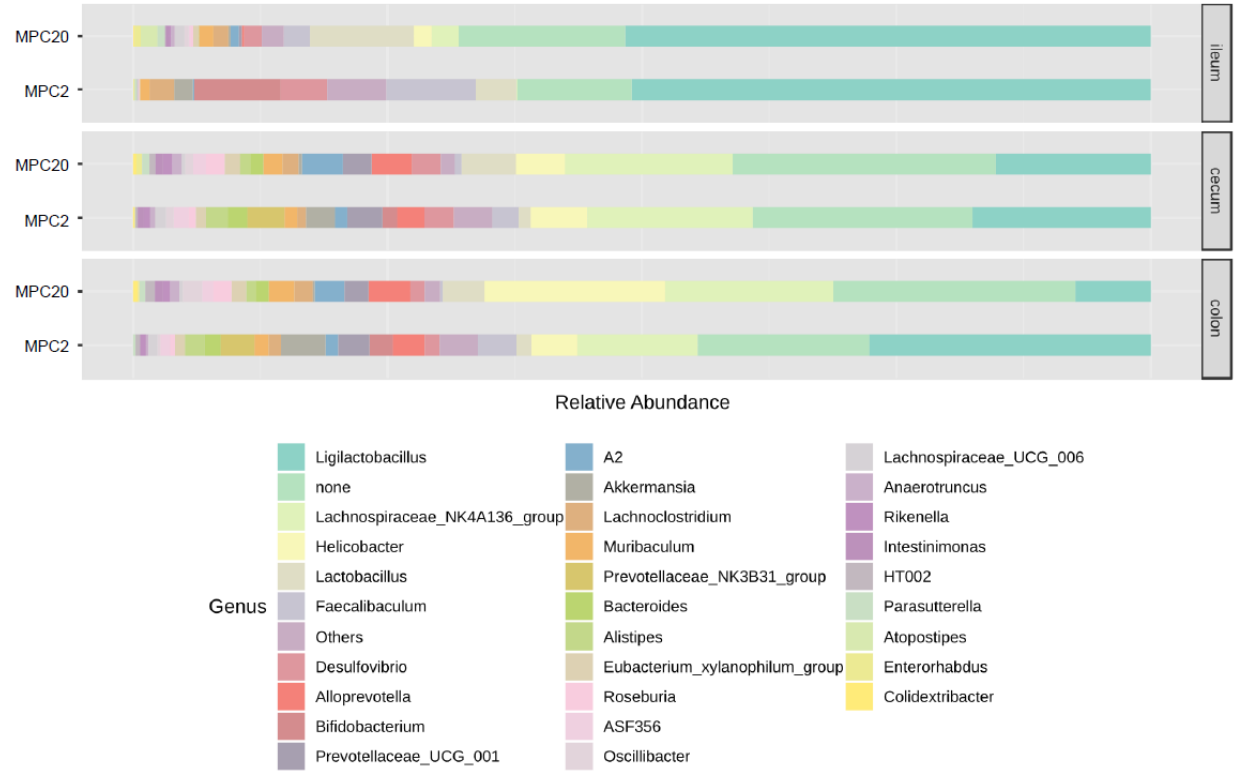

Fig. S2. Microbial composition in the intestinal regions of MPC2 and MPC20 at the genus level. Only the taxa with the top 30 relative abundances are shown.