Research Paper

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Antihyperalgesic properties of gut microbiota: *Parabacteroides distasonis* as a new probiotic strategy to alleviate chronic abdominal pain

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Abstract

The potential role of gut microbiota in pain modulation is arousing an emerging interest since recent years. This study investigated neuromodulatory properties of gut microbiota to identify next-generation probiotics to propose alternative therapies for visceral pain management. Neuromodulation ability of 10 bacterial strains isolated from a healthy donor was assessed both on ND7/23 immortalized cell line and primary neuronal cells from rat dorsal root ganglia. This screening highlighted the neuroinhibitory property of *Parabacteroides distasonis* (F1-2) strain, supported both by its intracellular content and membrane fraction, which was further investigated in visceral pain mouse models. Oral administration of F1-2 resulted in a significant decrease of colonic hypersensitivity (CHS) in dextran sulfate sodium (0.5%) model associated with low-grade inflammation and a significant decrease of CHS in *Citrobacter rodentium* postinfectious models. No effect of F1-2 oral administration on CHS was observed in a neonatal maternal separation stress model. Antihyperalgesic effect unlikely involved modulation of inflammatory processes or restoration of intestinal barrier. Exploration of direct dialogue mechanisms between this strain and nervous system, assessed by calcium imaging experiments, revealed that F1-2 interacts directly with nociceptors by reducing activation level on capsaicin, inflammatory soup, and bradykinin stimulations. Our study provides new insights about bacteria–host interaction and places *P distasonis* as a potential therapeutic strategy in the treatment of visceral pain observed in leaky gut–associated pathologies.

Keywords: Gut microbiota, Visceral pain modulation, Antihyperalgesia, *Parabacteroides distasonis*

1. Introduction

In addition to dysregulation of immune response and changes in stool consistency or frequency, the development of chronic abdominal pain is part of symptomatology associated with inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS).[11,30,63] Owing to the complex etiology of IBS, treatment aims at relieving patients rather than treating the pathology and is poorly effective.[47] Anti-inflammatory drugs or immunomodulators offer a large therapeutic arsenal to control the inflammation of IBD, but management of chronic abdominal pain remains challenging mainly because of the intestinal adverse effects associated with pharmacological analgesic therapies.[19]

Impairment of the tripartite microbiota–gut–brain communication is a well-established feature of many intestinal pathologies, and a dysbiotic microbiome is now recognized as a hallmark of IBS and IBD with a relative decrease in Firmicutes and an increase in Bacteroidetes and Enterobacteriaceae.[22,25,27,58] At the peripheral level, although the sensation of pain can may be initiated through the release of inflammatory mediators triggered by microbial signals, recent evidence shows that direct interaction between microbial cells and nervous system is possible and could lead to subsequent pain signaling and associated comorbidities.[6,10,17,18,34,37] Recent reports also suggest beneficial effects of some prevalent bacterial species with reduced abundancy in several pathologies. Some, such as *Faecalibacterium prausnitzii*,[7,44,54] *Akkermansia muciniphila*,[16,17] and *Parabacteroides distasonis*,[15,28,33] have anti-inflammatory or antinociceptive properties, but the mechanism of action driving the neuromodulatory effect of these species is poorly understood and seems to be dependent on the bacterial strain.

In view of the emerging role of the microbiota in pain processing, the modulation of its composition has become a novel strategy to relieve visceral pain. This modulation, which acts in several ways, can be effective in the more or less long term. Therapies using "biotic" products are gaining great interest[12,16,24,39] because they rely on the administration of commensal bacteria that are generally well tolerated, compared with pharmacological therapies, which often cause adverse effects. Interests have recently focused on "Live Biotherapeutic

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Products," defined as live microorganisms with the ability, unlike most of the current probiotics used as dietary supplements, to treat or cure symptoms or diseases.[1] In this study, we compared the neuromodulatory ability of several bacterial fractions from different species isolated from healthy donors to identify a potential candidate to reduce visceral pain in mouse models with different etiologies characterized by colonic hypersensitivity (CHS). This screening process identified *P distasonis* reference strain F1-2 as a potent neuroinhibitory probiotical strain with antihyperalgesic properties in pain models associated with increased intestinal permeability. Further experiments were then performed to determine the molecular mechanisms and cellular pathways involved in the effect of the F1-2 strain.

2. Materials and methods

2.1. Bacterial cells

2.1.1. Bacterial strain isolation, identification, growth conditions, and fractioning

Bacterial strains were isolated from feces of a male healthy donor, (age 34 years, 178 cm tall, BMI: 22.1 $kg{\cdot}m^{-2}$). He was free of gastrointestinal symptoms, had received no antibiotic treatment over the past 6 months, and provided a written informed consent. Feces samples were processed within one hour of collection in anaerobic conditions. To limit oxygen exposure, they were transferred to an anaerobic chamber filled with N_2 (85%), H_2 (5%), and CO_2 (10%). YBHI culture medium used for anaerobic bacteria isolation, containing Brain Heart Infusion (37 $g{\cdot}L^{-1}$) and yeast extract (5 $g{\cdot}L^{-1}$), was supplemented with D-maltose (1 $g{\cdot}L^{-1}$), D-cellobiose (1 $g{\cdot}L^{-1}$), L-cysteine hydrochloride (0.5 $g{\cdot}L^{-1}$), hemin (10 $mg{\cdot}L^{-1}$), and agar (14 $g{\cdot}L^{-1}$) and was adjusted to pH 6.9. After 48 hours of incubation at 37°C in strict anaerobic conditions, the bacterial isolates were purified on fresh agar medium and tested for their oxygen susceptibility. Any colony unable to develop after 1 hour of oxygen exposure was selected and identified by 16s rRNA sequencing. Ten anaerobic bacterial isolates were characterized and stocked at −80°C with 20% of glycerol.

For bacterial screening, all anaerobic bacterial isolates were cultivated in complex anaerobic medium containing neutralized soya peptone (10 $g{\cdot}L^{-1}$ OXOID), yeast extract (15 $g{\cdot}L^{-1}$ OXOID), KH_2PO_4 (0.9 $g{\cdot}L^{-1}$), K_2HPO_4 (0.9 $g{\cdot}L^{-1}$), NaCl (0.9 $g{\cdot}L^{-1}$), sodium acetate (2.7 $g{\cdot}L^{-1}$), $MgSO_4$ $7H_2O$ (0.09 $g{\cdot}L^{-1}$), L-cysteine hydrochloride (1 $g{\cdot}L^{-1}$), D-glucose (20 $g{\cdot}L^{-1}$), $NaHCO_3$ (4 $g{\cdot}L^{-1}$), maltose (0.5 $g{\cdot}L^{-1}$ Sigma), and cellobiose (0.5 $g{\cdot}L^{-1}$ Sigma), adjusted to pH 6.9 at 37°C for 24 to 48 hours. The biomass of each bacterial isolate was harvested by centrifuging whole culture 30 minutes at 5000*g* at 4°C and suspended in physiological solution composed of NaCl (140 mM), KCl (3 mM), $MgCl_2$ (1 mM), $CaCl_2$ (2 mM), D-glucose (10 mM), and HEPES (10 mM) adjusted to pH 7.4. Cell suspensions were mechanically lysed by high-pressure homogenizer at 2.6 kbar (One shot cell disrupter from Constant systems Ltd) to allow efficient lysis of bacterial cells. The lysed biomass was centrifuged at 5000*g* for 15 minutes at 4°C to separate insoluble fraction (bacterial membranes or biomass) from intracellular content (also called lysate). Intracellular content was sterilized by filtration (0.2 μm). Each bacterial fraction was stocked at −80°C before testing for its neuromodulatory potential.

2.1.2. Parabacteroides distasonis *growth conditions*

Parabacteroides distasonis (isolate F1-2, CNCM I-5828) and the corresponding reference strain from the ATCC collection (ATCC8503) were cultivated in a 5-L bioreactor containing complex anaerobic medium as described earlier. Anaerobic conditions were maintained by continuous injection of a gas mixture, N_2 (85%), H_2 (5%), and CO_2 (10%), which ensured low redox potential (−400 mV), and homogenization was achieved by 2 Rushton blades with an agitation rate of 150 rpm. Bioreactor culture was conducted for 24 hours with a controlled temperature of 37°C and reached OD_{600nm} of 6. The biomass of *P distasonis* was harvested by centrifuging whole culture for 30 minutes at 5000*g* at 4°C in anaerobic condition to maintain cell viability and conserved in PBS supplemented with 20% of glycerol. Bacterial titer was estimated between 1.52×10^{10} $CFU{\cdot}mL^{-1}$ and 2.06×10^{10} $CFU{\cdot}mL^{-1}$ according to batch production by count on brucella blood agar medium.

2.2. In vivo experiments

2.2.1. Animals and ethical statements

Male and female C57Bl/6 mice (6-9 weeks old) and male Wistar rats were obtained from Janvier laboratories (Le Genest-Saint-Isle, France). Male C57Bl/6 mice Nav1.8-Cre-GFP were obtained by crossing mT/mG mice with Nav1.8-Cre mice[45] (from Jackson Laboratory). Mice and rats were housed in a temperature-controlled room (21 ± 1°C) under standard conditions, with access to food and water ad libitum. For the dextran sulfate sodium (DSS)–induced CHS model and neonatal maternal separation (NMS) mouse model, mice were housed in specific pathogen-free (SPF) facilities. For postinfectious IBS mouse model, mice were housed in the biosafety level 2 (ABSL2) facility of the University of Clermont Auvergne (Clermont-Ferrand, France). All experiments were performed according to the ethical guidelines set out by the International Association for the Study of Pain,[65] complied with the European Union regulation, and were approved by the local C2EA-02 ethics committees of Clermont-Ferrand (approvals DSS: protocol number APAFIS 02498.01; NMS: protocol number CE110-12 and CE111-12; PI-IBS: protocol number EU0116-3460). The authors read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

2.2.2. Mouse models of colonic hypersensitivity and bacterial treatments

Three models of CHS were used. First, low-grade inflammation was induced by 0.5% (w/v) of DSS (MW = 36,000-50,000 Da) dissolved in drinking water ad libitum for 12 days. The control group received only drinking water. A postinfectious IBS (PI-IBS) model induced by *Citrobacter rodentium* inoculation was also performed, as described by Meynier et al. *4 4* [44] Finally, an NMS model was induced by pups separation from their mothers 3 hours a day from day 2 to day 14, as previously described.[38,44] The pups were then left with their mothers until weaning. Before the start of any treatment, colonic sensitivity was assessed in 9-week-old male mice or 11-week-old female mice to discriminate hypersensitive mice. As previously described, among NMS mice, only a subset developed CHS in comparison with nonhandled (= nonseparated) mice. Therefore, 2 groups of NMS mice were defined: NMS mice with CHS, hereafter called NMS-sensitized (NMS-S), and the normosensitive mice, hereafter called NMS-nonsensitized (NMS-NS).

In all CHS animal models, mice were daily gavaged with 0.2 mL of 1.52×10^{10} $CFU.mL^{-1}$ of *P distasonis* F1-2 strain for 8 to 12 days according to the model. In the DSS-induced CHS mouse model, animals were daily gavaged with the *P distasonis* F1-2 strain or *P distasonis* ATCC8503 strain (0.2 mL of 2.06×10^{10}

CFU.mL^{-1}), which had been produced in the same growth conditions over 12 days (**Fig. 1A**). In the PI-IBS model, mice were treated with F1-2 during the postinfectious phase for 8 days, from day 16 until day 23 (**Fig. 1B**). Finally, hypersensitive NMS mice were gavaged for 10 days (**Fig. 1C**).

2.2.3. Colonic sensitivity

Colonic sensitivity was evaluated by quantifying intracolonic pressure variations in response to CRD at the end of experiment in each CHS mouse mode, as previously described.[43] In brief, mice were habituated 30 minutes before experiments to reduce motion artefacts caused by restraint stress. A polyethylene balloon with a connecting catheter was inserted into the distal colon 1 cm from the base of the balloon to the anus of mice during light isoflurane anesthesia. The catheter was attached to the tail by tape. The balloon was connected to a pressure transducer to control intraballoon pressure during the CRD procedure. Barostat was used to manage air infiltration and balloon pressure control. The CRD procedure assesses visceral pain–related responses and consisted of repeated phasic distensions of 20, 40, 60, and 80 mm Hg lasting 20 seconds and spaced apart by 4 minutes.

2.2.4. Anxiety-like behavior

Anxiety-like behavior was assessed by the Elevated Plus Maze (EPM) test (ViewPoint Behavior Technology, Lissieu, France) at 21 days postinfection, as previously described.[43] Mice were acclimated to the room at least 45 minutes before the test. Individual animals were placed in the central zone and allowed to explore the maze for 5 minutes. They were filmed with a camera, and data were manually scored. The perform distance in the apparatus was recorded (Ethovision XT 15, Noldus). Anxiety was characterized through the number of entries in each arm (considered when the 4 taws are located within the arm) and time spent in open arms.

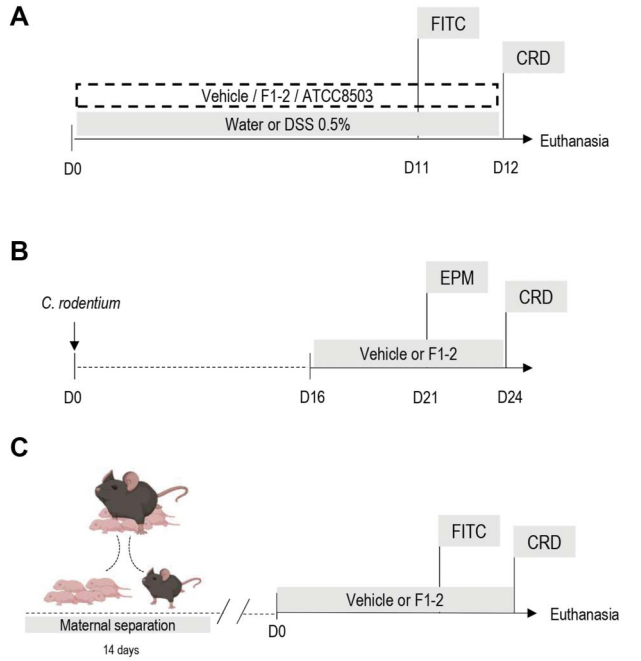

Figure 1. Experimental procedure of colonic hypersensitivity–induced mouse model. (A) DSS 0.5% CHS-induced mouse model. (B) *Citrobacter rodentium* postinfectious IBS mouse model and (c) neonatal maternal separation (NMS) mouse model. F1-2, *Parabacteroides distasonis F1-2 bacterial strain*; CRD, colorectal distension; DSS, dextran sulfate sodium; EPM, elevated plus maze test; FITC, fluorescein isothiocyanate dextran.

2.2.5. Intestinal permeability

In vivo intestinal permeability was assessed using fluorescein dextran (FITC-dextran 4000 Da, TdB labs). In brief, fasting mice were orally gavaged with 0.6 mg·g^{-1} of FITC-dextran per mouse body weight after a gavage period with *P distasonis* F1-2. Three-and-a-half hours after FITC-dextran gavage, blood samples were collected from the retro-orbital venous plexus, and plasma FITC levels were determined on serum fraction by fluorometry at 488 nm with a Flexstation 3 Multi-Mode Microplate Reader (Molecular Devices).

2.2.6. Colonic inflammation

Colonic sections of DSS-treated mice were harvested at the end of experiment and were homogenized in potassium phosphate buffer (50 mM) containing HTAB at 0.5% (wt/vol) (SIGMA). Supernatants were collected and frozen at −20°C. Inflammatory mediator levels were measured by ELISA using the Duoset kit (R&D System), according to the manufacturer's instructions. Inflammatory level was assessed by measuring the inflammatory mediators lipocalin-2, IL-1β, and IL-8. Th1-Th2 (Bio-Plex Pro Mouse Cytokine TH1-TH2 kit, BIO-RAD) and Th17 (Bio-Plex Pro Mouse Th17 Panel kit, Bio-Rad) inflammatory pathways were monitored by LUMINEX technology (BIOPLEX Luminex 200 Bio-Rad), according to the supplier's recommendations. Data acquisition and/or data analysis were performed on the Department of Biochemistry and Molecular Genetics at Clermont-Ferrand University Hospital.

2.2.7. Bacterial quantification in colonic samples

Genomic DNA was extracted from 50 mg of distal colonic tissues using the NucleoSpin Tissue XS kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer's instructions. DNA was stored at −20°C until analysis. Then, DNA was subjected to quantitative PCR using F1-2 specific primers: forward primer 5′-ACGTTTGATTATGCTCCAGCC-3′ and reverse primer 5′-CAG-TACCCGTAGGTCCAAGT -3′. An F1-2 strain culture was used as control for the absolute quantification.

2.2.8. Colonic 16S RNAscope

RNAscope in situ hybridization detection of PGP9.5 and EB-16S was performed on colon from perfused mice that were postfixed, sectioned, and stored at −80°C.[53] Neurons expressing PGP9.5 mRNA were identified using Mm-Uchl1-C2 probe (Cat No. 561861-C2, ACDBio; San Francisco, CA) and bacteria with EB-16S-rRNA-C1 (Cat No. 464461, ACD Bio; San Francisco, CA) using RNAscopeFluorescent Assay (ACD Bio, San Francisco, CA). Sections (12-15 μm) were mounted onto Superfrost Plus microscope slides (ThermoFisher Scientific) and air-dried. Slides were rinsed in 1X PBS and ethanol dehydrated in 50%, 70%, and 100%, with 5 minutes incubation time each. After target retrieval, slides were rinsed, dipped in 100% ethanol, and air-dried before creating a hydrophobic barrier using Immedge hydrophobic barrier pen (ACDBio, San Francisco, CA). RNAscope in situ hybridization detection was performed according to Multiplex Fluorescent v2 manual. In brief, incubations were performed at 40°C within a HybEZTM Oven, using the HybEZTM Humidity

Control Tray. For each section, 6 to 8 drops of each of the following reagent solutions were used to cover tissue sections, followed by 3 minutes washing in 1 X wash buffer at room temperature. Sections were incubated with Protease III for 30 minutes and washed in deionized water 3 times for 2 minutes each. Sections were then incubated with both Mm-Uchl1-C2 and EB-16S-rRNA probes for 2 hours at 40°C, followed sequentially by amplification steps with Amp1 for 30 minutes and Amp2 for 30 minutes. Sections were incubated with HRP-Ch1 for 15 minutes, TSA-Cy3 - (1:1500, PerkinElmer) for 30 minutes, and HRP blocker for 15 minutes. Slides were then incubated with HRP-C2 for 15 minutes, TSA-Fluorescein (1:1500, PerkinElmer) for 30 minutes, and HRP blocker for 15 minutes. The slides were further counterstained with DAPI and cover slipped.

2.2.9. Genomic analysis

The genome of the F1-2 strain was sequenced by short read sequencing technologies and compared against the genome of the ATCC-8503 strain available in the RefSeq database (GCF_000012845.1). The average nucleotide identity (ANI) analysis score was performed in pairwise method with OrthoANI tool (v1.2).[35] The genome annotation is performed with Bakta tool (v1.5.1), and then, the annotations are used into a pan-genomic analysis made with PPanGGOLiN tool (v1.2.105).[23,52]

2.3. In vitro experiments

2.3.1. Primary culture of neuronal cells

Dorsal root ganglia (DRG) from male Wistar rat, male C57Bl/6 wild-type mice, and male Nav1.8-Cre-GFP C57Bl/6 mice were explanted, cleaned of anterior and posterior roots and connective tissue, and digested by an enzyme mix containing collagenase type III (5 mg·mL^{-1}; Worthington) and dispase (10 mg·mL^{-1}; Gibco), for 45 minutes to 1h 15 at 37°C. DRG suspension was centrifuged at 175 g for 15 seconds after which the supernatant was removed and replaced by 1 mL of Dulbecco modified Eagle medium (DMEM; SIGMA). The DRG suspension was triturated through fire-polished Pasteur pipettes and spun at 175*g* for 5 seconds. The supernatant containing dissociated cells was saved at each trituration step. Trituration was performed 6 times for mice DRG and 9 times for rat DRG using 3 fire-polished Pasteur pipettes of decreasing diameter. The cell solution was spun for 5 minutes at 175 g. The supernatant was removed and replaced by 400 μL of DMEM supplemented with 10% (vol/vol) fetal bovine serum, sodium pyruvate (110 mg·mL^{-1}), L-glutamine (2 mM), penicillin (100 μg·mL^{-1}), streptomycin (100 μg·mL^{-1}), vitamins, amino acids (MEM NEAA, Gibco), and NGF (6.25 pg·mL^{-1}). Four microliters of the cell suspension from rat DRG were plated in 96-well microplates (Krystal 96-well plate with black glass bottom, Dutsher) for bacterial screening experiments. For ratiometric calcium imaging experiments, 20 μL of cell suspension from mice DRG were plated on poly–L-lysine (100 μg·mL^{-1}) and laminin-coated (200 μg·mL^{-1}) glass coverslips. Cells were incubated for 30 minutes at 37°C before the addition of DMEM-supplemented medium. Cells were then incubated at 37°C overnight before experiments.

2.3.2. Global calcium imaging in cultured cells

To assess the neuromodulatory potential of bacterial fractions, the first approach was to evaluate the global signal of neuronal cells in culture. First, screening was performed on the ND7/23 immortalized neuronal cell line (from the European Collection of Authenticated Cell Culture, Merck, France) generated by fusion of cultured neonatal rat DRG neurons with N18TG2 mouse neuroblastoma cells.[62] This immortalized cell line has been characterized as a sensory neuron model.[26,31] ND7/23 cells were cultivated in 15 mL of DMEM supplemented with 10% (v/v) fetal bovine serum, sodium pyruvate (110 mg·mL^{-1}), L-glutamine (2 mM), penicillin (100 μg·mL^{-1}), and streptomycin (100 μg·mL^{-1}) in a 75-cm^2 culture flask. At 80% confluence, cells were detached from the substrate, centrifuged at 175*g* for 5 minutes and suspended in ND7/23 fresh culture medium at 8.0×10^5 cells·mL^{-1}. The ND7/23 suspension was plated on a 96-well microplate (Greiner bio-one 655096) at 8.0×10^4 cells/well and incubated at 37°C overnight. After 24 hours of incubation, the culture medium was removed and replaced by 81 μL of calcium probe (Kit FLIPR Calcium 6-QF, Molecular Devices, λ485 nm [excitation] and λ525 nm [emission]) and 81 μL of HBSS (Hanks buffered salt solution)–HEPES buffer 20 mM (adjusted to pH 7.4, 300 mOsm).

Cells were incubated with calcium probe for 2 hours at 37°C according to the supplier's recommendations. Bacterial fractions were injected in each microplate well after neuronal activation with a FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices) for 3 minutes after injection. ND7/23 were incubated with bacterial fractions (1.0×10^8 viable cells suspension) during 45 minutes before being stimulated with bradykinin (40 nM). Neuronal activation was monitored for 3 minutes after stimulatory injection (**Fig. 2A**). Data analysis consisted in maximum signal to stimulation determined on F-F0 normalized kinetic. Maximum signal to stimulatory solution relative to control was calculated. Based on the similar approach described earlier, neuromodulatory potential of bacterial fraction was also tested on DRG Wistar rat primary neuronal cells (from Janvier laboratories) stimulated with capsaicin (4 μM) instead of bradykinin.

2.3.3. Single-cell ratiometric calcium imaging

Another approach was adopted to explore the interaction that occurs between bacterial cells and DRG mice neuronal cells. The single-cell ratiometric calcium imaging technical approach was preferred to the global calcium imaging described earlier because of its ability to monitor calcium signaling of single and isolated neurons represented by single-spike resolution. In these experiments, intracellular free Ca^{2+} was monitored with Ca^{2+} ratiometric dye Fura-2 acetoxymethyl ester (Fura 2-AM, Invitrogen). DRG mice neurons were loaded with Fura-2 solution (4 μM) supplemented with pluronic acid (1 μg·mL^{-1}) for 45 minutes at 37°C with slight agitation (40 rpm). All imaging experiments were performed at 32°C in a dark room. After loading, a glass coverslip was mounted in an imaging/perfusion chamber equipped with perfusion valve system, which was mounted and viewed through an inverted microscope. Neuronal cells were alternately illuminated with 340 nm and 380 nm wavelengths. The exposure time to excitation was 400 milliseconds for each wavelength. Image pairs were acquired every 2 seconds. All calcium imaging experiments consisted on 2 stimulations spaced by 5 minutes of incubation with 2.4×10^8 *P distasonis* F1-2 viable cells suspension after rinsing with Tyrode buffer solution (**Fig. 2B**). Capsaicin (SIGMA), an inflammatory soup consisting of bradykinin acetate salt (5 μM) (SIGMA), histamine dichlorhydrate (5 μM) (SIGMA), serotonin hydrochloride (5 μM) (SIGMA), PGE2 (5 μM) (Sellesckchem), and modified tyrode with KCl (10 mM) adjusted to pH 6.0 or potassium chloride (30 mM) were tested. A final KCl (50 mM) stimulation was assessed at the end to evaluate cell viability. Tyrode buffer solution was composed as follows: NaCl

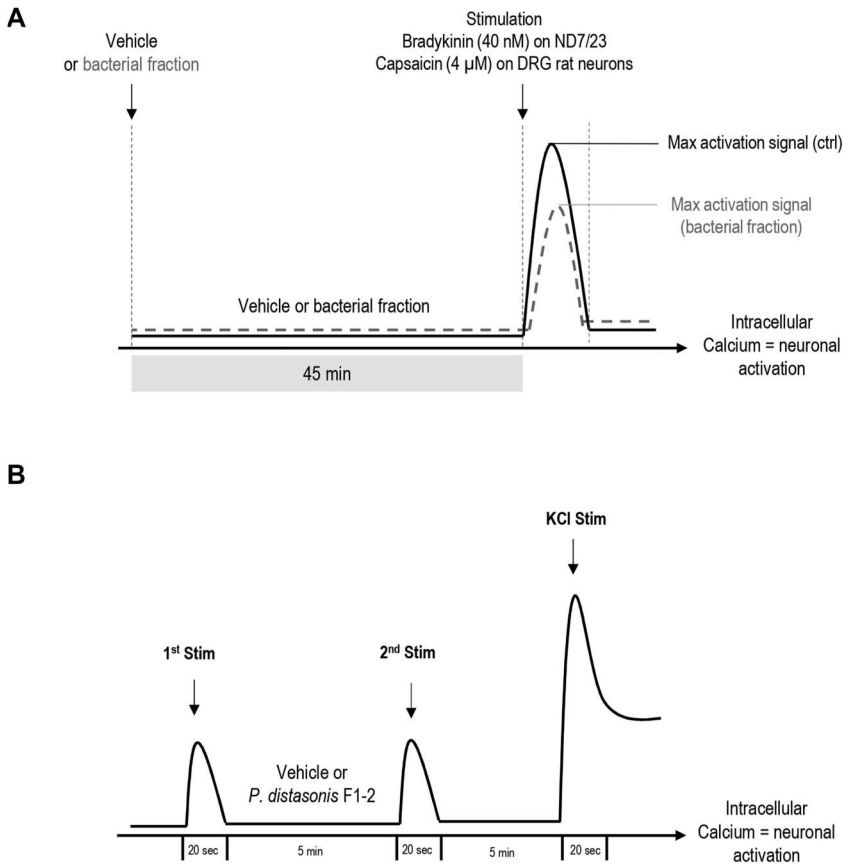

Figure 2. Experimental procedure of in vitro evaluation of the neuromodulatory properties of bacterial strains. (A) Screening protocol for neuromodulatory properties on ND7/23 immortalized neuronal cells and on primary neuronal cells from rat DRG. (B) Evaluation of neuronal cell activation by ratiometric calcium imaging on mice DRG neurons.

(140 mM), KCl (3 mM), $MgCl_2$ (1 mM), $CaCl_2$ (2 mM), D-Glucose (10 mM), and HEPES (10 mM) adjusted to pH7.4. Osmolarity was also adjusted at 300 mOsm. KCl solution was composed of NaCl (93 mM), KCl (50 mM), $MgCl_2$ (1 mM), $CaCl_2$ (2 mM), D-Glucose (10 mM), and HEPES (10 mM) adjusted to pH7.4. Osmolarity was adjusted to 300 mOsm.

For data analysis, kinetic stimulation was studied for each viable neuronal cell, which is a neuronal cell responding to the final KCl (50 mM) stimulation (**Fig. 2B**). The percentage of neuronal cells responding to each stimulation (before and after vehicle or *P distasonis* F1-2 incubation) over the total viable neuronal cells was calculated. The fold change to the different stimulation (KCl [30 mM], capsaicin, inflammatory soup, histamine, serotonin, or bradykinin) response intensity at the second stimulation over the response intensity to the first stimulation was also calculated.

2.4. Statistical analysis

Results were expressed as mean ± SEM. Statistical analyses were performed with GraphPad Prism 9 software (GraphPad, La Jolla). The D'Agostino–Pearson test and Shapiro–Wilk test were used to check whether data followed a normal distribution. The Mann–Whitney test and Student *t* test were used to compare 2 groups. One-way analysis of variance (ANOVA), the Kruskal–Wallis test, or 2-way ANOVA was used for intergroup comparisons with Tukey, Dunn, and Dunnett tests to compare more than 2-groups. A P value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Strictly anaerobic commensal bacterial fractions modulate neuronal responses to nociceptive stimulation through direct interaction

The isolation step made it possible to constitute a collection of 10 bacterial strains selected on the basis of their oxygen susceptibility (**Table 1**). We then tested 2 different compartments of the cultured bacteria, the biomass (= insoluble fractions) and the intracellular fraction, on neuron excitability. Among all the tested bacterial biomasses, screening results showed that only F1-2 biomass had an inhibitory effect on calcium response after bradykinin stimulation of ND7/23 cells in culture (**Figs. 3A and B**). Most intracellular bacterial cell contents inhibited calcium response to bradykinin on ND7/23 cells in culture, with a strong reduction observed after incubation with F1-2, NEO0037, and F1-1 soluble fractions (**Figs. 3C and D**).

In rat DRG neuronal cells primary culture, 4 biomasses had an inhibitory effect on calcium response after capsaicin stimulation, with the strongest reduction observed after incubation with F1-2 insoluble fraction (**Figs. 3E and F**). However, most of the intracellular contents that had an inhibitory effect in ND7/23 cells also reduced rat DRG neuron response to capsaicin (**Figs. 3G and H**).

The F1-2 bacterial strain was the only one to carry inhibitory activity both in its biomass and in its intracellular content in ND7/23 cells and rat DRG neurons. This particular strain, identified as *P*

Table 1

Bacterial strains isolated from the feces of healthy male donor.

Isolate denomination	Species identity (16s rRNA sequencing)
A2-3	*Escherichia/Shigella coli* group
F1-2	*Parabacteroides distasonis*
NE00037	*Bifidobacterium adolescentis*
A2-2	Unknown (not identified)
F1-1	*Bifidobacterium longum*
G2-1	*Clostridium citroniae*
G1-2	*Bifidobacterium animalis*
NE00060	*Hafnia alvei*
A1-7	Unknown (not identified)
G1-1	*Actinomyces turinencis*

Ten bacterial isolates were selected based on their oxygen susceptibility on agar medium and identified by 16S rRNA sequencing.

distasonis, was further investigated to test its effect on colonic sensitivity in CHS mouse models.

3.2. Administration of P distasonis F1-2 alleviates colonic hypersensitivity independently of intestinal barrier reinforcement or proinflammation resolution in a DSS-induced CHS model

Significant CHS was developed in male mice treated with DSS ($P < 0.001$ at 40, 60, and 80 mm Hg) (**Figs. 4A and B**). Oral administration of *P distasonis* F1-2 significantly reduced CHS (**Fig. 4A**) (DSS + veh vs DSS + F1-2: $P < 0.05$ at 40 and 60 mm Hg; $P < 0.0001$ at 80 mm Hg) as evidenced by 1.37-fold decrease in area under the curve (AUC) between 60 and 80 mm Hg in F1-2 treated mice compared with DSS-treated male mice (**Fig. 4B**). The antihypersensitive effect exerted by F1-2 was compared with that of the reference strain ATCC8503, which belongs to the same species and that shares 99.51% 16s RNA sequence homology with F1-2 (supplemental digital content, Fig. S1, available at http://links.lww.com/PAIN/B928). This observation was confirmed by the calculation of the orthoANI score between our *P distasonis* strain reference F1-2 and the reference strain ATCC8503, which revealed a score of 98.6. Despite the strong homology between these 2 strains, CRD results suggested that, unlike F1-2 strain, reference strain had no effect on CHS induced by DSS treatment (supplemental digital content, Fig. S2a and b, available at http://links.lww.com/PAIN/B928). Finally, a pan-genomic analysis made with PPanGGOLiN tool revealed the presence of 1020 genes and the absence of 520 genes in our *P distasonis* reference strain F1-2 in comparison with the reference strain ATCC8503 (supplemental digital content, Tables S1 and S2, available at http://links.lww.com/PAIN/B928).

Measurement of intestinal barrier disruption showed a higher permeability after DSS treatment ($P < 0.001$) that was not significantly reduced by administration of *P distasonis* F1-2 (**Fig. 4C**). In addition, an increase in intracolonic bacteria was observed by 16S RNAscope staining in the DSS-treated mice (**Fig. 4D**), and a colon-associated F1-2 quantification using qPCR demonstrated a significant increase in F1-2 bacteria presence in the colon of DSS-treated mice after F1-2 daily gavage (**Fig. 4E**). Colonic inflammation, evaluated by lipocalin 2 (Lcn2), CXCL1/KC, and IL-1β levels, was increased in DSS-treated mice but remained unchanged after F1-2 treatment (**Figs. 4F, G and H**). Colonic inflammation severity was further assessed by LUMINEX technology. Th1-Th2 and Th17 inflammatory pathways were targeted, and none of the 18 cytokines measured was significantly increased after DSS treatment (supplemental digital content, Fig. S3, available at http://links.lww.com/PAIN/B928).

Finally, to verify whether the mouse sex affected the efficacy of *P distasonis* F1-2 treatment, female mice were treated with DSS to induce the development of a CHS (**Fig. 4I**). Oral administration of *P distasonis* F1-2 significantly reduced CHS (**Fig. 4J**), as evidenced by a 1.39-fold decrease in AUC between 60 and 80 mm Hg in F1-2–treated mice compared with DSS-treated female mice (**Fig. 4J**).

3.3. P distasonis F1-2 alleviates colonic hypersensitivity but not anxiety-like behavior in postinfectious irritable bowel syndrome mouse model

As previously reported,[43] infection with *C rodentium* induces CHS after pathogen clearance by increasing intracolonic pressure to colorectal distension in male mice ($P < 0.05$ at 60 mm Hg and $P < 0.01$ at 80 mm Hg) (**Figs. 5A and B**). Oral administration of F1-2 for 8 days significantly reduced chronic colonic hypersensitivity in this model ($P < 0.01$ at 80 mm Hg) (**Fig. 5A**) as shown by a 1.98-fold decrease in AUC between 60 and 80 mm Hg in F1-2–treated mice compared with that in the infected mice group (**Fig. 5B**). As described earlier, a colon-associated F1-2 quantification using qPCR demonstrated a significant increase in F1-2 bacteria presence in the colon of *C rodentium*–infected mice after pathogen clearance (**Fig. 5C**).

Postinfectious anxiety-like behavior in *C rodentium*–infected mice has been documented elsewhere.[43] This observation was confirmed in our study by an increase in the number of entries ($P < 0.01$) (**Fig. 5D**) and time spent in open arms ($P < 0.01$) (**Fig. 5E**) during EPM test. F1-2 treatment had no beneficial effect on anxiety-like behavior in this model (**Figs. 5D and E**).

Finally, to verify whether the mouse sex affected the efficacy of *P distasonis* F1-2 treatment, female mice were also infected with *C rodentium* to induce the development of a CHS (**Fig. 5F**). Oral administration of *P distasonis* F1-2 significantly reduced CHS (**Fig. 5F**), as shown by the by 1.65-fold decrease of AUC between 60 and 80 mm Hg in F1-2–treated mice compared with that in *C rodentium*–infected female mice (**Fig. 5G**).

3.4. Administration of P distasonis F1-2 does not modify colonic hypersensitivity in a noninflammatory irritable bowel syndrome mouse model

Previous experiments by our team showed the efficacy of F1-2 treatment in 2 models of CHS associated with intestinal hyperpermeability. To assess the impact of the F1-2 strain in a model with a different etiology, we used an NMS paradigm protocol to induce long-term CHS.[41] Results of CRD showed that F1-2 has no effect on CHS in NMS male mice (**Figs. 6A and B**), suggesting that intestinal barrier disruption may be required to observe a beneficial effect of the F1-2 strain (**Fig. 6C**). In addition, a 16S RNAscope staining demonstrated that no intracolonic bacteria were observed in the sensitized NMS-S–treated mice (**Fig. 6D**), and a colon-associated F1-2 quantification using qPCR demonstrated no significant increase in F1-2 bacteria presence in the colon of NMS-S mice after F1-2 daily gavage (**Fig. 4E**).

Finally, to verify whether the mouse sex affected the efficacy of *P distasonis* F1-2 treatment, female mice were also used to perform an NMS stress paradigm. Oral administration of *P distasonis* F1-2 had no effect on NMS-induced CHS (**Figs. 6F and G**).

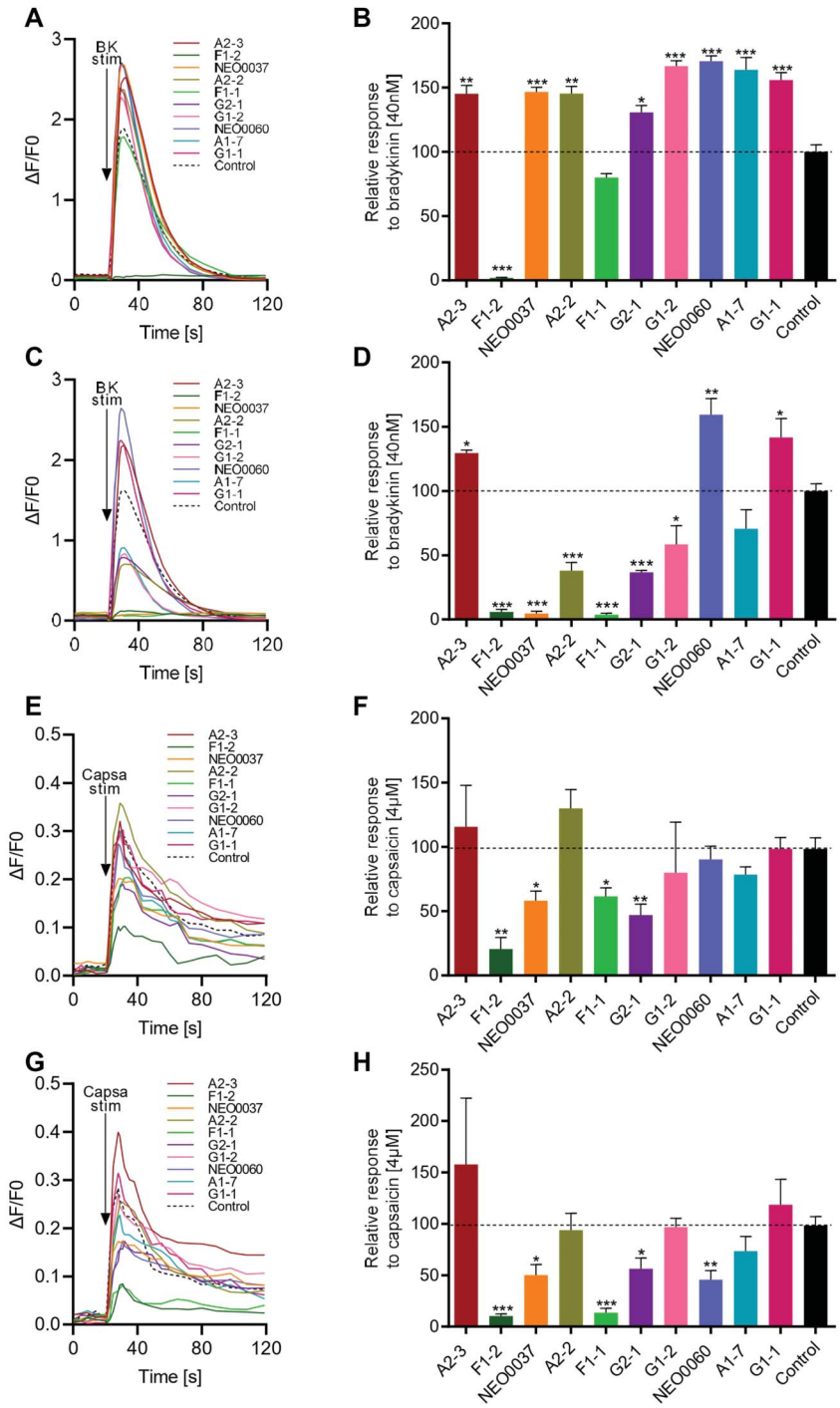

Figure 3. Strictly anaerobic commensal bacterial fractions modulate neuronal responses to nociceptive stimulation through direct interaction. (A–D) Ca^{2+} responses of ND7/23 neuronal cells loaded with calcium probe (FLIPR Calcium 6 QF) stimulated with bradykinin (40 nM) after 45 minutes of incubation with (A–B) insoluble fraction (biomass) or (C–D) intracellular bacterial cell content. (E–H) Ca^{2+} responses of primary neuronal cells from DRG rat loaded with calcium probe (FLIPR Calcium 6 QF), stimulated with capsaicin (4 μM), after 45 minutes of incubation with insoluble fraction (E–F) or intracellular bacterial cell content (G–H). For each experiment, representative calcium responses are shown (left panels), and bar graphs represent maximum peak values on normalized F-F0 relative to control (right panels). Each point represents an average of 3 to 4 independent readings for each bacterial fraction and 15 to 16 independent readings for control. Results are presented as mean ± SEM. Statistical analysis through a one-way ANOVA followed by a Kruskal–Wallis test. *$P < 0.05$, ** $P < 0.01$, and ***$P <$ 0.001 vs control group.

3.5. P distasonis *F1-2 decreases activation level of nociceptors*

Mouse DRG neurons in culture were submitted to 2 challenges with KCl (30 mM), capsaicin (1 μM), or inflammatory soup. In between, cells were incubated either with the F1-2 strain or with the vehicle. Incubation of mouse DRG neurons with F1-2 strain for 5 minutes did not affect the percentage of cells activated by a KCl (30 mM) used as a pan-neuronal stimulation (**Fig. 7A**) or the intensity of their activation response (**Figs. 7B and C**). Of interest, whereas vehicle treatment does not significantly affect the percentage of responding cells to

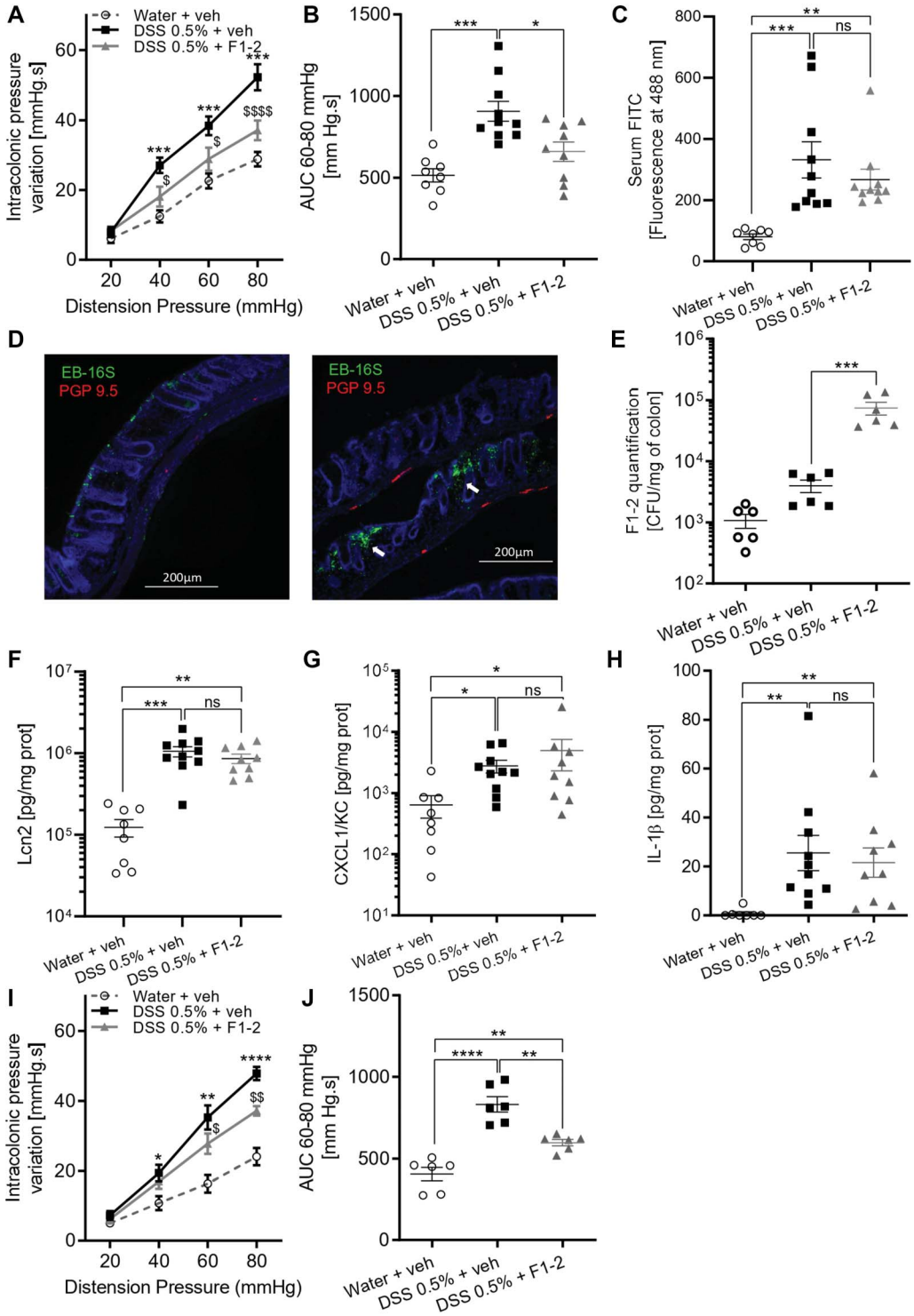

Figure 4. Administration of *Parabacteroides distasonis* F1-2 alleviates colonic hypersensitivity independently of intestinal barrier reinforcement or proinflammation resolution in a DSS-induced CHS model. CHS was induced using 0.5% DSS for 12 days. Mice were daily gavaged with vehicle or *P distasonis* F1-2. (A) Intracolonic pressure variation in response to colorectal distension in control (water + vehicle) male mice (n = 8), DSS 0.5% + vehicle-treated male mice (n = 10), DSS 0.5% + *P distasonis* F1-2– treated male mice (n = 9) were assessed at D12. (B) Area under the curve (AUC) was calculated between 60 and 80 mm Hg for the different groups. (C) Intestinal permeability was assessed at D11 using FITC-dextran tracer. (D) RNAscope staining of 16S rRNA (EB-16S-rRNA-C1 probe) and of PGP9.5 (Mm-Uchl1-C2 probe) in the colon of water-treated (left panel) or DSS-treated (right panel) mice. EB-16S-rRNA in green, PGP9.5 in red, and DAPI in blue. (E) The F1-2 strain bacteria were quantified by qPCR in colonic samples of water-treated or DSS-treated mice. (F) Lipocalin 2, (G) CXCL1/KC (IL-8), and (H) IL-1β colonic level were measured by enzyme-linked immunosorbent assay (ELISA) in DSS visceral pain mouse model. (I) Intracolonic pressure variation in response to colorectal distension in control (water + vehicle) female mice (n = 6), DSS 0.5% +-vehicle treated female mice (n = 6), DSS 0.5% + *P distasonis* F1-2–treated female mice (n = 6) was assessed at D12. (J) Area under the curve (AUC) was calculated between 60 and 80 mm Hg for the different groups. Results are presented as mean ± SEM. Statistical analysis through a Two-way ANOVA followed by a Dunnett test for multiple comparisons or through a One-way ANOVA followed by a Kruskal–Wallis test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ for water + veh vs DSS 0.5% + veh. $\$P < 0.05$, $^{\$\$}P < 0.01$, and $\$\$\$\$P < 0.0001$ for DSS 0.5% + veh vs DSS 0.5% + F1-2.

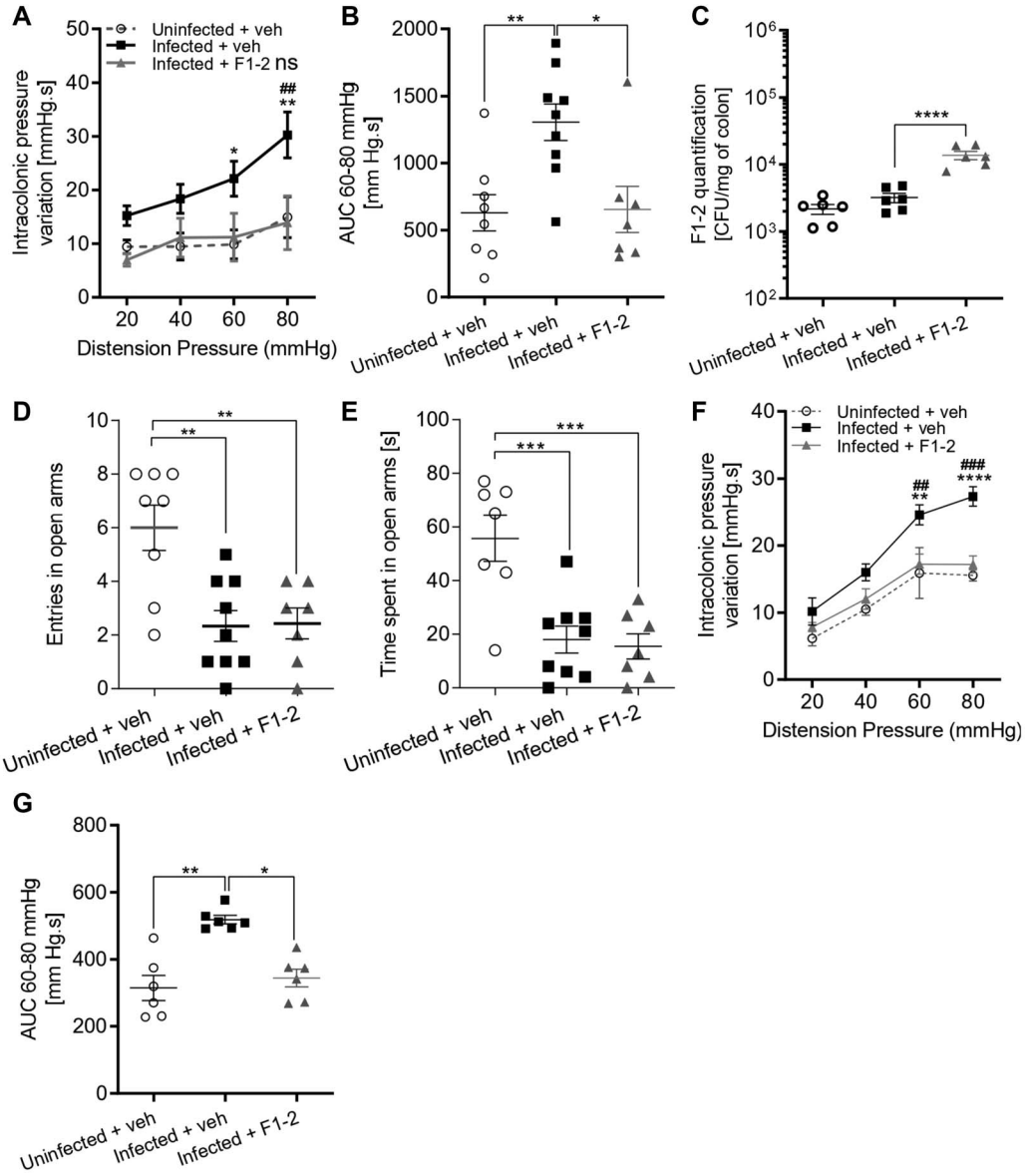

Figure 5. *Parabacteroides distasonis* F1-2 alleviates colonic hypersensitivity but not anxiety-like behavior in postinfectious IBS mouse model. Mice were infected with *C. rodentium* at D0 before being gavaged with *P distasonis* F1-2 or vehicle from D16 to D24. (A) Intracolonic pressure variation in response to colorectal distension in uninfected male mice (n = 8), infected + vehicle male mice (n = 9), and infected + *P distasonis* F1-2 male mice (n = 7) was assessed at D24. (B) Area under the curve between 60 and 80 mm Hg for the different groups. Behavioral effect on anxiety-like behavior was evaluated with the Elevated Plus Maze (EPM) test at day 21. (C) The F1-2 strain bacteria were quantified by qPCR in colonic samples of water-treated or DSS-treated mice. (D) Entries in open arms and (E) Time spent in open arms was measured for 5 minutes. (F) Intracolonic pressure variation in response to colorectal distension in uninfected female mice (n = 6), infected + vehicle female mice (n = 6), and infected + *P distasonis* F1-2 female mice (n = 6) was assessed at D24. (G) Area under the curve between 60 and 80 mm Hg for the different groups. Results are presented as mean ± SEM. Statistical analysis through a Two-way ANOVA followed by a Dunnett test for multiple comparisons or through a One-way ANOVA followed by a Kruskal–Wallis test. *$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ for uninfected + veh vs infected + veh. ##$P < 0.01$ and ###$P < 0.001$ for infected + veh vs infected + F1-2.

capsaicin stimulation, F1-2 incubation for 5 minutes significantly reduced the number of responding cells from 15.8% ± 2.2% for the first capsaicin stimulation to 6.8 ± 1.6% for the second capsaicin stimulation (**Fig. 7D**). Moreover, the fold change of the neuronal activation intensity induced by capsaicin between the 2 stimulations is significantly reduced with the F1-2 treatment (0.37 ± 0.01 A.U.) in comparison with the vehicle (0.74 ± 0.02 A.U.) (**Figs. 7E and F**). Another neuronal stimulation using an inflammatory soup, a different algogenic substance, demonstrated a similar neuromodulatory effect of F1-2 bacterial strain whether on the percentage of responding cells to inflammatory soup stimulation (**Fig. 7G**) or on their neuronal activation intensity fold change induced by inflammatory soup between the 2 stimulations (**Figs. 7H and I**).

To further explore the effect of the F1-2 strain on the activation of nociceptors, the same kind of experiments was performed on mouse DRG neuron cultures derived from Nav1.8-Cre-GFP mice. F1-2 treatment significantly reduced the percentage of responding cells to capsaicin or inflammatory soup stimulation only in Nav1.8-expressing cells compared with that in the control group (**Figs. 8A and C**), but it significantly reduced the neuronal activation intensity

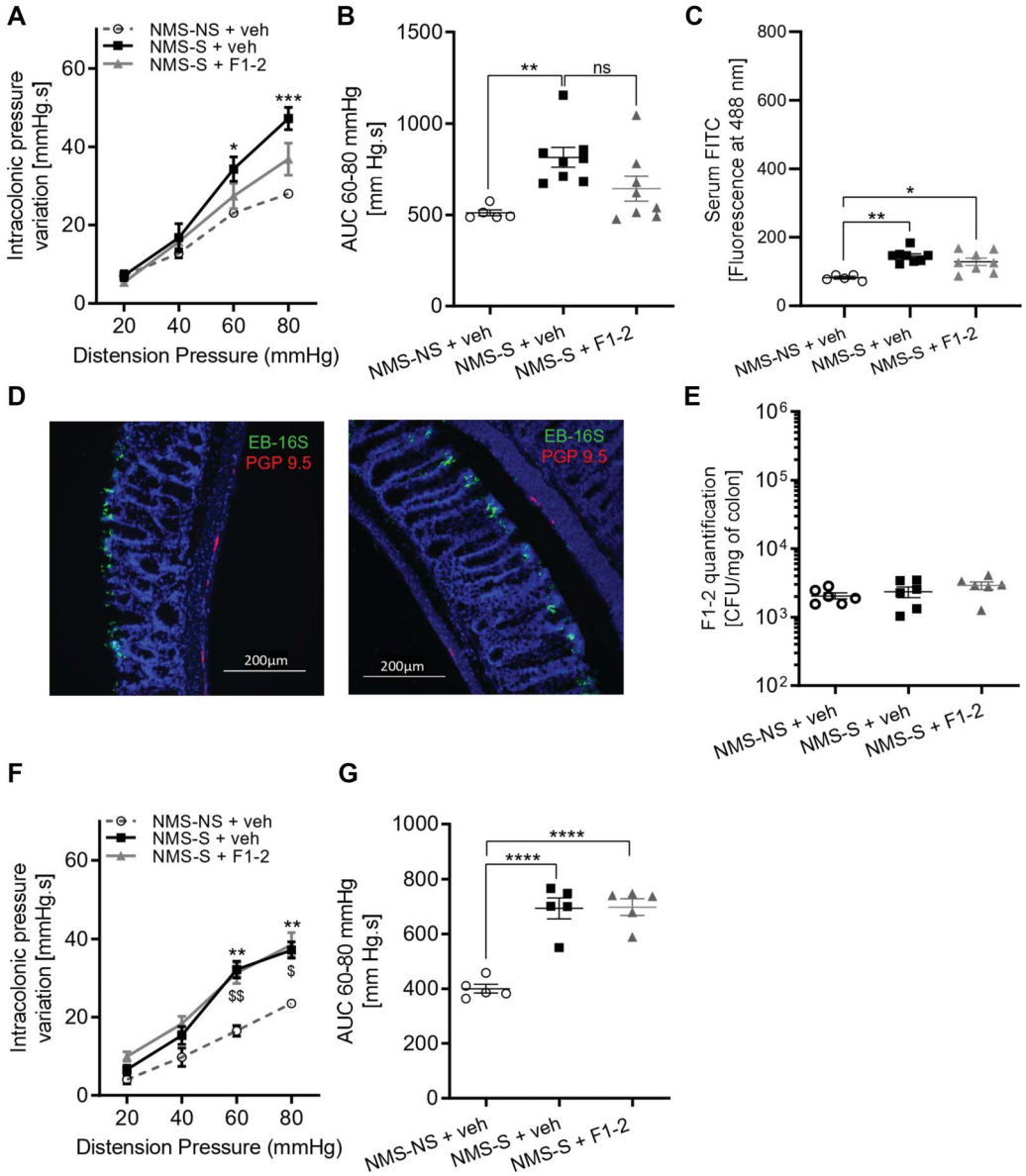

Figure 6. Administration of *Parabacteroides distasonis* F1-2 does not modify colonic hypersensitivity in a model of neonatal stress. *P distasonis* F1-2 strain was tested in neonatal maternal separation (NMS) visceral pain mouse model. Colonic hypersensitivity was assessed by colorectal distension (CRD) at the end of the experiment. (A) Intracolonic pressure variation in response to colorectal distension in NMS mouse model: NMS-nonsensitized (NMS-NS) + vehicle (n = 5), NMS-sensitized (NMS-S) + vehicle (n = 8), and NMS-S + F1-2–treated male mice (n = 8). (B) Area under the curve (AUC) was calculated between 60 and 80 mm Hg for the different groups. (C) Intestinal permeability was assessed at D10 using FITC-dextran tracer and compared with that of DSS mouse model. (D) RNAscope staining of 16S rRNA (EB-16S-rRNA-C1 probe) and of PGP9.5 (Mm-Uchl1-C2 probe) in the colon of NMS-NS (left panel) or NMS-S (right panel) mice. EB-16S-rRNA in green, PGP9.5 in red, and DAPI in blue. (E) The F1-2 strain bacteria were quantified by qPCR in colonic samples of water-treated or DSS-treated mice. (F) Intracolonic pressure variation in response to colorectal distension in NMS mouse model: NMS-NS + vehicle (n = 5), NMS-S + vehicle (n = 5), and NMS-S + F1-2–treated female mice (n = 5). (G) Area under the curve (AUC) was calculated between 60 and 80 mm Hg for the different groups. Results are presented as mean ± SEM. Statistical analysis through a Two-way ANOVA followed by a Dunnett test for multiple comparisons or through a One-way ANOVA followed by a Kruskal–Wallis test. *$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ for NMS-NS + veh vs NMS-S + veh. \$$P < 0.05$ and \$\$ $P < 0.01$ for NMS-NS + veh vs NMS-S + F1-2.

induced by both algogenic substances in both Nav1.8-expressing cells and Nav1.8-negative neurons compared with that in the control group (**Figs. 8B and D**).

3.6. P distasonis *F1-2 decreases the activation signal of neuronal cells stimulated with bradykinin*

As previously reported, F1-2 has a very potent effect in ND7/23 cells after bradykinin stimulation and in mouse DRG neurons after inflammatory soup challenge containing bradykinin. Hence, we focused the next experiment on the neuromodulatory properties of the F1-2 strain after bradykinin stimulation in mouse DRG neurons. In a first experiment, we assessed the effect of F1-2 strain on primary neuronal cells from mouse DRG stimulated by inflammatory soup containing (IS (+) BK) or deprived (IS (−) BK) of bradykinin by single-cell calcium imaging. Results indicated that 5 minutes contact with F1-2 bacterial strain significantly reduced the neuronal activation intensity induced by inflammatory soup containing bradykinin (IS (+) BK) stimulation, but this neuromodulatory

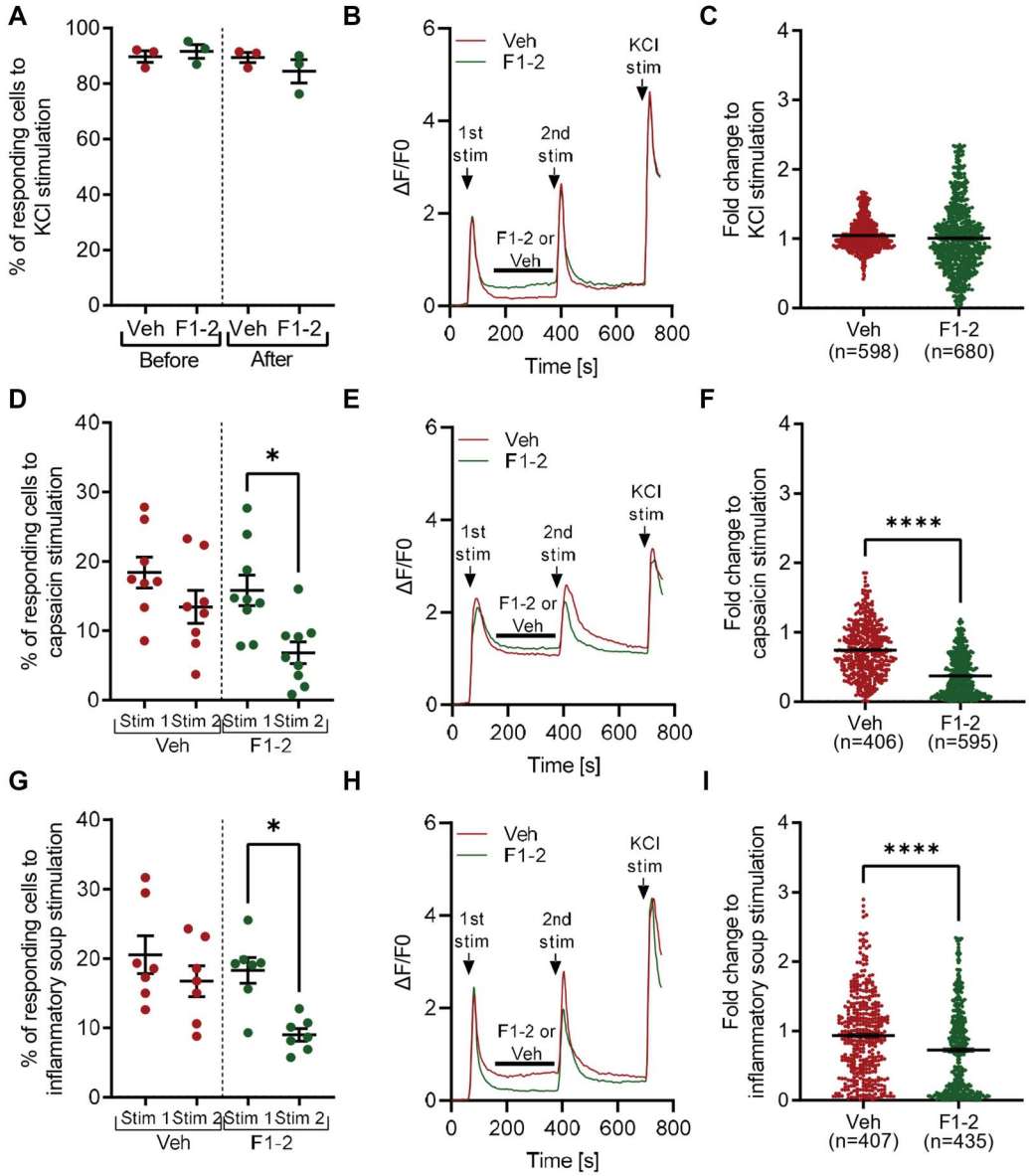

Figure 7. *Parabacteroides distasonis* F1-2 decreases the activation level of mouse DRG neurons in culture. Neuronal cells were stimulated a first time with KCl (30 mM), capsaicin (1 μM), or IS before being incubated with vehicle or *P distasonis* F1-2. Neuronal cells were stimulated a second time with capsaicin or IS mixed with *P distasonis* F1-2 before being stimulated with KCl (50 mM). IS was composed of bradykinin, serotonin, histamine, PGE2, and KCl. The percentage of responding cells to (A) KCl, (D) capsaicin, or (G) IS stimulation was calculated. (A) Representative calcium response is presented for each (B) KCl, (E) capsaicin, or (H) IS stimulation. (A) Fold change of the neuronal activation intensity between the 2 stimulations was calculated for each (C) KCl, (F) capsaicin, or (I) IS stimulation. Results are presented as mean ± SEM. Statistical analysis through a One-way ANOVA followed by a Kruskal-Wallis test or through an unpaired Student *t* test when used to compare 2 groups. *$P < 0.05$ and ****$P < 0.0001$. IS, inflammatory soup.

effect was lost when inflammatory soup was deprived of bradykinin (IS (−) BK) (**Fig. 9A**). To confirm the importance of bradykinin pathway in the neuromodulation observed with the F1-2 strain, a second experiment was performed to assess its effect on primary neuronal cells from mouse DRG stimulated by bradykinin, serotonin, or histamine, the 3 main algogenic substances that make up the inflammatory soup. The F1-2 stain incubation was associated with a significant reduction of the neuronal activation intensity fold change only after bradykinin stimulation (**Fig. 9B**), but not after serotonin or histamine stimulation (**Figs. 9C** and D).

Finally, the dose–response curve of bradykinin on immortalized ND7/23 neuronal cells was assessed after 45 minutes of incubation with F1-2 or vehicle (**Fig. 9E**). *P distasonis* F1-2 treatment significantly reduced maximum fluorescence intensity after bradykinin stimulation for every dose tested. Accordingly, EC50 changed from 6.1 nM in the control group to 22 nM in presence of F1-2 (**Fig. 9E**).

4. Discussion

This study highlights the neuroinhibitory effect of bacterial strain *P distasonis* F1-2 in the ND7/23 immortalized neuronal cell line and primary culture of rat or mouse DRG neurons after stimulation with several algogenic substances. In addition, the reference strain F1-2 strongly reduced CHS solely in mice models associated with intestinal hyperpermeability, thus supporting the hypothesis of a direct "bacteria–nervous system" interaction.

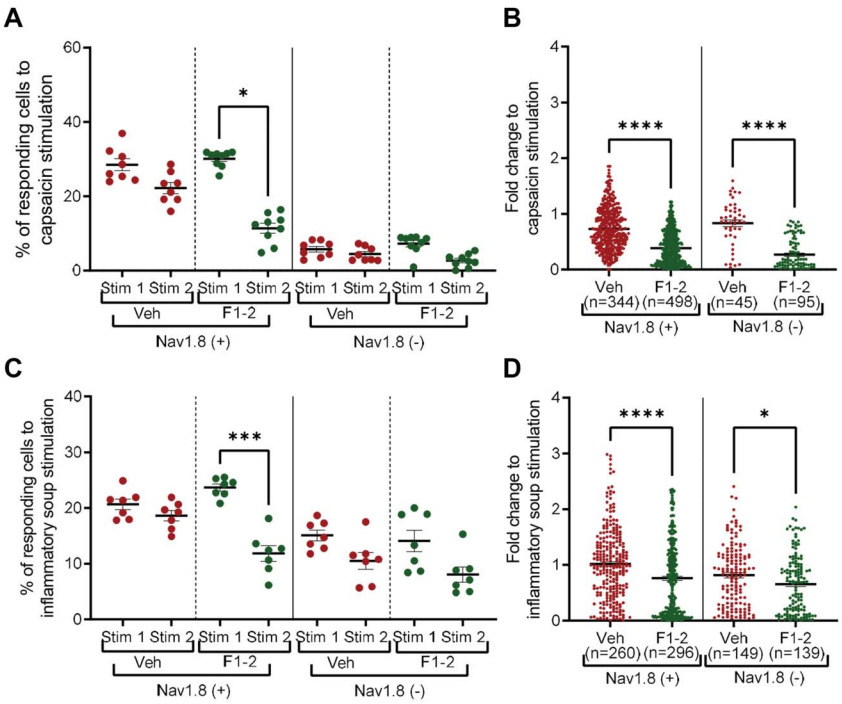

Figure 8. *Parabacteroides distasonis* F1-2 decreases the activation level of nociceptors in mouse. Neuronal cells were stimulated a first time with capsaicin (1 μM) or IS before being incubated with vehicle or *P distasonis* F1-2. Neuronal cells were stimulated a second time with capsaicin or IS mixed with *P distasonis* F1-2 before being stimulated with KCl (50 mM). Inflammatory soup was composed of bradykinin, serotonin, histamine, PGE2, and KCl. Nociceptors were identified as neurons expressing Nav1.8 channel. The percentage of responding cells to (A) capsaicin or (C) IS stimulation was calculated. A Fold change of the neuronal activation intensity between the 2 stimulations was calculated for each (B) capsaicin or (D) IS stimulation. Results are presented as mean ± SEM. Statistical analysis through a One-way ANOVA followed by a Kruskal–Wallis test or through an unpaired Student *t* test when used to compare 2 groups. $^{*}P < 0.05$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. IS, inflammatory soup. Nav1.8 (+): neuronal cells expressing Nav1.8 channel. Nav1.8 (−): neuronal cells not expressing Nav1.8 channel.

An isolation step and identification by 16s rRNA sequencing of fecal anaerobic bacteria led to the creation of a 10-strain collection. First, we started our study by testing whether the biomass and intracellular content fractions extracted from our anaerobic bacterial species were able to modulate activation of the ND7/23 immortalized cell line. In our experimental approach using in vitro "global calcium imaging," these cells were activated only by bradykinin but not by other algogenic substances as capsaicin, serotonin, or histamine. Most of the soluble and insoluble fractions extracted from our 10-strain collection bacterial species were able to modulate activation of the ND7/23 immortalized cell line. Then, to confirm their neuromodulatory effects, all bacterial fractions were also tested on primary cultures of DRG neuronal cells on stimulation with algogenic substances. Unfortunately, because only a small amount of DRG neuronal cells can be activated by bradykinin, serotonin, or histamine, we could not detect a significant increase in fluorescence using our "global calcium imaging." Only capsaicin, which is a commonly used algogenic substance, stimulation was able to induce a significant increase in fluorescence detected in our "global calcium imaging" on primary cultures of DRG neuronal cells. Nevertheless, we were able to confirm that most of our bacterial fractions can modulate activation of DRG neuronal cells either by increasing or lowering intracellular calcium flows upon stimulation with an algogenic substance. There has been increasing interest in the past few years in direct neuromodulation by bacteria. In most cases, an activation of afferent neurons was evidenced after exposure to pathogenic strains such as *Staphylococcus aureus*,[10] *Porphyromonas gingivalis*,[21] or *Mycobacterium tuberculosis*.[51] However, the mechanisms of the dialogue between bacteria and neurons are still poorly understood. Regarding the diversity of gut microbiota, we hypothesized that besides neuronal activation induced by a subset of pathogenic strains, some species could, by contrast, modulate neuronal activation to inhibit pain signal arising from the gut. Previous reports documented the antihyperalgesic properties of several bacterial species including *Lactobacillus acidophilus*, *F prausnitzii*, and some *Bifidobacteria* sp. in models of visceral pain in rodents, but to date, no direct inhibition of neuronal activation has been established.[33]

P distasonis is a highly prevalent bacterium and a member of core microbiota that is found in 91.7% of healthy subjects[2] and might represent up to 38% of *Bacteroidetes* sp. present in the gut.[49,66] Because of its lowered abundance in diverse pathological contexts, and more particularly in IBD,[34,36] this strain was believed to provide beneficial effects to the host[59] through probiotic properties that depend on the bacterial strain.[8,20] However, deleterious effects have also been associated with end product fermentation of *P distasonis* such as succinic acid, which acts as a proinflammatory signaling molecule in immune cells.[57] Based on genomic analysis and the calculation of the orthoANI score (>95%), our F1-2 isolated strain and the ATCC reference strain (ATCC8503) can be considered as part of the same bacterial species, *P distasonis*. Despite this strong homology, no effect of the ATCC8503 reference strain was observed on CHS in our study compared with our F1-2 isolated strain. This paradoxical effect is consistent with the variability observed between the species and reported in the literature.[8,28,64] This discrepancy can be explained by genomic differences between these 2 strains because our pan-genomic analysis made with

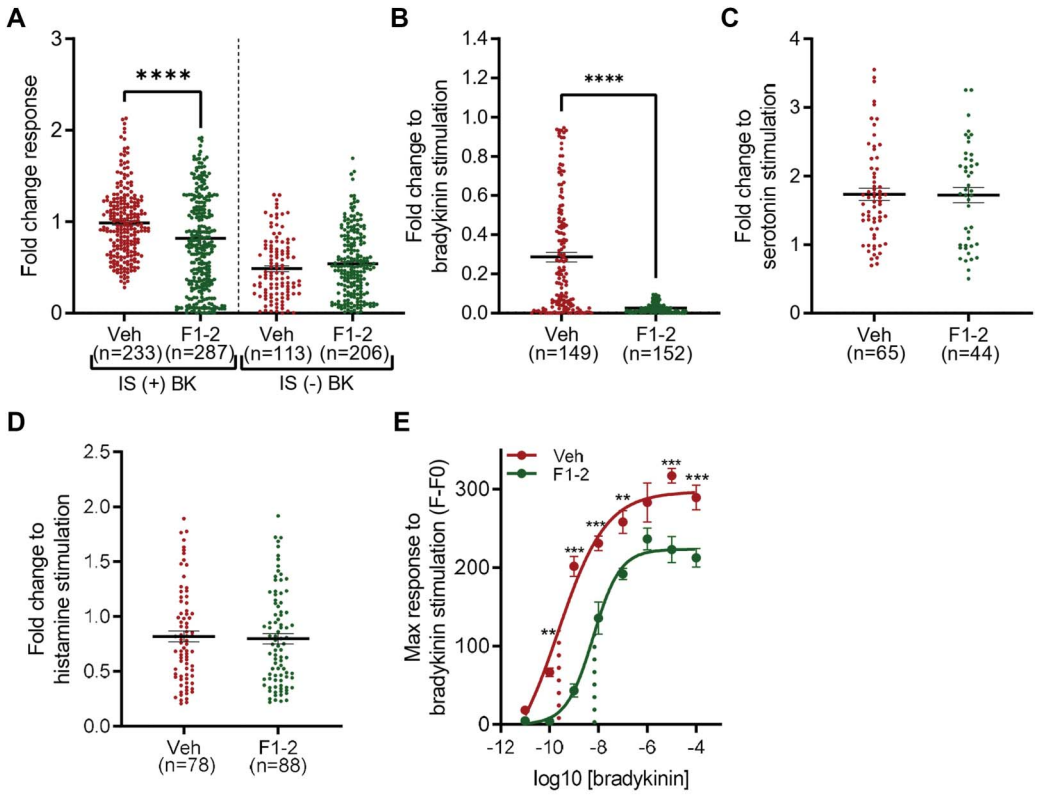

Figure 9. *Parabacteroides distasonis* F1-2 decreases the activation signal of neuronal cells stimulated with bradykinin. Neuronal cells were stimulated a first time with IS (+) BK, IS (−) BK, bradykinin, serotonin, or histamine before being incubated with Tyrode or *P distasonis* F1-2. Neuronal cells were stimulated a second time with the same substance mixed with *P distasonis* F1-2 before being stimulated with KCl (50 mM). A Fold change of the neuronal activation intensity between the 2 stimulations was calculated for each (A) IS (+) BK or IS (−) BK, (B) bradykinin (5 μM), (C) serotonin (15 μM), or (D) histamine (15 μM) stimulation. (E) Ca^{2+} activation signal of ND7/23 immortalized cells loaded with calcium probe (FLIPR Calcium 6 QF) stimulated with various doses of bradykinin (10^{-4} to 10^{-11} M) after 45 minutes of incubation with *P. distasonis* F1-2 or vehicle. Dose–response curve of bradykinin on ND7/23 cells expressing bradykinin receptor incubated with *P distasonis* F1-2 or vehicle. Results are presented as mean ± SEM. Statistical analysis through an unpaired Student *t* test when used to compare 2 groups or through a 2-way ANOVA followed by a Bonferroni test for multiple comparisons. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

PPanGGOLiN tool revealed the presence of 1020 genes and the absence of 520 genes in our *P distasonis* reference strain F1-2 in comparison with the ATCC8503 reference strain. Further genomic and functional in silico analyses will be needed to better understand the role of these genomic differences in the discrepancy between these 2 strains.

Of the 3 models of CHS used in our work, the isolated F1-2 strain reduced colonic sensitivity in the DSS-induced CHS model associated with low-grade inflammation and in the postinfectious IBS model using *C rodentium* but not in a neonatal stress model of the maternal separation paradigm. The degree of intestinal permeability is one of the parameters clearly differentiating those 3 models. A "leaky gut" is a hallmark of the DSS-induced CHS model, and we previously demonstrated that intestinal barrier integrity is also impaired in both infectious and postinfectious phases of this model.[43] Of interest, no effect of the F1-2 strain on CHS was observed in the NMS model, in which we previously observed only a slight increase in colonic permeability, but to a much lesser extent than in the 2 previous models.[38]

Because translocation of bacteria or their metabolites beyond the gastrointestinal tract is one of the hallmarks of the intestinal hyperpermeability,[60] we confirmed in our study that there is an increase of intracolonic bacteria, reveled by 16S RNAscope staining, in the DSS-treated mice but not in the sensitized NMS mice. Our result is in accordance with another study where they demonstrated by RNAscope staining that bacteria can be found in colon from IBS mice but not from healthy control mouse. Those intracolonic bacteria can be found in close proximity to colonic mast cells, thus suggesting occurrence of direct bacteria–mast cell interactions.[53] In addition, a colon-associated F1-2 strain quantification demonstrated a significant increase in F1-2 bacteria presence in the mouse colon from models with hyperpermeability as DSS-treated mice or the *C rodentium* infection model, but not in the NMS model. Thus, we hypothesized that F1-2 strain can survive to gastric pH and travel through the gastrointestinal tract as a bacterium simply in transit, but in a pathological context in which the intestinal barrier is disrupted, direct bacteria–neurons contact could be enhanced and would contribute to the antihyperalgesic characteristics of the F1-2 strain.

In an attempt to decipher the beneficial effects of the F1-2 strain, we further investigated its impact on intestinal permeability in the DSS-induced CHS model. *P distasonis* F1-2 was not able to restore normal intestinal permeability compromised in this model. Only a few strains belonging to the species have been recognized for their ability to reinforce the epithelial barrier, especially by increasing production of proteins involved in tight junction such as OCCLUDIN and ZO-1 in the TNBS colitis-induced mouse model.[15,28,32]

The antihyperalgesic effect of *P distasonis* F1-2 may involve different mechanisms at both peripheral and central levels. To further characterize these mechanisms, we assessed the modulation of inflammatory parameters by the F1-2 strain in the DSS-induced CHS model, characterized by a low-grade colonic

inflammation induced by 0.5% DSS. As expected, Lcn-2, CXCL1, and IL-1β measurements showed a low-grade inflammation caused by DSS treatment, in line with results in the literature.[9] Some *P distasonis* strains have already demonstrated beneficial effects by reducing proinflammatory cytokine release in vitro[28] and in mouse models of colitis and colorectal cancer[15,33] by reducing gene expression of IL-4 and TNF-α at the colonic level.[32] By contrast, *P distasonis* F1-2 did not modify inflammatory parameters in our study. Hence, and unlike for many probiotics strains, these results evidenced the antihyperalgesic potency of *P distasonis* F1-2 despite the lack of marked inflammation.

In pathological disorders such as IBD or IBS, most patients are diagnosed with various comorbidities including anxiety or depression. To study the effect of the F1-2 strain at the central level, a PI-IBS mouse model was used. The murine pathogen *C rodentium* induces the development of CHS accompanied by anxiety-like behavior, even after pathogen clearance.[43] The EPM test indicated that the treatment using *P distasonis* F1-2 strain did not reduce anxiety-like behavior induced in this PI-IBS model. In conclusion, F1-2 strain treatment has no impact on CHS-associated comorbidities. However, a modulation of the signal in central structures involved in the pain matrix by the F1-2 strain cannot be ruled out and warrants further investigation.

To study the peripheral modulation of the pain signaling as a potential mechanism of the antihyperalgesic action of the F1-2 strain, we investigated neuronal activation in primary cultures of mouse DRG neurons after stimulation with several algogenic substances. *P distasonis* F1-2 was able to desensitize a subset of DRG neurons stimulated with algogenic substances such as capsaicin or an inflammatory soup. This neural inhibition was peculiarly marked in Nav1.8-expressing neurons because these cells were, as expected, more prompt to be activated by stimulation with capsaicin or inflammatory soup. However, a subset of Nav1.8-negative cells was also desensitized by F1-2 treatment after exposure to capsaicin or inflammatory soup, suggesting that the effect of the strain is not restrained to nociceptors. Of interest, when inflammatory soup was deprived of bradykinin, the inhibitory effect of F1-2 strain was lost. Concomitantly, bradykinin, serotonin, and histamine, the 3 main algogenic substances that make up the inflammatory soup, were separately tested on primary neuronal cells from mouse DRG. The most potent inhibition induced by the F1-2 strain was observed after bradykinin challenge because the F1-2 stain incubation was associated with a significant reduction of the neuronal activation intensity fold change only after bradykinin stimulation, but not after serotonin or histamine stimulation. Interaction between the F1-2 strain and the bradykininergic system could therefore be a possible mechanism of action involved in the antihyperalgesic effect of the strain and requires further investigation. Bradykinin is a well-documented proinflammatory mediator that excites and sensitizes nociceptors and finally evokes pain. Its contribution to visceral pain has been reported both in rodents[3,56,61] and humans.[29,36,55] Of interest, a cross talk between bradykinin receptors and TRPV1 channels has been documented in previous studies demonstrating a sensitization of the channel after activation of bradykinin receptors.[42] Conversely, blocking B2 through a receptor antagonist can abolish C-fiber responses, whereas blocking B1 has no effect.[4] Of interest, response to capsaicin was also strongly reduced in nociceptors after F1-2 incubation.[5] However, whether this effect relies on a direct interaction between the strain and TRPV1 channels or whether it is a downstream effect through bradykinin receptors remains to be determined.

The molecular mechanisms by which the F1-2 strain exerts its beneficial effects need to be explored. Bacterial cell components or metabolites such as polysaccharides,[40,50] pili-like protein membrane,[46] butyrate, or long-chain fatty acids[48] have been identified as key molecules that confer anti-inflammatory properties of several intestinal commensal strains. In our study, although no anti-inflammatory action was observed, identifying molecules deriving from the F1-2 strain and that could interact with neurons, and more specifically with bradykinin receptors, would be of great interest to characterize the neuroinhibitory mechanisms induced by these bacteria. Regarding regulatory limitations in Europe,[13,14] it will take a long time before live biotherapeutic products is recognized as a new treatment, but the *P distasonis* F1-2 strain could be a potential novel therapeutic strategy to treat chronic abdominal pain observed in patients experiencing IBS or IBD in remission.

Conflict of interest statement

J.-Y. Berthon is the CEO of GREENTECH SA. The remaining authors have no conflict of interest to declare.

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