Lactococcus lactis CNCM I-5388 versus NCDO2118 by its GABA hyperproduction ability, counteracts faster stress-induced intestinal hypersensitivity in rats

Pedro Gomes, Valérie Laroute, Catherine Beaufrand, Marie-Line Daveran Mingot, Nathalie Aubry, Chloé Liebgott, Nathalie Ballet, Sophie Legrain-Raspaud, Vassilia Théodorou, Muriel Mercier-Bonin, et al.

Received: 5 August 2023 | Revised: 21 September 2023 | Accepted: 4 October 2023

DOI: 10.1096/fj.202301588R

RESEARCH ARTICLE

The FASEB Journal

Lactococcus lactis CNCM I-5388 versus NCDO2118 by its GABA hyperproduction ability, counteracts faster stress-induced intestinal hypersensitivity in rats

Pedro Gomes[1,2] | **Valérie Laroute**[1] | **Catherine Beaufrand**[2] | **Marie-Line Daveran-Mingot**[1] | **Nathalie Aubry**[1] | **Chloé Liebgott**[2] | **Nathalie Ballet**[3] | **Sophie Legrain-Raspaud**[4] | **Vassilia Theodorou**[2] | **Muriel Mercier-Bonin**[2] | **Muriel Cocaign-Bousquet**[1] | **Hélène Eutamene**[2]

[1]Toulouse Biotechnology Institute (TBI), Université de Toulouse, CNRS, INRAE, INSA, Toulouse, France

[2]Toxalim (Research Centre in Food Toxicology), Université de Toulouse, INRAE, ENVT, INP-Purpan, UPS, Toulouse, France

[3]Lesaffre International, Marcq-en-Barœul, France

[4]Gnosis by Lesaffre, Marcq-en-Barœul, France

Correspondence
Muriel Mercier-Bonin and Hélène Eutamene, Toxalim (Research Centre in Food Toxicology), Université de Toulouse, INRAE, ENVT, INP-Purpan, UPS, UMR INRAE 1331, 180 chemin de Tournefeuille, BP 93173, 31027 Toulouse cedex 3, France.
Email: muriel.mercier-bonin@inrae.fr and helene.eutamene@inrae.fr

Muriel Cocaign-Bousquet, Toulouse Biotechnology Institute (TBI), Université de Toulouse, CNRS, INRAE, INSA, 135 avenue de Rangueil, 31077 Toulouse cedex 04, France.
Email: cocaign@insa-toulouse.fr

Funding information
Lesaffre International, Grant/Award Number: CT010082 and CT011953

Abstract

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder characterized by its main symptom, visceral hypersensitivity (VH), which is aggravated by stress. Gut–brain interactions and gut bacteria may alleviate IBS symptoms, including VH. γ-amino butyric acid (GABA), produced notably by lactic acid bacteria (LAB), shows promising result in IBS symptoms treatment. In bacteria, GABA is generated through glutamate decarboxylase (GAD) metabolism of L-glutamic acid, maintaining intracellular pH. In mammals, GABA acts as an inhibitory neurotransmitter, modulating pain, stress, and anxiety. Therefore, utilizing GABA-producing LAB as a therapeutic approach might be beneficial. Our previous work showed that a GABA-producing *Lactococcus lactis* strain, NCDO2118, reduced VH induced by acute stress in rats after a 10-day oral treatment. Here, we identified the strain CNCM I-5388, with a four-fold higher GABA production rate under the same conditions as NCDO2118. Both strains shared 99.1% identical GAD amino acid sequences and in vitro analyses revealed the same optimal pH for GAD activity; however, CNCM I-5388 exhibited 17 times higher intracellular GAD activity and increased resistance to acidic pH. Additionally, in vivo experiments have demonstrated that CNCM I-5388 has faster anti-VH properties in

Abbreviations: CFU, colony forming unit; CRD, colorectal distension; EMG, electromyography; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; GI, gastrointestinal; GRAS, generally recognized as safe; IBS, irritable bowel syndrome; LAB, lactic acid bacteria; L-Glu, L-glutamic acid; PRS, partial restraint stress; VH, visceral hypersensitivity.

Muriel Mercier-Bonin, Muriel Cocaign-Bousquet, and Hélène Eutamene are co-last authors.

rats compared with NCDO2118, starting from the fifth day of treatment. Finally, CNCM I-5388 anti-VH effects partially persisted after 5-day treatment interruption and after a single oral treatment. These findings highlight CNCM I-5388 as a potential therapeutic agent for managing VH in IBS patients.

KEYWORDS

GABA, GAD activity, *Lactococcus lactis*, psychological acute stress, visceral hypersensitivity

1 | INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic heterogeneous multifactorial (physiological, genetic, psychosocial, and environmental) disorder of the gastrointestinal (GI) system. Its prevalence varies from 3% to 5% worldwide according to the Rome IV criteria; however, pathogenesis and pathophysiology of IBS are complex and not completely understood.[1] Patients report abnormal bowel habits, primarily with constipation, diarrhea (or both) and visceral hypersensitivity (VH), the main symptom of IBS exacerbated by anxiety and stressful psychological events, strongly affecting patients' quality of life.[1,2] First-line treatments for IBS symptoms include antispasmodics and tricyclic antidepressants; however, no pharmacological treatment is currently satisfactorily effective for these patients.[3]

In the last decade, clinical and preclinical studies on the use of psychobiotics have arisen for the treatment of IBS symptoms.[4] Indeed, recent studies have shown the anti-VH efficacy of certain bacteria, especially bifidobacteria and lactic acid bacteria (LAB).[5] The underlying mechanisms of action are complex and still fragmentarily understood. Among them, studies suggest gut microbiota modulation, as well as the reduction of motor disorders by the modulation of host's nociceptors through the production of neurotransmitters, such as serotonin and gamma(γ)-amino butyric acid (GABA).[6] In the GI tract, GABA acts mainly by binding to GABA A and B receptors. Metabotropic $GABA_B$ receptors are mainly present at the level of the fibers of vagal origin of the enteric nervous system and in the glandular part of the gastric mucosa with a number that decreases from the mouth to the anus. GABA-dependent activation of these receptors allows modulation of intestinal motility, gastric emptying, transient relaxation of the lower esophageal sphincter, and visceral sensation to painful colonic stimuli.[7] Accordingly, $GABA_B$ agonist baclofen has antinociceptive effects at the level of the spinal cord and can suppress VH responses to colorectal distension (CRD) stimuli.[8] At the sight of these properties, the use of GABA-producing bacterial strains could be a safe and promising adjuvant therapeutic solution.

In this context, GABA-producing LAB have received broad attention in recent years due to their generally recognized as safe (GRAS) status and their high application potential for fermented food products, but also for their health potential.[9] In LAB, the glutamate decarboxylase (GAD, EC 4.1.1.15) system, which is composed of the GAD enzyme (encoded by *gadA* and/or *gadB*, depending on the bacterial species) and the glutamate/GABA antiporter GadC (encoded by *gadC*), is responsible for the production of GABA. GadC is responsible for transporting L-glutamic acid (L-Glu) into the microorganism. Once inside the cytosol, L-Glu is decarboxylated by the pyridoxal-5′-phosphate-dependent GAD enzyme, leading to the formation of GABA while cytoplasmic H^+ is consumed and CO_2 is released as a by-product. Lastly, GABA is exported outside the cytoplasm by GadC.[10] Therefore, while GABA acts mainly as an inhibitory neurotransmitter in mammals, its production in bacteria represents one of the most important enzyme-based acid tolerance mechanisms. In LAB, particularly adapted to grow in acidic conditions, GABA production acts with another main system, the arginine deaminase (ADI) pathway, producing citrulline (and later ornithine) and the basic compound NH_3.[11] As pointed by Small & Waterman, many bacterial genera that either colonize the gut, becoming resident in microbiota, or temporarily integrate into the gut microbiome like transient food-borne bacteria, have similar GAD systems (e.g. *Escherichia*, *Shigella*, *Clostridium*, *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Lactococcus*).[10] Accordingly, a study has shown that *Lactococcus lactis* PU1, presenting a functional GAD system, was able to survive and synthesize GABA under simulated gastrointestinal conditions.[12] It is reasonable to postulate that the primary role of GAD is to facilitate bacterial transit and survival through the stomach harsh conditions.[10]

We have previously shown that *L. lactis* NCDO2118 grown in L-Glu-supplemented medium is a GABA producer in vitro[13] and that 10-day daily oral treatment by this strain is efficient to reduce acute stress-induced VH in response to CRD in a rat model.[14] Furthermore, our results have shown that GABA was produced by NCDO2118 under "stomach-like" conditions in vitro and in vivo in the stomach, raising the hypothesis that a dynamic "virtuous

circle" would be generated between *L. lactis* and the host. In this context, *L. lactis* GAD would be activated under acidic conditions in the host's stomach, activating $GABA_B$ receptors, widely distributed from the stomach to the ileum, increasing gastric secretion through vagal cholinergic and gastrin-dependent mechanisms, maintaining acidic conditions in the stomach.[14] In recent screening studies, we have identified the *L lactis* strain CNCM I-5388 as a promising candidate to produce GABA among 132 *L. lactis* strains isolated from various environments (Laroute et al., 2023, submitted). Here, we quantified the production of GABA in *L. lactis* CNCM I-5388 in vitro and demonstrated that it was significantly higher than that of NCDO2118 under similar culture conditions. We have also shown that CNCM I-5388 has faster antinociceptive properties than NCDO2118, starting from the fifth day of treatment, in acute stress-induced VH in the same rat model as in the work of Laroute and collaborators. Furthermore, we demonstrate that CNCM I-5388 tends to reduce VH after 5-day oral treatment interruption and also after a single oral treatment. We finally explained that the differences in CNCM I-5388 and NCDO2118 GAD activities could justify differences in GABA production in vitro and antinociceptive properties in vivo.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains

The *Lactococcus lactis* strains CNCM I-5388 and NCDO2118 (dairy and vegetable origin, respectively) were used throughout this study. CNCM I-5388 Δ*gadB* mutant was constructed by double-crossing over in the chromosome as previously described.[14,15] Briefly, two fragments upstream and downstream of the *gadB* coding sequence were PCR amplified, fused by overlapping PCR and cloned in the pGhost9 vector using Gibson assembly method (New Englang Biolabs). Primers are listed in Supplementary Table S1.

2.2 | Sequence analysis of GAD and GadC amino acid residues

The *gad* operon sequence for the NCDO2118 strain was taken from the chromosomal sequence available in the NCBI-GenBank database under the accession number CP009054. The CNCM I-5388 strain's genome was sequenced and entered into the NCBI-GenBank database with the accession number JASGWY000000000. Sequences of the GAD and GadC amino acid residues were determined based on the *gadCB* operon nucleotide sequences and compared using Clustal Omega alignment algorithm.[16]

2.3 | Cultures in bioreactor

Bacterial cultures were performed for each strain mentioned above with least three biological replicates, in 2-L Biostat B-plus bioreactor (Sartorius, Melsungen, Germany) in M17 supplemented with 55 mM (8 g/L) L-Glu, 29 mM (5 g/L) arginine, 250 mM (45 g/L) glucose, and 300 mM. Fermentations were carried out under oxygen-limiting conditions at 30°C. pH was maintained at 6.6 by KOH addition for 8 h, then pH was dropped and regulated at 4.6. Culture was inoculated with cells from precultures grown in Erlenmeyer flask on similar medium, harvested during the exponential phase and concentrated to obtain an initial optical density (OD) at 580 nm of 0.25 in the fermenter. Bacterial growth was monitored by spectrophotometric measurements at 580 nm (Libra S11, Biochrom, BIOSERV, Massy, France; 1 unit of optical density is equivalent to 0.3 g dry weight/L). Samples were collected every 30 min for HPLC measurement of GABA concentration in the growth medium. For in vivo assays, bacterial cells were harvested before the pH modification (i.e., at 7 h). The culture volume required for approximately 3×10^{11} CFU was centrifuged to pellet the bacterial cells. Then, cells were washed and suspended in 0.9% [w/v] NaCl containing 15% glycerol [v/v] to a final concentration of 10^9 CFU/mL. For GAD activity measurements, 150 mg of bacterial cells were harvested at 7 and 24 h of culture.

2.4 | Cytoplasmic and cell-bound GAD activities by *L. lactis* cells

For cytoplasmic GAD activity measurement, 150 mg cells were washed twice with 0.2% KCl [w/v] and suspended in 3 mL sodium acetate buffer (100 mM, pH 4.6) containing 4.5 mM $MgCl_2$, 22% [v/v] glycerol and 1.5 mM dithiothreitol. This mixture was distributed into three tubes containing 6 mg of glass beads. Then, cells were disrupted in a FastPrep-24 homogenizer (MP Biomedicals, Illkirch, France) using 6 cycles of 30 s at 6.5 m/s interrupted by 1 min incubation on ice. Cell debris were removed by centrifugation for 15 min at 10 000 *g* and 4°C. The supernatant was used for enzyme assays, and the protein concentration of the extract was determined by the Bradford method. For the assays of optimal pH of cytoplasmic GAD activity, 240 mM NaCl was added to the sodium acetate buffer and pH was controlled with 100 mM acetic acid as follows: 4.0, 4.2, 4.4, 4.6, 4.8, 5.0 5.4 and 6.0 (n = 2 for CNCM I-5388 and

NCDO2118 strains). Enzyme assay was performed with 0.5 mL of substrate solution, consisting of 20 mM sodium glutamate, 2 mM pyridoxal phosphate (PLP) incubated at 30°C then mixed with 0.5 mL supernatant. Every 30 min until 4 h, 100 µL were sampled and inactivated by boiling for 5 min to stop the decarboxylation reaction. Reaction mixtures were subsequently analyzed for the presence of GABA using HPLC. Cell-bound GAD activities of CNCM I-5388 and NCDO2118 strains were determined by assessing the amount of GABA formed at 37°C in a reaction mixture containing 0.5 mL of each strain (10^9 CFU), 1 mL of 75 mM sodium acetate buffer (pH 4.6) and 0.5 mL 67 mM L-Glu. For in vitro HCl-induced acidic stress assay, the pH of the mixture acetate buffer and L-Glu was adjusted by addition of HCl prior to the addition of bacterial cells as follows: 4.6, 4.0, 3.5, 3.0, and 2.5 ($n = 3$ for CNCM I-5388 and NCDO 2118 strains). Bacterial viable counts after 2 h of acidic stress exposure were assessed by plating samples on M17 broth medium supplemented with 20 g/L glucose and incubated for 48 h at 30°C to determine CFU. In parallel, cell-bound GAD activity assay was carried out during 2 h with 100 µL sample recovery at regular time intervals 0, 10, 20, 30, 60, 90, and 120 min, next incubated at 95°C for 5 min for enzyme inactivation. Proteins were precipitated by adding four volumes of methanol followed by incubation on ice and supernatants were recovered after centrifugation for HPLC analysis. Cell-bound GAD activity was defined as the production of 1 µmol of GABA in 1 min under the above conditions. GABA concentration in culture supernatant or in reaction mixtures, associated with assays for GAD activity (cytoplasmic or cell-bound) and GABA production, was measured by HPLC (Agilent Technologies 1200 Series, Waldbronn, Germany) as previously described.[17] In both CNCM I-5388 and NCDO2118 strains, cell-bound GAD activities from pH 4.0 to 2.0 were normalized by the mean value of the control (pH 4.6).

2.5 | Animals and surgical procedure

Adult female Wistar rats (200–225 g) were purchased from Janvier Labs (Le Genest St Isle, France) and individually housed in polypropylene cages under standard conditions (temperature 22 ± 2°C and a 12-h light/dark cycle) with free access to water and food (standard pellets 2016, Envigo RMS SARL, Gannat, France). The experiments were carried out according to the requirements laid down in European directive 2010/63/EU on the protection of animals used for scientific purposes. The experimental procedures and protocols were approved by the local ethics committee of Toulouse Midi-Pyrénées and authorized by the French Ministry of Higher Education and Research under the references APAFIS#5577-201606061639777v3 (for NCDO2118 in vivo experiments) and APAFIS#14898-2018043016033142 6v2 (for CNCM I-5388 in vivo experiments).

2.6 | CRD and acute stress procedures

Under general anesthesia by intraperitoneal administration of 0.6 mg/kg acepromazine (calmivet, Vetoquinol, Lure, France) and 120 mg/kg ketamine (Imalgene 1000, Merial, Lyon, France), rats were equipped with NiCr wire electrodes implanted in the abdominal striated muscle for electromyography (EMG) recording.[18] Animals were then accustomed to be in polypropylene tunnels for several days before CRD. A 4-cm long latex balloon, fixed on a rigid catheter was used. CRD was performed after insertion of the balloon in the rectum at 1 cm from the anus. The tube was fixed at the basis of the tail. Isobaric distensions of the colon were performed from 0 to 60 mmHg using a Distender Series IIR Barostat (G&J Electronics Inc, Toronto, Canada) with each distension step lasting 5 min. The striated muscle spike bursts, related to abdominal cramps, were recorded by electromyography (EMG) (Mini VIII, Alvar, Paris, France).

Partial restraint stress (PRS), a relatively mild non-ulcerogenic model of stress, was performed as previously described.[14] Briefly, rats were sedated with diethyl-ether and their fore shoulders, upper forelimbs, and thoracic trunk were wrapped in a confining harness of paper tape to restrict, but not to prevent body movements. Rats were then placed in their home cage for 2 h.

2.7 | Experimental protocol for in vivo assays

Series of experiments, based on a 10-day treatment by oral gavage, were conducted using for each series three groups of 7–13 female rats equipped for EMG as previously described.[14]

In the first series of experiments, rats were divided into two groups and orally treated for 10 days (1 mL/rat) with: (a) *L. lactis* NCDO2118 (10^9 CFU/day) plus L-glutamic acid (L-Glu) in its monosodium salt hydrate form (0.2% [w/v]) or (b) vehicle (NaCl 0.9% [w/v] + glycerol 15% [v/v]). Noteworthy, this form chemically indistinguishable from its non-monosodium salt counterpart,[19] was used for its solubilization in non-acidified solutions (i.e., vehicle used for rats' oral treatments). In the second series, rats were divided into four groups and orally treated for 10 days (1 mL/rat) with: (a) *L. lactis* CNCM I-5388 (10^9 CFU/day) plus L-Glu monosodium salt (0.2% [w/v]) or (b) CNCM I-5388 (10^9 CFU/day) plus L-Glu

monosodium salt (0.2% [w/v]) and $GABA_B$ receptor antagonist (2S)(+)-5,5-dimethyl-2-morpholineacetic acid (SCH-50911, Sigma-Aldrich SML1040; 3 mg/kg body weight, IP 20 min before the PRS session) or (c) CNCM I-5388 Δ*gadB* (10^9 CFU/day) plus L-Glu monosodium salt (0.2% [w/v]) or (d) vehicle (NaCl 0.9% [w/v] + glycerol 15% [v/v]). In the third series, rats were divided in into two groups and orally treated for 5 days (1 mL/rat) (1 mL/rat) with: (a) *L. lactis* CNCM I-5388 (10^9 CFU/day) plus L-Glu in its monosodium salt hydrate form (0.2% [w/v]) or (b) vehicle (NaCl 0.9% [w/v] + glycerol 15% [v/v]), followed by 5 days of treatment interruption. In a final series of experiment, rats were divided into two groups and orally treated (1 mL/rat) with a single dose of: (a) *L. lactis* CNCM I-5388 (10^9 CFU/day) plus L-Glu monosodium salt (0.2% [w/v]) or (b) vehicle (NaCl 0.9% [w/v] + glycerol 15% [v/v]). Responses to CRD were recorded a day before treatment and post-PRS (20 min after the 2-h PRS session) abdominal responses were recorded 1 h after single oral treatment. For all other oral treatments used, abdominal responses to CRD were recorded on Day 4 and Day 9 for basal conditions, and post-PRS (20 min after the 2-h PRS session) on Day 5 and Day 10.

2.8 | Fecal bacterial microbiota composition using 16S rRNA gene sequencing

Feces were collected at the fifth and tenth day of oral treatment, just before the 2-h PRS session (see Section 2.6). Fecal samples were stored at −80°C until DNA was extracted using the ZymoBIOMICS DNA Miniprep Kit (D4300, Zymo Research) following manufacturer's instructions. The 314F/805R primers (5′ GACTACHVGGG TATCTAATCC-Forward primer, 5′ GACTACHVGGG TATCTAATCC-Reverse primer) were used to amplify the V3–V4 variable regions of the 16S rRNA gene. Forward primer and reverse primer carried overhang adapters (5′ CTTTCCCTACACGACGCTCTTCCGATCT-Forward primer, (5′ GGAGTTCAGACGTGTGCTCTTCCGATCT-Reverse primer) for Illumina index and sequencing adapters. First round of PCR was carried in a single-step 30 cycle using the MTP Taq DNA Polymerase Kit (D7442, Sigma-Aldrich) under the following conditions: 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min, after which a final elongation step at 72°C for 10 min was performed. Second-round amplicons libraries and sequencing were performed at the Sequencing Platform of Toulouse (GeT-Biopuces) on an Illumina-MiSeq following the manufacturer's guidelines.

Bioinformatic analysis of all samples was performed with the Find Rapidly OTUs (operational taxonomic unit) with Galaxy Solution (FROGS) pipeline.[20] Briefly, reads were contigued with the VSEARCH software and sequences with sizes inferior to 300 bp or superior to 700 bp were eliminated. SWARM algorithm for clustering was used to define OTUs and chimeric sequences were also removed. Sequences were then filtered using the phiX contaminant databank and the OTUs presenting a frequency inferior to 0.005% on all samples and present in less than three samples were also removed.[21] The R package ANOMALY[22] was used for the assignment of the taxonomic classification of the representative sequences of each OTU. For that, the algorithm IDTAXA and the reference databases SILVA 138 16S and GTDB bac120_arc22 were used.[23,24] Alpha (α) and beta (β) diversities analyses were performed in R using the Phyloseq package.[25] Alpha diversity metrics (i.e., observed OTUs and Shannon Index) were calculated based on the genus level.

2.9 | Statistical methods

The software GraphPad Prism 9.1 (GraphPad, San Diego, CA) was used for statistical analyses. For the results of CFU/mL and cell-bound GAD activity under HCl-induced stress, Mann–Whitney U test was performed to compare the control and treatment conditions for CNCM I-5388 and NCDO2118 strains ($n = 3$). Mann–Whitney U test was also used to compare maximum specific growth rate, specific consumption rate of glucose, specific lactate production rate, specific L-Glu consumption rate, and specific GABA production rate between strains ($n = 3$ for both strains). One-way ANOVA, followed by Tukey's multiple comparison test, was performed between different groups of animals (at least seven per treatment). Data are reported as the means ± SEM and *p*-values lower than .05 were considered as significant. For fecal microbiota, one-way ANOVA was used to test α diversity metric dissimilarities with post hoc Tukey's test. The Bray-Curtis distance was used to reveal β diversity metrics. We explored the community structure of the samples with PERMANOVA.[26]

3 | RESULTS

3.1 | *L. lactis* CNCM I-5388 is a higher GABA producer than NCDO2118

In the interest of comparing GABA production under the same culture conditions, CNCM I-5388 and NCDO2118 were grown for 24 h in batch bioreactor with a complex medium (see Experimental section). Biomass production of CNCM I-5388 and NCDO2118 and their maximum specific growth rates were very similar (Figure 1A and

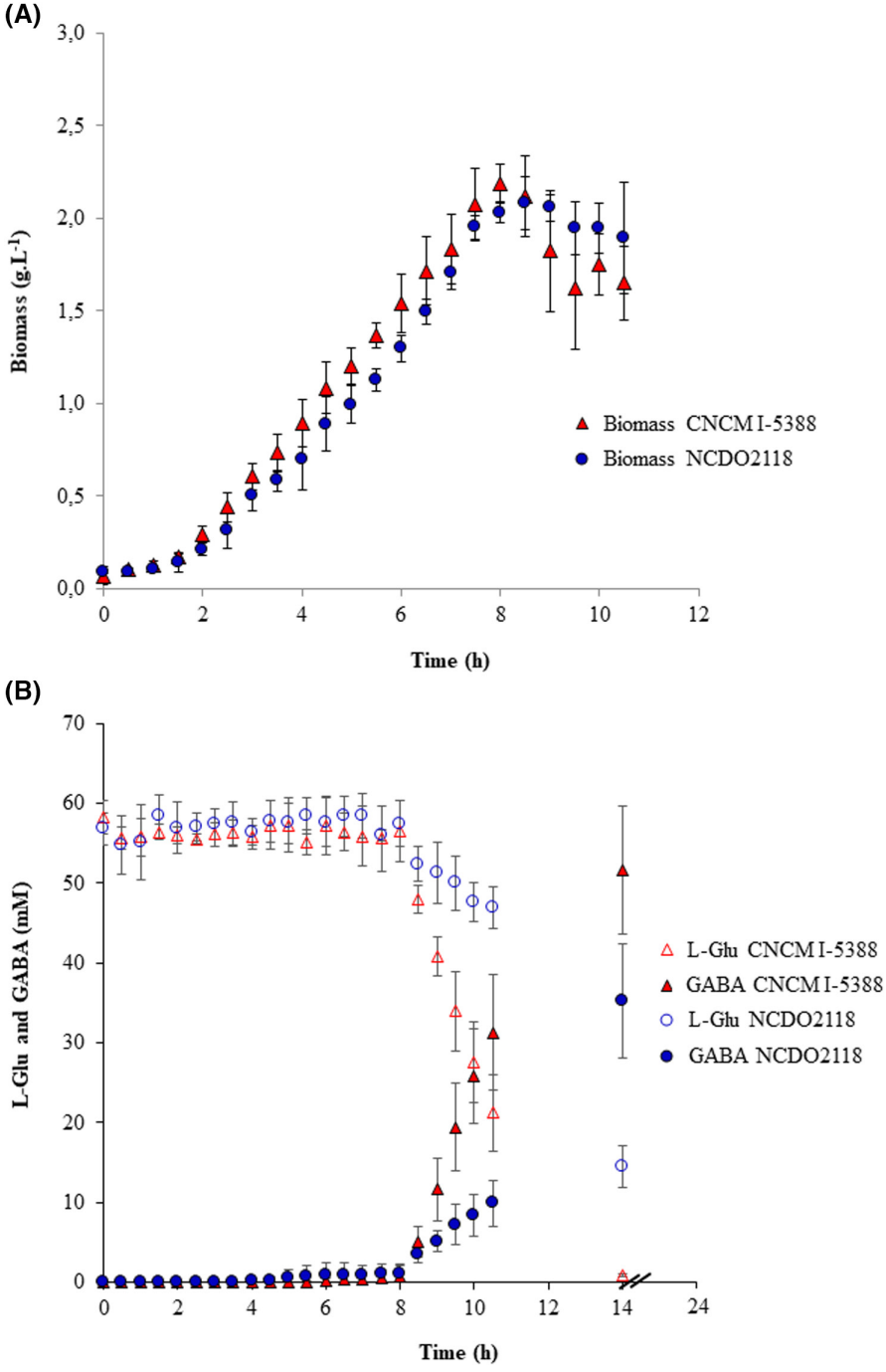

FIGURE 1 (A) Evolution of biomass (g/L) and (B) GABA production and L-Glu consumption (mM) during 10.5-h growth of *L. lactis* CNCM I-5388 and NCDO2118 in bioreactor.

Table 1). Both strains consumed glucose and produced lactate in a similar manner during growth. A slight metabolic acceleration, associated with a small but not statistically significant ($p > .05$) increase in the specific rates of glucose consumption and lactate production, were observed for CNCM I-5388 compared with NCD2118 (Table 1). GABA and L-Glu concentrations were also measured all along the culture (Figure 1B). Both strains had their GABA production and L-Glu consumption started at 8 h of fermentation (Figure 1B), once pH was changed from 6.6 to 4.6; However specific rate of GABA production for CNCM I-5388 (7.2 ± 2.1 mmol/g.h) and specific rate of L-Glu consumption (7.9 ± 1.8 mmol/g.h) were approximately four times higher ($p < .05$) than the values obtained for NCDO2118 (1.8 ± 0.7 and 1.6 ± 0.4 mmol/g.h, respectively) (Figure 1B and Table 1). At 10.5 h of fermentation (i.e., 2.5 h after the pH change), CNCM I-5388 GABA concentration reached values approximately three times higher ($p < .05$) than NCDO2118 (31.1 ± 7.2 and 9.9 ± 2.9 mM, respectively), which can be justified by their differences in the specific rate of GABA production (Figure 1B and Table 1). Accordingly, at 10.5 h of fermentation, less than 25 mM of L-Glu were detected for CNCM I-5388 against 47 mM for NCDO2118 (Figure 1B and Table 1). These

results demonstrated that CNCM I-5388 is a higher GABA producer in comparison to NCDO2118, generally characterized as the GABA producer reference for *L. lactis*.[13] We have also verified that this very high GABA production was related to the GAD enzyme since growth of mutant strain CNCM I-5388 Δ*gadB*, in which GABA pathway was interrupted, was unable to produce GABA although growth was similar (Supplementary Figure S1).

TABLE 1 *L. lactis* NCDO2118 and CNCM I-5388 maximum specific growth rate, specific consumption rate of glucose, specific lactate production rate, specific L-Glu consumption rate, specific GABA production rate and concentration of L-Glu and GABA at 10.5-h fermentation.

L. lactis strain	CNCM I-5388	NCDO2118
μ_{max} (h^{-1})	0.86 ± 0.06	0.81 ± 0.05
$q_{glucose}$ (mmol/g.h)	18.5 ± 2.6	15.1 ± 1.1
$\nu_{lactate}$ (mmol/g.h)	36.4 ± 4.5	30.3 ± 1.4
$q_{L\text{-}Glu}$ (mmol/g.h)	7.9 ± 1.8	1.6 ± 0.4*
ν_{GABA} (mmol/g.h)	7.2 ± 2.1	1.8 ± 0.7*
$[L\text{-}Glu]_{10.5\,h}$ (mM)	21.3 ± 4.8	46.9 ± 2.6*
$[GABA]_{10.5\,h}$ (mM)	31.1 ± 7.2	9.9 ± 2.9*

*$p < .05$.

3.2 | Both antinociceptive effects of CNCM I-5388 and NCDO2118 are found at 10 days of oral treatment, but only higher GABA producer CNCM I-5388 is efficient at 5 days

To compare in vivo anti-VH properties of *L. lactis* higher GABA producer CNCM I-5388 and GABA producer NCDO2118, rats were given each strain by oral gavage once daily with the same number of bacterial cells (10^9 CFU per day) supplemented with L-Glu monosodium salt hydrate and the responses to CRD under PRS were measured after 10 or 5 days of treatment. We have first verified that NCDO2118 and CNCM I-5388 had no impact on basal CRD sensitivity (i.e., measured 1 day before the stress session) after either 9-day treatment

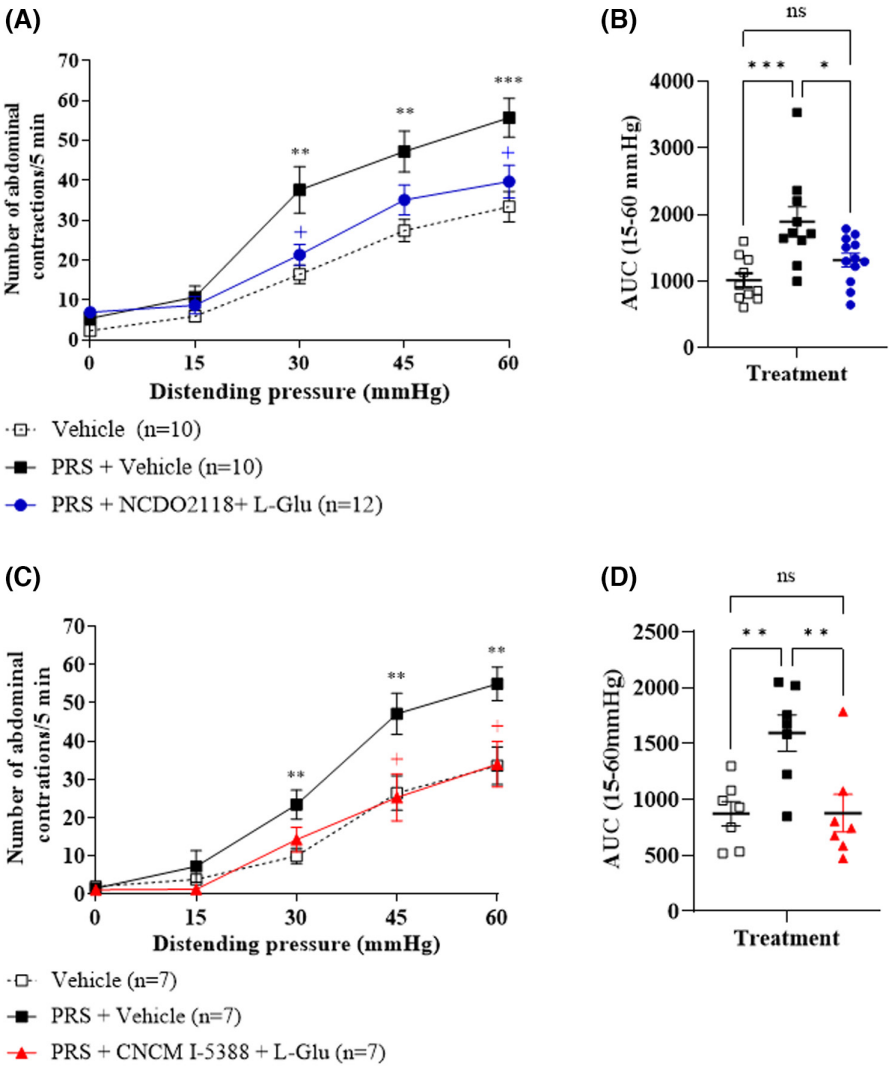

FIGURE 2 Effect of 10-day oral administration of *L. lactis* in the presence of L-Glu monosodium salt hydrate on VH induced by PRS in response to colorectal distension. (A) Effect of GABA-producing NCDO2118 on PRS-induced VH to all colorectal distension pressures (15 to 60 mmHg) and respective (B) area under the curve. (C) Effect of higher GABA producer CNCM I-5388 on PRS-induced VH to all colorectal distension pressures (15 to 60 mmHg) and respective (D) area under the curve. Data are expressed as mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$ versus basal values for animals treated with vehicle. $^{+}p < .05$, $^{++}p < .01$, $^{+++}p < .001$ versus values for stressed animals treated with vehicle. ns = non-significant ($p > .05$).

(Supplementary Figure S2A,B, respectively) or 4-day treatment (Supplementary Figure S2C,D, respectively).

Next, we confirmed that NCDO2118 10-day oral treatment was capable to restore a quasi-basal sensitivity to CRD, significantly decreasing the number of abdominal contractions of stressed rats at 30 and 60 mmHg ($p < .05$) compared with vehicle-treated animals (Figure 2A), fully in accordance with the results previously published by our group.[14] These results were here consolidated once the area under the curve (AUC) for all distension pressures was calculated (Figure 2B). Similarly, CNCM I-5388 10-day oral treatment was capable to restore a quasi-basal sensitivity to CRD, significantly decreasing the number of abdominal contractions of stressed rats at 45 mmHg and 60 mmHg ($p < .05$) compared with vehicle-treated animals (Figure 2C,D). After 5 days of oral treatment, NCDO2118 was no more efficient because no statistical differences ($p > .05$) were detected between the numbers of abdominal contractions of stressed rats that received NCDO2118 or the vehicle, either comparing all distension pressures (Figure 3A,B). Interestingly, this was not the case for the CNCM-I 5388 5-day oral treatment, since it was capable to restore a quasi-basal sensitivity to CRD, significantly decreasing the number of abdominal contractions at 30 mmHg ($p < 0.05$) and 60 mmHg ($p < .001$) (Figure 3C,D). Additionally, the efficacy of CNCM I-5388 treatment on stress-induced VH was abolished when animals received not only the $GABA_B$ receptor antagonist SCH-50911, but also the mutant strain CNCM I-5388 *ΔgadB* either for 10 (Supplementary Figure S3A,B) or 5 days of oral treatment (Supplementary Figure S3C,D). Finally, CNCM I-5388 treatment had no impact on the fecal bacterial microbiota composition at the level of alpha (α) and beta (β) diversities for 10 (Supplementary Figure S4A–D) or 5 days (Supplementary Figure S5A–D).

3.3 | CNCM I-5388 antinociceptive properties start partially from the first day of treatment

Since *L. lactis* CNCM I-5388 has shown antinociceptive efficacy after a 5-day treatment, independently of the fecal microbiota influence, we wondered if this strain could have a pharmacological-like effect, with antinociceptive efficacy after a single-day treatment. We have first verified that there were no impacts on basal CRD sensitivity a day before CNCM I-5388 single-dose treatment (Supplementary Figure S2E). Then, rats were given a single-dose of CNCM I-5388 (10^9 CFU) supplemented with L-Glu monosodium salt hydrate 1 h prior to partial restraint stress (PRS) followed by the measurement of abdominal contractions in response to CRD. We detected no statistical differences ($p > .05$) between the numbers of abdominal contractions of stressed rats that received either CNCM I-5388 or the vehicle, in response to all distension pressures from 15 to 60 mmHg (Figure 4A). Interestingly, no statistical differences were also detected between non-stressed rats treated with the vehicle and stressed rats treated with CNCM I-5388, pointing toward a moderate effect, which was also the case for AUC (Figure 4A,B, respectively). This result suggests that a single dose of CNCM I-5388 tends to reduce VH in response to a PRS session.

3.4 | CNCM I-5388 antinociceptive properties last partially after 5-day treatment interruption

To evaluate if *L. lactis* CNCM I-5388 anti-VH properties would persist after treatment interruption, rats received 5-day daily oral treatment of CNCM I-5388 (10^9 CFU) supplemented with L-Glu monosodium salt hydrate, followed by 5-day washout. We have first verified that there were no impacts of either 5-day treatment or 5-day washout on basal CRD sensitivity (Supplementary Figure S2F,G, respectively). As here before presented, 5-day oral treatment restored a quasi-basal sensitivity to CRD, significantly diminishing the number of abdominal contractions at 45 mmHg ($p < .01$) and 60 mmHg ($p < .001$) (Figure 5A,B). We detected no statistical differences ($p > .05$) between the numbers of abdominal contractions of stressed rats that received either CNCM I-5388 or the vehicle, in response to all distension pressures from 15 to 60 mmHg (Figure 5C). Interestingly, no statistical differences were also detected between non-stressed rats treated with the vehicle and stressed rats treated with CNCM I-5388, indicating an intermediate impact, which was also the case for the AUC (Figure 5C,D, respectively). This result suggests that 5-day washout after 5-day oral treatment of CNCM I-5388 also tends to reduce VH in response to a PRS session.

3.5 | Intracellular GAD activity of higher GAB producer CNCM I-5388 is 17-fold higher than GABA-producing NCDO2118

Aiming to better understand the CNCM I-5388 GABA hyperproduction in vitro and its faster performance in vivo compared with the reference NCDO2118 strain, we have measured the cytoplasmic GAD activity of both strains. A high cytoplasmic GAD activity was obtained for CNCM I-5388 (813.0 ± 183.7 μmol/min.mg), consistently with its GABA hyperproduction ability, compared with NCDO2118 (48.7 ± 10.7 μmol/min.mg) ($p < .05$) (Table 2). This very high level of GAD

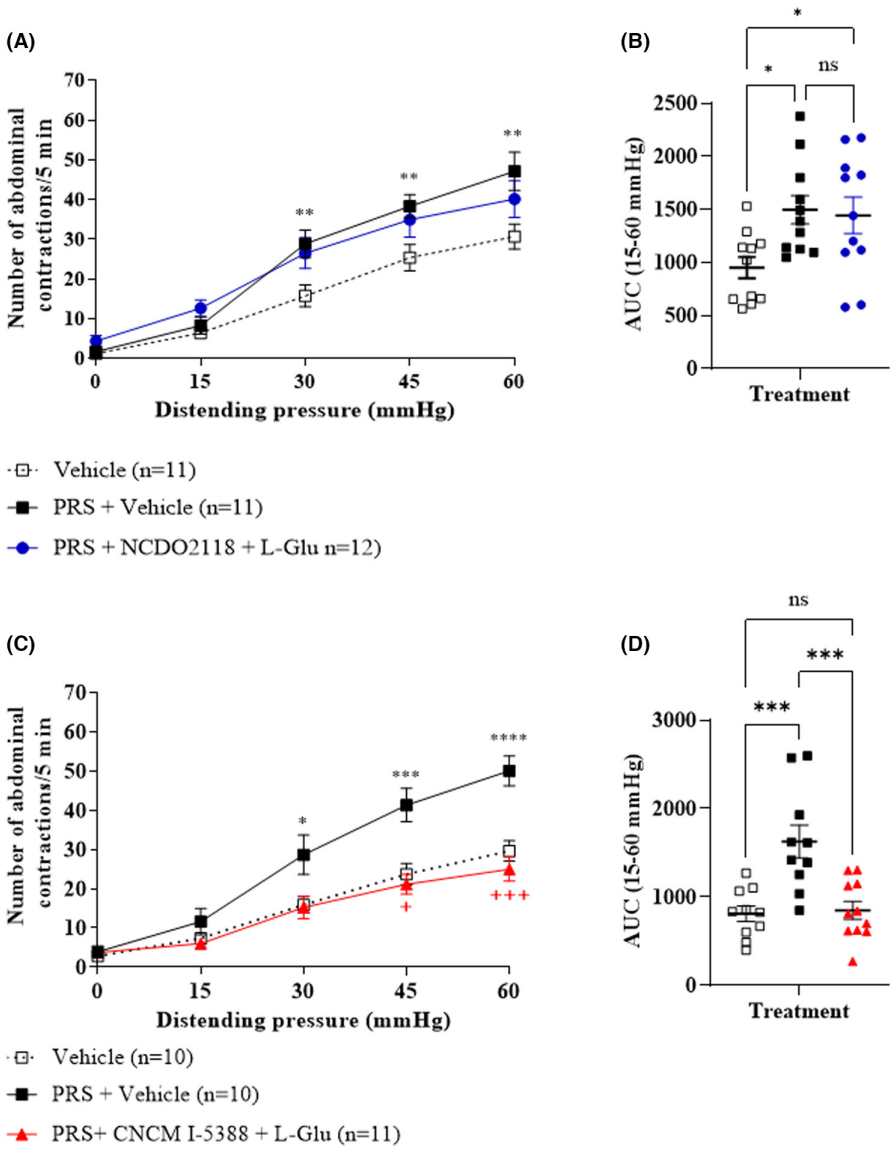

FIGURE 3 Effect of 5-day oral administration of *L. lactis* in the presence of L-Glu monosodium salt hydrate on VH induced by PRS in response to colorectal distension. (A) Effect of GABA-producing NCDO2118 on PRS-induced VH to all colorectal distension pressures (15–60 mmHg) and respective (B) area under the curve. (C) Effect of higher GABA producer CNCM I-5388 on PRS-induced VH to all colorectal distension pressures (15–60 mmHg) and respective (D) area under the curve analysis. Data are expressed as mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$ versus basal values for animals treated with vehicle. $^{+}p < .05$, $^{++}p < .01$, $^{+++}p < .001$ versus values for stressed animals treated with vehicle. ns, non-significant ($p > .05$).

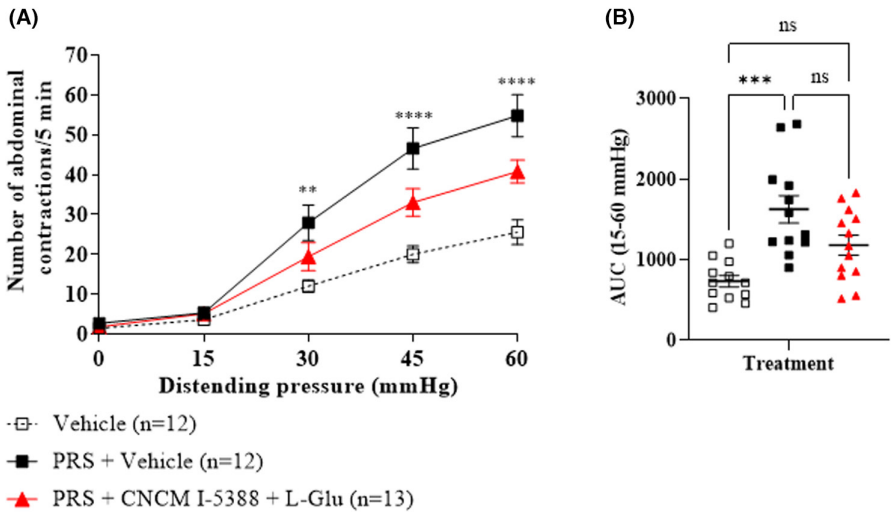

FIGURE 4 Effect of a single oral administration of *L. lactis* CNCM I-5388 in the presence of L-Glu monosodium salt hydrate on PRS-induced VH induced by stress in response to colorectal distension. (A) Treatment response to all colorectal distension pressures (15 to 60 mmHg) and respective (B) area under the curve. Data are expressed as mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$ versus basal values for animals treated with vehicle. ns, non-significant ($p > .05$).

activity was associated with a weak activity in the ADI pathway relative to NCDO2118 (i.e., significant reduction in arginine and ornithine consumption rate and citrulline production rate ($p < .05$), Table 2). To go further in the understanding of this higher GABA production in CNCM I-5388 than in NCDO2118, we have

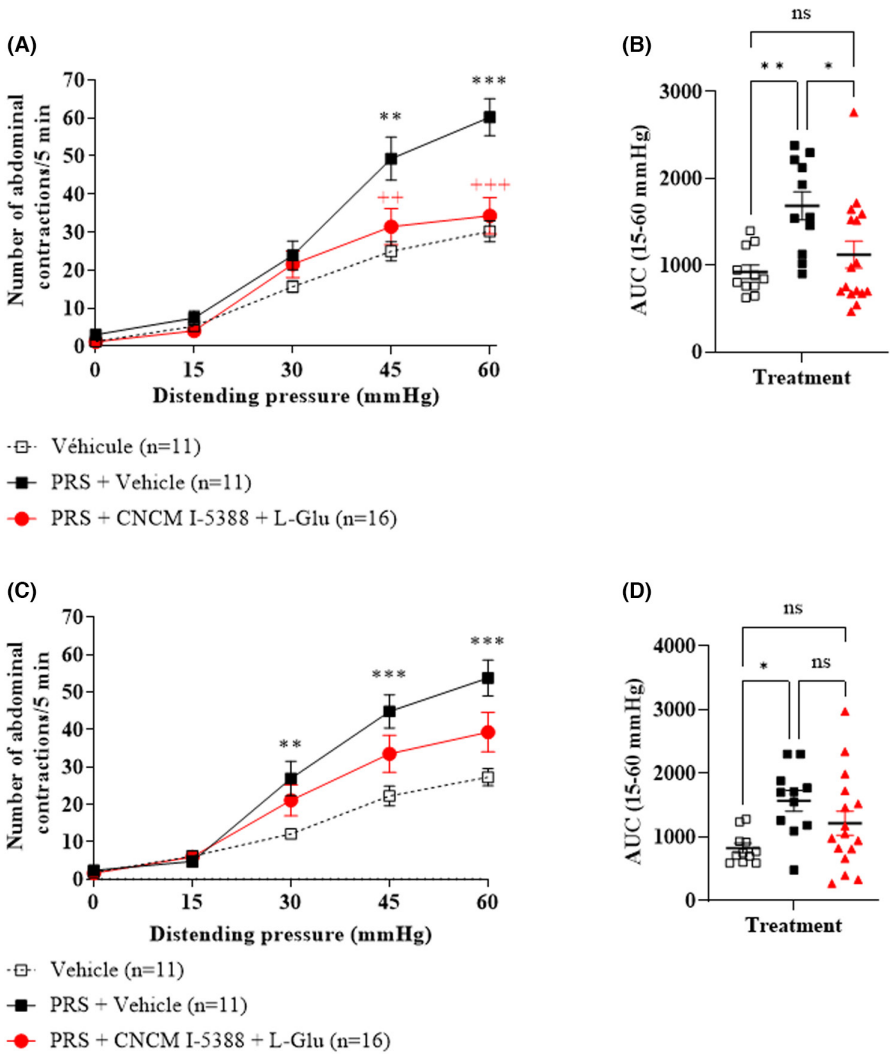

FIGURE 5 Effect of 5-day oral administration followed by 5-day treatment interruption of *L. lactis* CNCM I-5388 in the presence of L-Glu monosodium salt hydrate on VH induced by PRS in response to colorectal distension. (A) 5-day treatment response to all colorectal distension pressures (15–60 mmHg) and respective (B) area under the curve. (C) 5-day treatment followed by 5-day interruption after response to all colorectal distension pressures (15–60 mmHg) and respective (D) area under the curve. Data are expressed as mean ± SEM. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, versus basal values for animals treated with vehicle. $^{++}p < .01$, $^{+++}p < .001$ versus values for stressed animals treated with vehicle. ns, non-significant ($p > .05$).

TABLE 2 *L. lactis* NCDO2118 and CNCM I-5388 cytoplasmic GAD activity at 7-h fermentation, specific arginine and ornithine consumption rate and specific citrulline production rate.

L. lactis strain	GAD 7 h (µmol/min.mg)	$q_{arginine}$ (mmol/g/h)	$q_{ornithine}$ (mmol/g/h)	$\nu_{citrulline}$ (mmol/g/h)
CNCM I-5388	813.0 ± 183.7	3.6 ± 0.5	3.5 ± 0.7	0.5 ± 0.3
NCDO2118	48.7 ± 10.7*	9.2 ± 0.1*	5.3 ± 0.5*	2.8 ± 0.3*

*$p < .05$.

compared in silico their GAD amino acid sequences to reveal any potential polymorphisms that could have an impact on the enzyme structure and activity. GAD sequences alignment revealed that amino acid composition in CNCM I-5388 differs from NCDO2118 in only one polymorphism in position 185, presenting a residue of His and Arg, respectively, both with electrically charged side chains that should not impact GAD structure (Supplementary Figure S6A). This high homology between the two GADs at the level of amino acid composition was also observed at the level of their physicochemical properties. GAD enzymes are indeed similarly affected by the pH and share the same pH for optimal cytoplasmic activity between 4.6 and 4.8 in both CNCM I-5388 and NCDO2118 (Figure 6).

3.6 | Only CNCM I-5388 cell-bound GAD activity is enhanced in HCl-induced acidic stress conditions in vitro

Our present results indicate that *L. lactis* CNCM I-5388 has higher intracellular GAD activity than the NCDO2118. However, intracellular GAD activity does not necessarily reflect GABA production in vivo, since animals are not treated with the enzyme GAD itself but with intact

bacterial cells that count with a selective gate for the entry of L-Glu and exit of GABA (GadC). Therefore, we compared amino acid residues of GadC in CNCM I-5388 and NCDO2118 aiming to reveal any possible polymorphisms and differences in hypothetical physicochemical properties (i.e., hydrophobicity, electrical charge, and isoelectric point) that could have an impact on GABA delivery by *L. lactis* in vivo. The 466 GadC amino acid residues were strictly the same for both strains with the exception of four polymorphisms detailed in Supplementary Figure S6B, three of them representing substitutions of amino acid groups of similar properties and one with weakly similar properties. Therefore, there is little chance that the polymorphisms detected have an impact on the L-Glu/GABA antiporter action of the GadC of both strains.

We have also previously indicated that GABA production in vivo by *L. lactis* would be preferentially triggered at the host's stomach, since acidic pH is essential for GAD activation and GABA production.[14] Such acidic stress might also reduce or extinguish *L. lactis* survival, affecting bacterial cell membrane permeability and consequently impacting cell-bound GAD activity. For that reason, we compared CNCM I-5388 and NCDO2118 cell-bound GAD activity and the number of colony forming units (CFU)/mL in acetate buffer in which pH was controlled with HCl (see the experimental section) in a range from 4.6 to 2.0 as a first attempt to simulate in vitro the acidic stress encountered in vivo by *L. lactis*. The pH 4.6 was set as the control, as it is the one with maximum cytoplasmic GAD activity for both strains (see Figure 6). Figure 7A shows

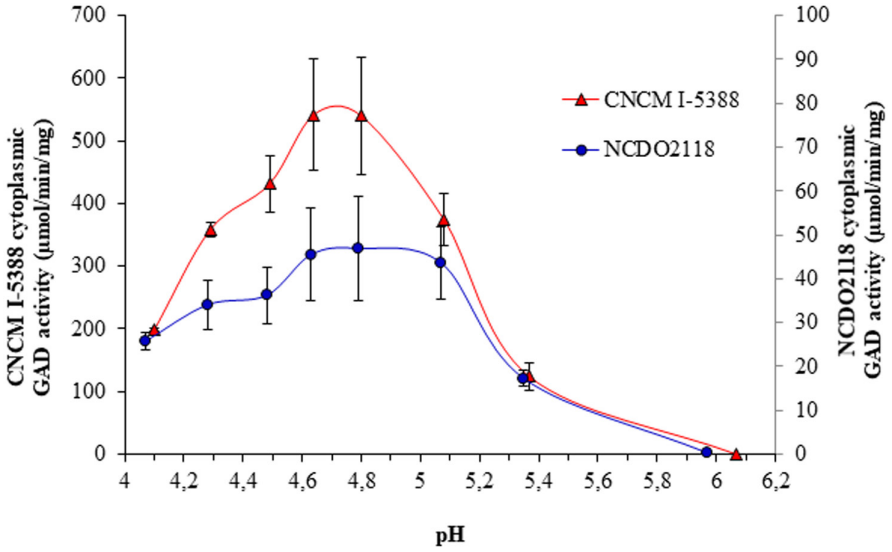

FIGURE 6 The optimal pH for cytoplasmic GAD activity for *L. lactis* NCDO2118 and CNCM I-5388.

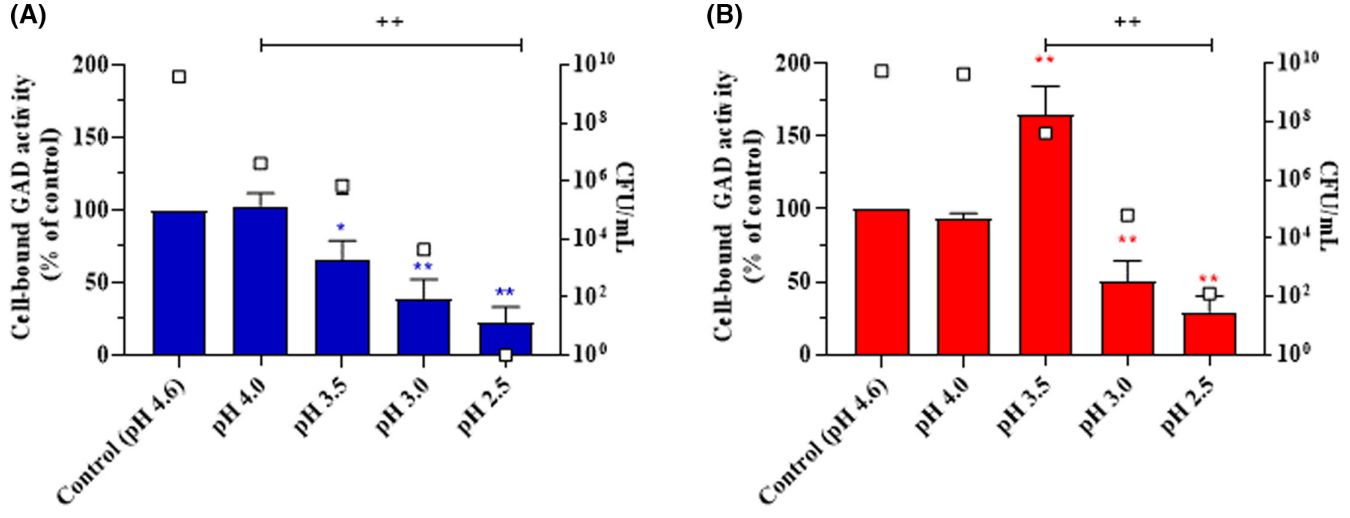

FIGURE 7 *L. lactis* cell-bound GAD activity and bacterial viable counts after HCl-induced acidic stress. (A) NCDO2118. (B) CNCM I-5388. Bars represent percentage values for cell-bound GAD activity (% of control (pH 4.6)) and squares represent values for CFU/mL. Data are expressed as mean ± SEM. $^{*}p < .05$, $^{**}p < .01$ vs control cell-bound GAD activity. $^{++}p < .01$ versus control bacterial cultivability (pH 4.6).

that 2-hour exposure of NCDO2118 to pH 4.0 did not significantly affect cell-bound GAD activity compared with the control ($p > .05$). However, it started to decline significantly with pH decrease, with 34%, 61%, and 78% less cell-bound GAD activity than the control for pH 3.5 ($p < .05$), 3.0, and 2.5 ($p < .01$), respectively. Bacterial viable counts also diminished significantly with increase in acidity from pH 4.0 to 2.5 ($p < .01$). Figure 7B shows that for CNCM I-5388 neither cell-bound GAD activity nor bacterial viable counts were significantly affected by the decrease of pH from 4.6 to 4.0 ($p > .05$). Interestingly, at pH 3.5 cell-bound GAD activity was 70% higher than the control ($p < .01$) while at pH 3.0 and 2.5, it was, respectively, 46% and 69% inferior to the control ($p < .01$). Finally, bacterial viable counts were significantly reduced from pH 3.5 to 2.5 ($p < .01$). Altogether, our results indicate that CNCM I-5388 presents higher survival than NCDO2118 under acidic conditions. Additionally, CNCM I-5388 has enhanced cell-bound GAD activity at pH 3.5, contrarily to NCDO2118.

4 | DISCUSSION

LAB are among the best-characterized GABA-producing microorganisms due to their active GAD system and GRAS status. We were the first group to demonstrate that transient food-borne LAB *L. lactis* NCDO2118 has GAD and GABA-dependent anti-VH effects in vivo.[14] Interestingly, here we were able to qualify the strain CNCM I-5388 as a higher GABA producer than NCDO2118 while having similar biomass profile evolution under the same fermentation conditions. To survive in acidic environments, such as those found in fermented foods and successfully transit through the gastric compartment, LAB must be able to perceive and respond to acid stress. Hence, the GAD system provides protection under acidic conditions by lowering the proton content in the cytoplasm converting L-Glu into GABA.[15,16,17,19,20,21,22,23,24,25,26,27] Yogeswara and colleagues summarized the biochemical properties of GAD of numerous bacterial strains, with optimal pH for intracellular activity ranging from 4.5–5.2. This is consistent with our results, considering that CNCM I-5388 and NCDO2118 share similar GAD amino acid sequences and both have maximum cytoplasmic GAD activity in the pH range of 4.6–4.8. Thus, this does not explain why CNCM I-5388 is a higher GABA producer, while having similar physicochemical properties and under the same growth conditions as NCDO2118. Worthy of notice, CNCM I-5388 arginine and ornithine consumption rate and citrulline production rate were significantly lower than that observed for NCDO2118. Those metabolites are key indicators of the activity of the ADI system in bacteria, an important enzyme-based acid tolerance mechanism.[11] It is not trivial to speculate that a downregulation in the ADI system in CNCM I-5388 would represent a decrease in acid resistance of the strain, culminating in a compensation through other systems, such as the GAD pathway. Indeed, a recent study on *E. coli* has shown that these acid resistance systems allow "division of labor" in the bacterial population and ensure its survival over a wide range of low pH values. Consequently, the lack of one of these pathways directly affects the others.[28]

Accordingly, NCDO2118 viable counts and cell-bound GAD activity decreased starting from pH <4.0 and pH <3.5, respectively, while CNCM I-5388 viable counts started to decrease at pH 3.5 whilst its cell-bound GAD activity was increased. One might perceive such an increase in CNCM I-5388 cell-bound GAD activity at pH 3.5 as contradictory considering the observation of an optimal cytoplasmic activity of the enzyme GAD at pH 4.6–4.8 for both strains. However, the latter was obtained from a bacterial lysate for the evaluation of intracellular (or specific) enzymatic activity, contrary to the results of that analysis based on cell-bound GAD activity via measurement on whole cells. This difference between optimal pH for cell-bound and specific GAD activities might be explained by the existence of a disparity between the extracellular pH and the intracellular pH of bacteria, vastly observed in the literature. For instance, a ΔpH (intracellular pH – extracellular pH) of approximately 1.0 was also found during the deceleration phase of *L. lactis* MG1363 growth, whereas the external pH was 4.7 and the intracellular pH was 5.7.[29] *Lactobacillus acidophilus* 3532 cells in stationary phase were found to have an internal pH of 4.5 while the pH of growth medium was 3.5.[30] *Lactococcus cremoris* NCDO 712 growth medium acidified to an external pH at 5.0 triggered the intracellular pH to reach a value of 5.9.[31] Therefore, it is reasonable to postulate that CNCM I-5388 cell-bound GAD activity might be enhanced with an external pH of 3.5 if we consider an intracellular pH of approximately 4.6, optimal for specific GAD activity. Another prospect to consider is that CNCM I-5388 lower viable cell counts measured at pH 3.5 suggest an enhancement in bacterial lysis and subsequent cell membrane and wall permeabilization. Consequently, the extracellular L-Glu would be more encountered by GAD once it would be less submissive to bacterial cell permeability barrier and to the antiporter action of GadC, increasing GAD activity and GABA production. Finally, the scenarios here described can be easily reflected in vivo, considering the acidic nature faced by *L. lactis* in the stomach of rats with a pH varying from 2.0 to 5.0, in light of several factors including age, sex, and specially feeding.[32,33] However, we should keep in mind that the HCl-induced acidic stress used here was over-simplified, operated under static conditions and

only based on the adjustment of pH. To simulate a more physiologically relevant gut environment, similar analyses should be considered in dynamic models, like the TNO gastrointestinal model (TIM).[34] This model reproduces the main physicochemical conditions of the upper GI tract of humans by considering not only pH and its temporal and spatial variations, but also other variables (e.g., digestive enzymes, bile salts) that might influence *L. lactis* cell-bound GAD activity and stress resistance.

We have also demonstrated that a 10-day daily oral administration of CNCM I-5388 is equally efficient as NCDO2118 in reducing VH induced by acute stress; however, only the first one was shown to reduce VH after 5-day oral treatment. Only few studies have investigated the anti-VH potential of LAB, but most of them concern lactobacilli applied to preclinical rat and mouse models. Through different mechanisms, these LAB have been shown to reduce VH from 7 to 15 days of oral treatment in such rodent models.[35-39] To our knowledge, only a Bifidobacterium strain was reported to have antinociceptive properties against VH after only 5 days of treatment, as seen in our study.[40] The antinociceptive effect at either 10 or 5-day treatment was proven to be GAD-dependent and mediated by the host's $GABA_B$ receptors. Interestingly, a single dose of CNCM I-5388 tended to reduce VH after treatment indicating that treatment efficacy started on the first day but became optimal from the fifth day on. In the rat mucosal epithelium layer, $GABA_B$ receptor-positive cells are present throughout the GI tract from the stomach to the colon with a decreased number of cells morphologically similar to enteroendocrine cells from the oral to anal direction.[7] Gastric $GABA_B$-immunoreactive positive cells also contain somatostatin. A group has demonstrated that $GABA_B$ receptor-induced acid secretion raised in the presence of somatostatin.[41] Thanks to this acid secretion in the stomach, which is conducive to the GAD enzyme activity and GABA production, we hypothesized in a previous work, a dynamic "virtuous circle" between *L. lactis* NCDO2118 and the host that induced a significant level of GABA production in the gastric lumen[14]; such an increase in CNCM I-5388 cell-bound GAD activity at pH 3.5 was observed in vitro in our study. Although activation of $GABA_B$ receptors accelerates their internalization,[42,43] this phenomenon is counterbalanced by their enhanced recycling to the cell surface.[42,44] Moreover, activation of $GABA_B$ receptors appears to stabilize them at the cell surface and a sustained activation of $GABA_B$ receptors by the agonist baclofen appears to stabilize the number of receptors on the cell neuronal surface.[45] In this work, the authors suggested that either sustained activation of remaining $GABA_B$ receptors or persistent activation of the receptors stabilizes them at the cell surface and reduces their downregulation, making the baclofen treatment more efficient. Based on these observations, we suggest that rapidly over time of daily administration, CNCM I-5388 according to its ability to produce high levels of GABA in acidic conditions contributes to switch on the activation and the stabilization of $GABA_B$ receptors on the gastric epithelial cells and/or on the enteric plexus as well as on the vagus nerve. Indeed, $GABA_B$ receptors are also involved in the gastric vagal and enteric cholinergic neural signaling pathway that contributes to the regulation of gastric acid secretion. Besides, another hypothesis related to the mRNA expression of GABAergic receptor subunits can be evoked. A study in mice has indicated that the administration of *Lactobacillus rhamnosus* (JB-1) consistently modulated the mRNA expression of $GABA_A$ and $GABA_B$ receptor subunits in the CNS[34]. Therefore, the partial antinociceptive effect exerted by the higher GABA producer CNCM I-5388 from the first day of oral treatment can be explained by combined and progressive GABAergic receptor modulatory effects that can go from mRNA expression to the stabilization of $GABA_B$ receptors. These hypotheses concerning expression and/or stabilization of $GABA_B$ receptors can be extended to explain the partial persistence effect of CNCM I-5388 under wash-out conditions where $GABA_B$ receptor regulations might still be in place in host's GIT after 5-day treatment interruption. We suggest that the $GABA_B$ receptor activation persists under these conditions by GABA originated from rats' diet and/or produced by GIT microbiota from L-Glu, which is also present in the diet. In addition, as observed for the NCDO2118 strain,[14] even though we did not observe any impact of CNCM I-5388 treatment on the α and β diversities of the fecal microbiota we cannot rule out different activities of the microbiota that can contribute via a favorable environment in the stomach generated by the CNCM I-5388 to the antinociceptive properties against VH induced by stress. However, from today, our results seem to be insufficient to correlate changes in the fecal composition of the microbiome with an enhancement of the anti-VH effect of either CNCM I-5388 or NCDO2118 and further works should target distinct gut compartment microbial functional changes rather than compositional, and particularly at the stomach level.

In conclusion, through in vivo tests and by simulating in vitro the type of acid stress encountered within the host's stomach, our data firmly suggest that CNCM I-5388 may be qualified as a higher GABA producer in vivo, the efficacy of which in reducing VH has now to be further elucidated and extended in order to fully assess its potential for clinical studies concerning the treatment of VH in IBS individuals.

AUTHOR CONTRIBUTIONS

The contributions of all authors encompassed designing the experiments, analyzing the results, drafting and

revising the article. PG, VL, CB, NA, and CL were responsible for implementing the experiments and acquiring data. NB, SLR, VT, MMB, MCB, and HE provided supervision for the study and secured funding. Ultimately, all authors gave their approval for the final version of the article.

ACKNOWLEDGMENTS

The authors wish to thank the Sequencing Platform of Toulouse (GeT-Biopuces) and especially Etienne Rifa for gut microbiota analyses. The authors wish to thank Sébastien Nouaille (TBI, Toulouse, France) for the *gadB* mutant strain construction and Hervé Robert (Toxalim, Toulouse, France) for helpful discussion.

FUNDING INFORMATION

This work received the financial support of Lesaffre International (Marcq-en-Barœul, France, contracts CT010082 and CT011953).

DISCLOSURES

NB and SLR are full-time employees of Lesaffre. The other authors report no conflict of interest.

DATA AVAILABILITY STATEMENT

Relevant additional data files are provided as source data files. For Supplementary Figures S3A–C and S4A–C, all data (raw and treated) can be found in this link: https://forgemia.inra.fr/umrf/exploremetabar. For all other data, the authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials.

ORCID

Pedro Gomes https://orcid.org/0009-0007-9508-2609
Valérie Laroute https://orcid.org/0000-0003-1108-7714
Catherine Beaufrand https://orcid.org/0000-0002-1906-3699
Marie-Line Daveran-Mingot https://orcid.org/0000-0001-6884-1840
Nathalie Aubry https://orcid.org/0000-0002-5722-0253
Chloé Liebgott https://orcid.org/0009-0005-2142-8561
Nathalie Ballet https://orcid.org/0000-0002-6474-7678
Sophie Legrain-Raspaud https://orcid.org/0000-0001-7747-5766
Vassilia Theodorou https://orcid.org/0000-0003-0801-264X
Muriel Mercier-Bonin https://orcid.org/0000-0001-8398-2529
Muriel Cocaign-Bousquet https://orcid.org/0000-0003-2033-9901
Hélène Eutamene https://orcid.org/0000-0002-2983-1938

REFERENCES

1. Camilleri M. Testing the sensitivity hypothesis in practice: tools and methods, assumptions and pitfalls. *Gut*. 2002;51:34-41.
2. Hamarashid BR, Dalkiliç S, Kadiouglu Dalkiliç L, Saleh KK, Kirbag S. Irritable bowel syndrome (IBS): a review Bahra. *J Adv lab Res Biol*. 2020;11:36-52.
3. Farzaei MH, Bahramsoltani R, Abdollahi M, Rahimi R. The role of visceral hypersensibility in irritable bowel syndrome: pharmacological targets and novel treatments. *J Neurogastroenterol Motil.* 2016;22:558-574.
4. Simon E, Florina L, Mitrea L. Beneficial effects against irritable bowel syndrome. *Nutrients*. 2021;13:1-27.
5. Theodorou V, Belgnaoui AA, Agostini S, Eutamene H. Effect of commensals and probiotics on visceral sensitivity and pain in irritable bowel syndrome. *Gut Microbes*. 2014;5:430-436.
6. Strandwitz P, Kim KH, Terekhova D, et al. GABA-modulating bacteria of the human gut microbiota. *Nat Microbiol*. 2019;4:396-403.
7. Hyland NP, Cryan JF. A gut feeling about GABA: focus on GABAB receptors. *Front Pharmacol*. 2010;4:1-9.
8. Hara K, Saito Y, Kirihara Y, Yamada Y, Sakura S, Kosaka Y. The interaction of antinociceptive effects of morphine and GABA receptor agonists within the rat spinal cord. *Anesth Analg*. 1999;89:422-427.
9. Cui Y, Miao K, Niyaphorn S, Qu X. Production of gamma-aminobutyric acid from lactic acid bacteria: a systematic review. *Int J Mol Sci*. 2020;21(3):995.
10. Small PLC, Waterman SR. Acid stress, anaerobiosis and gadCB: lessons from *Lactococcus lactis* and *Escherichia coli*. *Trends Microbiol*. 1998;6:214-216.
11. Guan N, Liu L. Microbial response to acid stress: mechanisms and applications. *Appl Microbiol Biotechnol*. 2020;104:51-65.
12. Siragusa S, de Angelis M, di Cagno R, Rizzello CG, Coda R, Gobbetti M. Synthesis of γ-aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. *Appl Environ Microbiol*. 2007;73:7283-7290.
13. Mazzoli R, Pessione E, Dufour M, et al. Glutamate-induced metabolic changes in *Lactococcus lactis* NCDO 2118 during GABA production: combined transcriptomic and proteomic analysis. *Amino Acids*. 2010;39:727-737.
14. Laroute V, Beaufrand C, Gomes P, et al. Lactococcus lactis NCDO2118 exerts visceral antinociceptive properties in rat via GABA production in the gastrointestinal tract. *Elife*. 2022;11:1-18.
15. Maguin E, Prévost H, Ehrlich SD, Gruss A. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. *J Bacteriol*. 1996;178:931-935.
16. Sievers F, Higgins DG. Clustal omega, accurate alignment of very large numbers of sequences. *Methods Mol Biol*. 2014;1079:105-116.
17. Laroute V, Yasaro C, Narin W, et al. GABA production in *Lactococcus lactis* is enhanced by arginine and co-addition of malate. *Front Microbiol*. 2016;7:1050.
18. Morteau O, Hachet T, Caussette M, Bueno L. Experimental colitis alters visceromotor response to colorectal distension in awake rats. *Dig Dis Sci*. 1994;39:1239-1248.
19. Borissova A et al. Modeling the precipitation of L-glutamic acid via acidification of monosodium glutamate. *Cryst. Growth Design*. 2005;5:845-854.

20. Escudié F, Auer L, Bernard M, et al. FROGS: find, rapidly, OTUs with galaxy solution. *Bioinformatics*. 2018;34:1287-1294.
21. Bokulich NA, Subramanian S, Faith JJ, et al. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods*. 2013;10:57-59.
22. Fisch ATM, Eckley IA, Fearnhead P. A linear time method for the detection of point and collective anomalies. *Stat Anal Data Min*. 2022;15:494-508.
23. Parks DH et al. A complete domain-to-species taxonomy for bacteria and archaea. *Nat Biotechnol*. 2020;389(38):1079-1086.
24. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res*. 2013;41:D590-D596.
25. McMurdie PJ, Holmes S. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE*. 2013;8:e61217.
26. Kelly BJ, Gross R, Bittinger K, et al. Power and sample-size estimation for microbiome studies using pairwise distances and PERMANOVA. *Bioinformatics*. 2015;31:2461-2468.
27. Yogeswara IBA, Maneerat S, Haltrich D. Glutamate decarboxylase from lactic acid bacteria—a key enzyme in GABA synthesis. *Microorg*. 2020;8:1923.
28. Brameyer S, Schumacher K, Kuppermann S, Jung K. Division of labor and collective functionality in Escherichia coli under acid stress. *Commun Biol*. 2022;5:1-14.
29. Even S, Lindley ND, Loubière P, Cocaign-Bousquet M. Dynamic response of catabolic pathways to autoacidification in *Lactococcus lactis*: transcript profiling and stability in relation to metabolic and energetic constraints. *Mol Microbiol*. 2002;45:1143-1152.
30. Kashket ER. Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. *FEMS Microbiol Lett*. 1987;46:233-244.
31. O'Sullivan E, Condon S. Intracellular pH is a major factor in the induction of tolerance to acid and other stresses in *Lactococcus lactis*. *Appl Environ Microbiol*. 1997;63:4210-4215.
32. McConnell EL, Basit AW, Murdan S. Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments. *J Pharm Pharmacol*. 2008;60:63-70.
33. Merchant HA, Rabbie SC, Varum FJO, Afonso-Pereira F, Basit AW. Influence of ageing on the gastrointestinal environment of the rat and its implications for drug delivery. *Eur J Pharm Sci*. 2014;62:76-85.
34. Minekus M. The TNO gastro-intestinal model (TIM). *Impact Food Bioact Heal Vitr Ex Vivo Model*. 2015;5:37-46.
35. Ait-Belgnaoui A, Han W, Lamine F, et al. Lactobacillus farciminis treatment suppresses stress induced visceral hypersensitivity: a possible action through interaction with epithelial cell cytoskeleton contraction. *Gut*. 2006;55:1090-1094.
36. Ait-Belgnaoui A, Payard I, Rolland C, et al. Bifidobacterium longum and lactobacillus helveticus synergistically suppress stress-related visceral hypersensitivity through hypothalamic-pituitary-adrenal Axis modulation. *J Neurogastroenterol Motil*. 2018;24:138-146.
37. Darbaky Y, Evrard B, Patrier S, et al. Oral probiotic treatment of lactobacillus rhamnosus Lcr35® prevents visceral hypersensitivity to a colonic inflammation and an acute psychological stress. *J Appl Microbiol*. 2017;122:188-200.
38. Kamiya T, Wang L, Forsythe P, et al. Inhibitory effects of lactobacillus reuteri on visceral pain induced by colorectal distension in Sprague-Dawley rats. *Gut*. 2006;55:191-196.
39. Rousseaux C, Thuru X, Gelot A, et al. Lactobacillus acidophilus modulates intestinal pain and induces opioid and cannabinoid receptors. *Nat Med*. 2007;13:35-37.
40. McKernan DP, Fitzgerald P, Dinan TG, Cryan JF. The probiotic Bifidobacterium infantis 35624 displays visceral antinociceptive effects in the rat. *Neurogastroenterol Motil*. 2010;22:1029-e268.
41. Piqueras L, Martinez V. Peripheral GABA B agonists stimulate gastric acid secretion in mice. *Br J Pharmacol*. 2004;142:1038-1048.
42. Zhang Z, Zhang W, Huang S, et al. GABAB receptor promotes its own surface expression by recruiting a Rap1-dependent signaling cascade. *J Cell Sci*. 2015;128:2302-2313.
43. Wilkins ME, Li X, Smart TG. Tracking cell surface GABAB receptors using an α-bungarotoxin tag. *J Biol Chem*. 2008;283:34745-34752.
44. Grampp T, Notz V, Broll I, Fischer N, Benke D. Constitutive, agonist-accelerated, recycling and lysosomal degradation of GABAB receptors in cortical neurons. *Mol Cell Neurosci*. 2008;39:628-637.
45. Hleihil M, Vaas M, Bhat MA, Balakrishnan K, Benke D. Sustained baclofen-induced activation of GABAB receptors after cerebral ischemia restores receptor expression and function and limits progressing loss of neurons. *Front Mol Neurosci*. 2021;14:1-13.

SUPPORTING INFORMATION

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

How to cite this article: Gomes P, Laroute V, Beaufrand C, et al. *Lactococcus lactis* CNCM I-5388 versus NCDO2118 by its GABA hyperproduction ability, counteracts faster stress-induced intestinal hypersensitivity in rats. *The FASEB Journal*. 2023;37:e23264. doi:10.1096/fj.202301588R