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The extracellular vesicle of depressive patient-derived *Escherichia fergusonii* induces vagus nerve-mediated neuroinflammation in mice

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Abstract

Background Gut microbiota dysbiosis is closely associated with psychiatric disorders such as depression and anxiety (DA). In our preliminary study, fecal microbiota transplantation from volunteers with psychological stress and subclinical symptoms of depression (Vsd) induced DA-like behaviors in mice. *Escherichia fergusonii* (Esf) was found to be more abundant in the feces of Vsd compared to healthy volunteers. Therefore, we investigated the effect of Esf on DA-like behavior and neuroinflammation in mice with and without celiac vagotomy.

Methods and results Orally gavaged Esf increased DA-like behaviors, tumor necrosis factor (TNF)- α , and toll-like receptor-4 (TLR4) expression, and NF- κ B^{+Iba1+} and lipopolysaccharide (LPS)^{+Iba1+} cell populations, while decreasing serotonin, 5-HT_{1A} receptor, and brain-derived neurotrophic factor (BDNF) expression in the hippocampus and prefrontal cortex. However, celiac vagotomy attenuated Esf-induced DA-like behavior and neuroinflammation. Orally gavaged extracellular vesicle (EV) from Vsd feces (vfEV) or Esf culture (esEV) induced DA-like behavior and inflammation in hippocampus, prefrontal cortex and colon. However, celiac vagotomy attenuated vfEV- or esEV-induced DA-like behaviors and inflammation in the brain alone, while vfEV- or esEV-induced blood LPS and TNF- α levels, colonic TNF- α expression and NF- κ B-positive cell number, and fecal LPS level were not. Although orally gavaged fluorescence isothiocyanate-labeled esEV was translocated into the blood and hippocampus, celiac vagotomy decreased its translocation into the hippocampus alone.

Conclusions esEVs may be translocated into the brain via the vagus nerve and bloodstream, subsequently inducing TNF- α expression and suppressing serotonin, its receptor, and BDNF expression through the activation of TLR4-mediated NF- κ B signaling, thereby contributing to DA pathogenesis.

Keywords Gut dysbiosis, Depression, *Escherichia fergusonii*, *Enterococcus faecium*, Vagus nerve

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Background

Depression and anxiety (DA) are the most common mental disorders [1]. They often co-occur: approximately 90% of individuals with anxiety disorders also exhibit symptoms of depression, and 85% of those with depression exhibit symptoms of anxiety [2, 3]. Excessive exposure to stressors, such as social defeat and pathogens, triggers the adrenal release of glucocorticoids, which can lead to anxiety and/or depression and subsequently induce systemic inflammation, including neuroinflammation [4, 5]. Stresses also cause gut inflammation-mediated gut dysbiosis including bacterial lipopolysaccharide (LPS) overproduction [6, 7]. Overproduced LPS causes toll-like receptor 4-involved NF- κ B-mediated inflammation including neuroinflammation [8–12]. Neuroinflammation suppresses the release of serotonin, brain-derived neurotrophic factor (BDNF), and γ -aminobutyric acid (GABA) in the brain, resulting to psychiatric disorder [13–16]. Nevertheless, understanding the role of gut dysbiosis in the development of DA is still elusive.

The gut microbiota communicates with systemic organs, including the hippocampus, hypothalamus, amygdala, and prefrontal cortex of the brain, through intricate interactions involving the nervous, endocrine, and immune systems located within the intestinal environment. [17, 18]. Metabolites from gut microbiota such as short-chain fatty acids, lipopolysaccharides (LPS), and extracellular vesicle (EV) play crucial roles in maintaining host health [19–21]. Patients with inflammatory bowel disease (IBD) often exhibit the dysbiosis of gut microbiota along with comorbid DA [22, 23]. Fecal microbiota transplantation (FMT) from individuals with or without IBD into mice induces depressive-like symptoms and intestinal inflammation [24, 25]. Compared to healthy volunteers, patients with IBD and depression (IBD/D) show higher abundances of *Enterococcaceae*, *Enterobacteriaceae*, and *Lactobacillaceae* [25]. *Escherichia coli* isolated from gut microbiota of IBD/D patients causes DA and gut inflammation in conventional and germ-free mice [12, 26]. Therefore, gut dysbiosis is suggested to be closely connected with the occurrence of DA and gut inflammation. Nevertheless, understanding how gut bacteria cause depression is still a challenge.

In our preliminary study, FMT from volunteers with psychological stress and subclinical symptoms of DA (Vsd) caused DA-like behavior in mice. The population of *Proteobacteria* and *Firmicutes* including *Escherichia fergusonii* was higher in the feces of Vsd than in the feces of healthy volunteers (Vh). Therefore, we isolated *E. fergusonii* from the feces of Vsd and examined its effects on vagus nerve-involved DA-like symptom in mice.

Methods

Bacterial strains

E. fergusonii NK3001, a gut bacterium, was isolated from freshly collected feces of Vsd. These bacteria were cultured in anaerobic conditions at 37 °C in standard broth (GAM broth, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) for 16–20 h until reaching an optical density (OD) of 0.7–0.9. Cultures were then centrifuged (4 °C, 4000 g, 10 min) and washed twice with distilled water. For in vitro experiments, collected bacteria were heat-treated (75 °C, 15 min) to tyndallize them and suspended in sterile distilled water. For in vivo experiments, collected bacteria were suspended in saline.

Preparation of extracellular vesicle (EV)

The EV was prepared from Vsd fecal suspensions or *E. fergusonii* culture supernatants as previously described [26]. Briefly, the Vsd fecal suspension or *E. fergusonii* culture was centrifuged at 10,000 g for 30 min at 4 °C. The supernatant was filtered sequentially through 0.45 μ m and 0.22 μ m microporous filters. The filtered supernatant was then centrifuged at 120,000 g for 3 h at 4 °C. The resulting pellet was resuspended in saline and purified using an iodixanol density gradient medium (Sigma) to isolate *E. fergusonii* EV (esEV).

The purified esEV was characterized by transmission electron microscopy (Hitachi), revealing a size range of 45–70 nm (spherical vesicle structures without intact *E. fergusonii*). The purified esEV contained 33.48% protein, 0.21% lipopolysaccharide (LPS), and 0.02% nucleic acids (comprising 42.7% DNA and 57.3% RNA). Protein concentration was determined using the Bradford protein assay kit. Nucleic acid content was quantified using DNA and RNA purification kits from Qiagen, with the total amount calculated as the sum of DNA and RNA. Protein analysis of esEV was performed by SDS-PAGE and LC-MS/MS using a Thermo Fisher Orbitrap XL with Easy nLC 1000.

For in vivo experiments, esEV (2 mg of protein) was labeled with fluorescence isothiocyanate (FITC) using the FITC antibody labeling kit (50 μ g), following the manufacturer's instructions [26]. The FITC-labeled esEV (FEV) solution (1.5 mL) was then used for subsequent experiments.

Volunteers

Vsd (age, 39.2 \pm 12.1 [25–52] years; Stress Response Inventory (SRI) score; and Beck Depression Inventory-II (BDI-II) score, 30.0 \pm 6.7: $n=6$ [male, 2; female, 4]) and Vh (age, 32.3 \pm 11.9 [20–46] years; Stress Response Inventory (SRI) score, <15; and Beck Depression Inventory-II (BDI-II) score, <5: $n=6$ [male, 3; female, 3]) were enrolled in the sleep clinic of Seoul National University Bundang Hospital and Kyung Hee National University

Medical, respectively. Participants were included if they had not received antibiotic treatment within the past 3 months and had no acute gastrointestinal symptoms such as diarrhea or abdominal pain within the week prior to fecal collection. The study protocol and informed consent for fecal sample collection were approved by the Medical School of Kyung Hee University Clinical Research Ethics Committee (IRB No.: KMC-202103102) and the Institutional Review Board of Seoul National University Bundang Hospital (IRB No.: B-1807/483-005). All experimental procedures were conducted in accordance with the principles of the Declaration of Helsinki and the Korean Good Clinical Practice guidelines.

Animals

Male C57BL/6 mice (7 weeks old, 18–21 g) were purchased from Koatech Inc. (Gyeonggi-do, Korea). The mice were housed in well-ventilated plastic cages under controlled conditions (temperature 20–22 °C, humidity 50% ± 10%, 12-hour light/dark cycle), as previously described [26]. All animal experiments were approved by the Kyung Hee University Institutional Animal Care and Use Committee (IACUC No.: KUASP(SE)-21451) and conducted in accordance with the university's guidelines for the care and use of laboratory animals, adhering to ethical standards.

First, to investigate whether the gut microbiota from Vsd could induce DA-like behaviors in mice, the mice were divided into three groups: NC (normal control), Vsd, and Vh. Each group consisted of 7 mice. Mice in the Vsd and Vh groups were orally administered suspensions of Vsd or Vh fecal microbiota (1×10^9 CFU per mouse, suspended in 0.1 mL saline) daily for 5 consecutive days. The NC group received saline instead of Vsd or Vh suspensions.

Fecal microbiota suspensions from Vsd and Vh were prepared as follows. Fresh fecal samples from Vsd or Vh were collected and immediately suspended in saline within 2 h. The suspension was filtered through sterile gauze and centrifuged at 500 g for 5 min at 4 °C. The resulting supernatant was diluted with saline to a concentration of 1×10^{10} CFU/mL, forming the fecal microbiota suspension. The bacterial counts in the fecal suspensions were determined by culturing on GAM agar plates.

Second, to investigate the role of the vagus nerve pathway in the pathogenesis of depression induced by Vsd, mice were divided into five groups: NC (normal control), Sham (sham-operated mice treated with saline), vEv (sham-operated mice treated with Vsd extracellular vesicle (vEv)), Vx (mice with vagotomy treated with saline), and VvEv (mice with vagotomy treated with vEv). Each group consisted of seven mice. Mice in the vEv treatment groups (vEv and VvEv) received oral administration of vEv (20 µg per mouse, dissolved in 0.2 mL saline)

daily for 5 consecutive days. The weight of EV was determined based on protein content using the Bradford protein assay.

Third, to elucidate the mechanisms by which gut bacteria induce depression-like symptoms, mice were divided into four groups: NC (normal control), Es (mice gavaged with *E. fergusonii*), Vx (vagotomized mice gavaged with saline), and VEs (vagotomized mice gavaged with *E. fergusonii*). Each group comprised seven mice. *E. fergusonii* (1×10^9 CFU in 0.2 mL saline) was orally gavaged in mice with or without celiac vagotomy daily for 5 consecutive days.

Fourth, to investigate the role of the vagus nerve in the pathogenesis of esEV-induced depression, mice were divided into four groups: NC (normal control), eEv (mice treated with esEV), Vx (vagotomized mice treated with saline), and VeEv (vagotomized mice treated with esEV). Each group comprised seven mice. esEV (20 µg per mouse per day, dissolved in 0.2 mL saline) was orally gavaged in mice with or without celiac vagotomy daily for 5 consecutive days.

Fifth, to investigate whether esEV can translocate to the brain, mice were divided into four groups: NC (normal control), FEv (mice treated with FITC-labeled esEV), Vx (vagotomized mice treated with saline), and VFEv (vagotomized mice treated with FITC-labeled esEV). Each group consisted of seven mice. Mice in the FEv and VFEv groups received oral gavage of FITC-labeled esEV (20 µg per mouse per day, dissolved in 0.2 mL saline) daily for 5 consecutive days.

Celiac vagotomy was performed, as previously described [27]. In brief, a midline abdominal incision was made to expose the anterior esophageal wall. The celiac branch of the vagus nerve was identified and transected subdiaphragmatically. Sham-operated mice underwent the same midline abdominal incision without nerve transection.

DA-like behaviors (open-field test [OFT], elevated plus maze test [EPMT], and tail suspension test [TST]) were measured after the final treatment with gut bacteria on the following day (between 1:00 and 5:00 pm). Mice were euthanized in a CO₂ chamber 18 h after the final behavioral tasks, followed by the cervical dislocation. Blood was collected and sera were prepared, as previously reported [12]. Brain and colon were collected for the enzyme-linked immunosorbent assay (ELISA) and stored at -80 °C.

Behavioral tasks

The DA-like behavior tasks were conducted in a room equipped with a recording camera and analyzed using EthoVision XT software, as previously reported [8–12]. The OFT was conducted in a chamber measuring 40 × 40 cm, with a defined center zone of 20 × 20 cm. Each

test lasted 10 min, during which the movements of the subjects were recorded and analyzed to assess their activity levels and anxiety-related behaviors.

The EPMT consists of a cross-shaped platform with two open arms and two enclosed arms (30×7×20 cm walls), elevated 40 cm above the floor. Mice were placed on the central platform facing one of the open arms. Behavior was recorded for 5 min. Entries into the open arms (OE) and closed arms (CE) were recorded. The time spent in the open arms (OT) and closed arms (CT) was measured. An entry was defined as all four paws entering an arm. The following calculations were performed to quantify behavior: $OT (\%) = 100 \times [OT / (OT + CT)]$, $OE (\%) = 100 \times [OE / (OE + CE)]$.

The TST was performed by suspending the mice by the tail from the edge of a table, 30 cm above the surface, for a duration of 5 min. Mice were considered immobile if they did not move and hung passively. The duration of immobility was recorded to assess depressive-like behavior.

Assay of cytokines and LPS

Hippocampal, prefrontal cortex, and colonic tissues were lysed and centrifuged in RIPA lysis buffer as previously described [27]. ELISA kits were utilized to measure levels of tumor necrosis factor (TNF)- α (R&D, DY410), IL-1 β (R&D, DY401), IL-6 (R&D, DY406), IL-10 (R&D, DY417), corticosterone (Elabscience), serotonin (Eagle Biosciences), myeloperoxidase (R&D, DY3667), and brain-derived neurotrophic factor (BDNF, R&D, DY248) in hippocampal and colonic supernatants and sera. LPS levels were determined using the Limulus Amebocyte Lysate (LAL) assay kit (Cape Cod Inc.) [27].

Immunofluorescence staining

Brain and colon tissues of mice transcardially perfused with paraformaldehyde were sectioned (thickness, 25 μ m). [12]. The sections were then subjected to immunostaining. After washing with PBS, antigen retrieval was performed by incubating the sections in citrate buffer (pH 6.0) at 95 °C for 10 min. Subsequently, the sections were blocked in 10% normal donkey serum (Abcam) for 1 h and incubated overnight at 4 °C with the following primary antibodies: Iba1 (1:800, Abcam), NF- κ B (1:800, Cell Signaling Technology), CD11c (1:250, Cell Signaling Technology), NeuN (1:1000, Abcam), and BDNF (1:800, Abcam). The sections were then incubated with secondary antibodies (Alexa-conjugated secondary antibodies, diluted 1:800, Jackson ImmunoResearch Laboratories) for 1 h at room temperature. Fluorescent sections were mounted with anti-fade mounting medium containing DAPI and analyzed using confocal microscopy.

Quantitative real-time polymerase chain reaction (qPCR)

Bacterial DNA was purified from the fresh feces of mice using QIAamp Fast DNA stool mini kit (Qiagen) and its cDNA was prepared using cDNA synthesis kit [26]. qPCR for *E. fergusonii* and 16 S rRNA were performed using SYBER premix Ex Taq II (TaKaRa).

mRNAs (2 μ g) from the prefrontal cortex and thalamus (PT) were isolated using a RNeasy Mini kit (Qiagen) and their cDNAs were prepared using cDNA synthesis kit (TaKaRa) [28]. qPCR for N-methyl-D-aspartate receptor (NMDAR) R1/R2, serotonin 1 A receptor (5-HT_{1A}R), toll-like receptor 4 (TLR4), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using SYBER premix Ex Taq II (TaKaRa). Primers used for qPCR are listed in Supplementary Table S1.

Microbiota analysis

Microbial genomic DNA was extracted from mouse fecal samples using the QIAamp DNA Stool Mini Kit. Subsequently, amplification and sequencing of the 16 S rRNA gene were conducted according to previously described methods [12]. Sequencing data have been deposited in NCBI (PRJNA1122947).

Statistics

Data were presented as mean \pm standard deviation (SD) using GraphPad Prism 9. For multi-group comparisons of parametric data, either one-way analysis of variance (ANOVA) with Tukey's multiple comparisons post hoc test or two-way ANOVA with Tukey's post hoc test was employed. Non-parametric data were analyzed using the Kruskal-Wallis test with Dunn's multiple comparisons test ($p < 0.05$).

Results

FMT from Vsd caused depression-like symptoms in mice

To understand whether gut microbiota dysbiosis is associated with the onset of depression, we transplanted fecal microbiota from Vsd or Vh into mice and measured DA-like symptoms (Fig. 1a-h, Supplement Figure S1). FMT from Vsd (FVsd) decreased OT in the EPMT to 20.3% ($F_{2,18} = 12.32$, $p < 0.001$) of NC and increased immobility time (IT) in the TST to 123.0% ($F_{2,18} = 7.701$, $p = 0.003$) of NC. However, FMT from Vh (FVh) did not significantly alter DA-like behaviors in EPMT and TST. FVsd resulted in decreased expression of serotonin, BDNF, and IL-10, along with a reduction in the BDNF⁺NeuN⁺ cell population in the hippocampus. Furthermore, FVsd led to increased expression of TNF- α , IL-1 β , and IL-6 and an upregulation of the NF- κ B⁺Iba1⁺ cell population. However, oral gavage of FVh did not significantly affect DA-related biomarker levels.

FVsd also increased the levels of IL-6 and corticosterone in the blood and the expression of myeloperoxidase,

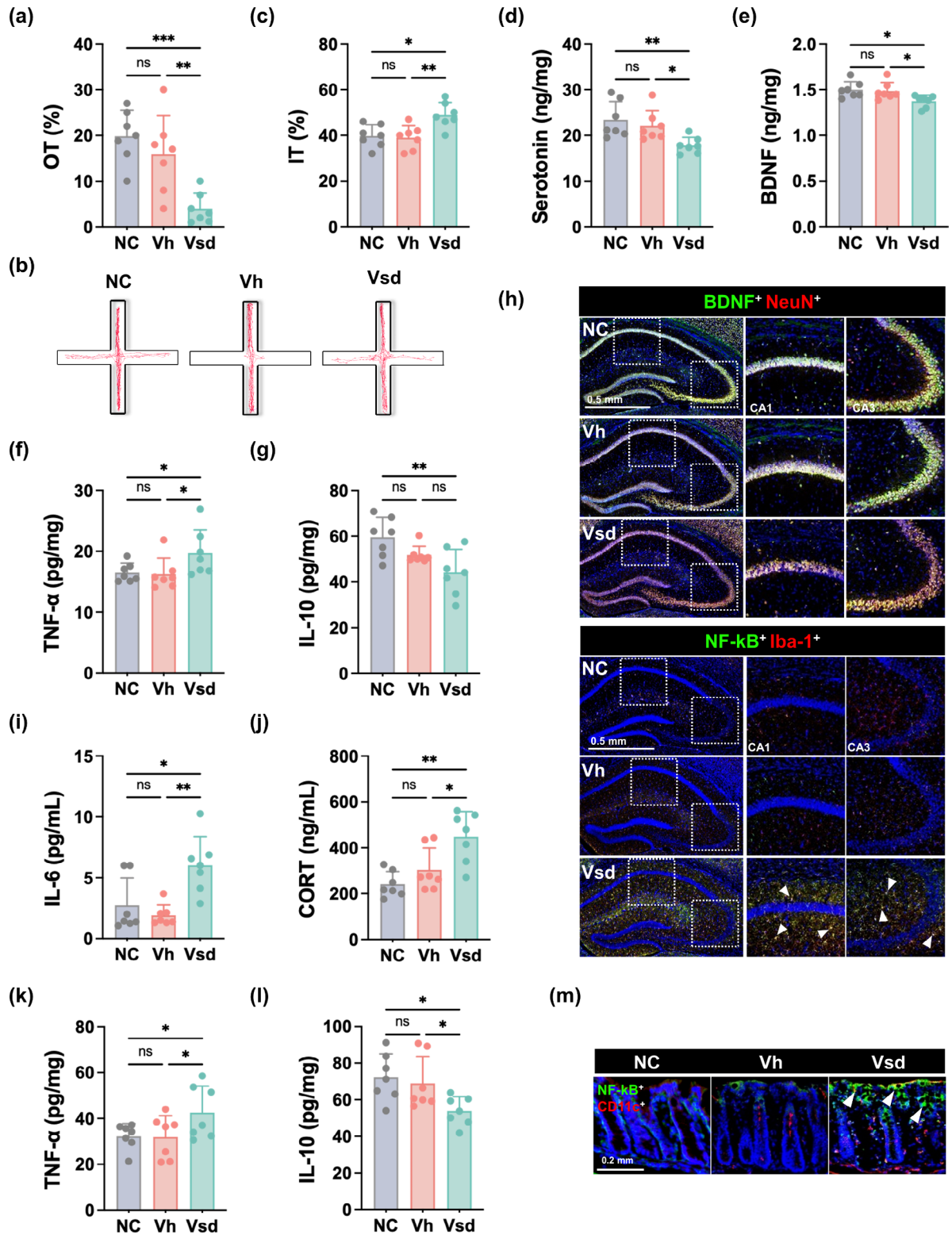


Fig. 1 Fecal microbiota transplantation (FMT) from Vsd caused DA-like behavior and neuroinflammation in mice. Effects of FVsd and FVh on OT (a) and track path (b) in the EPMT and IT in the TST (c). Effects on serotonin (d), BDNF (e), TNF-α (f), and IL-10 (g) expression and BDNF⁺NeuN⁺ and NF-κB⁺Iba-1⁺ cell populations (h) in the hippocampus. Effects on IL-6 (i) and corticosterone (CORT) levels (j) in the blood. Effects on TNF-α (k) and IL-10 expression (l) and NF-κB⁺CD11c⁺ cell populations (m) in the colon. NC, vehicle in normal control mice; Vsd, fecal microbiota of Vsd; Vh, the fecal microbiota of Vh. Data values were indicated as mean ± SD (n = 7). *p < 0.05. **p < 0.01. ***p < 0.001. ns, no significant

TNF- α , IL-1 β , and IL-6, and the NF- κ B⁺CD11c⁺ cell population in the colon, while decreasing IL-10 expression (Fig. 1i-m). In contrast, FVh had no significant impact on these measures.

Bacterial EVs exposed in the intestine are suggested to be able to cause psychiatric disorder through the vagus nerve [27]. Therefore, to understand the role of Vsd fecal EVs (vFEV) in the onset of depression, we investigated their effects on DA-like behavior and neuroinflammation in mice subjected to either sham or celiac vagotomy (Fig. 2, Supplement Figure S2). Oral gavage of vFEV decreased total travelled distance (TD), distance moved in the center area (DC), and time spent in the center area (TC) in mice in the OFT to 85.6% ($F_{4,30}=3.792$, $p=0.013$), 76.7% ($F_{4,30}=3.486$, $p=0.018$), and 73.0% ($F_{4,30}=3.737$, $p=0.013$) of NC, respectively. vFEV also decreased OT in the EPMT to 54.1% ($F_{4,30}=3.619$, $p=0.016$) of NC and increased IT in the TST to 126.0% ($F_{4,30}=3.746$, $p=0.013$) of NC. However, celiac vagotomy recovered vFEV-suppressed TD, DC, and TC in the OFT to 95.0% ($F_{4,30}=3.792$, $p=0.013$), 94.4% ($F_{4,30}=3.486$, $p=0.018$), and 88.7% ($F_{4,30}=3.737$, $p=0.013$) of NC, respectively. Furthermore, celiac vagotomy significantly recovered vFEV-suppressed OT in EPMT to 78.1% of NC and vFEV-induced IT in the TST to 107.6% of NC. Celiac vagotomy also decreased vFEV-induced TNF- α and IL-6 expression, as well as NF- κ B⁺Iba1⁺ and LPS⁺Iba1⁺ cell populations in the hippocampus. Conversely, celiac vagotomy increased the expression of BDNF and IL-10 suppressed by vFEV, along with the BDNF⁺NeuN⁺ (BDNF-positive neuron) cell population.

Oral administration of vFEV resulted in increased levels of TNF- α , IL-6, and LPS in the blood, and upregulated the expression of myeloperoxidase, TNF- α , IL-1 β and IL-6 and an increased NF- κ B⁺CD11c⁺ cell population in the colon. Additionally, vFEV elevated fecal LPS levels (Fig. 3, Supplement Figure S3). Celiac vagotomy did not significantly alter vFEV-induced biomarker levels in the blood, colon, and feces.

To investigate the influence of gut microbiota on the occurrence of DA, we analyzed the fecal microbiota composition of Vsd and Vh (Fig. 4, Supplement Tables S2 and S3). Alpha-diversity, measured by estimated operational taxonomic units (OTUs), was not significantly different between the two groups, whereas β -diversity showed the significant difference. The populations of *Proteobacteria* and *Firmicutes* including *Lachnospiraceae*, *Veillonellaceae*, and *Enterobacteriaceae* were higher in the Vsd than in the Vh, while the populations of *Actinobacteria* and *Verrucomicrobia* including *Coriobacteriaceae*, *Rikenellaceae*, and *Barnesiellaceae* were lower in the Vsd. Culturing on selective DHL agar plates, as well as qPCR analysis, revealed the higher abundance of *E. fergusonii* in Vsd feces compared to those of Vh feces. In particular,

exposure to *E. fergusonii* significantly induced TNF- α expression in Caco-2 cells and suppressed BDNF expression in SH-SY5Y cells (Supplement Figure S4).

Effect of *E. Fergusonii* on the occurrence of DA and colitis in mice with or without celiac vagotomy

To understand whether *E. fergusonii*, which was significantly more abundant in the feces of Vsd than in those of Vh, might contribute to the occurrence of depression, we assessed their impact on DA-like behavior and neuroinflammation in mice (Fig. 5, Supplement Figure S5). Oral gavage of *E. fergusonii* caused DA-like behaviors. In particular, *E. fergusonii* decreased OT in EPMT to 55.3% ($F_{3,24}=10.43$, $p<0.001$) of NC and TD, DC, and TC in the OFT to 86.7% ($F_{3,24}=6.861$, $p=0.002$), 61.3% ($F_{3,24}=5.746$, $p=0.004$), and 68.1% ($F_{3,24}=3.133$, $p=0.044$) of NC. They increased IT in the TST to 123.8% ($F_{3,24}=15.56$, $p<0.001$) of NC. Oral gavage of *E. fergusonii* upregulated the expression of TNF- α , IL-1 β , and IL-6 and the activation of NF- κ B in the hippocampus, along with NF- κ B⁺Iba1⁺ and LPS⁺Iba1⁺ cell populations, while reducing the expression of BDNF, serotonin, and IL-10, as well as the BDNF⁺NeuN⁺ cell population. Furthermore, *E. fergusonii* decreased BDNF, serotonin, and 5-HT_{1A}R expression in the prefrontal cortex, while TNF- α , TLR-4, and NF- κ B expression increased. *E. fergusonii* did not significantly affect NMDAR R1 and R2 expression.

However, celiac vagotomy significantly attenuated *E. fergusonii*-induced DA-like behaviors: it recovered TD, DC, and TC in the OFT to 96.4%, 90.7%, and 89.1% of NC, respectively, OT in the EPMT to 66.7% of NC, and IT in the TST to 98.2% of NC. Celiac vagotomy also reduced *E. fergusonii*-induced expression of TNF- α , IL-1 β and IL-6 and activation of NF- κ B, as well as NF- κ B⁺Iba1⁺ and LPS⁺Iba1⁺ cell populations in the hippocampus. It also recovered *E. fergusonii*-suppressed IL-10 expression and BDNF⁺NeuN⁺ cell population. Furthermore, celiac vagotomy increased *E. fergusonii*-suppressed decreased BDNF, serotonin, and 5-HT_{1A}R expression in the prefrontal cortex, while *E. fergusonii*-induced TNF- α , TLR4, and NF- κ B expression decreased. Celiac vagotomy did not alter the NMDAR R1 and R2 expression in the prefrontal cortex.

Next, we examined the effect of *E. fergusonii* on the occurrence of gut inflammation in mice with or without vagotomy (Fig. 6, Supplement Figure S6). Orally gavaged *E. fergusonii* increased levels of IL-6, corticosterone, and LPS in the blood, as well as myeloperoxidase, TNF- α , and IL-6 expression and NF- κ B⁺Iba1⁺ cell population in the colon. Additionally, it elevated LPS levels in the feces. However, celiac vagotomy did not significantly alter these biomarker levels in the blood, colon, and feces.

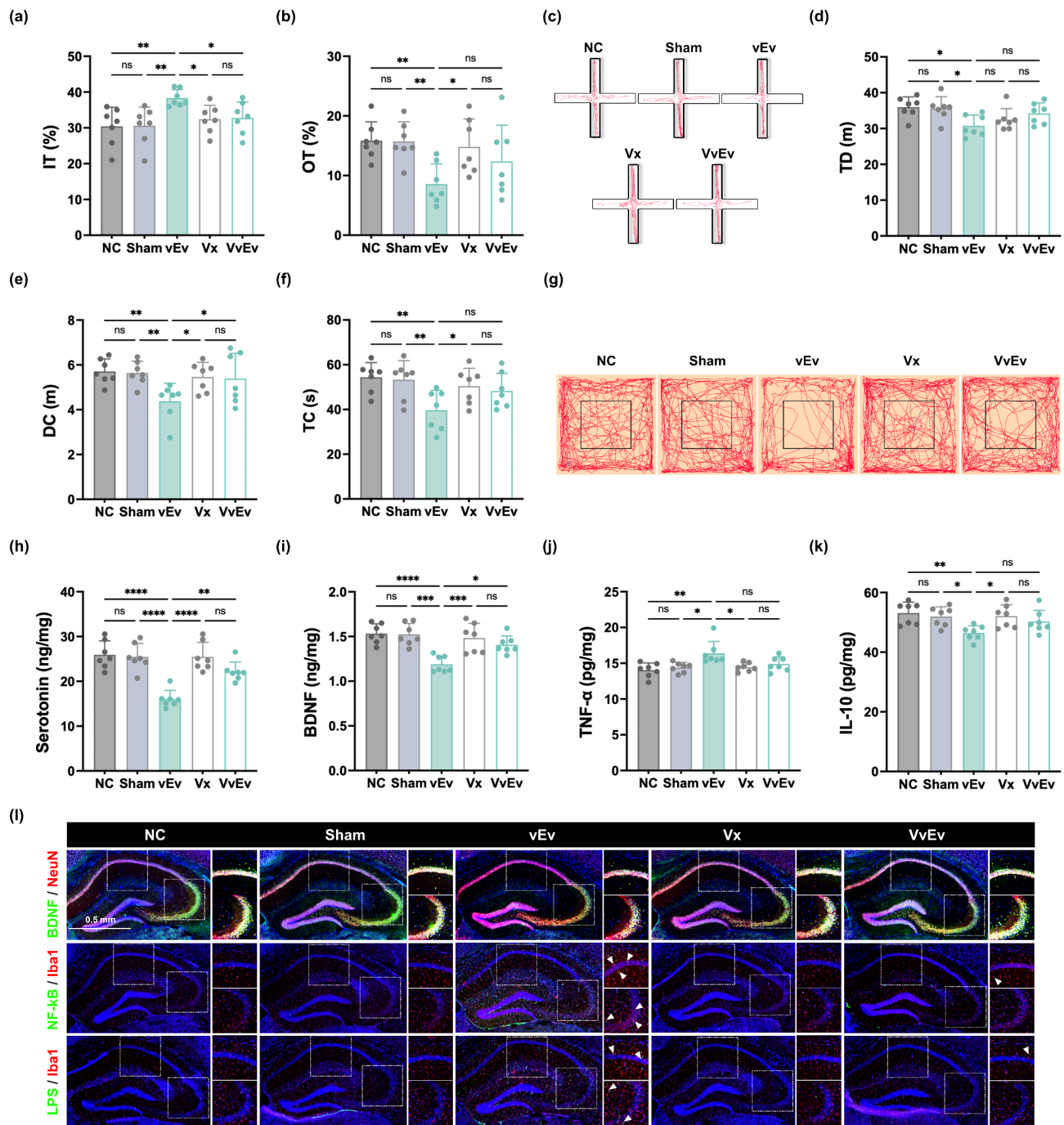


Fig. 2 EV from FvSD (vEv) caused DA-like behavior and neuroinflammation in mice. Effect on IT in the TST (a), OT (b) and track path (c) in the EPMT, and total travelled distance (TD, d), distance travelled in the center (DC, e), and time spent in central area (TC, f), and track path (g) in OFT. Effect on serotonin (h), BDNF (i), TNF-α (j), and IL-10 expression (k), and BDNF+NeuN+, NF-κB+Iba1+, and LPS+Iba1+ cell populations (l) in the hippocampus. NC, vehicle in normal control mice; Sham, vehicle; vEv, vEV in mice; Vx, vehicle in mice with vagotomy; VvEv, vEV in mice with vagotomy. Data values were indicated as mean ± SD (n = 7). *p < 0.05. **p < 0.01. ***p < 0.001. ****p < 0.0001. ns, no significant

Effect of *E. Fergusonii* EV (esEV) on the occurrence of depression and colitis in mice with or without celiac vagotomy

esEV isolated from the culture of *E. fergusonii* in GAM broth was round, vesicular structures of 30–70 nm,

which consisted of proteins, lipopolysaccharide, and nucleic acid (Supplement Figure S7a-c). Of proteins, ompA, ompC, and LpoB (outer membrane lipoprotein B) were main components. The esEV significantly induced the expression of TNF-α in Caco-2 cells and decreased

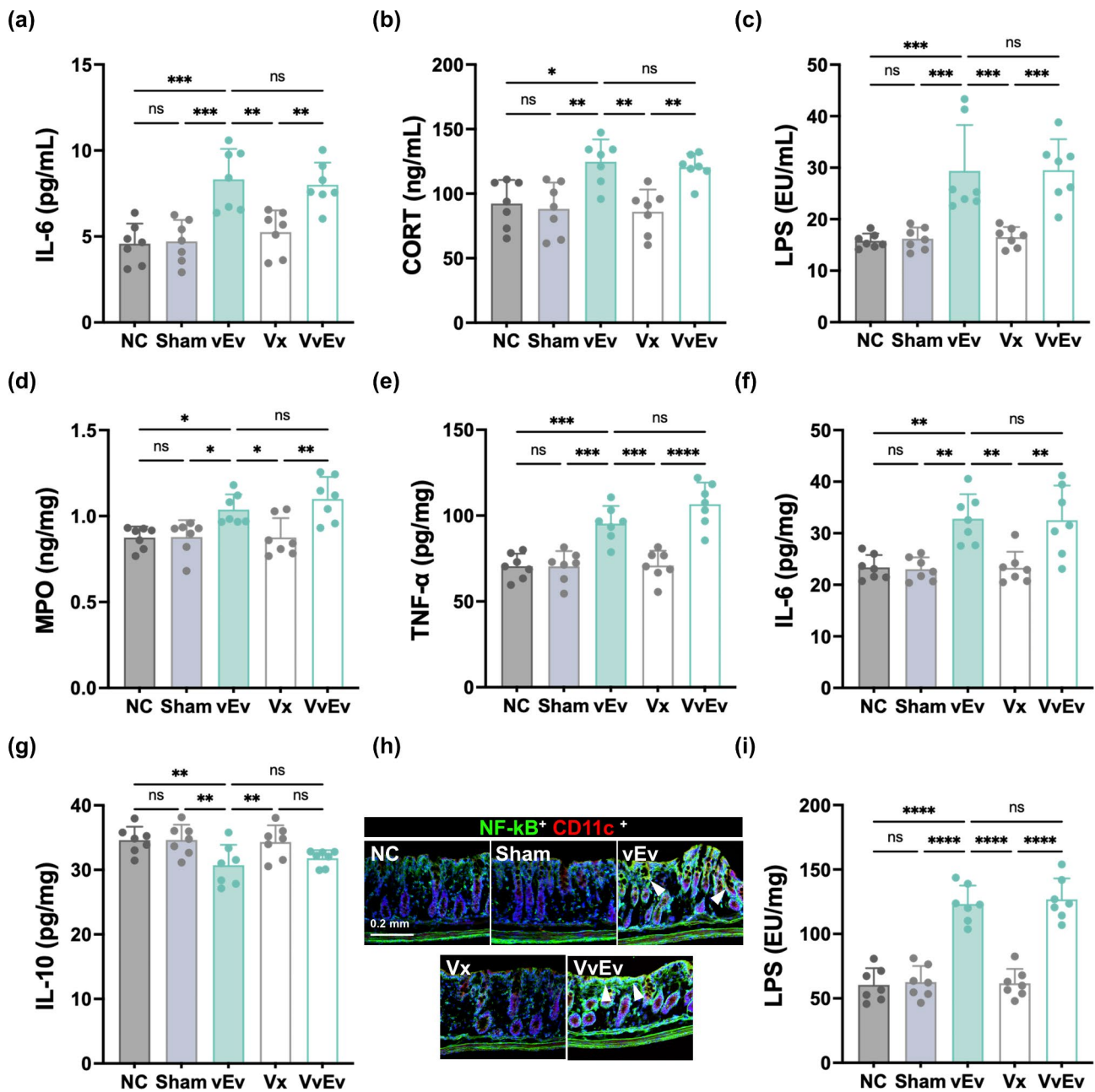


Fig. 3 vEv increased inflammation-related marker levels in the blood and colon of mice. Effect of vEv on IL-6 (a), corticosterone (CORT, b), and LPS levels (c) in the blood. Effect on myeloperoxidase (MPO, d), TNF- α (e), IL-6 (f), and IL-10 expression (g) and NF- κ B⁺CD11c⁺ cell populations (h) in the colon. (i) Effect on fecal LPS level. NC, vehicle in normal control mice; Sham, vehicle; vEv, vEv in mice; Vx, vehicle in mice with vagotomy; VvEv, vEv in mice with vagotomy. Data values were indicated as mean \pm SD ($n = 7$). * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$. ns, no significant

the expression of BDNF in SH-SY5Y cells (Supplement Figure S7d, e).

Next, we examined the effects of esEV on DA-like behavior and neuroinflammation in mice (Fig. 7, Supplement Figure S8). Orally gavaged esEV decreased it decreased OT in EPMT to 61.8% ($F_{3,24} = 4.998$, $p = 0.007$) of NC and TD, DC, and TC in the OFT to 85.2% ($F_{3,24} = 3.639$, $p = 0.027$), 73.4% ($F_{3,24} = 4.481$, $p = 0.012$) and 70.0% ($F_{3,24} = 5.062$, $p = 0.007$) of NC,

respectively, and increased IT in the TST to 129.9% ($F_{3,24} = 4.272$, $p = 0.015$) of NC. esEV treatment upregulated the expression of TNF- α , IL-1 β , and IL-6 and the activation of NF- κ B in the hippocampus, as well as the NF- κ B⁺Iba1⁺ and LPS⁺Iba1⁺ cell populations, while reducing the expression of BDNF and IL-10, as well as the BDNF⁺NeuN⁺ cell population. Furthermore, esEV decreased BDNF, serotonin, and 5-HT_{1A}R expression in the prefrontal cortex, while increasing the expression of

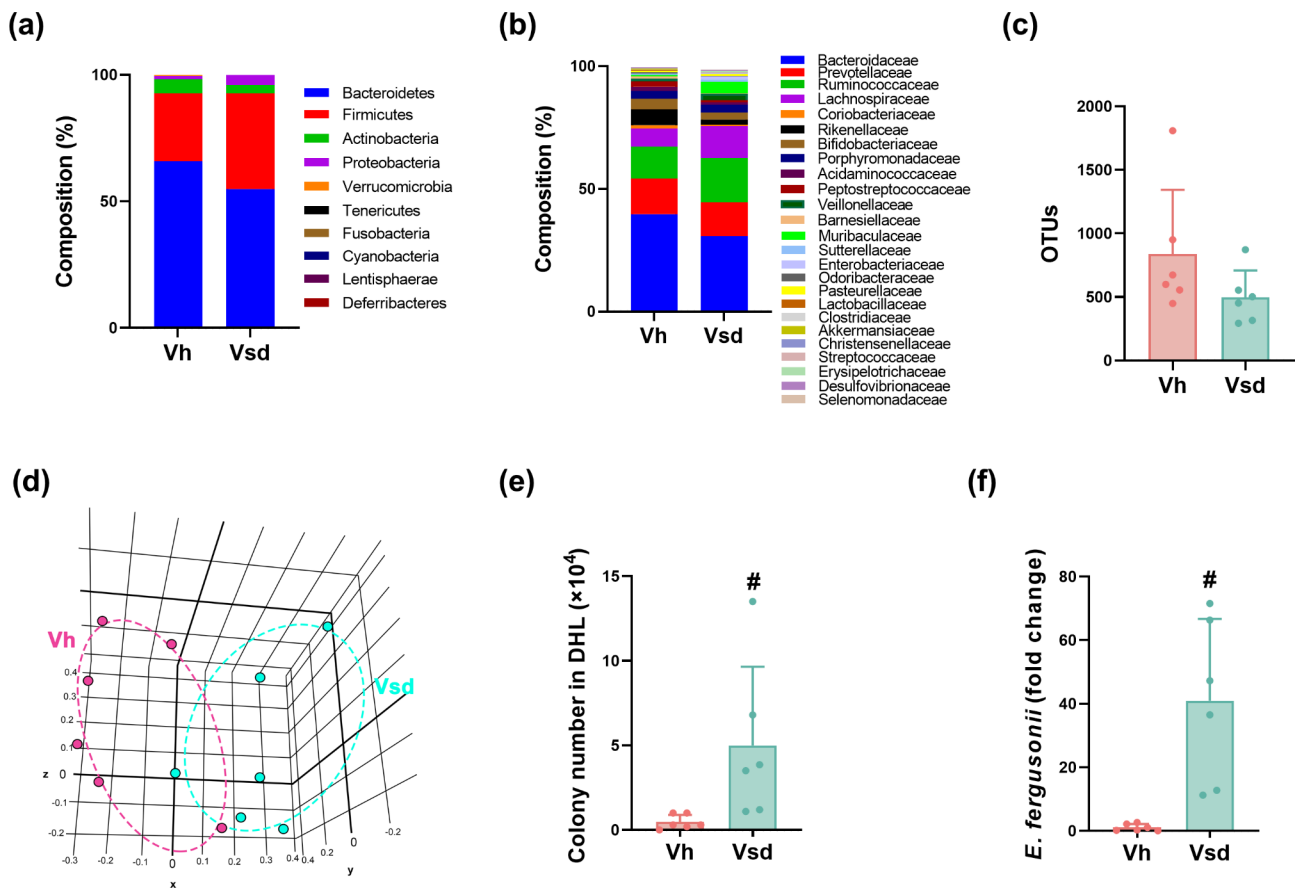


Fig. 4 The fecal microbiota composition of Vsd and Vh. **(a)** The composition at the phylum level **(a)** and family level **(b)**. The α -diversity (OTU richness, **c**) and β -diversity (principal coordinate analysis plot based on Jensen-Shannon analysis, **d**). The number of gut bacteria *Enterobacteriaceae* and *Enterococcaceae* grown in DHL **(e)** agar plates (inoculating Vsd and Vh feces). The levels of *E. fergusonii* **(f)**, assessed Vsd and Vh feces by qPCR. Fmci, the feces of volunteers with MCI; Fhv, the feces of healthy volunteers. Vsd, $n=6$; Vh, $n=6$. # $p < 0.05$ vs. Vh

TNF- α , TLR-4, and NF- κ B expression. The expression of NMDAR R1 and R2 was not affected by exposure to esEV. Celiac vagotomy partially recovered esEV-induced DA-like behaviors: it increased OT in the EMPT to 86.5% of NC and TD, DC, and TC in the OFT to 93.3%, 89.0%, and 87.7% of NC, respectively, and decreased IT in the TST to 109.4% of NC. Furthermore, celiac vagotomy decreased esEV-induced hippocampal TNF- α , IL-1 β and IL-6 expression and NF- κ B^{Iba1+} and LPS^{Iba1+} cell populations. The vagotomy also attenuated esEV-mediated suppression of hippocampal IL-10 expression and BDNF^{NeuN+} cell population. Celiac vagotomy partially recovered esEV-suppressed BDNF, serotonin, and 5-HT_{1A}R expression in the prefrontal cortex and expression, while decreasing esEV-induced TNF- α , TLR-4, and NF- κ B expression. Celiac vagotomy did not alter the NMDAR R1 and R2 expression in the prefrontal cortex.

Oral gavage of esEV increased IL-6, corticosterone, and LPS levels in the blood, myeloperoxidase, TNF- α , IL-1 β , and IL-6 expression and the NF- κ B^{Iba1+} cell population in the colon, and LPS level in the feces (Fig. 8,

Supplement S9). Celiac vagotomy did not significantly affect these biomarker levels in the blood, colon, and feces.

esEV caused depression-like behavior and neuroinflammation through the vagus nerve. Therefore, to confirm whether orally gavaged esEV could be translocated into the brain, we prepared FITC-labelled esEV (FEV), and orally gavaged it in mice with or without celiac vagotomy (Fig. 9). When FEV was gavaged in mice (without vagotomy), it was detectable in the hippocampal pyramidal region and blood. However, when orally administered to mice that had undergone celiac vagotomy, FEV was undetectable in the hippocampus although it remained detectable in the blood.

Discussion

The gut microbiota communicates bidirectionally with the brain via the immune, endocrine, and nervous systems, which control the expression and turnover of neurotransmitters and cytokines [13, 17, 29, 30]. Exposure to stressors such as social defeat disrupts the expression of neurotransmitters and cytokines through the

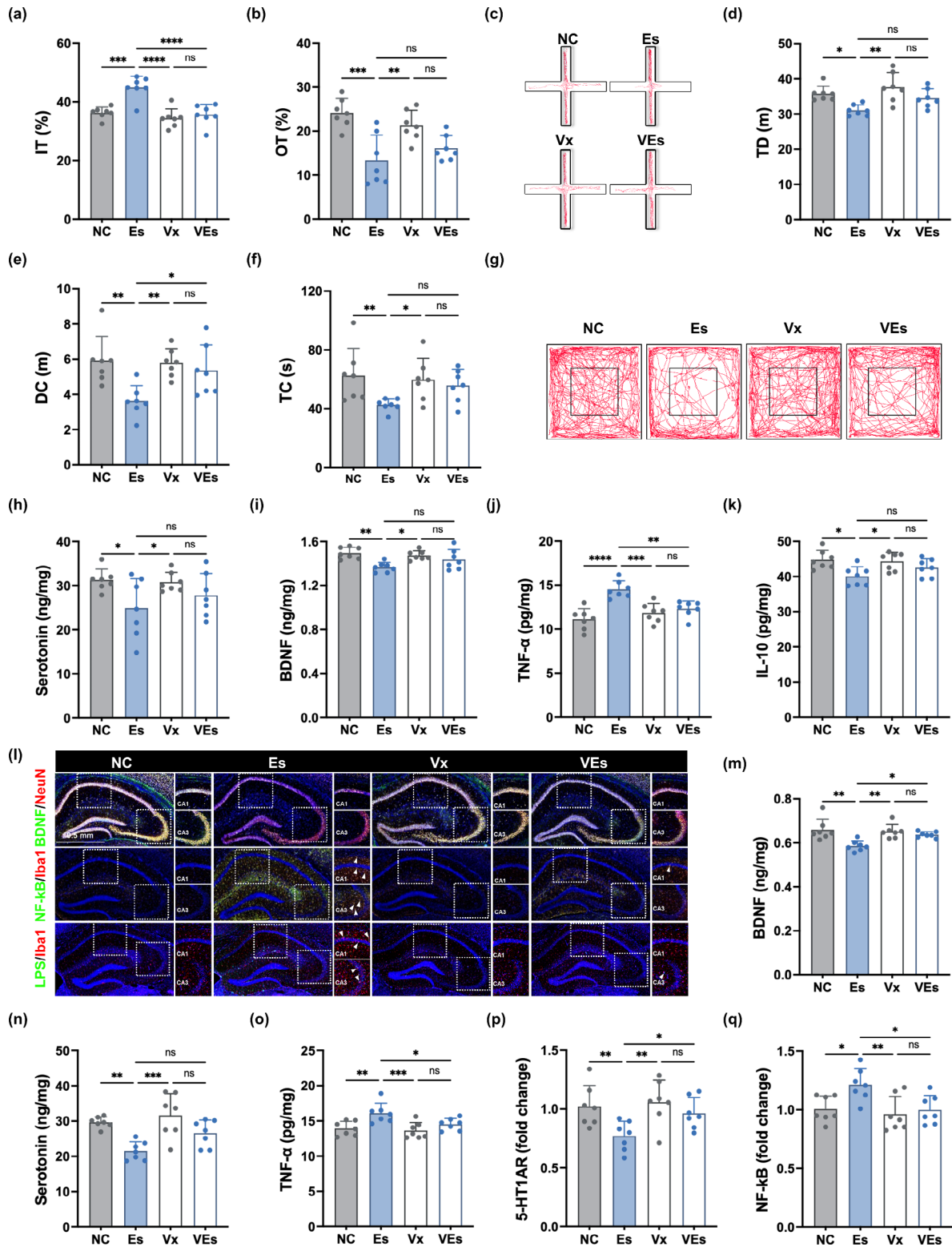


Fig. 5 Effect of *E. fergusonii* on DA-like behavior and neuroinflammation in mice with or without celiac vagotomy. Effect on IT in the TST (a), OT (b) and track path (c) in the EPMT, and TD (d), DC (e), TC (f), and track path (g) in OFT. Effect on serotonin (h), BDNF (i), TNF- α (j), and IL-10 expression (k) and BDNF+NeuN⁺, NF- κ B⁺Iba1⁺, and LPS⁺Iba1⁺ cell populations (l) in the hippocampus. Effect on BDNF (m), serotonin (n), TNF- α (o), 5-HT_{1A}R (p), and NF- κ B (q) in the prefrontal cortex. NC, vehicle in normal control mice; sham, vehicle; Es, *E. fergusonii* in mice; Vx, vehicle in mice with vagotomy; VEs, *E. fergusonii* in mice with vagotomy. ** $p < 0.05$. *** $p < 0.01$. **** $p < 0.001$. ***** $p < 0.0001$. ns, no significant

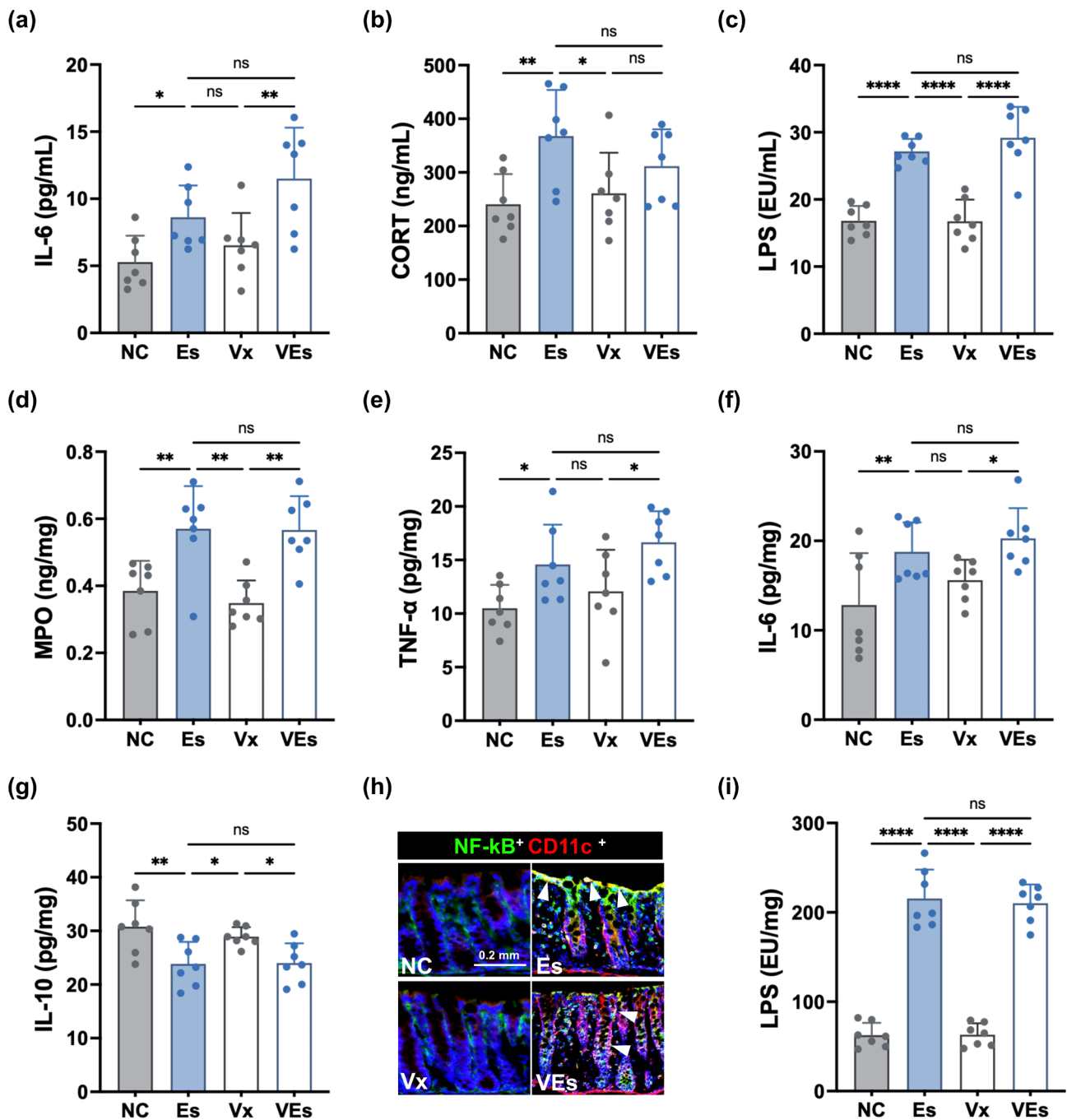


Fig. 6 Effects of *E. fergusonii* on DA-related biomarker levels in the blood and colon of mice with or without celiac vagotomy. Effects on IL-6 (a), corticosterone (CORT, b), and LPS levels (c) in the blood. Effects on myeloperoxidase (MPO, d), TNF-α (e), IL-6 (f), and IL-10 expression (g) and NF-κB⁺CD11c⁺ cell populations (h) in the colon. (i) Effects on fecal LPS level. NC, vehicle in normal control mice; sham, vehicle; Es, *E. fergusonii* in mice; Vx, vehicle in mice with vagotomy; VEs, *E. fergusonii* in mice with vagotomy. Data values were indicated as mean ± SD (n = 7). *p < 0.05. **p < 0.01. ****p < 0.0001. ns, no significant

hypothalamic-pituitary-adrenal axis, leading to DA [31, 32]. Oral exposure to pathogens such as *Klebsiella oxytoca* and *Escherichia coli* alters gut microbiota and its byproducts, which induces proinflammatory cytokine expression in gut immune cells, leading to gut dysbiosis and inflammation. [26, 33]. Gut dysbiosis-mediated intestinal inflammation induces NF-κB activation and

suppresses BDNF expression in the brain, thereby contributing to the development of DA [12, 25]. However, understanding the pathogenesis of gut pathogen-mediated depression is still elusive.

In the present study, the fecal microbiota from Vsd induced DA-like behaviors in mice. Vsd feces exhibited the higher abundance of *Proteobacteria* population

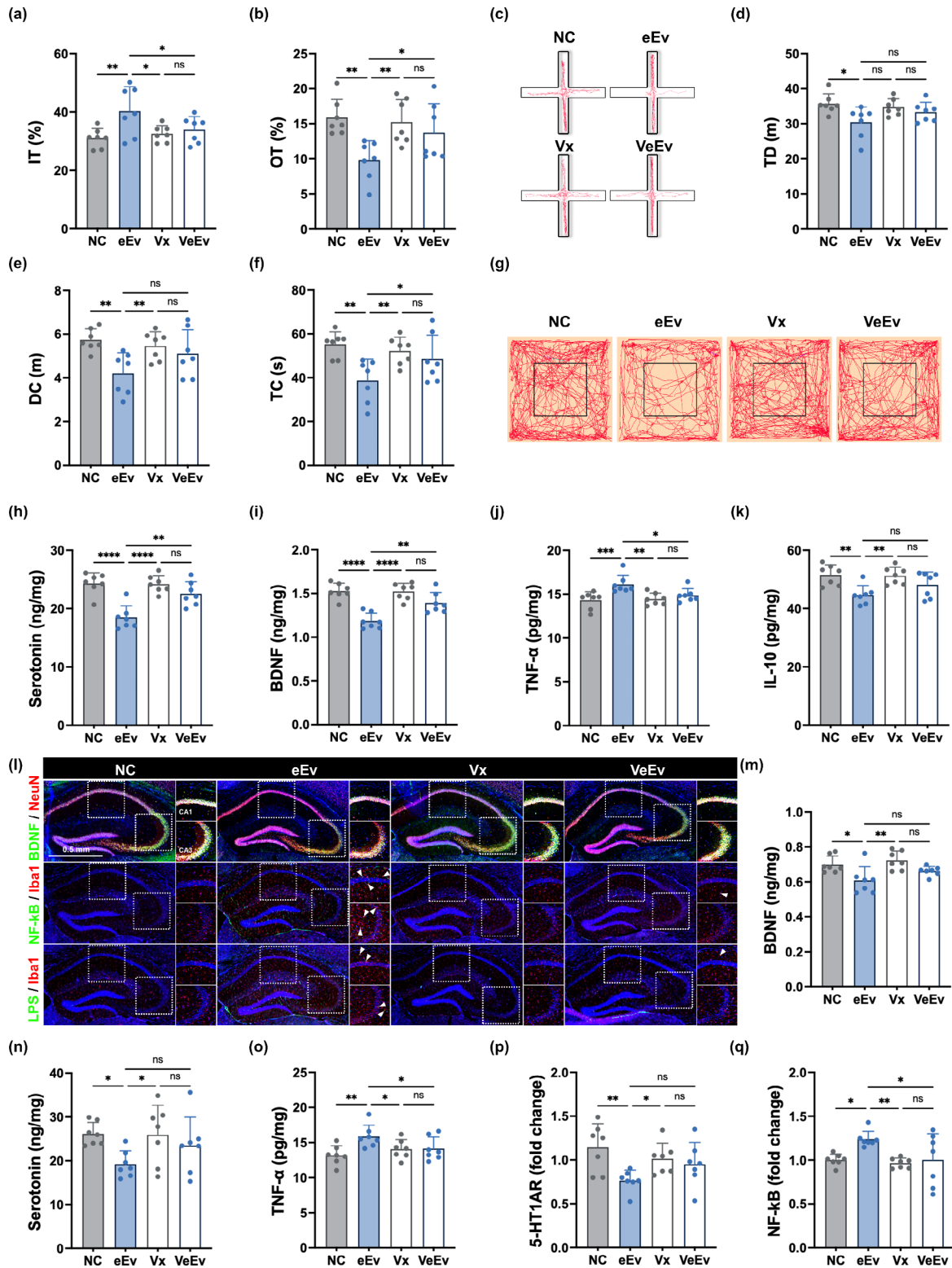


Fig. 7 Effect of esEV on DA-like behavior and neuroinflammation in mice with or without celiac vagotomy. Effects on IT in the TST (a), OT (b) and track path (b) in the EPMT, and TD (d), DC (e), TC (f), and track path (g) in OFT. Effects on serotonin (h), BDNF (i), TNF-α (j), and IL-10 expression (k) and BDNF+NeuN+, NF-κB+Iba1+, and LPS+Iba1+ cell populations (l). Effect on BDNF (m), serotonin (n), TNF-α (o), 5-HT_{1A}R (p), and NF-κB (p) in the prefrontal cortex. NC, vehicle; eEv, esEV in mice; Vx, vehicle in mice with vagotomy; VeEv, esEV in mice with vagotomy. Data values were indicated as mean ± SD (n = 7). *p < 0.05. **p < 0.01. ***p < 0.001. ****p < 0.0001. ns, no significant

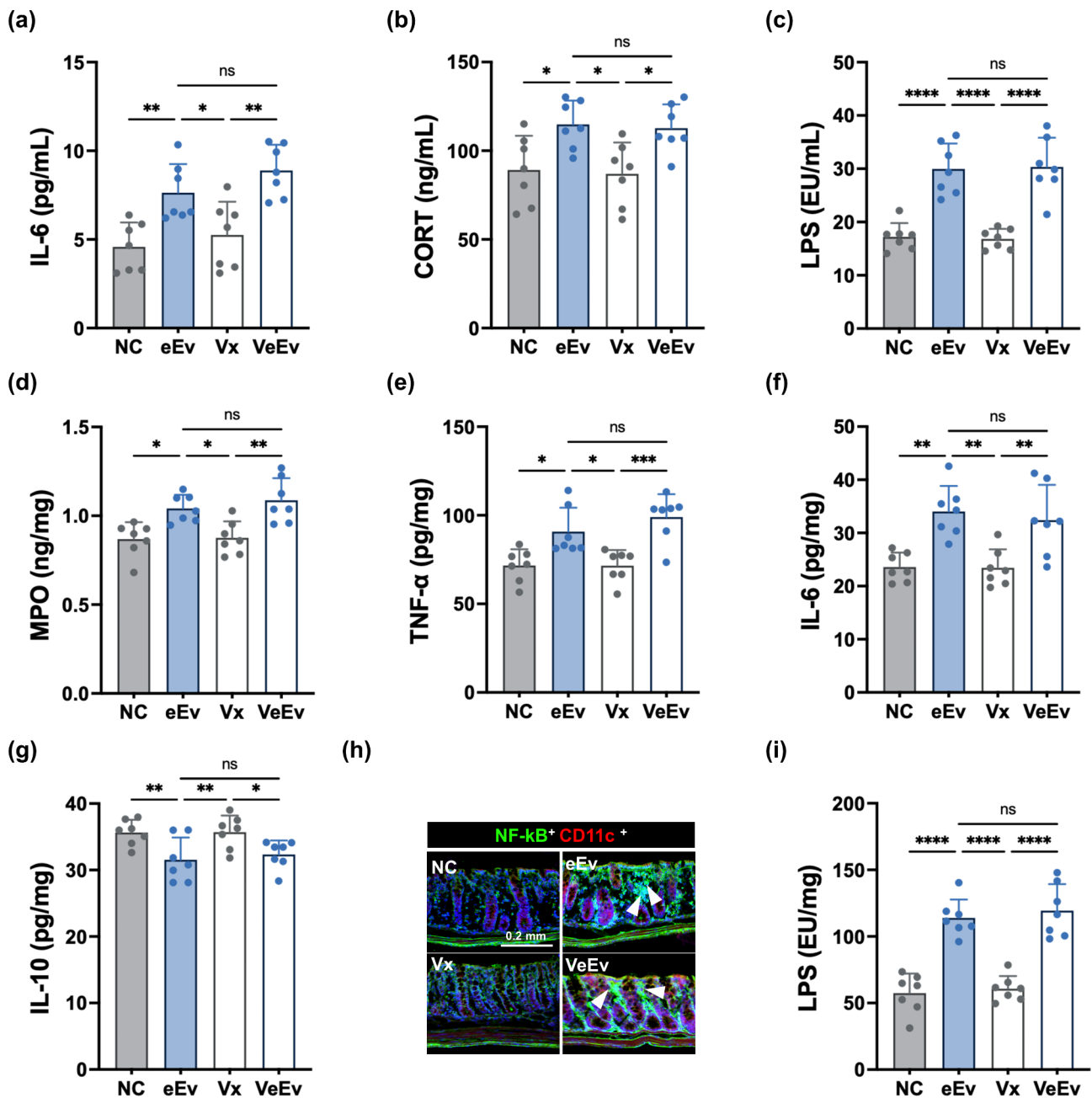


Fig. 8 Effect of esEV on inflammation-related marker levels in the blood and colon mice. Effects on IL-6 (a), corticosterone (CORT, b), and LPS levels (c) in the blood. Effects on myeloperoxidase (MPO, d), TNF-α (e), IL-6 (f), and IL-10 expression (g) and NF-κB⁺CD11c⁺ cell populations (h) in the colon. (i) Effect on fecal LPS level. NC, vehicle; eEv, esEV in mice; Vx, vehicle in mice with vagotomy; VeEv, esEV in mice with vagotomy. Data values were indicated as mean ± SD (n = 7). *p < 0.05. **p < 0.01. ***p < 0.001. ****p < 0.0001. ns, no significant

compared to Vh feces, as previously reported [25]. Of Vsd feces-rich bacteria, we isolated gram-negative *E. fergusonii*, which induced the expression of TNF-α in Caco2 cells and suppressed the expression of BDNF in SH-SY5Y cells. When *E. fergusonii* was orally gavaged in mice, TD, DC, and TC in the OFT were decreased. Of these, DC the most strongly decreased, followed by TC. *E. fergusonii* also strongly increased IT in the TST and decreased OT in the EPMT. These suggest that *E.*

fergusonii infection can cause DA-like behavior. Orally gavaged *E. fergusonii* increased TNF-α, IL-6, and TLR4 expression levels, and NF-κB⁺ and LPS⁺ microglial cell number in the hippocampus and prefrontal cortex, while decreasing the expression of BDNF, serotonin, and its receptor 5-HT_{1A}R, but not NMDAR. Oral gave of *E. fergusonii* also increased TNF-α and LPS levels in the blood and elevated TNF-α and IL-6 expression and NF-κB-positive immune cell numbers in the colon and

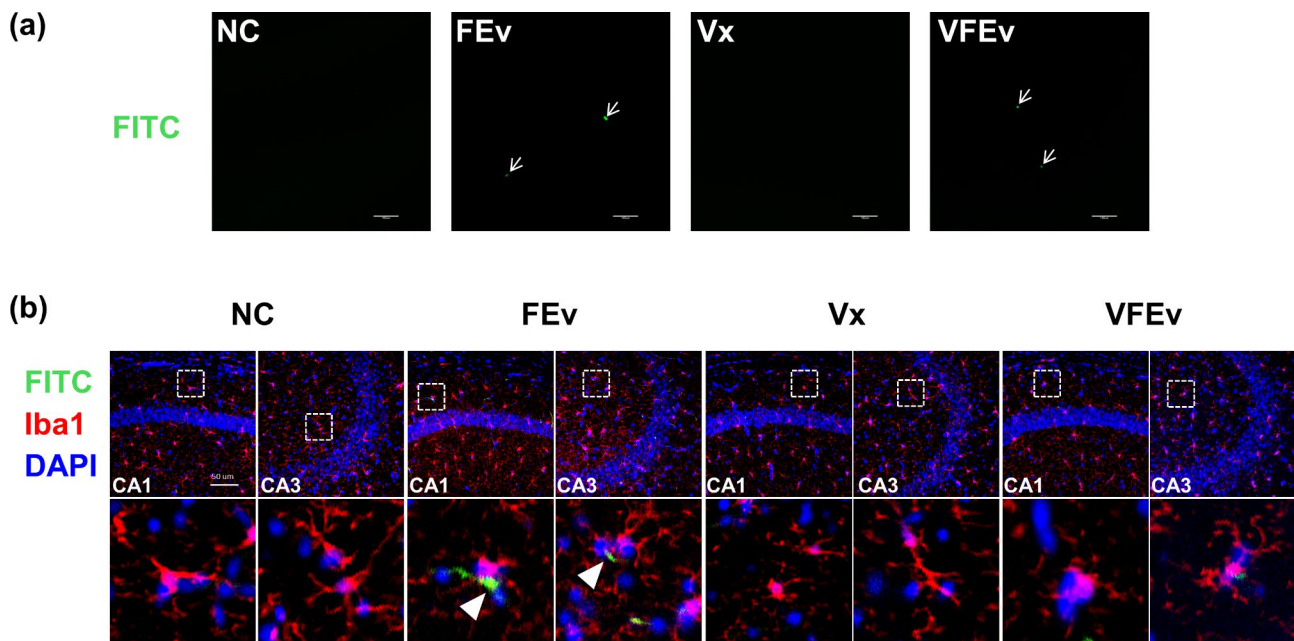


Fig. 9 Orally gavaged FITC-labeled esEV (FEV) was translocated into the blood (a), hippocampus CA1 and CA3 (b). FITC-labeled esEV and vehicle were orally gavaged. NC, vehicle in normal mice; FEV, FITC-labeled esEV in mice; Vx, vehicle in normal mice; VFEV, FITC-labeled esEV in mice with vagotomy.

bacterial LPS production levels in the feces. These findings suggest that *E. fergusonii* overgrown in Vsd can induce gut inflammation through excessive production of pro-inflammatory bacterial metabolites (e.g., LPS) and facilitate their translocation via the bloodstream to the brain, thereby triggering DA-like behaviors and neuroinflammation by suppressing BDNF and serotonin/5-HT receptor (5-HTR) expression and inducing TNF- α expression through the activation of TLR4-mediated NF- κ B signaling.

However, gram-negative *E. fergusonii*-induced DA-like behaviors, hippocampal TNF- α , IL-1 β , IL-6 expression levels, and NF- κ B⁺ and LPS⁺ microglial cell numbers were attenuated by celiac vagotomy. The vagotomy also attenuated the suppression of serotonin, BDNF, and 5-HTR expression by oral exposure to *E. fergusonii*. Nevertheless, celiac vagotomy did not affect *E. fergusonii*-induced TNF- α and LPS levels in the blood, expression levels of TNF- α and IL-6, number of NF- κ B-positive immune cells in the colon, and LPS production levels in the feces. These findings suggest that gram-negative gut bacteria such as *E. fergusonii* may produce transmittable metabolites that can reach the brain via the vagus nerve, potentially inducing neuroinflammation and contributing to DA-like behavior by upregulating TNF- α expression and downregulating BDNF and serotonin/5-HTR expression through the activation of TLR4-mediated NF- κ B signaling.

EV was easily isolated from the culture of gram-negative *E. fergusonii*. However, EV could not be isolated from the culture of gram-positive *E. faecium*, which

was isolated from human gut microbiota, under various extraction conditions. Although no representative, these results suggest that gram-negative bacteria may produce EVs in abundance compared to gram-positive bacteria. Oral gavage of esEVs isolated from the culture of *E. fergusonii* were induced DA-like behavior, hippocampal TNF- α and IL-6 expression levels, and NF- κ B⁺ and LPS⁺ microglial cell numbers and suppressed hippocampal BDNF and serotonin levels in mice. Interestingly, celiac vagotomy significantly attenuated esEVs-induced DA-like behavior, TNF- α , IL-6, TLR4, and NMDAR expression levels and increased esEVs-suppressed BDNF, serotonin, and 5HT_{1A}R expression levels in the hippocampus and prefrontal cortex. Furthermore, esEV, which consisted of LPS, proteins containing outer membrane proteins (omps), and nucleic acid, induced the expression of TNF- α in Caco-2 cells and the expression of BDNF in SH-SY5Y neuronal cells. LPS and omps induce inflammatory responses in vivo [12, 34]. In particular, omps such as ompA contribute to invasion of intestinal epithelial cells and brain microvascular endothelial cells (the brain-blood-barrier) [34–36]. Moreover, orally gavaged FITC-labeled esEV was translocated into the blood and the brain. However, it was hardly detected in the hippocampus of mice with celiac vagotomy. Celiac vagotomy attenuates gram-negative *Paenacaligenes hominis*- or its EV-induced cognitive impairment in mice [27]. Gram-negative *Porphyromonas gingivalis*- and its EV-induced cognitive impairment are attenuated by trigeminal neurectomy [37]. These results suggest that gram-negative *E. fergusonii* can produce EV, which may be translocated

into the brain from the gut through the vagus nerve and blood, and may cause neuroinflammation by inducing NF- κ B activation, which can suppress BDNF, serotonin, and 5-HTR expression and induce TNF- α expression in the brain, resulting in DA (Supplementary [Graphic abstract](#)).

Conclusions

E. fergusonii overpopulated in Vsd can cause DA and neuroinflammation by overproducing inflammation-inducible gut bacteria byproducts such as LPS and EV, inducing gut inflammation, and accelerating their translocation into the brain, resulting in DA. Gram-negative *E. fergusonii* may produce inflammation-inducible EV, which may be translocated into the brain through the vagus nerve and blood and then suppress BDNF, serotonin, and 5-HTR expression and induce TNF- α expression in the brain through the NF- κ B activation, resulting in depression and neuroinflammation. Finally, gram-negative *E. fergusonii* and its EV may be the risk factor for DA and neuroinflammation.

Abbreviations

DA	Depression and anxiety
DAPI	4',6-diamidino-2-phenylindole
DC	Distance moved in the center area
EC	Number of entries in the center area
ELISA	Enzyme-linked immunosorbent assay
EMPT	Elevated plus maze test
esEV	EV of Esf culture
Esf	Escherichia fergusonii
EV	Extracellular vesicle
FEV	FITC-labelled esEV
FITC	Fluorescence isothiocyanate
FMT	Fecal microbiota transplantation
GABA	γ -aminobutyric acid
IBD	Inflammatory bowel disease
IT	Immobility time
LAL	Limulus Amebocyte Lysate
LPS	Lipopolysaccharide
OE	Number of open arm entries
OFT	Open-field test
OT	Time spent in open arm
PBS	Phosphate-buffered saline
SD	Standard deviation
SRI	Stress Response Inventory
TNF	Tumor necrosis factor
vFEV	EV of Vsd feces
Vh	Healthy volunteers
Vsd	Volunteers with psychological stress and subclinical symptoms of depression
TST	Tail suspension test
TD	Total travelled distance
TC	Time spent in the center area

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12974-024-03211-7>.

Supplementary Material 1

Supplementary Material 2

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Author contributions

XM, IYY, and DHK conceived and designed experiments and wrote the main manuscript text. MX, HSP, YJS, JKK, JKH, SWH, IYY prepared Figs. 1, 2, 3 and 4. MX and HSP prepared Figs. 5, 6, 7, 8 and 9. All authors contributed to reviewing the paper and agreed on the present version for submission.

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Data availability

The data that support the findings of this study are openly available in figures, table and NCBI under accession number PRJNA913477. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Declarations

Ethical approval

The study protocol and consent forms for the collection of stool samples were approved by the Committee for the Care and Use of Clinical Study in the Medical School of Kyung Hee University (IRB No., KMC-202103102) and the Institutional Review Board of Seoul National University Bundang Hospital (IRB No., B-1807/483-005). All animal experiments were approved by the Institutional Animal Care and Use Committee of Kyung Hee University (IACUC No., KUASP(SE)-21451) and ethically performed according to the University Guide for Laboratory Animals Care and Usage.

Competing interests

The authors declare no competing interests.

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