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Health potential of GABA-producing *Lactococcus lactis* strains: towards novel therapeutic strategies in visceral hypersensitivity management

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Université
de Toulouse

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M. PEDRO GOMES

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RESUME

Le syndrome de l'intestin irritable (SII) est une maladie chronique invalidante, récemment classée dans le nouvel intitulé des « désordres des interactions de l'axe cerveau-intestin », anciennement appelés « troubles fonctionnels intestinaux ». Sur le plan clinique selon les critères de ROME IV, le SII se caractérise par une sensibilité accrue et une perception renforcée de la douleur dans l'intestin, phénomène appelé hypersensibilité viscérale, associée à des troubles du transit sans cause organique identifiable. Les symptômes du SII ont de fortes répercussions en termes de coûts et de qualité de vie des patients et entraînent et/ou aggravent le stress et l'anxiété. La physiopathologie du SII reste aujourd'hui mal comprise et les traitements prescrits se font en fonction des symptômes prédominants et de la gravité des plaintes. Dans ce cadre, certaines souches probiotiques sont capables d'influencer la communication entre le microbiote intestinal, l'intestin et le cerveau et de ce fait de moduler la sensibilité viscérale à travers la libération de neuromédiateurs spécifiques issus de ces bactéries. Parmi ces médiateurs, l'acide γ -aminobutyrique (GABA) est un neurotransmetteur inhibiteur du système nerveux central qui joue un rôle clé dans la perception et allègement de la douleur chez les mammifères. Chez plusieurs bactéries, un système de résistance au stress acide, les permet de produire du GABA après la décarboxylation de l'acide L-glutamique par l'enzyme glutamate décarboxylase (GAD). Ce système est notamment observé chez les bactéries lactiques (LAB) comme *Lactococcus lactis* (*L. lactis*), largement utilisé dans l'industrie agro-alimentaire pour son profil reconnue comme sûre pour la santé humaine (du terme « generally recognized as safe » en anglais). Bien qu'il ait été observé que la bactérie commensale *Bifidobacterium dentium* soit capable de réduire la HV induite par stress chez le rat via la production de GABA, la capacité des LAB à moduler la sensibilité viscérale via leur capacité à produire du GABA reste à définir. En conditions identiques de culture en bioréacteur, nous avons caractérisé trois souches de *Lactococcus lactis* productrices de GABA à des niveaux différents (de faiblement à fortement productrices) en relation avec leur activité GAD, et nous

avons évalué leurs capacités à réduire la HV en réponse à un stress aigu chez le rat, dans un modèle mimant la symptomatologie du SII. Nous avons ainsi qualifié la souche CNCM I-5388 comme hyper-productrice de GABA vis-à-vis de la souche de référence NCDO2118 et montré que ses propriétés antinociceptives apparaissent dès le 1^{er} jour de traitement par voie orale pour être significative après 5 jours de traitements contre 10 jours pour NCDO2118. Nous avons également établi que ces propriétés, pour les deux souches, sont GAD/GABA-dépendantes, agissant sur les récepteurs GABA_B de l'hôte indépendamment des modifications de l'alpha et de la bêta-diversité du microbiote fécal. De plus, nous montrons que cet effet ne dépend pas de la viabilité cellulaire de *L. lactis* pour la souche CNCM I-5388. En effet, sous sa forme postbiotique (non viable, mais pour laquelle l'enzyme GAD est toujours active) nous observons un effet similaire à celui de la souche sous sa forme probiotique après 5 et 10 jours de traitement. Finalement, via des analyses des contenus gastrointestinaux des rats et des modèles statique ou dynamique qui miment le compartiment gastrique humain, Nos résultats suggèrent qu'un pH similaire à celui de l'estomac pourrait jouer un rôle majeur dans l'activation de la GAD bactérienne et la production de GABA pour exercer des propriétés antinociceptives *in vivo*. Nos conclusions indiquent que les souches de *L. lactis* productrices de GABA pourraient être utilisées à des fins thérapeutiques dans le traitement symptomatologique de la douleur viscérale invalidante dans la pathologie du SII et d'autres pathologies.

Mots clés : GRAS Bactéries lactiques, *Lactococcus lactis*, GAD, GABA, syndrome de l'intestin irritable, hypersensibilité viscérale

ABSTRACT

Irritable bowel syndrome (IBS) is a disabling chronic disease, recently classified under the new term "disorders of the gut-brain axis," formerly known as "functional gastrointestinal disorders." Clinically, according to the ROME IV criteria, IBS is characterized by heightened sensitivity and amplified pain perception in the gut, a phenomenon referred to as visceral hypersensitivity (VH), associated with bowel disturbances without identifiable organic causes. IBS symptoms have significant implications in terms of patient quality of life and costs, and they can trigger or exacerbate stress and anxiety. The pathophysiology of IBS remains poorly understood, and prescribed treatments are based on predominant symptoms and the severity of complaints. In this context, certain probiotic strains have the capacity to influence communication between the gut microbiota, the intestine, and the brain, thereby modulating visceral sensitivity through the release of specific neuromodulators produced by these bacteria. Among these modulators, γ -aminobutyric acid (GABA) is an inhibitory neurotransmitter of the central nervous system that plays a key role in pain perception and relief in mammals. In several bacteria, an acid stress resistance system allows them to produce GABA after the decarboxylation of L-glutamic acid by the enzyme glutamate decarboxylase (GAD). This system is notably observed in lactic acid bacteria (LAB) like *Lactococcus lactis* (*L. lactis*), widely used in the food industry for its generally recognized as safe (GRAS) status. Although it has been observed that the commensal bacterium *Bifidobacterium dentium* is capable of reducing stress-induced VH in rats through GABA production, the ability of LAB to modulate visceral sensitivity via their capacity to produce GABA remains to be defined. Under identical culture conditions in a bioreactor, we characterized three strains of *L. lactis* with varying levels of GABA production (from low to high production) in relation to their GAD activity. We evaluated their abilities to reduce VH in response to acute stress in a rat model that mimics IBS symptoms. We qualified the *L. lactis* strain CNCM I-5388 as a hyperproducer of GABA compared to the reference strain NCDO2118 and we demonstrated that its antinociceptive

properties become significant after only 5 days of oral treatment, compared to 10 days for NCDO2118. We also established that these properties, for both strains, are GAD/GABA-dependent, acting on the host's GABA_B receptors independently of changes in fecal microbiota alpha and beta diversities. Furthermore, we show that this effect does not depend on the cellular viability of *L. lactis* for strain CNCM I-5388. Indeed, in its postbiotic form (non-viable but with active GAD enzyme), we observe a similar effect to that of the strain in its probiotic form after 5 and 10 days of treatment. Finally, through analyses of rats' gastrointestinal contents and static or dynamic models that mimic the human gastric compartment, our results suggest that a stomach-like pH might be the major player in bacterial GAD activation and GABA production to exert antinociceptive properties *in vivo*. Our conclusions indicate that GABA-producing *L. lactis* strains could be used therapeutically for the symptomatic treatment of disabling visceral pain in IBS and other pathologies.

Keywords: Lactic acid bacteria, *Lactococcus lactis*, GAD, GABA, irritable bowel syndrome, visceral hypersensitivity

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“Pare de superestimar as coisas ruins do mundo e, sobretudo, não subestime o amor dos teus próximos”.

“Stop overestimating the bad things in the world and, above all, do not underestimate the love of those around you”

Plínio Moisés Gomes

★1990 - †2020

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

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●CHAPTER 3

Figure 1. Effect of EtOH treatment on bacterial inactivation and cell-bound GAD activity of *L. lactis* CNCM I-5388.

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Figure 3. Effect of a single oral administration of EtOH-treated *L. lactis* CNCM I-5388 in the presence of L-Glu monosodium salt on PRS-induced VH induced by stress in response to colorectal distension.

Figure 4. Effect of 5-day oral administration followed by 5-day treatment interruption of EtOH-treated *L. lactis* CNCM I-5388 in the presence of L-Glu monosodium salt hydrate on VH induced by PRS in response to colorectal distension.

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Figure 1. Main findings concerning *L. lactis* and GABA production *in vitro*.

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Figure 4. Main findings in *L. lactis* anti-VH properties.

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ABBREVIATIONS LIST

5-HT: 5-hydroxytryptamine or serotonin;	HPA: hypothalamic-pituitary-adrenal
ACTH: Adrenocorticotrophic hormone	HPLC: High performance liquid chromatography
ADI: Arginine decarboxylase	IBD: Inflammatory bowel disease
AdiC: Arginine/agmatine antiporter	IBS: Irritable bowel syndrome
AR: Acid resistance	IECs: intestinal epithelial cells
ATP: Adenosine triphosphate	IL: Interleukin
AUC: Area under the curve	ISAP: International Scientific Association for Probiotics and Prebiotics
BDNFs: Brain-derived neurotrophic factors	LAB: Lactic acid bacteria
CAD: Lysine decarboxylase	L-Glu: L-glutamic acid or L-glutamate
cAMP: Cyclic adenosine monophosphate	NE: norepinephrine
CFU: Counting Forming Units	OCD: Putrescine decarboxylase
CNS: Central nervous system	OD: Optical density
CRD: Colorectal distension	ODHC: 2-oxoglutarate dehydrogenase complex
CRH: Corticotrophin releasing hormone	PLP: Pyridoxal phosphate
DGBI: Disorders of the gut-brain interaction	PNS: Peripheral nervous system
DSS: Dextran sodium sulfate	PotE: Ornithine/putrescine transporter
EMG: Electromyography	PRS: Partial restraint stress
ENS: Enteric nervous system	SCFAs: Short-chain fatty acids
EtOH: Ethanol	TCA: Tricarboxylic acid cycle
FDA: Food and Drug Administration	TNBS: 4,6-trinitrobenzenesulfonic acid
FODMAPs: Fermentable oligosaccharides, disaccharides, monosaccharides and polyols	VH: Visceral hypersensitivity
GABA: gamma (γ)-aminobutyric acid	WGO: World Gastroenterology Organization
GABA-AT: GABA aminotransferase	WHO: World Health Organization
GAD or GadA/GadB: Glutamate decarboxylase	WO: Washout
GadC: glutamic acid/GABA antiporter	
GIT: Gastrointestinal tract	
GRAS: Generally recognized as safe	

FOREWORD

CONTEXT OF THE PH.D.

1. Host laboratories

1.1. Toulouse Biotechnology Institute: Team BLADE

The Toulouse Biotechnology Institute (TBI) at INSA-Toulouse hosts the BLADE (BacteriaL Adaptation, Diversity and Engineering) team, headed by Dr. Muriel COCAIGN-BOUSQUET. The team's research activities are situated within the realm of bacterial physiology, with the primary goal of delving into the intricate mechanisms by which bacteria adapt to their surroundings, ultimately for enhanced control over their utilization. The team's objectives are twofold: 1) to amass foundational and mechanistic insights into bacterial operations, and 2) to elevate their efficacy in biotechnology through strategic engineering approaches that effectively address the challenges posed by the bio-economy. The team's focal points lie notably with two prototype bacteria, namely *Lactococcus lactis* and *Escherichia coli*, both of which find applications spanning the domains of food production, human health, and ecological biotechnology. The team's scientific approach aligns with the methodologies and principles of systems biology. It involves the meticulous characterization of various tiers of cellular processes, encompassing genes, mRNA molecules, proteins, and metabolites, through the application of "omics" tools. Subsequently, these datasets are integrated within mathematical models, thereby yielding a holistic and hierarchical perspective on the multifaceted regulatory networks at play. Bacterial subjects are subjected to genetic perturbations (mutants) or environmental fluctuations (such as stress, alterations in carbon sources, nutrient scarcities, etc.), either in isolation or in synergy with other bacteria or heterogeneous subpopulations, as part of the team's investigative protocol.

1.2. ToxAlim: Team NGN

The Neuro-Gastroenterology & Nutrition (NGN) team, situated within the ToxAlim-INRAE (Research Centre in Food Toxicology) in Toulouse and headed by Dr. Vassilia

THEODOROU and H  l  ne EUTAMENE, boasts a robust expertise in the realm of fundamental research in digestive pathophysiology. Its scientific theme is delineated across three axes, spanning from the perinatal period to adulthood. The first axis revolves around the study of the regulation of intestinal barrier function by environmental factors such as diet type, food contaminants, toxic substances, or bacterial metabolites generated within the digestive tract, in conjunction with psychological or nutritional stress. The second axis delves into/focus in ? the involvement of the gut-brain axis in regulating intestinal barrier function. The third axis pertains to nutritional remediation aimed at averting physiopathological consequences stemming from a compromised intestinal barrier function and its interplay with the brain. The team has garnered expertise and skills, with a strong collaborative network, aimed at exploring and comprehending: the role of intestinal epithelium integrity; the structure and function of the mucus layer; the composition and function of the microbiota; the immune response of the intestinal mucosa; visceral pain and behavioral changes associated with disruptions in intestinal barrier function homeostasis. The favored approach involves a dialogue among the various players within the intestinal barrier, both among themselves and with the nervous system, enabling an integrated understanding of the system. Lastly, through academic collaborations with French and international clinicians in the field of neuro-gastroenterology, the team conducts translational studies to gauge the relevance of mechanistic approaches across the intestinal-brain barrier continuum in humans.

1.3. Lesaffre

Born in the Hauts-de-France in 1853, Lesaffre has been innovating in the field of fermentation and micro-organisms for 170 years (<https://www.lesaffre.com>). By placing fermentation at the core of its activities, Lesaffre is engaged in biotechnological advancements and innovative solutions for a healthier and more sustainable future, regarding health, well-being and nutrition of humans, animals, and plants.

Four entities of the Lesaffre group supported this Ph.D. project:

- Lesaffre International, and more particularly Lesaffre Institute of Science & Technology (LIST), Research and Innovation department of Lesaffre;
- Gnosis by Lesaffre, a business unit dedicated to human nutrition & health;
- Phileo by Lesaffre, a business unit dedicated to animal nutrition & health;
- Biospringer by Lesaffre, a business unit specializing in yeast extracts and dedicated to food taste.

Additionally, Lesaffre's Nutrition, Microbiota & Health Center of Excellence and its expertise in dynamic digestion models were of particular interest in this Ph.D. project.

2. Ph.D. background

I began my studies in France in 2017, after completing my 4-year bachelor's degree in Biotechnology in Brazil. During the final semester of my 2-year master's program in molecular microbiology at Paul Sabatier University (Toulouse, France), I had the opportunity to undertake my final year internship with the BLADE team at TBI, focusing on the heterogeneity of *Escherichia coli* populations during sugar transitions. Towards the end of this project, I became acquainted with the collaborative work between the BLADE team and the NGN team at ToxAlim, where they explored the health potential of GABA-producing *Lactococcus lactis* strains in the context of stress-induced visceral hypersensitivity in rats. The results gathered by both teams were the subject of a patent application of title “*Lactococcus lactis* strains for the prevention and/or the treatment of visceral pain” (publication number WO/2020/157297). This project has later received funding from Lesaffre International, which not only granted me a one-year research contract in 2020 but also paved the way for the initiation of my three-year doctoral thesis in early 2021. Since then, I have been amalgamating my microbiology expertise gained from the inception of my higher education journey, along with delving into the new and challenging realm of neurogastroenterology. This Ph.D. was a multidisciplinary work that

harmonized various domains within life sciences, encompassing physiology and preclinical assessments, analytical biochemistry and enzymology, genomics, metagenomics and fermentation technology, seamlessly integrating academic and industrial research.

3. Manuscript organization

Section I comprises a literature review, offering a comprehensive overview of the existing knowledge on irritable bowel syndrome (IBS) and its cardinal symptom, visceral pain, with a special emphasis on exploring the health potential of lactic acid bacteria (LAB) for individuals with IBS. In **Chapter 1**, a meticulous depiction of IBS is provided, focusing on its diagnosis, associated biological factors, and the impact of VH on the quality of life for those affected by IBS. Additionally, a thorough examination of current research models and treatments for VH is conducted, highlighting the notable potential of GABA-producing bacteria within the gut microbiota and ingested bacteria, notably generally recognized as safe (GRAS) lactic acid bacteria (LAB). Moving on to **Chapter 2**, attention is centered on bacterial physiology and their acid resistance systems, with a particular focus on the glutamate decarboxylase (GAD), responsible for GABA production in bacteria, particularly LAB. Following this, the food-borne GABA-producing *Lactococcus lactis* strains are presented as potential agents against VH in IBS patients, not only due to their GABA production capabilities but also their widely recognized safe status and their utilization in the dairy industry.

Section II unveils the outcomes of the experimental work in the format of scientific publications. In **Chapter 1**, a comparative analysis is undertaken between a GABA-producing *L. lactis* strain (NCDO2118) and a GABA low-producing counterpart (NCDO2727), assessing the effects on anti-VH properties under acute stress conditions, the influence of GAD activity in this context, and the involvement of gut bacterial microbiota at the level of its composition and alpha and beta diversities. Expanding these anti-VH properties (i.e. duration treatment, single-dose administration, wash-out phase) to a higher GABA-producing strain (CNCM I-

5388) and evaluating the effects of *in vitro* “stomach-like” acidity enhancement on GAD enzyme activity, GABA production, and bacterial survival are the focus of **Chapter 2**. Moving to **Chapter 3**, the assessment narrows down to a comparison between anti-VH properties of metabolically active and inactive high GABA-producing CNCM I-5388 strain, with a particular emphasis on GABA accumulation within the host's gastrointestinal tract.

Worthy of notice, concerning **section II**, I've contributed to the first chapter with rat's fecal DNA extraction and metabarcoding analysis and journal publication writing, all other experiments date from before the start of my Ph.D. In the other two chapters of this section, I've participated directly in all experimentations and publication writing.

The final segment, **Section III**, constitutes a comprehensive discussion and provides a forward-looking perspective on the current research.

SECTION I – LITERATURE REVIEW

CHAPTER 1**IRRITABLE BOWEL SYNDROME (IBS) – A COMMON, DEBILITATING AND
COSTLY DISORDER****1. Irritable bowel syndrome (IBS)****1.1. Definition**

Irritable bowel syndrome (IBS) is categorized as a disorder of the gut-brain interaction (DGBI), commonly characterized by a range of intestinal issues like abdominal pain, diarrhea, constipation, bloating, gas and swelling ¹⁻³. Although DGBIs are of current interest, credible accounts resembling the present-day IBS can be traced back to the early 19th century ⁴. The term "irritable intestine" was likely first mentioned by Peters and Barger in 1944 ⁵. Since then, numerous studies have been conducted in an effort to gain a deeper understanding of this syndrome and, consequently, to attain a more precise definition and classification.

1.2. Rome IV diagnostic criteria

The original concept definitions of functional gastrointestinal disorders (FGIDs) initially emphasized the absence of organic disease, which sometimes led to the misconception that these conditions were primarily psychosomatic in nature ². However, scientific research has demonstrated that many FGIDs exhibit organic changes that are not routinely addressed in clinical practice ². Additionally, there is emerging evidence of alterations in gut microbiota in IBS and changes in brain processing and structure in various FGIDs ⁶⁻⁸. Based on these advancements, the Rome committees have proposed a new definition for FGIDs, now termed disorders of gut-brain interaction (DGBI) ². The complete consensus definition can be found in

Table 1.

Table 1: Definition of disorders of gut-brain interaction according to the Rome IV working teams. Adapted from ².

Functional GI disorders: disorders of gut–brain interaction

A group of disorders classified by GI symptoms related to any combination of:

- Motility disturbance
- Visceral hypersensitivity
- Altered mucosal and immune function
- Altered gut microbiota
- Altered central nervous system (CNS) processing

With the aim of establishing a practical diagnostic criterion, a dedicated working group for DGBI known as the Rome Foundation was formed in 1989 ⁹. This group, following formal consensus, developed the Rome criteria, which have undergone three subsequent revisions (I, II, III and IV). The most recent iteration, Rome IV was introduced in 2016 and replaced the Rome III diagnostic criteria published 10 years earlier ^{1,2,10}. The diagnostic criteria of Rome IV for identifying IBS are more stringent in comparison to Rome III, mainly due to their stipulation of more frequent occurrences of abdominal pain than the earlier criteria. Under the Rome IV criteria, abdominal pain needs to transpire on average at least once a week for three months 1 day/week in the last 3 months, associated with two or more of the following criteria: (i) related to defecation; (ii) associated with a change in frequency of stool; (iii) associated with a change in form (appearance) of stool ^{1,3,11}.

1.2.1. Subtypes of IBS

The four subclassifications are IBS based on predominant bowel habits and stool form, as indicated by Bristol stool chart (BSC) ³ (**Figure 1**). IBS subtypes are as follow:

IBS with predominant constipation (IBS-C): >25% of abnormal bowel movements with Bristol stool types 1 or 2 and <25% of bowel movements with Bristol stool types 6 or 7. Patient reports that abnormal bowel movements are usually constipation (like type 1 or 2 of BSC)

IBS with predominant diarrhea (IBS-D): >25% of abnormal bowel movements with Bristol stool types 6 or 7 and <25% of bowel movements with Bristol stool types 1 or 2. Patient reports that abnormal bowel movements are usually diarrhea (like type 6 or 7 of BSC)

IBS with mixed bowel habits (IBS-M): > 25% of abnormal bowel movements with Bristol stool types 1 or 2 and >25% of bowel movements with Bristol stool types 6 or 7. Patient reports that abnormal bowel movements are usually both constipation and diarrhea.

IBS unclassified (IBS-U): Patients who meet diagnostic criteria for IBS but whose bowel habits cannot be accurately categorized into 1 of the 3 groups above should be categorized as having IBS-U. Patient reports that abnormal stools (both diarrhea and constipation) are rare.

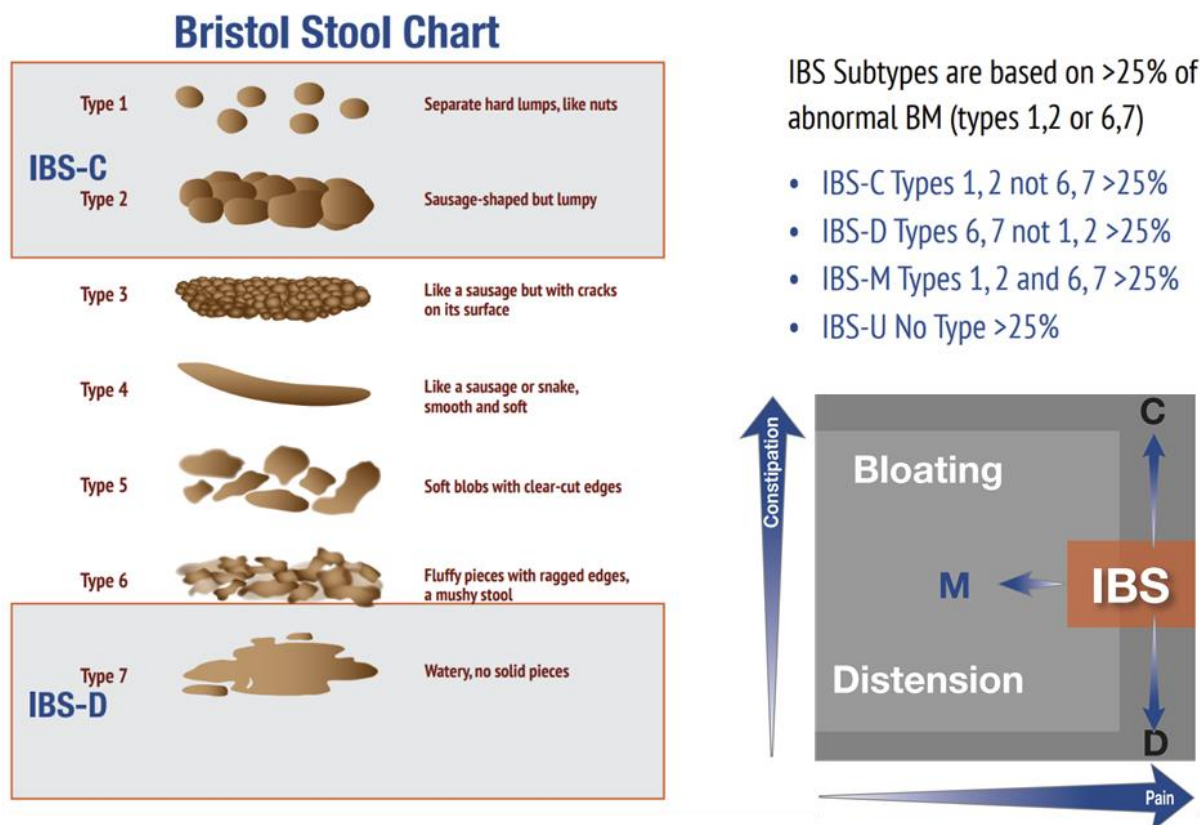


Figure 1. Bristol stool chart and the subtypes of IBS in the Rome IV criteria. BM, bowel movement; IBS-C, IBS with predominant constipation; IBS-D, IBS with predominant diarrhea; IBS-M, IBS with mixed bowel habits; IBS-U, IBS unclassified. Adapted from (Rome Foundation, 2016).

1.2.2. Global prevalence of IBS

The alterations concerning abdominal pain was the key factor leading to a decrease in the projected prevalence of IBS from 11.7% using Rome III to 5.7% using Rome IV ^{1,12}. Furthermore, the presence of comorbidity between IBS and functional constipation, functional diarrhea, and functional dyspepsia also played a role in the potential misclassifications ¹. Accordingly, a recent study conducted a comprehensive analysis, including a systematic review and meta-analysis, of cross-sectional surveys based on population samples ¹³. The global prevalence of IBS, based on 53 studies, which were done in 38 countries and comprised 395 385 participants, was 3.8% following Rome IV criteria ¹³. In the early 2000's, A nationally representative sample of 20 000 French subjects, aged 18 years and over indicated that prevalence of IBS in France was 4.7% ¹⁴. More recently, a study of 288 103 participants in 41 countries has shown that the mean prevalence of IBS in France was 1.1% ¹⁵. Worthy of notice, Rome criteria used for these studies were II and III, respectively, therefore, there's currently no information on IBS prevalence in French population following Rome IV criteria.

The assessment of IBS prevalence is influenced by the clarity of evaluation criteria that differentiate between various DGBIs and other chronic gastrointestinal conditions ^{11,16}. No discernible structural anomaly, definitive biological marker, or specific bacterial imbalance has thus far been pinpointed as the root cause of IBS, resulting in symptoms that may appear “medically unexplainable” ^{1,17}. Unsurprisingly, the process of diagnosing IBS can be accompanied by sentiments of misunderstanding, embarrassment, and societal stigma ¹³, which explains why a significant number of patients opt not to seek medical assistance. With a subset of individuals enduring symptoms in silence, it is plausible that the scope of the health issue posed by IBS is even more substantial than what statistics might indicate ¹³.

The compilation and analysis of global data on IBS prevalence underscore the substantial burden this disorder poses on populations, and consequently, the implications for

healthcare services worldwide, including in lower-income nations ¹³. From an economic perspective, the disorder correlates with three times the average rate of work absenteeism, leading to a notable burden on the healthcare system through visits to primary-care physicians, gastroenterologists, outpatient facilities, and hospital admissions. Additionally, there is the associated cost of pharmaceuticals commonly employed to alleviate presenting symptoms ¹⁸. For instance, in the United States, the direct expenses associated with managing IBS have been previously estimated to exceed US\$1 billion annually, while indirect costs stemming from productivity loss surpass \$200 million. In a meta-analysis incorporating 11 European datasets with over 2700 patients, the overall yearly cost attributed to IBS was nearly €3000 per patient ¹³. Noteworthy, IBS is not exclusive to humans and it has been reported in dogs, cats and horses, associated with stress, bloating, abdominal pain, and/or constipation ¹⁹⁻²¹. However, there is currently no global prevalence data concerning IBS in animals.

2. The biological factors associated with IBS

The origins and underlying mechanisms of IBS remain uncertain. Nevertheless, an increasing body of literature suggests the involvement of various factors in its onset and persistence. According to the biopsychosocial framework, it is believed that a combination of biological predispositions, environmental factors, and psychophysiological elements contribute to the development of IBS^{2,22}. It is conceivable that a combination of visceral hypersensitivity (VH), genetic predisposition, psychological stress, compromised intestinal barrier function, and alterations in the microbiota may play a role in the development of IBS. These factors are associated with alterations in the neuroendocrine and immune systems, as well as the enteric nervous system (ENS) and central nervous system (CNS). Noteworthy, while this chapter focuses on biological mechanisms, it is important to note the implicit interaction with sociological and psychological factors ²³.

2.1. Stress and anxiety

The stress response can be activated by a range of internal and personal factors,

including fear and aggression, as well as by external and objectively identifiable elements such as changes in the environment, traumatic events, and social isolation. Many individuals with IBS report that their symptoms first emerged during periods of heightened stress, such as going through a relationship breakup, emotional abuse or the loss of a loved ^{24,25}. Indeed, generalized anxiety frequently occurs in individuals with IBS and is linked to heightened intensity of gastrointestinal symptoms. This correlation may arise from the distressing nature of IBS symptoms for certain patients, similar to other chronic illnesses, leading to anxiety as a secondary response to the physical condition ²⁶. Accordingly, a recent review has indicated that patients with IBS have three-fold-increased odds of either anxiety or depression, compared to healthy subjects ²⁷. Additionally, anxiety might impact the perception of pain by disrupting central mechanisms responsible for processing and regulating distressing visceral sensory signals ²⁶. The underlying mechanisms behind this observed association likely encompass genuine psychological elements, as well as the interactions within the gut-brain and the hypothalamo-pituitary-adrenal (HPA) axis. Increased secretion of corticotrophin releasing hormone (CRH), adrenocorticotrophic hormone (ACTH), and cortisol are the final effects of activation of the HPA axis. Increased CRH release is thought to encourage central sensitization, whereas ACTH and cortisol are thought to cause peripheral sensitization by activating resident immune cells and extrinsic primary afferents in the gastrointestinal tract ²⁸.

Concerning the HPA axis, the pathophysiology of IBS has been explained by two models: top-down and bottom-up ²⁹ (**Figure 2**). The top-down model contends that the brain is where the majority of pathophysiological changes first take place. This concept proposes that the basic illness processes in the brain communicate with the peripheral organs via the gut-brain axis to produce changes in the gut that result in the clinical manifestation of IBS ²⁹. Therefore, this model proposes that the symptoms of IBS are brought on by changes in the central nervous system brought on by numerous stressors that are directed at the brain like unfortunate life events, anxiety and depression ^{28,30}. It is thought that the brain's several neural networks

intricately interact with one another to produce symptoms. Interactions between the central executive network (involving attention, working memory planning, and response selection), the salient network (responding to external and internal stimuli that reach the brain), the sensory motor network, and the autonomic networks (central control of autonomic function) have been reported in studies done on adult patients with IBS ²⁹.

The bottom-up model proponents contend that peripheral variables in the gut play a crucial role and that changes in cerebral functioning are a result of gut-brain interactions ²⁹. According to the bottom-up paradigm, certain stressors that are focused on the gut can affect the CNS and change how the cortex brain responds to the visceral inputs that cause IBS symptom ²⁹. In this model, abnormal responses of the enteric nervous system (ENS) to gut stimuli (for example, changes in neurotransmitters like serotonin) and intestinal infections, mucosal inflammation, gut distension, immune mediated reactions, food allergies, altered gut microbial flora, increased intestinal permeability, and abnormal ENS responses all together or separately cause symptom generation. The intrinsic primary afferent neurons, whose cell bodies are found in the cranial and dorsal nerve root ganglia, may have an impact on the brain. While vagal afferents are mostly thought to be involved in non-nociceptive sensations (such as local reflexes, stomach accommodation, etc.), the sympathetic afferents from the gut are thought to be the primary mediator of nociceptive stimuli ²⁹.

There is a clear correlation between a higher occurrence of negatively perceived life events in adulthood and an elevated likelihood of experiencing IBS, along with more severe symptoms, lower quality of life related to the disease, and a disrupted stress response in IBS patients. Grasping the significance of stressful life events in adulthood, as well as their impact on the HPA axis, could provide valuable insights for tailoring IBS treatment approaches.

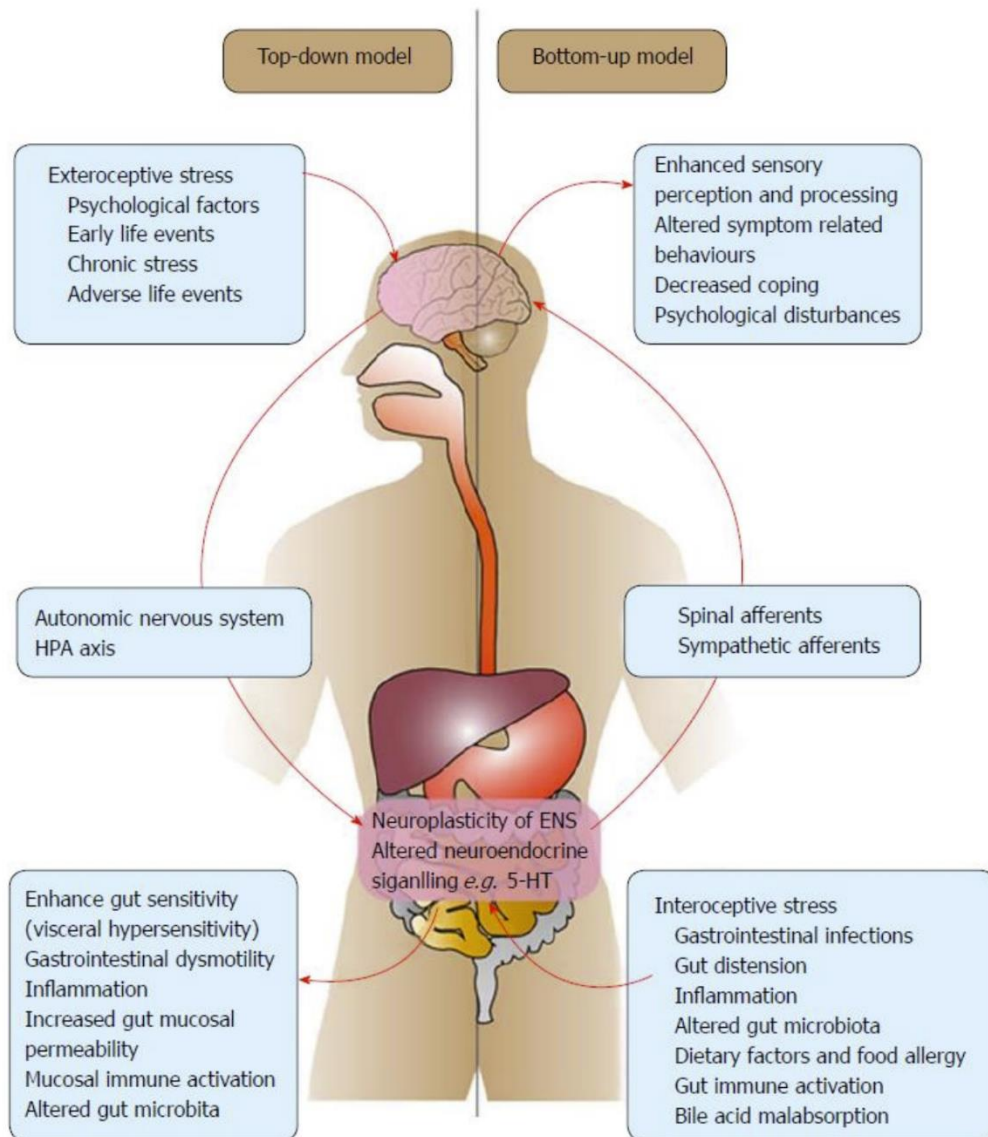


Figure 2. Top-down and bottom-up models of pathophysiology of irritable bowel syndrome. ENS: Enteric nervous system; 5-HT: 5-hydroxytryptamin; HPA axis: Hypothalamo-pituitary-adrenal axis. Adapted from ²⁹.

2.2. Motility disturbance

Certain symptoms associated with IBS, like diarrhea, constipation, or the sensation of incomplete rectal evacuation, point toward a disruption in motor function. In the context of IBS, there are no identifiable structural abnormalities present in the gastrointestinal tract; rather, the issues are tied to its proper functioning. In certain subsets of individuals with IBS, there is an observed increase in the motility of the small intestine. IBS-D, for instance, showcases an accelerated intestinal transit, while IBS-C demonstrates delayed transit. These variations have been identified in response to internal cues such as food intake, chemical activation of

corticotropin-releasing hormone (CRH), and the inflation of a balloon within the bowel, as well as external factors including emotional and physical stress^{31–33}.

Moreover, issues concerning motility in the small bowel and colon are not exclusive to IBS; often, they arise as a secondary effect of heightened sensitivity and amplified pain perception in the gut, a phenomenon referred to as visceral hypersensitivity (VH)^{34–37}. The variations in transit observed in IBS symptoms are predominantly attributed to irregularities in motility. Yet, for the majority of individuals, intestinal contractions are linked with discomfort and pain, a phenomenon not solely accountable by motility factors. Studies employing both painful and non-painful visceral stimulation have discovered heightened activation of the anterior cingulate cortex in IBS patients. This region in the brain plays a pivotal role in processing pain signals. As a result, it seems evident that individuals with IBS possess an amplified sensitivity to pain within the brain-gut^{11,38}.

2.3. Gut permeability

Studies suggest that alterations in gut epithelial permeability play a significant role in the altered communication between the gut and brain³⁹. These changes in permeability are believed to be a key factor in the development of central sensitization in IBS, as indicated elsewhere^{40,41}. Previous research has implicated increased gut permeability in the sensitization of viscerosensory pathways and the development of the chronic visceral hypersensitivity (VH) commonly observed in IBS^{40,41}. Accordingly, it has been demonstrated that heightened visceral sensitivity in response to acute stress in rats, such as restraint, is linked to an increase in intestinal permeability. When this rise in permeability is prevented, it mitigates the impact on sensitivity⁴². In essence, the activation of stress pathways triggers mast cell activation, resulting in the production of mediators that open tight junctions and elevate paracellular permeability. Consequently, this leads to increased absorption of bacterial fragments and toxins in the mucosa, activating the mucosal immune system. In conjunction with mast cell mediators, this process contributes to VH⁴². Thus, it's been shown in humans that enhanced small bowel

permeability effect can be blocked by the mast-cell stabilizer cromoglicate, underscoring that corticotropin releasing factor (CRF) impacts intestinal permeability through mast cell activation^{43,44}. When volunteers were administered CRF, it resulted in increased small intestinal permeability. However, when both CRF and cromoglicate were used in treatment, the permeability did not increase⁴⁴. Therefore, mediators like mast cells seem like potential targets in reducing gut permeability and IBS symptoms like VH.

2.4. Genetic factors

Certain genes have been linked to IBS, particularly polymorphisms in the serotonin receptor genes^{45,46}, polymorphisms in genes involved in the anti-inflammatory action of interleukin-10⁴⁷, adrenergic receptor genes^{48,49}, among others notably related to GIT motility and heightened sensibility. A recent genome-wide analysis of 53 400 people with IBS highlights shared genetic pathways with mood and anxiety disorders⁵⁰. Given the multifactorial pathophysiological mechanisms linked to IBS, it is possible to consider that a group of genes, rather than a singular gene, might be involved in the onset of IBS. Alternatively, other factors, such as environmental influences, could play a role in mediating the connection between genotype and phenotype, given their ability to modify gene expression⁵¹.

Newly gathered data indicates that epigenetic processes, which encompass alterations at the molecular level unrelated to changes in gene sequence, may play a role in how environmental factors influence both central and peripheral functions⁵². There is a growing body of evidence indicating epigenetic alterations in individuals with IBS, including modifications in DNA methylation observed in animal models and IBS patients, as well as the involvement of various miRNAs associated with IBS traits like heightened visceral sensitivity and increased intestinal permeability⁵². Of particular interest is DNA methylation, an emerging area in the study of complex diseases, which holds promise in shedding light on the underlying causes of IBS and potentially uncovering targets for therapeutic interventions⁵².

2.5. Diet

Food plays a significant role in the context of IBS. Various pathways can potentially connect food intolerance with IBS symptoms, including mechanisms like osmotic and fermentation effects, alterations in neurohormonal or intestinal immune activity, shifts in the intestinal microbiome, and changes in intestinal permeability. Food is composed of a multitude of different components, many of which have the potential to influence the functioning and sensation of the intestine ⁵³.

A consensus exists that initial dietary management of IBS will primarily involve food and lifestyle recommendations. Healthcare professionals typically offer advice on healthy eating and lifestyle adjustments as part of IBS dietary management ⁵⁴. Several studies have indicated that irregular eating habits among IBS patients could impact colonic motility and contribute to the manifestation of IBS symptoms. In general, these recommendations are centered on maintaining regular eating patterns and minimizing the consumption of potential dietary triggers, such as alcohol, caffeine, spicy foods, and fatty foods. Adequate hydration and daily physical activity are also incorporated into these recommendations. As a second-line approach to IBS dietary management, more advanced dietary manipulations can be employed to alleviate symptoms. One such approach is the utilization of a low FODMAP (fermentable oligosaccharides, disaccharides, monosaccharides and polyols) diet. FODMAPs are substances that are not readily absorbed in the colon. They increase luminal water through osmotic effects and lead to gas production due to fermentation by colonic bacteria. This can result in luminal distension and gastrointestinal symptoms in individuals with IBS ⁵⁴.

2.6. Immune alterations

Considering the relevance of altered gastrointestinal motility in affecting bowel habits for patients with IBS, it is worth noting the established interaction between the nervous and immune systems during inflammation ⁵⁵. This dynamic is of potential interest for understanding IBS. Additionally, the communication between luminal microbiota and the intestinal immune

system, which has been shown to impact gastrointestinal motility in animal models, is an area of significance for IBS. To illustrate, in mouse models, the absence of TLR, a receptor commonly found on innate immune cells that recognizes lipopolysaccharide from Gram-negative bacteria, leads to a substantial decrease in gastrointestinal motility⁵⁶. Furthermore, intestinal macrophages play a role in regulating the peristaltic activity of the colon. They achieve this by altering the pattern of contractions in smooth muscle cells in the presence of luminal microbiota^{57,58}.

The precise mechanism by which altered immune function contributes to symptoms in IBS patients is not fully understood. Nevertheless, numerous studies now provide support for the significance of neuroimmune interactions in generating symptoms not only in IBS but also in other functional gastrointestinal disorders⁵⁹. This theory is grounded in anatomical findings from studies demonstrating that individuals with IBS exhibit heightened counts of sensory nerve fibers expressing the capsaicin receptor TRPV1 in the rectosigmoid region. Additionally, there is an increased presence of mast cells in close proximity to nerves in the colon, along with a heightened density of intestinal mucosal nerve fibers forming clusters around mast cells⁷. Furthermore, these studies have revealed positive correlations between the severity of abdominal pain and both the number of nerve fibers expressing the TRPV1 receptor and the number of mast cells in close proximity to colonic nerves⁷.

3. Visceral hypersensitivity: the cardinal symptom of IBS

Visceral hypersensitivity (VH), which involves an heightened perception of stimuli originating from the viscera, is a defining characteristic of IBS and is currently recognized as the primary contributor to abdominal pain experienced by individuals with IBS¹. The nociceptive system encompasses pain, a term characterized by the International Association for the Study of Pain as an uncomfortable amalgamation of sensory and emotional perceptions intertwined with real or potential harm to tissues⁶⁰. It constitutes an unwelcome and

displeasing sensation ⁶¹, operating to safeguard the body against plausible adverse incidents ^{62,63}. The principal objective of pain, rooted in survival, involves prompting the body to respond by evading the noxious stimulus intentionally. Nociceptive pain can also be categorized into somatic and visceral pain ⁶⁴. Somatic pain is identifiable and localized more easily, whereas visceral pain is challenging to pinpoint to a specific body area and is frequently characterized as "generalized" ⁶⁵. Visceral pain often triggers more pronounced automatic reflexes than somatic pain ⁶⁵.

The process of nociception starts when the tissue is exposed to intense thermal, mechanical, or chemical stimuli (**Figure 3**). When the tissue is injured, it releases inflammatory substances like globulin, protein kinases, arachidonic acid, histamine, nerve growth factor, substance P, and calcitonin gene-related peptide ⁶⁶. These substances activate channels similar to voltage-gated channels, which leads to the generation of electrical signals (receptor potentials). The receptor potentials then trigger electrical signals (action potentials) in sensory nerve fibers. These action potentials travel as signals through sensory nerve fibers to the dorsal root ganglia and dorsal horn of the spinal cord (transmission). From there, the signal travels up the spinal cord to the brain stem and thalamus, where it undergoes significant processing (modulation). Finally, the signal reaches the somatosensory cortex (central perception) ⁶⁶.

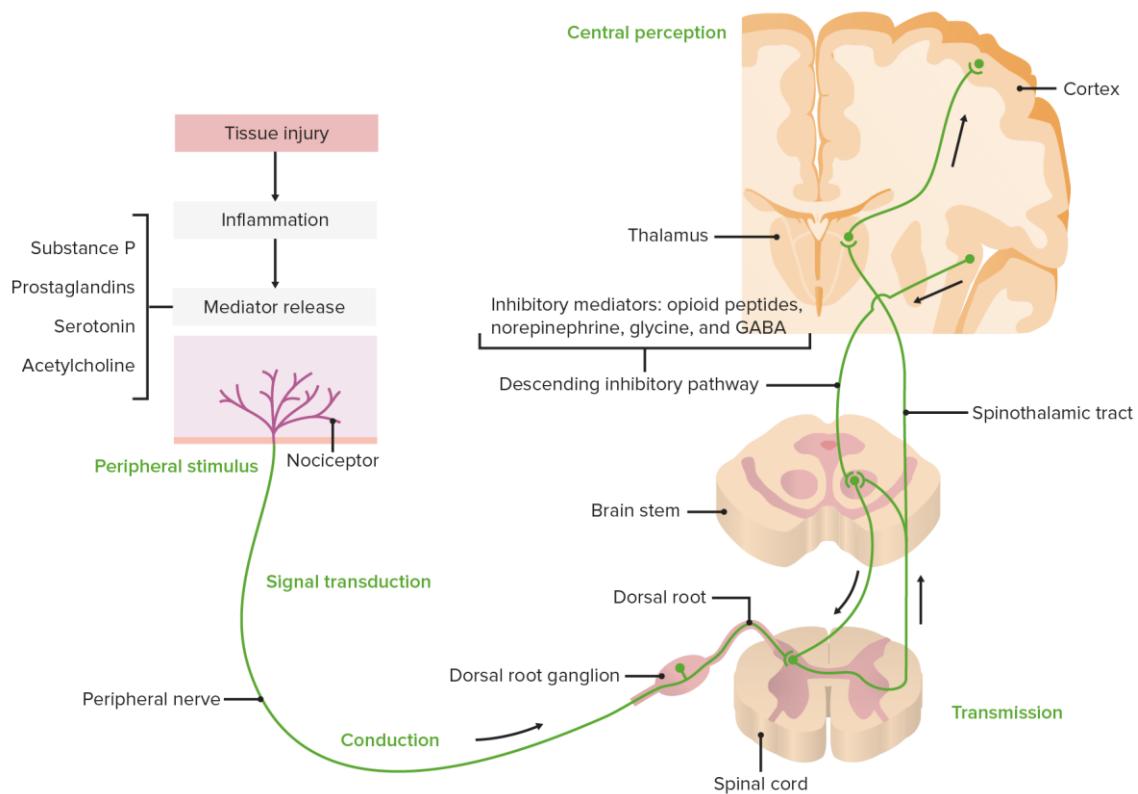


Figure 3. Pathway of pain transduction, transmission, modulation and central perception. Adapted from ^{23,66–68}.

Pain signals are regulated (their transmission from nociceptive afferents is reduced) by natural opioid substances like endorphins, dynorphins, and enkephalins)⁶⁶. This regulation occurs in the "descending" or "inhibitory" pathways, found in the spinal cord and dorsal root ganglia⁶⁸. These opioids work by activating specific receptors, which leads to reduced calcium influx, resulting in less release of glutamate and substance P. Additionally, there is an increase in potassium conductance in dorsal horn neurons. Other substances that play a role in pain perception and modulation include norepinephrine (NE), glycine, and GABA^{66,68} (**Figure 3**). The phenomenon of hypersensitivity encompasses both hyperalgesia and allodynia, as depicted in **Figure 4**^{23,69}. Hyperalgesia refers to an intensified response to a stimulus that is normally painful. Allodynia, on the other hand, signifies the experience of pain in response to a stimulus that is typically innocuous⁷⁰.

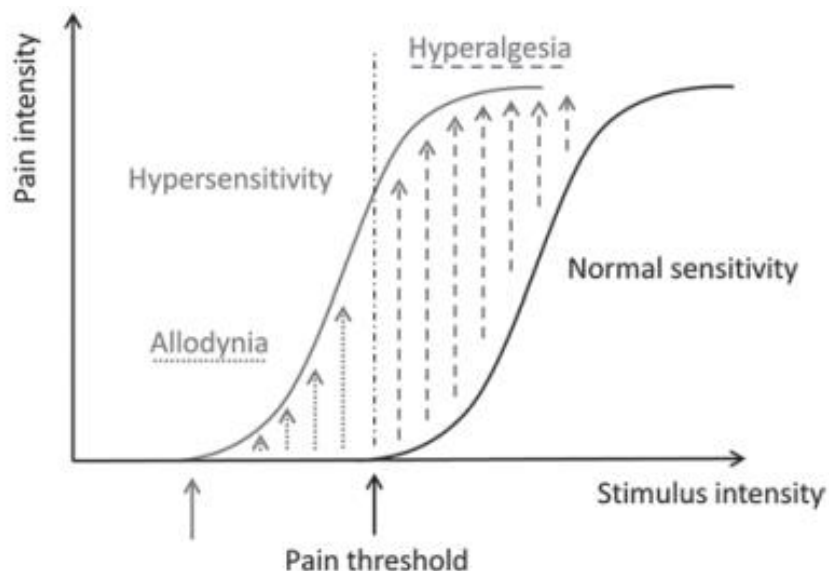


Figure 4. Concept of hypersensitivity. The black curve represents the normal pain perception. Only when the pain threshold is exceeded (full thickness arrow) does the stimulus-induced pain. The grey curve demonstrates hypersensitivity, characterized by a decreased pain threshold (grey arrow), allodynia (dotted arrows) and hyperalgesia (dashed arrows). Adapted from ²³.

In clinical trials, the presence of VH or altered stimulus perception is frequently measured using balloon distension. This procedure involves inserting a balloon into the distal colon or rectum and progressively inflating it to engage the sensory nerve endings within the colon wall ⁷¹. Following each distension, patients rate their perceived pain on a visual analog scale. In comparison to healthy individuals, IBS patients consistently report lower pain thresholds and higher pain ratings in response to these distensions ⁷¹. Consistently, Bouin et al. (2002) ⁷² demonstrated that individuals with IBS, as opposed to controls or patients with functional constipation (without pain), exhibit a reduced pain threshold when subjected to barostat testing, indicating a hypersensitivity of the rectum (**Table 2**).

Table 2. Rectal balloon distension by barostat. Adapted from ⁷².

	Controls	IBS	Functional constipation
Pain threshold (mmHg)	44.5 ± 5	30.4 ± 6.7	45.4 ± 5.3

Moreover, Azpiroz et al. (2007) ⁷¹ identified three criteria indicating altered rectal

perception in individuals with IBS: (i) a reduced pain threshold (33 mmHg instead of 40 mmHg); (ii) heightened sensitivity to repeated distensions, with a threshold of 28 mmHg compared to 41 mmHg in controls, and (iii) the occurrence of pain referred to locations other than the sacral area, observed in 83% of IBS patients as opposed to 10% in controls. The authors attribute these findings to a potential dysregulation in the neural sensory system. Furthermore, they suggest that this dysregulation is likely peripheral in nature, as indicated by the presence of inflammatory cell infiltration into the gut mucosa or enteric plexuses, a phenomenon often observed in individuals with IBS ^{43,73}.

Various studies centered on IBS have employed intestinal balloon distension to induce VH. By employing techniques such as positron emission tomography and functional magnetic resonance imaging, researchers are able to gauge an individual's perception of pain, thereby offering some objective validation of subjective pain accounts ⁷⁴. In IBS subjects, there is an observed elevation in the activation of cortical and subcortical brain regions associated with emotions, arousal, attention, and autonomic responses ^{71,75}. Investigations delving into the impact of psychological processes on VH propose that factors like depression, anxiety, somatization, and other forms of psychological distress are more tightly linked to nociception reporting than abnormalities in neurosensory sensitivity ^{30,76,77}.

3.1. Rodent models for VH studies

Hypersensitivity can be induced in rodents in various ways; they are summarized in **Figure 5** ⁷⁸. The Wistar rat has been identified as exhibiting spontaneous colonic hypersensitivity, showing strain variation. Chronic hypersensitivity can be established through manipulation of neonatal pups, including maternal separation protocols or direct irritation of the colon, as well as through conditioning protocols ⁷⁸. Acute stress protocols can lead to colonic hypersensitivity of varying duration, depending on the type of stressor and the rodent

strain. Targeting the central nucleus of the amygdala with stress hormones can produce colonic hypersensitivity that persists beyond the exposure to the hormone. Restraint stress typically results in acute hypersensitivity (lasting less than a day), while exposing rats to variable stress or repeated water avoidance stress protocols can lead to colonic hypersensitivity that persists for several weeks. Acute sensitization of colonic afferents produces hypersensitivity that is either transient (lasting several hours) or short-lived (lasting days), depending on whether the irritant induced an inflammatory response. Substances like acetic acid and butyrate can sensitize afferents without causing inflammation⁷⁸. Capsaicin and mustard oil induce spontaneous noxious behaviors. Agents like Dextran sodium sulfate (DSS), 2,4,6-trinitrobenzenesulfonic acid (TNBS), and zymosan induce colonic inflammation of varying severity. Post inflammatory colonic hypersensitivity can occur following recovery from colitis induced by substances like acetic acid, DSS, or TNBS. Additionally, recovery from colitis induced by pathogenic bacteria (*Citrobacter rodentium*, *Campylobacter jejuni*) or nematodes (*Trichinella spiralis*) can also lead to chronic colonic hypersensitivity. For the purposes of **Figure 5**, mediators (including ligands, signaling pathways, processes, or receptors) were categorized as "central" if a therapeutic was administered centrally (in the brain or spinal cord) and/or if only changes within the brain and spinal cord were analyzed. Mediators were considered "peripheral" if a therapeutic was administered peripherally and/or if only tissues outside of the spinal cord (including the dorsal root ganglion) were investigated. Mediators were listed as "both" central and peripheral if a therapeutic was administered both centrally and peripherally, if the therapeutic was known to cross the blood-brain barrier with targets in both sites, and/or if the study analyzed both central and peripheral tissues⁷⁸.

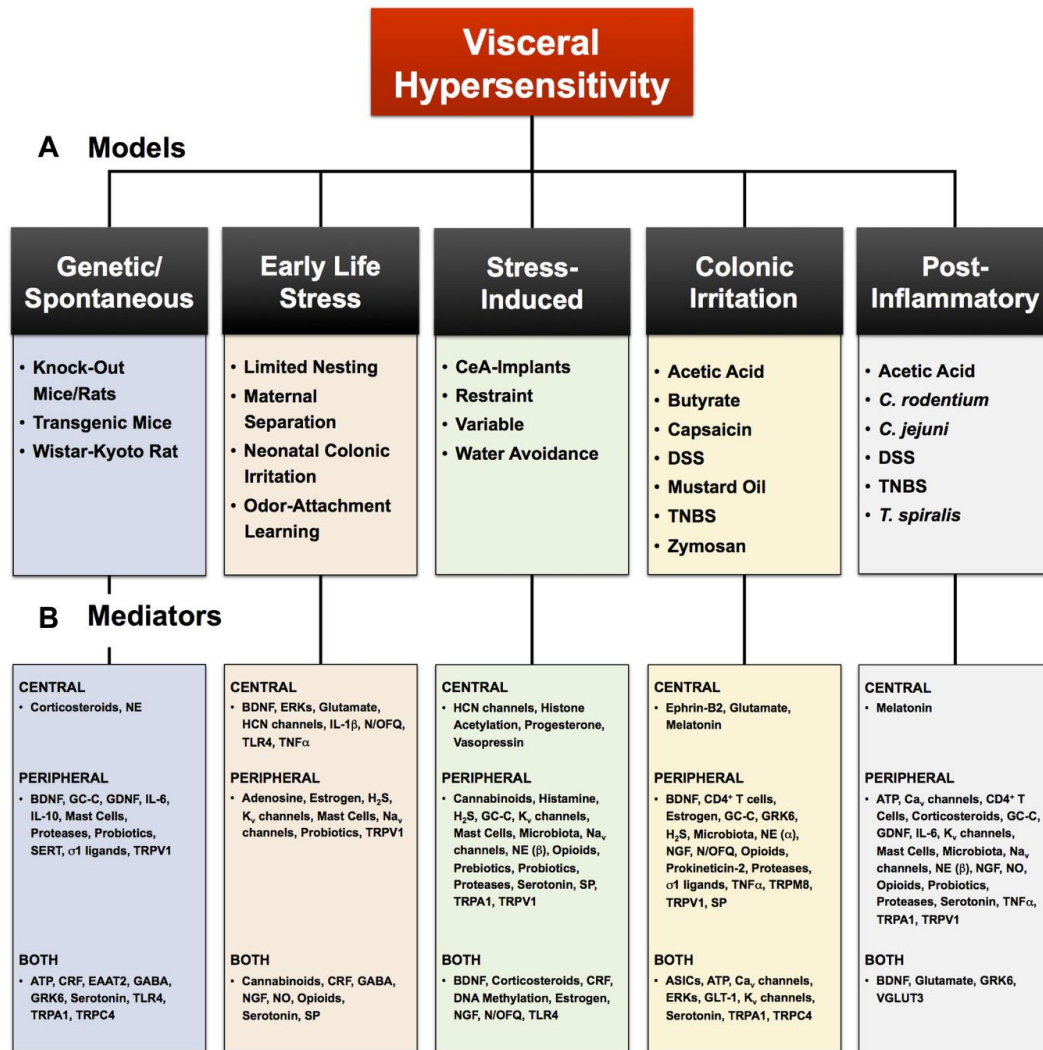


Figure 5. Rodent models and mediators of colonic hypersensitivity. ASICs, acid-sensing ion channels; ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; Cav, voltage-gated calcium; CRF, corticotropin releasing factor; EAAT2, excitatory amino-acid transporter type 2; ERKs, extracellular-signal-regulated kinases; GABA, -aminobutyric acid; GC-C, guanylate cyclase C; GDNF, glia cell line-derived neurotrophic factor; GLT-1, glutamate transporter-1; GRK6, G protein-coupled receptor kinase 6; HCN, hyperpolarization-activated cyclic nucleotide-gated; Kv, voltage-gated potassium; IL-1, interleukin 1; IL-6, interleukin 6; IL-10, interleukin 10; Nav, voltage-gated sodium; NE, norepinephrine; NGF, nerve growth factor; NO, nitric oxide; N/OFQ, nociception/orphanin FQ; SERT, serotonin reuptake transporter; SP, substance P; TLR4, Toll-like receptor 4; TNF, tumor necrosis factor; TRPA1, transient receptor potential cation channel, subfamily A, member 1; TRPC4, short transient receptor potential channel 4; TRPM8, transient receptor potential cation channel, subfamily M, member 8; TRPV1, transient receptor potential cation channel, subfamily V, member 1; VGLUT3, vesicular glutamate transporter 3. Adapted from ⁷⁸.

4. Current treatment options for IBS symptoms

Among the diverse range of treatment options available for IBS, none exhibit universal effectiveness, especially for individuals with severe symptoms. The most common approaches to treatment encompass pharmacological interventions (such as smooth muscle relaxants, bulking agents, anxiolytics, anti-depressants, and psychotropic agents), psychological strategies (including interpersonal psychotherapy, cognitive-behavioral therapy, cognitive therapy, and mindfulness training), dietary adjustments (such as increasing fiber intake and eliminating wheat, corn, dairy, coffee, and citrus), as well as alternative or additional methods (such as hypnotherapy, meditation, Chinese medicine, probiotics, peppermint oil, and melatonin)^{35,36,79–81}.

In the context of managing VH, innovative therapeutic substances encompass compounds that modify communication pathways between the gut and brain, as well as the nearby neuroimmune pathways. Given that gastrointestinal hyperalgesia frequently coincides with muscle spasms, substances that reduce the contractions of smooth muscles in the gut hold the potential to ameliorate VH. Furthermore, therapeutic agents capable of inhibiting nerve activation and signaling transmission from the gastrointestinal tract to the central nervous system play a crucial role in regulating VH. More details on the most popular pharmacological treatments for VH can be seen in **Table 3**. Among alternative therapies for IBS patients, probiotics and ingested bacteria are increasingly being suggested (further detailed in this chapter).

Table 3. Most popular treatments for visceral hypersensitivity (VH).

VH treatment	Mecanism	Efficacy	Safety	References
Antispasmodics	Antispasmodics function by either impeding the effects of acetylcholine at muscarinic or tachykinin NK2 receptors, or obstructing calcium channels on the smooth muscle of the gastrointestinal tract. This modulation influences gastrointestinal movement, thereby playing a role in alleviating pain and addressing disruptions in bowel patterns.	Comprehensive evaluations found limited substantiation regarding the efficacy of certain antispasmodics in terms of abdominal pain alleviation and overall symptom mitigation. Among the studies, notable enhancements in abdominal pain were observed in 7 out of 9 investigations, improvements in bowel symptoms were noted in 2 out of 9 studies, and reductions in global symptom severity were reported in 4 out of 9 studies.	Antispasmodics lead to a higher occurrence of side effects compared to placebo, with the most prevalent ones being dry mouth, dizziness, blurred vision, and constipation.	82-85
Peppermint oil	Menthol, the primary component of peppermint oil, functions by obstructing calcium influx, thereby inhibiting the contraction of smooth muscles in the gastrointestinal tract. Furthermore, menthol triggers analgesia by activating the transient receptor potential cation channel subfamily M member 8, a temperature-sensitive ion channel, which possesses anti-nociceptive characteristics in visceral sensory pathways.	Peppermint oil showed greater efficacy than placebo in a meta-analysis of four trials involving 392 IBS patients. In another meta-analysis of five RCTs involving 197 patients on peppermint oil and 195 on placebo, it improved IBS symptoms and abdominal pain across five studies.	Peppermint oil usage can lead to manifestations of gastroesophageal reflux, dry mouth and belching.	82,86-89
Anxiolytics and antidepressants	The justification for employing anxiolytics and antidepressants in IBS encompasses several factors: the concurrent presence of psychological conditions in IBS; the demonstrated influence of depression on the central nervous system's reaction to painful stimuli; the advantages of antidepressants in persistent painful conditions; and rectification of disrupted intestinal movement. The approach of antidepressants in addressing IBS operates through multiple facets, which might entail dampening activation in pain	Open-label trials have explored the impact of duloxetine, a serotonin and norepinephrine reuptake inhibitor, demonstrating effectiveness in IBS treatment, specifically visceral pain. The use of SNRI medications, like duloxetine, is established in pain management. However, rigorous double-blind, placebo-controlled, randomized clinical trials are imperative to establish the efficacy of these	Common adverse effects included drowsiness and dry mouth. Prolonged utilization of certain categories of psychotropic medications for non-psychiatric purposes has been associated with dementia in population-based investigations, although a definitive cause-and-effect connection has not been	90,91,100-103,92-99

	centers like the anterior cingulate cortex and central pain processing, as well as peripheral mechanisms that impact pain perception, including colonic compliance and visceral sensory function.	medications for IBS and pain relief, particularly in cases accompanied by comorbid depressive or generalized anxiety disorders.	established.
Drugs acting on opioid receptors	Agonists of opioid receptors decelerate gut and colonic movement, heighten fluid absorption, and diminish the perception of pain.	A 21-patient IBS-D trial found loperamide improved stool, pain, and urgency. Another 60-patient trial, including functional diarrhea or IBS-D, confirmed reduced frequency, better consistency, and fewer days of pain. In a third unselected IBS trial, loperamide enhanced stool frequency, consistency, and overall pain intensity, but nighttime abdominal pain increased. Loperamide is ideal for diarrhea or urgency. Eluxadoline, a novel κ - and μ -opioid receptor agonist and δ -opioid receptor antagonist, proved effective in relieving diarrhea and pain in three trials with 3000 IBS patients	Nausea and headaches as side effects. There have been infrequent instances of pancreatitis and sphincter of Oddi spasm reported. Following FDA guidance, eluxadoline should not be prescribed to individuals with a history of biliary obstruction, cholecystectomy, pancreatitis, severe hepatic impairment, severe constipation, or those consuming over three alcoholic drinks daily. ¹⁰⁴⁻¹⁰⁹
Off-label approaches			
Histamine H1 receptor antagonist, ebastine	Mast cells and their substances, particularly histamine, serotonin, and proteases, contribute to IBS development. In IBS patients, colonic biopsies release histamine that sensitizes TRPV1 on neurons in dorsal root ganglia (afferent pathways) and human submucosal neurons in rectal biopsies via H1 histamine receptors.	Ebastine, a non-sedating H1 receptor antagonist, has demonstrated the ability to decrease visceral hypersensitivity, overall IBS symptoms, and abdominal pain in IBS patients.	Drug non-approved in the United States and other countries ^{110,111}
Intestinal secretagogues chloride-channel related	Lubiprostone, a prostaglandin derivative, interacts with chloride channels on the apical surface of intestinal enterocytes. This interaction prompts chloride secretion, subsequently leading to the passive influx of sodium ions and water into the lumen. Consequently, stool consistency becomes more liquid, and there is an acceleration	Linaclootide and Plecanatide have shown general improvements in abdominal pain scores that parallel the improved straining and stool consistency	Nausea is the most common side effect, experienced by 8% of patients, ¹¹²⁻¹²⁰

in gastrointestinal transit.

Serotonin receptor agonists and antagonists	Intestinal enterochromaffin cells contain about 90% of the body's total serotonin (5-HT). Additionally, 5-HT functions as a neurotransmitter in the brain, where various classes of 5-HT receptors exist in both the brain and the gut.	5-HT ₃ receptor antagonists alosetron and ramosetron and 5-HT ₄ receptor agonist tegaserod reportedly reduced visceral pain	Risk of cardiovascular ischemic events	121–123
GABAergic receptor agonists	GABAergic receptor agonists decrease the release of various excitatory neurotransmitters implicated in pain processes, such as glutamate, noradrenaline, substance P, and calcitonin gene-related peptide	Collectively, these studies propose that these agents lower sensation without notable effects on compliance. GABAergic receptors agonist Baclofen was shown to reduce visceral nociception in multiple models	Reports of insomnia and rhinitis	124–128

5. Gut microbiota and ingested bacteria: potential therapies for IBS

5.1. Gut microbiota

As humans consume food, it traverses through the GIT carrying along microorganisms. In the stomach, digestive enzymes like pepsin are released, and hydrochloric acid is secreted, resulting in a decrease in the stomach's pH. The food then progresses through the small intestine, passing through the duodenum, jejunum, and ileum. During this journey, the pH rises from 6.6 to 7.5, and additional digestive enzymes aid in the breakdown and absorption of carbohydrates, proteins, and lipids. Subsequently, the luminal content moves into the colon, where the pH ranges from 5.4 to 6.9. The colon can be divided into ascending, transcending, descending, and sigmoidal regions. In the colon, water and salts are absorbed, and the remaining contents are expelled from the body as feces^{129–132}.

Various factors such as transit time, oxygen levels, pH levels, nutrient availability, and substrate changes can influence the microbial ecosystem within the human GIT. Consequently, diverse microorganisms accumulate, forming a complex microbial community known as the gut microbiota, which includes bacteria, yeast, fungi, archaea, and viruses. This community consists of an impressive number of microorganisms ranging from 10^{13} to 10^{14} , which dominate the gut¹³⁰. The composition of the gut microbiota changes along the GIT, with bacterial concentrations increasing from the stomach to the colon (**Figure 6**). In the stomach and duodenum, there are approximately 10^1 and 10^3 colony-forming units (CFU) of live bacterial cells per mL, while in the jejunum, it ranges from 10^4 to 10^8 CFU, and in the colon, it reaches 10^9 to 10^{12} CFU (**Figure 6**)^{130,133}. This intricate ecosystem is mainly represented by a few bacterial phyla, namely *Firmicutes*, *Bacteroidota*, *Proteobacteria*, *Fusobacteria*, *Verrucomicrobia*, and *Actinobacteria*. *Proteobacteria* and *Actinobacteria* initially dominate the gut microbiota of newborns, but as they grow, it shifts to be primarily dominated by *Firmicutes* and *Bacteroidetes*, which are the dominant representatives in the adult intestinal tract. Within

the *Firmicutes* phylum, key genera include *Faecalibacterium*, *Lactobacillus*, and *Roseburia*, while the *Bacteroidetes* phylum comprises genera such as *Bacteroides*, *Alistipes*, and *Prevotella*. Additionally, *Bifidobacterium* and *Collinsella* are among the genera present in the gut microbiota under the *Actinobacteria* phylum^{134,135}.

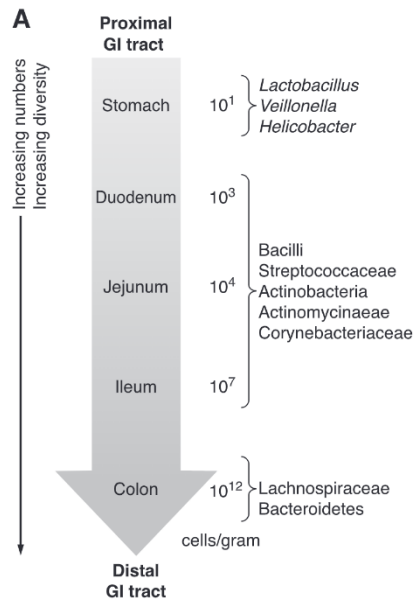


Figure 6. Variations in microbial numbers and composition across de length of the gastrointestinal tract. Adapted from 130.

5.2. Autochthonous and transiting bacteria of the GIT

In a specific intestinal environment, certain microbial members can be categorized as true residents or autochthonous species, forming stable and long-term associations with that habitat¹³⁶. On the other hand, there are bacteria that transit through the GIT along with the food and typically do not establish colonization¹³⁶. Fermented food products, like yogurt and cheese, are significant sources of these transiting bacteria, providing around 10^9 to 10^{10} live bacteria per serving. Studies investigating true resident and transient species, particularly within the lactic acid bacteria (LAB), a group commonly used in food fermentation, has been for long studied^{136,137}. Within the LAB group, species like *Lactobacillus plantarum*, *Lb. casei*, *Lb. paracasei*, *Lb. buchneri*, *Lb. brevis*, *Lb. rhamnosus*, *Lb. fermentum*, and thermophilic dairy

lactobacilli *Lb. delbrueckii* and *Lb. helveticus* seem not to establish stable communities and can be considered as transient or allochthonous species (Reuter 2001). There have been varying results reported for *Bifidobacterium* species. The presence and persistence of *B. adolescentis*, *B. longum*, and *B. bifidum* show significant variation among individuals¹³⁷, making it challenging to draw definite conclusions about their autochthonous or transient nature. The survival of allochthonous bacteria in the GIT depends on several factors, including their resistance to gastric acid, bile salts, and pancreatic juice¹³⁸. Allochthonous bacteria can have both positive and negative effects on their hosts. Transiting bacteria may include enteric food-borne pathogens that can invade and disrupt the intestinal epithelial barrier, in contrast to commensal microorganisms^{137,139}. On the other hand, some transiting bacteria are now recognized as probiotics, contributing to beneficial effects on human health. Further discussion on probiotics and their potential benefits for human health will be later presented here.

5.2.1. Influencing factors in gut colonization

Various studies have been conducted to investigate the bacterial colonization of the gut in newborn infants, revealing *Enterococcus* and *Streptococcus* as the dominant bacterial populations, with *E. faecalis* and *S. salivarius* being the most abundant species¹⁴⁰. Newborn infants are born with an underdeveloped GIT as they develop in a germ-free environment called the chorion. After birth, as they enter a more complex environment, they start getting colonized by microorganisms acquired from the mother and the surrounding environment. This colonization process continues until a more stable gut microbiota develops after weaning and persists throughout life into adulthood¹³¹. Several factors can influence the composition of the human gut microbiota. These factors include endogenic factors such as sex, gestational age, genetics, and immunity, as well as external factors like the mode of delivery (vaginal birth or cesarean section), mode of feeding (breastfed or formula-fed), diet, antibiotic usage, and environmental factors^{131,141,142}.

5.3. The role of microbiota in gut health and disease

Two primary mechanisms can influence the composition of the gut microbiota. The first involves host-bacteria interactions, which encompass immunological interactions occurring in the gut. The second mechanism involves bacterial-bacterial interactions, which result from metabolite production, oxygen consumption, and nutrient competition^{131,143}. Changes brought about by these interactions can lead to shifts in the bacterial population within the gut, potentially leading to dysbiosis. Dysbiosis refers to an imbalance in the gut microbiota, characterized by an increase in pathobiont bacteria, which can adversely impact the host's health. The rising incidence of dysbiosis has spurred interest among researchers in modulating the functions and composition of the gut microbiome to promote host health¹⁴⁴.

However, it is essential to note that the physiological state of the host is linked to the diversity and composition of the gut microbiota. For instance, studies have shown that obese individuals exhibit a lower ratio of Bacteroidetes to Firmicutes compared to those with normal weight^{145,146}. Dysbiosis in the gut microbiota has been associated with various diseases, including IBS, Alzheimer's, Crohn's disease, depression, anxiety, and autism. In response, researchers have explored several novel therapeutic approaches to regulate the gut microbiota, such as antibiotics, probiotics, prebiotics, synbiotics (probiotics + prebiotics), and fecal transplantation/bacteriotherapy^{147–150}.

The gut microbiota establishes a mutually beneficial and commensal connection with its host, inhabiting the intestinal environment through the consumption of available substrates that undergo saccharolytic or proteolytic fermentation¹⁵¹. This commensal relationship has been extensively researched to ascertain its impact on the host cells. These investigations have highlighted the gut microbiota's role in supporting crucial homeostatic functions, including immunomodulation, preservation of the intestinal mucosa, enhancement of cytoprotective genes, and the regulation of apoptosis^{152,153}.

They additionally serve a protective function against pathogenic bacteria seeking to establish themselves in the gut, competing for available resources, space, and receptors. Furthermore, they generate substances like fatty acids, peroxides, and bacteriocins that possess the capability to impede the growth of pathogens. Additionally, certain microbes produce proteases that aid in neutralizing toxins^{135,154}. In addition to their protective roles, the intestinal microbiota exhibits metabolic capabilities, including the synthesis of vitamins (such as folate and Vitamin K) and short-chain fatty acids that facilitate the growth of intestinal epithelial cells. Moreover, they may produce chemical compounds that function as neurotransmitters and neuromodulators, acting as psychobiotics^{135,144}.

6. Probiotics, prebiotics, postbiotics, psychobiotics and the gut-brain axis

As per the definition provided by the International Scientific Association for Probiotics and Prebiotics (ISAPP), probiotics are described as "live microorganisms that, when administered in sufficient quantities, confer health benefits on the host"¹⁵⁵. Health Canada has granted approval for certain probiotics, allowing non-strain-specific claims for *Bifidobacterium* species (*adolescentis*, *animalis*, *bifidum*, *breve*, and *longum*) and *Lactobacillus* species (*acidophilus*, *casei*, *fermentum*, *gasseri*, *johnsonii*, *paracasei*, *plantarum*, *rhamnosus*, and *salivarius*) when delivered in food at a level of 10⁹ colony forming units (CFU) per serving¹⁵⁵. On the other hand, the first definition of prebiotics dates back from 1995, when Gibson and Roberfroid defined them as "nondigestible food ingredients that have a beneficial impact on the host by selectively promoting the growth and/or activity of a specific group of bacteria in the colon, thereby contributing to improved host health"¹⁵⁶. This initial definition, however, has undergone several revisions over time^{157–160} until the current ISAPP consensus panel proposed the following definition of a prebiotic: "a substrate that is selectively utilized by host microorganisms conferring a health benefit"¹⁶¹. Finally, postbiotics are defined by the World

Gastroenterology Organization as “preparation of inanimate microorganisms and/or their components that confers a health benefit on the host”¹⁶².

In order to exert their beneficial effects, probiotic microorganisms must meet several criteria, including being nonpathogenic, safe, and capable of surviving and thriving in the conditions of the digestive tract¹⁶³. Therefore, various factors, such as safety, functional attributes, and technological properties, are taken into consideration when selecting potential probiotic candidates¹⁶⁴. These selection criteria encompass a range of evaluation tests, including assessing acid and bile salt tolerance, determining antibiotic resistance/susceptibility patterns, evaluating adhesion to intestinal epithelial cells, verifying the absence of virulence factors, testing survival ability in GIT, and ensuring viability during processing and storage¹⁶⁵. For instance, intrinsic resistance/sensitivity to antibiotics is an important characteristic used to identify potential probiotic strains that can be safely employed in various functional products¹⁶⁶. Similarly, the ability of potential probiotic bacteria to survive the acidic conditions during gastric transit and to grow in the presence of bile are critical functional properties to be considered¹⁶⁷. Probiotics play a significant role in immune modulation, neurotransmitter biosynthesis, and maintaining gut homeostasis by restoring microbial balance and composition, which involves intricate bidirectional interactions with the microbiota-gut-brain axis^{168–170}.

According to the guidelines established by the World Gastroenterology Organisation, the careful selection of probiotic therapy has the potential to alleviate symptoms and enhance the quality of life for individuals afflicted with irritable bowel syndrome. This positive impact is primarily attributed to the reduction of bloating and the improvement of bowel rhythm¹⁶². Notably, effective strains in addressing functional bowel disorders encompass *Lactiplantibacillus plantarum* 299v (DSM 9843), *Bifidobacterium infantis* 35624, *Lactobacillus rhamnosus* NCIMB 30174, and *Saccharomyces boulardii* CNCM I-745¹⁷¹. *Lactiplantibacillus plantarum* 299v, in addition to its therapeutic effects, has also been

demonstrated to contribute to the alleviation of anxiety and depressive states^{171–173}. Similar properties were also detected for *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175^{171,174,175}.

A novel category within the realm of probiotics is referred to as psychobiotics. These are strains of bacteria capable of exerting influence over brain function by impacting immune responses, hormonal signaling, and the modulation of neurotransmitter levels and availability¹⁷⁶. Psychobiotics carry out a range of pivotal functions, encompassing the safeguarding of intestinal barrier integrity, the reduction of pro-inflammatory cytokine synthesis, the production of neuroactive compounds like GABA, the modulation of neurotropic factors like brain-derived neurotrophic factors (BDNFs), 5-HT, histamine, norepinephrine and the influence on neurotransmitter synthesis (**Figure 7**)^{177,178}. In practical terms, the application of psychobiotics entails stress reduction, alleviation of depressive symptoms, mitigation of anxiety disorders, enhancement of cognitive function, and attenuation of stress-related gastrointestinal issues, among other benefits¹⁷⁹. Owing to their capacity to alleviate depression and anxiety symptoms, the use of psychobiotics as a supplementation strategy is considered a safe avenue for averting related mental health concerns. This approach boasts a markedly lower risk of side effects, such as allergies, addiction, or gastrointestinal discomfort, in comparison to psychotropic medications¹⁸⁰. Of particular relevance is the application of psychobiotics in addressing functional bowel disorders like IBS. This stems from their ability to curtail the intensity of VH, a pivotal factor in reducing the severity of this disorder's symptoms. This impact is closely intertwined with stress hormone concentrations such as norepinephrine, corticosterone, and adrenaline, and is likely modulated through glucocorticoid receptors¹⁷¹. Recent times have witnessed an increasing accumulation of reports highlighting the advantageous role of psychobiotics in alleviating stress-related gastrointestinal issues in individuals grappling with mood disorders^{181,182}. This underscores the substantial effectiveness of probiotics in alleviating

gastrointestinal symptoms, achieved not solely through the regulation of the microbiome's composition but also via their role in curbing inflammatory responses and facilitating emotional regulation.

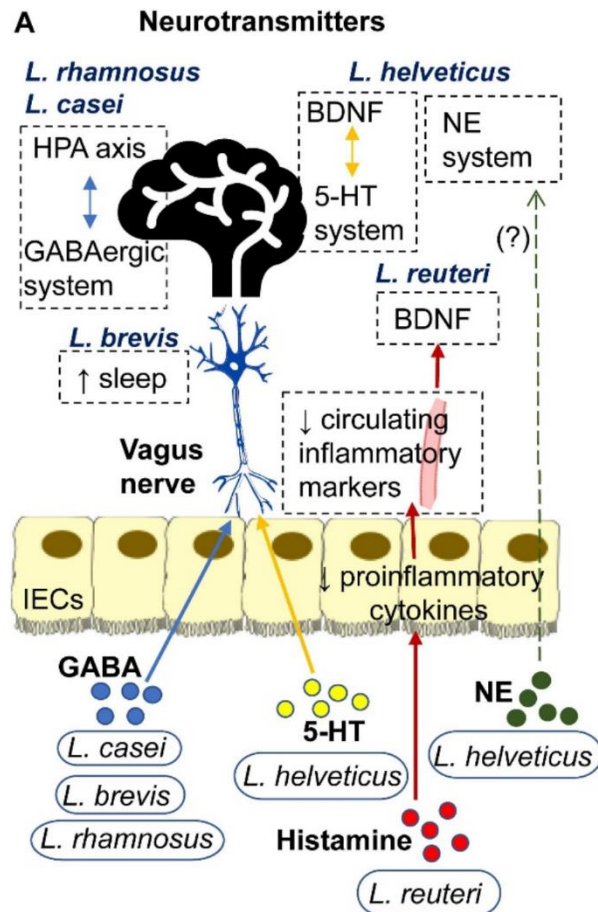


Figure 7. Signaling mechanisms underlying health effects of probiotics mediated through secretion of neurotransmitters. *L. rhamnosus* and *L. casei* secrete GABA that may signal central GABAergic system and HPA axis via the neural route. *L. brevis* secretes GABA that enhances sleep. *L. helveticus* secrete 5-HT that may signal the central 5-HT system via the neural route. *L. helveticus* also secretes NE that may affect the central NE system. *L. reuteri* secretes histamine that decreases secretion of proinflammatory cytokines by IECs. This may reduce circulating inflammatory markers, such as LPS, IL-6 and corticosterone, and subsequently prevent the inflammation-induced decrease in hippocampal BDNF. 5-HT, 5-hydroxytryptamine or serotonin; BDNF, brain-derived neurotrophic factor; GABA, γ -aminobutyric acid; HPA, hypothalamic-pituitary-adrenal; IECs, intestinal epithelial cells; NE, norepinephrine. Adapted from ¹⁷⁸.

Psychobiotics have the capacity to alter gut microbial composition and activity, leading to the production of various neuroactive compounds in the gut. These compounds can influence metabolic activity, psychological and physiological function, as well as the brain's biochemistry and behavior¹⁷⁸. Notably, psychobiotics can enhance neurotransmitter production, and studies have shown that GABA-producing *Lactobacillus plantarum* 90sk and *Bifidobacterium adolescentis* 150 are associated with reduced depressive-like behavior in mice, likely achieved by modulating GABAergic signaling via host enteric neurons' GABA receptors¹⁸³. Similar effects on depression and anxiety behaviors have been observed for *L. rhamnosus* JB-1, which alters GABA receptor expression levels¹⁸⁴. Moreover, GABA-producing bacteria may impact the gut-brain axis by modulating enteric nervous system (ENS) signaling and regulating neural excitation-inhibition levels¹⁸⁵. Psychobiotics can also indirectly influence the brain through the vagal pathway and neuroendocrine systems, leading to alterations in neurotransmission in the central nervous system and expression of inhibitory GABA receptors in the brain^{186,187}.

7. Physiological functions of GABA in mammals

GABA is a biogenic amine with four carbon atoms, predominantly serving as an inhibitory neurotransmitter in the mammalian brain¹⁸⁸. Research has demonstrated that orally administered GABA possesses tranquilizing and diuretic properties, as well as the ability to alleviate depression, anxiety, sleeping disorders, and visceral hypersensitivity^{167,189–192}. Due to these beneficial physiological functions, several GABA-enriched fermented foods, such as yogurt, cheese, green tea, and rice germ^{193–197}, are considered commercially promising functional foods¹⁹⁸. Functional foods, as described by Roberfroid, are foods that resemble conventional food and are intended to be consumed as part of the normal diet but have been modified to serve physiological roles beyond simple nutrient provision¹⁹⁹. The health benefits of fermented functional foods are achieved either directly through the interactions of ingested

live microorganisms with the host (probiotic effect) or indirectly through the ingestion of microbial metabolites synthesized during fermentation (biogenic effect) ²⁰⁰.

In order to exert its neurotransmitter functions, GABA must activate GABAergic receptors, which are described below.

7.1. GABA receptors and the enteric nervous system

Over the past three decades, there has been a growing body of data exploring the role of GABA in modulating gastrointestinal (GI) functions, potentially through its involvement in the circuitry of the enteric nervous system (ENS). The ENS is a sophisticated neuronal network situated within the gut wall, responsible for autonomously regulating various GI functions, including motility, secretions, blood flow, and the enteric immune system, independent of the central nervous system (CNS) ²⁰¹. Nevertheless, the precise and functional significance of GABA within the ENS remains largely unclear. GABA in the GI tract is sourced from neurons containing the GABA-synthesizing enzyme (L-glutamate decarboxylase, GAD), with the highest activity reported in the myenteric plexus. Additionally, mucosal endocrine-like cells also contribute to GABA production, implying a dual role for GABA as both a neural and endocrine mediator in the GI tract ²⁰¹. GABAergic neuronal cells, primarily interneurons, are distributed in both the submucosal and myenteric plexus throughout the GI tract ²⁰². GABA has the potential to impact both secretory and motor GI function, exhibiting either a stimulating or inhibitory effect on neuronal activity through the activation of GABA receptors. In the context of GABA receptors, there are three types identified in **Figure 8**: GABA_A, GABA_B, and GABA_C ²⁰³. GABA_A and GABA_C receptors are ionotropic receptors, specifically pentameric chloride channels. Due to the maintenance of elevated intracellular Cl⁻ concentration in enteric neurons through a specific sodium-potassium-chloride symporter, the activation of GABA_A receptors in the ENS leads to an excitatory effect, which is in contrast to the well-known inhibitory effect observed in the CNS ^{204–206}. GABA_B receptors are metabotropic G protein-coupled receptors

consisting of heterodimers. They exert negative modulation on presynaptic voltage-activated Ca^{2+} channels and positive modulation on postsynaptic inwardly rectifying K^+ channels^{207,208}.

The main characteristics of GABA receptors can be seen in **Table 4**.

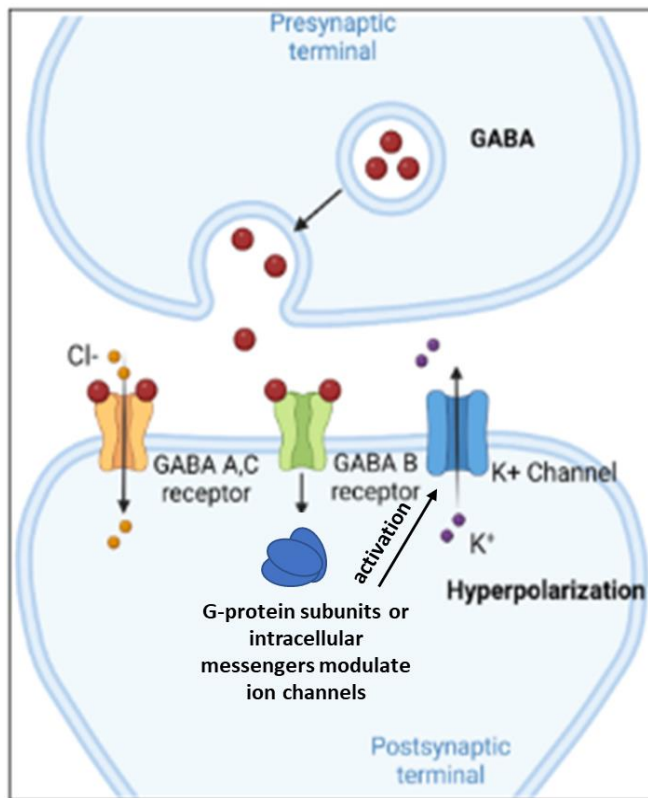


Figure 8. The role of GABA receptors in neuronal hyperpolarization. Adapted from (Kim & Yoon, 2023).

While GABA exerts a notable modulatory effect on GI motility across the entire gastrointestinal tract, its significance becomes particularly pronounced in the concluding segment, specifically the colon²⁰⁹. To illustrate, numerous studies have illustrated the antinociceptive effects of baclofen, which serves as the prototypical GABA_B receptor agonist, both *in vitro* through its impact on colonic afferent nerve firing and *in vivo* through its influence on visceromotor responses^{124,210–212}. C12AsnGABAOH, a lipopeptide, has been shown to inhibit VH in mouse models through the activation of GABA_B receptors²¹³. Additionally, studies have demonstrated that augmenting the efficacy of endogenous GABA by positively modulating the

GABA receptor ²¹⁴ effectively diminishes the visceromotor response to colorectal distension in healthy control rats ²¹¹. More recently, peripheral GABA_A receptor signaling contributes to visceral hypersensitivity in a mouse model of colitis ²¹⁵. Therefore, the widespread impact of GABA in the enteric system suggests that targeting the GABAergic system may present a novel approach in developing drugs aimed at reinstating normal GI motility in various motor GI disorders ²⁰⁹.

Table 4. Characteristics of GABA receptors. Adapted from ²⁰³.

Characteristics	GABA _A and GABA _C	GABA _B
Type of receptor	Ligand-gated ion channel that binds with GABA (ionotropic receptor)	G protein-coupled receptor (metabotropic receptor)
Function	Postsynaptic inhibition by Cl ⁻ influx	Pre-synaptic inhibition by reducing Ca ²⁺ entry and post-synaptic inhibition by enhancing K ⁺ permeability
Molecular weight	Approximately 300 kDa	Approximately 80 kDa
Distribution	Mainly postsynaptic membrane of CNS	Mainly pre and postsynaptic membranes of central and autonomic division of the PNS
Structure	Pentamer	Dimer
Speed of synaptic response	Milliseconds	Seconds or minutes

Moreover, in the rat mucosal epithelium, cells expressing GABA_B receptors were observed throughout the GIT, ranging from the gastric body to the colon. Their prevalence decreased in the direction from the mouth to the anus. These GABA_B receptor-positive cells

resembled enteroendocrine cells in morphology²¹⁶. Both gastric and intestinal regions exhibited GABA_B immunoreactivity in the mucosa. However, GABA_B-positive cells in the gastric region tended to contain somatostatin, whereas those in the duodenal region showed positive staining for serotonin, as reported by Nakajima et al. in 1996²¹⁶. Consequently, the functional effects of GABA_B receptors are likely to vary along the GI tract and depend on their coexistence with key enteroendocrine cell mediators such as somatostatin and serotonin²¹⁶.

7.2. Microbiota, ingested bacteria and GABA

The gut microbiota plays a crucial role in maintaining human health and has significant involvement in the activation of both the immune system and the CNS. Notably, there exists a strong gut-brain connection, which has become a prominent area of research, particularly in neuroscience. Studies in this field have demonstrated that the development of the brain system is reliant on the gut microbiota²¹⁷. The gut microorganisms have the capability to produce and release neuroactive substances like γ -aminobutyric acid (GABA), which influences the gut-brain axis, a bidirectional communication pathway between the GIT and the CNS. Research has revealed that the gut microbiota can impact mood, behavior, and cognitive function in humans¹⁸⁵.

To enhance GABA production, fermentation has been explored as a viable approach using microorganisms, particularly lactic acid bacteria (LAB). Compared to GABA obtained from plant sources, the fermentation process led by microorganisms like LAB has proven to be more efficient and convenient for food processing and production²¹⁸. Various microorganisms with a glutamate decarboxylase system, including LAB, yeast, and fungi, have been extensively studied for GABA production^{219–221}. Among these, LAB hold particular significance in the food industry due to their "generally recognized as safe" (GRAS) status and their vital role in fermentation, especially in dairy product production, owing to their functional and probiotic

properties²²². Given the positive health effects associated with GABA, its production by LAB can be considered an important probiotic property with potential benefits for human well-being.

LAB strains have the ability to influence the gut microbiota and the functioning of the gastrointestinal tract by modulating the production of short-chain fatty acids, including butyrate, and neuroactive metabolites like GABA. Additionally, they promote the development of a diverse and intricate bacterial community, leading to increased GABA production through communication with resident gut bacteria²²³. Of particular interest is the observation that *Bacteroides fragilis* KL1758, which resides in the human gut, plays a vital role in producing GABA. This GABA production is crucial for supporting the growth of the commensal KLE1738, an uncultured bacterium listed among the "most wanted" by the National Institutes of Health. Remarkably, KLE1738 relies solely on GABA as its carbon source, underscoring the significance of GABA in facilitating the growth of specific gut bacteria and maintaining the gut ecosystem²²⁴.

Due to these significant physiological effects, there has been a growing interest in producing GABA-enriched functional foods. Consequently, scientists have been investigating the bacterial glutamate decarboxylase system (GAD) (responsible for GABA production), further developed in the next chapter of this manuscript.

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CHAPTER 2**BIOCHEMICAL INSIGHTS ON GLUTAMATE****DECARBOXYLASE (GAD) AND GABA-PRODUCING BACTERIA****1. Acid resistance (AR) in ingested bacteria**

Acid stress poses a significant challenge to bacterial cells. Slight changes in intracellular pH can have severe consequences on cell growth, which is why most bacteria have the ability to maintain a relatively constant internal pH when cultured in diverse media at different external pH levels ^{1,2}. For instance, in *Escherichia coli* (*E. coli*), the intracellular pH changes only slightly from 7.2 to 7.8 over an extracellular pH range of 5.5 to 9 ³. As a result, intracellular pH is consistently maintained at a higher level compared to acidic extracellular pH and lower compared to alkaline pH. Fluctuations of intracellular pH are deemed undesirable, as evidenced by the fact that bacteria have limitations in their ability to grow within certain ranges of acidic pH values and display transcriptional and translational responses to a pH drop. Indeed, most biological molecules function optimally only within a narrow pH range ⁴. These considerations are particularly significant for neutrophilic bacteria acquired through oral routes. In order to colonize the mammalian host's gastrointestinal tract (GIT), these bacteria must withstand exposure to the highly acidic secretions (pH < 2.5) of the stomach, which act as a major bactericidal barrier in the GIT ⁵. Moreover, additional acid stress occurs in the distal gut, where resident microbiota produces short-chain fatty acids (SCFAs) like acetic, propionic, butyric, and lactic acid through anaerobic fermentation ⁶. Notably, SCFAs can penetrate the cell membrane in their undissociated form and subsequently dissociate within the cytoplasm, leading to acidification of the cytoplasm. Hence, the capacity to perceive and respond to acid stress is crucial for the successful colonization or transition through the GIT and survival in other acidic environments, such as fermented foods.

In bacteria, the response to acid stress might involve several components: (i) passive mechanisms, which rely on the buffering capacity of amino acids, proteins, polyamines, polyphosphate, and inorganic phosphate present in the cytoplasm; (ii) physiological adaptations, including membrane modifications, such as adjusting the content of fatty acids and outer membrane porins to reduce proton influx and also activity of periplasmic chaperones triggered by acid pH; (iii) metabolic responses, particularly during aerobic growth under mild acid stress, that facilitate proton efflux through components of the electron transport chain; (iv) proton-consuming and ammonia-producing reactions, which depend on the activation of acid-inducible amino acid-dependent decarboxylases and deaminases^{7,8}. In the latter case, five acid resistance (AR) systems have been characterized (namely AR1, AR2, AR3, AR4 and AR5) and are here further described as well as in **Figure 1**, where AR1 is distinguished as the sole system that doesn't necessitate an amino acid and is governed by an oxidative (induced by absence of glucose) mechanism overseen by the alternate σ -factor (RpoS) and the cAMP receptor protein^{9,10}. RpoS plays an important role regulating gene expression as an adaptive response to a variety of stressful conditions, including acid shock. For instance, RpoS was found to impact on the expression of genes related to the lysine decarboxylase and glutamate decarboxylase pathways in *E. coli*¹¹. AR2, AR3, AR4 and AR5 are based on two components: a cytoplasmic pyridoxal 5'-phosphate (PLP)-dependent decarboxylase that catalyzes the relevant amino acid's reaction, and an inner membrane antiporter that exchanges the incoming substrate for the exported product of the reaction^{8,12}.

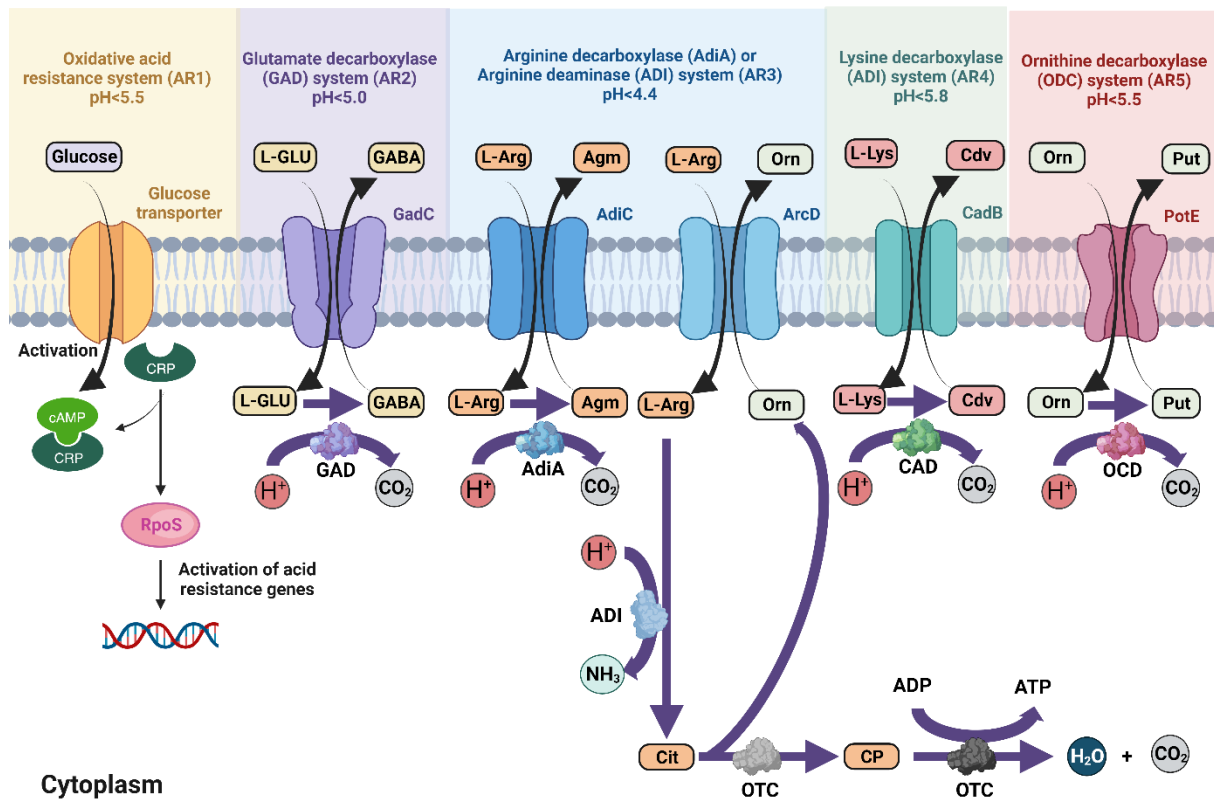


Figure 1. Representation of bacterial acid resistance systems (AR1-AR5). ADI, arginine deaminase; AdiA, arginine decarboxylase; AdiC, arginine decarboxylase; Agm, agmatine; ArcD, Arginine/ornithine antiporter; CAD, lysine decarboxylase; cAMP, cyclic adenosine monophosphate; Cdv, cadaverine; Cit, citrulline; CK, carbamate kinase; CP, carbamyl phosphate, CRP catabolite repressor protein; GABA, gamma-aminobutyric acid; GAD, glutamate decarboxylase; GadC, L-glu/GABA antiporter; L-Arg, L-arginine; L-Glu, L-glutamic acid; PotE, Ornithine/putrescine antiporter; Put, putrescine; ODC, putrescine decarboxylase; Orn, ornithine; OTC, ornithine carbamoyltransferase; RpoS, regulator of proteolysis of the stationary-phase sigma factor. Adapted from ^{4,12-17}.

The major AR systems encompass the glutamate decarboxylase (GAD) system, the arginine decarboxylase (AdiA) and deaminase (ADI) systems, and the lysine decarboxylase (CAD) system (referred to as AR2, AR3, and AR4 respectively) ^{7,9,18} while the ornithine decarboxylase (ODC) system (AR5) holds limited significance in *E. coli* MG1655, it assumes a more crucial function in avian pathogenic *E. coli* ¹⁹. The fundamental constituents within these major AR systems consist of an amino acid decarboxylases or deaminases that consume protons and a corresponding antiporter that expels the decarboxylated and/or deaminated

compounds (which are more alkaline) while simultaneously importing the respective extracellular amino acid. This orchestrated process leads to an elevation in both intracellular and extracellular pH values ^{7,15,20}. The functionality of each system is dependent on distinct external pH conditions and stages of growth, with their activation being governed by a complex interplay involving regulators embedded within the membrane as well as soluble regulators.

The GAD system becomes active when bacterial cultures undergo a transition to the stationary phase and during periods of exponential growth in media with lowered pH ^{9,21–23}. The *E. coli* GAD system utilizes GadA and GadB, two decarboxylases reliant on pyridoxalphosphate, which catalyze the proton-utilizing decarboxylation process of L-glutamate, resulting in the generation of γ -aminobutyric acid (GABA). Accompanying this, the system includes GadC, the corresponding antiporter responsible for importing L-glutamate and exporting GABA ^{22,24} (**Figure 1**).

The CAD system becomes operational when *E. coli* encounters a pH of 5.8 along with lysine exposure. This system relies on external lysine as a substrate, which, under the influence of the lysine decarboxylase (CAD), transforms into cadaverine while consuming a proton. The lysine/cadaverine antiporter CadB facilitates the importation of lysine while simultaneously expelling the more alkaline cadaverine, thereby causing an increase in the extracellular pH ²⁵ (**Figure 1**).

The AdiA system transforms arginine into agmatine, involving the utilization of a single proton. The pivotal constituents of this system encompass the arginine decarboxylase ADI and the arginine/agmatine antiporter AdiC. Optimal induction of the AdiA system occurs when conditions involve an acidic pH (pH 4.4), anaerobic conditions, and a nutrient-rich medium ²⁶. The operation of AdiC is subject to regulation in response to acidic pH, and its functionality remains fully effective at a pH equal to or less than 6.0 ²⁷ (**Figure 1**). In parallel, another arginine-based system exists in bacteria (especially lactic acid bacteria) functioning in similar

pH as the AdiA the arginine deaminase (ADI) system¹³. To begin with, L-arginine is transported into the cell via the ArcD transporter, where it undergoes conversion into citrulline and ammonia by ADI. Following this, ornithine carbamoyltransferase facilitates the phosphorolysis of citrulline into ornithine and carbamoyl phosphate. Ornithine is subsequently transported out of the cell, while carbamoyl phosphate is further transformed into carbon dioxide and ammonia through the action of carbamate kinase. This enzymatic cascade not only neutralizes protons through the generation of ammonia and carbon dioxide but also yields ATP from ADP¹³ (**Figure 1**).

The ODC system facilitates the liberation of the carboxyl group from the amino acid ornithine via the ornithine decarboxylase. Consequently, putrescine, a primary diamine with alkalizing properties, is generated, influencing the environmental pH. Both ornithine and putrescine can enter or exit bacterial cytosol through the transporter PotE. The activation of ornithine decarboxylase occurs in response to anaerobic conditions and acidic pH lower than 5.5¹⁹ (**Figure 1**).

The ADI and specially the GAD acid resistance systems provide the most robust protection against extreme acid stress while the lysine- and ornithine-dependent systems offer less extensive protection against extreme acid stress due to the higher optimal pH of their decarboxylases (between 5.5-6.0)^{22,28-30}. As a result, they are unable to provide sufficient protection when the intracellular pH drops to <5, which can occur when the external pH is lower than 2.5⁷.

2. Glutamate decarboxylase: a key enzyme for bacterial pH regulation and GABA production

Glutamate decarboxylase (GAD; EC 4.1.1.15) is an enzyme found in both eukaryotes and prokaryotes, each of which assigns distinct physiological functions to it³¹. Its primary function involves catalyzing the irreversible α -decarboxylation of L-Glu into GABA with

pyridoxal-5'-phosphate (PLP) as coenzyme (**Figure 2**). It plays an important role as an acid resistance mechanism preserving the viability of microorganisms^{32,33}. Undoubtedly, the GAD system serves as the primary acid resistance system in various food-borne pathogens and ingested bacteria, including *E. coli*, *Shigella flexneri*, *Listeria monocytogenes*, and *Lactococcus lactis*^{29,30,34–36}.

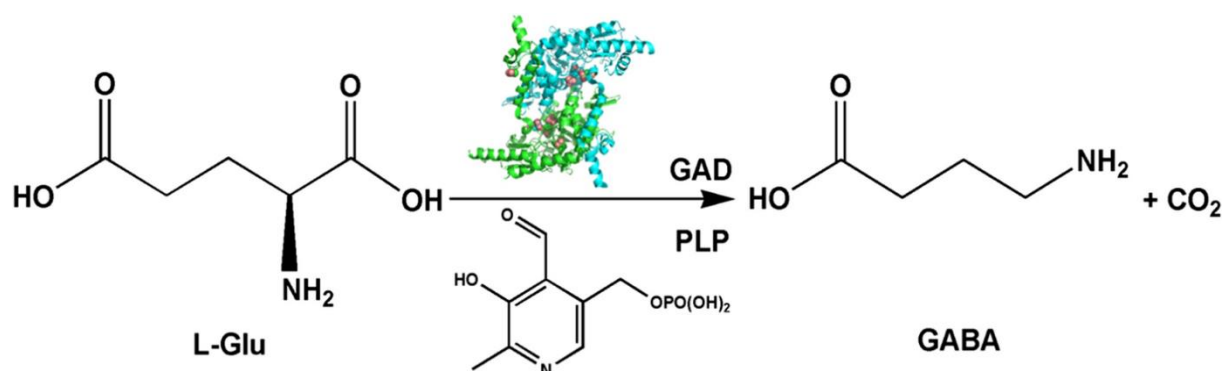


Figure 2. The synthesis of GABA through the action of glutamate decarboxylase (GAD) with pyridoxal-5'-phosphate (PLP) as a coenzyme. Adapted from³⁷.

GAD also holds significant relevance in mammals within the central nervous system (CNS), particularly due to GABA's role as a prominent inhibitory neurotransmitter³⁸. GAD is notably distinct among enzymes engaged in neurotransmitter synthesis due to the remarkable feature that both its substrate and resultant product function as neurotransmitters with contrasting effects³¹. In the context of the neuronal system, L-Glu operates as an excitatory neurotransmitter, whereas GABA fulfills an inhibitory function. These neurotransmitters are integral components of glutaminergic and GABAergic synapses, respectively³⁹. As depicted in **chapter 1**, GABAergic receptors are interesting targets in disorders of gut-brain interaction, including irritable bowel syndrome (IBS). Therefore, biosynthesis of GABA has been gaining growing attention.

Studies encompass documented structural investigations conducted on GADs from

bacterial, plant, insect, and mammalian sources, delving into the distinctions within the GAD structures and their correlation with chemical reactions and physiological roles. Notably, *Escherichia coli* GAD offers intriguing insights among bacterial GADs. The cloned *E. coli* GAD has unveiled the existence of two biochemically identical isoforms of GAD forms of GAD enzymes: GadA, encoded by the gene *gadA* and GAD (or GadB) encoded by *gadB*. Those isoforms have been extensively described in the literature for several bacterial species^{38,40,41}, and limited sequence identity with their mammalian counterparts has been observed³¹. Despite the congruence in the decarboxylation reaction between mammalian bacterial GADs, substantial variations exist in terms of subunit composition, molecular mass and optimal pH for the activity of the enzyme³¹. Low sequence identity (12%) between the vertebrate (human GAD65) and bacterial (*E. coli* GAD) proteins was demonstrated but plant (*Arabidopsis thaliana* Gad1) and bacterial enzymes (*E. coli* GAD) exhibit higher similarity in terms of sequence identity (39%) and oligomeric assembly⁴².

2.1. Benefits of GABA-producing bacteria

Several studies have explored diverse chemical methods for the synthetic production of GABA. One intricate approach involves a five-step process encompassing nitrile reduction, ester hydrolysis, and deethoxycarbonylation. This method synthesizes GABA from a functionally modified intermediate, which is derived by alkylating diethyl cyanomalonate with ethyl bromoacetate⁴³. In an alternative strategy, GABA synthesis is achieved through the carboamination reaction of alkenes, catalyzed by copper complexes^{44,45} have reported a method for GABA production using glutamic acid, sourced from waste gluten. This process incorporates isophorone as an inducer reagent and employs microwave-assisted decarboxylation, resulting in GABA with a short reaction time and a yield of 63%⁴⁶. However, it's worth noting that these chemical synthesis routes are accompanied by challenges such as

the generation of unwanted side-products, the use of hazardous reagents, and the complexity of multi-step chemical reactions, which has led to a preference for microbial biosynthetic routes⁴⁷.

A wide variety of fruits, edible plants, roots, and botanicals are rich in neurotransmitters, including GABA, which can be synthesized through different metabolic processes, ecological interactions, or microbial food fermentation⁴⁸. However, L-Glu is more abundant in dietary sources compared to GABA, making the conversion of glutamate to GABA a cost-effective approach⁴⁸. Additionally, the presence of dietary GABA in edible plants varies based on factors such as plant parts, morphological and chemotype differences, environmental adaptation, geographical location, seasonal growth and harvest, weather conditions, contaminants, agricultural practices, and processing methods⁴⁹.

Furthermore, GABA has been identified as a growth co-factor for beneficial gut microbes, particularly the *Bacteroidetes* population, which can be promoted by the presence of competitive GABA-producing strains that adhere to epithelial cells and continuously release GABA in the intestinal environment⁵⁰. Numerous studies have highlighted the potential benefits of consuming GABA-rich foods fermented using probiotics on mental health disorders such as social anxiety, cognition, and pain sensitivity, largely attributed to their interactions with the gut microbiota^{51,52}.

2.2. Prokaryotic GADs

E. coli glutamate decarboxylase GAD is undoubtedly the most extensively studied bacterial GAD. The purified enzyme is composed of six identical subunits, each weighing 53 kDa^{53,54}. Within each subunit, one pyridoxal 5-phosphate (PLP) is present at the active site. The *E. coli* enzyme is prone to dissociation at pH values of 6.0 and above⁵³⁻⁵⁵ and its optimal activity is sustained within the pH range of 4.0 to 4.5^{56,57}. While L-Glu stands as the most

suitable substrate for the *E. coli* GAD enzyme, it also possesses decarboxylation capabilities for other amino acids, such as γ -methylene-L-glutamate, threo- β -hydroxy-L-glutamate, and L-homocysteine sulfinic acid. Interestingly, GAD appears to maintain specific size and optical activity preferences, evident in its inability to utilize D-glutamate as substrate^{56,58,59}.

GadC, on the other hand, is a notable constituent of the APC (amino acid/polyamine/organocation) superfamily of membrane transporters⁶⁰. The proposed model for L-Glu and GABA through GadC in *E. coli* is described in **Figure 3**. When the extracellular proton concentration sharply rises (during its passage through the host stomach notably), protons (and likely undissociated HCl) passively enter the inner membrane, causing a drop in intracellular pH. This drop triggers the import of L-Glu with no net charge (Glu^0) via GadC^{15,61,62} (**Figure 3**). Once inside the cell, Glu^0 remains protonated on the γ -carboxylate, facilitating its binding to GAD for decarboxylation. The α -carboxyl group departs as CO_2 , and a cytoplasmic proton replaces it, resulting in the formation of positively charged GABA (GABA^{+1}). GABA^{+1} is then exported via the functionally associated antiporter GadC in exchange for a new Glu^0 molecule. This export of GABA^{+1} leads to a net export of positive charges (0.9 H^+ /exchange cycle), helping to mitigate intracellular acidification⁶³. Investigations in *E. coli* cells have demonstrated that the GAD system not only plays a role in maintaining pH homeostasis but also employs a unique strategy to counteract the unwanted influx of protons. By transiently accumulating GABA within the cell, it effectively reverses the membrane potential, employing a similar strategy observed in extreme acidophiles¹².

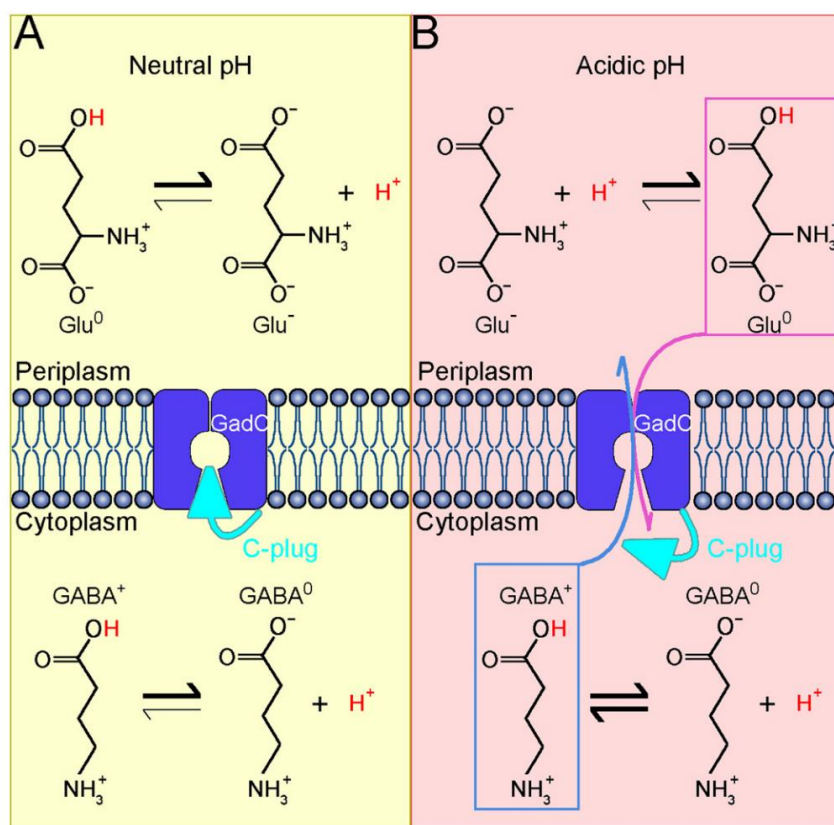


Figure 3. Proposed mechanism of pH-dependent substrate transport by GadC. (A) GadC is inactive at neutral pH. Most of the substrate molecules (L-glutamic acid (Glu) and GABA) are deprotonated, thus are unfavorable for transport. In addition, the substrate transport path is blocked by the C-plug of GadC. Under these conditions, no substrate exchange occurs. B, GadC is activated at acidic pH. When the extracellular pH is extremely acidic (pH 2 ~ 3), the cytoplasmic pH in *E. coli* drops to a value between 3.5 and 5.0. In the cytoplasm, GABA exists in two forms, with no net charge (GABA⁰) and with one positive charge (GABA⁺), whereas the majority of Glu in the extracellular space carries no net charge (Glu⁰). At acidic pH, the C-plug of GadC may be displaced, allowing influx of Glu⁰ and efflux of GABA⁺. Adapted from ⁶².

There have been only a few reports focusing on the biochemical properties of other bacterial GADs, as shown in **Table 1**. For instance, GAD from *Clostridium perfringens*, a significant cause of food poisoning in developed countries, has been purified to homogeneity and partially characterized ⁶⁴. Its spectroscopic and kinetic properties, as well as its oligomeric assembly, resemble those of the *E. coli* enzyme. In the last two decades, the growing interest in high GABA-producing strains of lactic acid bacteria (LAB) for biotechnological applications has led to the purification and functional analysis of GADs from *Lactococcus lactis* ³²,

Lactobacillus paracasei ⁶⁵, *Lactobacillus brevis* ^{65–67} and *Latilactobacillus curvatus* ⁶⁸. Additionally, highly heat-stable GADs from the hyperthermophilic archaea *Pyrococcus horikoshii* ⁶⁹ and *Pyrococcus furiosus* ⁷⁰ have been purified as recombinant proteins and characterized for potential industrial applications. Compared to the *E. coli* enzyme, the GADs from *Pyrococcus* species exhibit different biochemical features, including an unusual monomeric assembly, a pH optimum in the neutral-alkaline range, and broader substrate specificity (**Table 1**)

Table 1. Prokaryotic GADs. Adapted from Grassini (2013). MW, molecular weight.

Species	Subunit MW	No. of subunits	pH optimum	Reference
<i>Escherichia coli</i>	50	6	3.8-4.6	71–74
<i>Clostridium perfringens</i>	48-50	6	4.7	64
<i>Listeria monocytogenes</i>	54	-	-	75
<i>Lactobacillus brevis</i> <i>IFO12005</i>	54	2-4	4.2-4.6	65,76
<i>Lactococcus lactis</i>	54	-	4.7	32
<i>Lactobacillus paracasei</i>	57	2	5	67
<i>Latilactobacillus curvatus</i>	-	-	5	68
<i>Streptomyces toxytricini</i> <i>NRRL15443</i>			5.2	77
<i>S. toxytricini</i> <i>MJ654-NF4</i>	-	-	3.8	
<i>S. chromofuscus</i>			4.2	
<i>Pyrococcus horikoshii</i>	42-45	1	8	69
<i>Pyrococcus furiosus</i>	41	1	6	69

2.2.1. Conservation of GAD within bacteria

A ClustalW alignment, which included 20 bacterial GADs predominantly from Gram-positive and Gram-negative enteric bacteria, revealed that 84 residues (18% of the *E. coli* GadB sequence) are strictly conserved ²⁴. Many of these residues occupy crucial positions in *E. coli* GAD, either within or near the active site or at locations where conformational changes occur.

In addition to the 8 residues shared with group II decarboxylases, which are primarily involved in anchoring PLP and serve structural roles, the presence of the residues listed in bold in **Table 2** can be considered the characteristic signature for bacterial GAD. These residues fulfill different roles, including offering sites for substrate binding or decarboxylation, PLP interaction decarboxylation, pH-shift detection and structural roles ²⁴.

.2. GABA-producing lactic acid bacteria (LAB)

GABA is commonly synthesized through fermentation utilizing various microorganisms such as yeast, and bacteria ⁷⁸. Among these, lactic acid bacteria (LAB) have gathered substantial attention as GABA producers. LAB gained more prominence following the dissemination of Metchnikoff's "Theory of Longevity" in 1910. Metchnikoff related the high daily consumption of yogurt to the extended lifespan of Bulgarian people who lived beyond 100 years. Thus, the longevity was attributed to the fermenting bacteria present in yogurt ⁷⁹. LAB's high fermentation capacity allows for the production of end products that not only contribute to the sensory properties and preservation of food but also play a significant role in creating an antimicrobial environment within the food matrix ⁸⁰.

Phylogenetically, LAB belong to the group of Gram-positive bacteria that are non-spore forming, facultative anaerobes, tolerant to acidic conditions, and lack catalase enzyme activity. Morphologically, they can be categorized into two main shapes, rods and cocci. Additionally, their major metabolic fermentation end-products can be classified into two types: a mixture of lactic acid, carbon dioxide, and acetic acid/ethanol (heterofermentative), or exclusively lactic acid (homofermentative) (**Figure 4**) ⁸¹⁻⁸³. Due to their significant role in fermentation and their "Generally Regarded as Safe" (GRAS) status, LAB have gained immense importance in various industries, including food/feed, medical, pharmaceutical, and chemical industries. They are widespread and predominantly thrive in environments abundant in carbohydrates. They are widely used as functional ingredients in the production of enzymes, low-calorie sweeteners,

and starter cultures for fermentation. Additionally, they form an integral part of the natural microbiota found in both human and animal organisms ⁸¹. Moreover, they might serve as valuable antimicrobial agents, produce exopolysaccharides, and act as carriers for vaccine delivery ^{38,40}.

Table 2. Amino acid residues composing the bacterial GAD signature. Adapted from ²⁴.

Residue	Role
^a Thr62 (side chain OH)	Binding of substrate g-carboxylate
^a Phe63 (amide N)	Binding of substrate g-carboxylate
^a Phe63 (side chain)	Prevents Arg422 binding to substrate a-carboxylate
^a Asp86* (b-carboxylate)	Binding of substrate g-carboxylate
^a Glu89* (side chain)	Significant change in orientation upon pH-shift
^a Gly120b	Structural role
^{a/b} Ser126 (amide N)	Hydrogen bond with PLP phosphate oxygen
^{a/b} Ser127 (side chain OH)	Hydrogen bond with PLP phosphate oxygen
^a Gln163 (alkyl chain)	PLP stacking
^b Cys168	PLP stacking
^a Gly210	Probable structural role
^b Ile211	PLP stacking
^a Thr212 (side chain OH)	Interacting with PLP OH (intramolecular proton transfer?)
^a His241 (side chain p N)	Hydrogen bond with Ser269
^a Asp243 (b-carboxylate)	Interacting with the pyridine N of PLP
^a Ala245 (side chain)	Hydrophobic contact with the pyridine ring of PLP
^a Ser269 (side chain OH)	Hydrogen bond with His241
^a His275 (side chain t N)	Hydrogen bond with PLP phosphate oxygen
^a Lys276 or ^b Ser276 (e-amino group)	Forming the Schiff-base with the C4' of PLP
^b His278	PLP stacking
^a Tyr305* (side chain OH)	Interacts with residues 461–463 of the C-terminus (reprotonation?)
^a Leu306*	Interacts with residues 461–463 of the C-terminus
^a Gly307*	In the b-turn of b-hairpin 300–313
^b Tyr308*	Critical role in decarboxylation of L-Glu
^a His465 (side chain t N)	Covalent bond with PLP-Lys276 Schiff base

a. Numbering refers to Gram-negative *E. coli* GAD ⁷³.

b. Numbering refers to Gram-positive *Lb. brevis* GAD ⁶⁶.

Residues in plain text are the residues strictly conserved in the decarboxylases of the PFAM PF00281

* residues contributed by the neighboring subunit in the functional dimer.

Apart from benefiting the food industry, LAB are also known for their ability to enhance gut health. The lactate, acetic acid, hydrogen peroxide, carbon dioxide, and bacteriocins produced by LAB ensure a balance between the growth of pathogenic and beneficial bacteria in the gut, contributing to the microbiota's homeostasis⁸⁴. Another advantageous characteristic for the gastrointestinal tract is that LAB are acidophilic, with an optimal growth pH range between 3.5 and 6.5. They can enhance their resistance to the stomach's acidic pH by producing alkaline metabolites and through acid resistance systems, specially the GAD system³⁸.

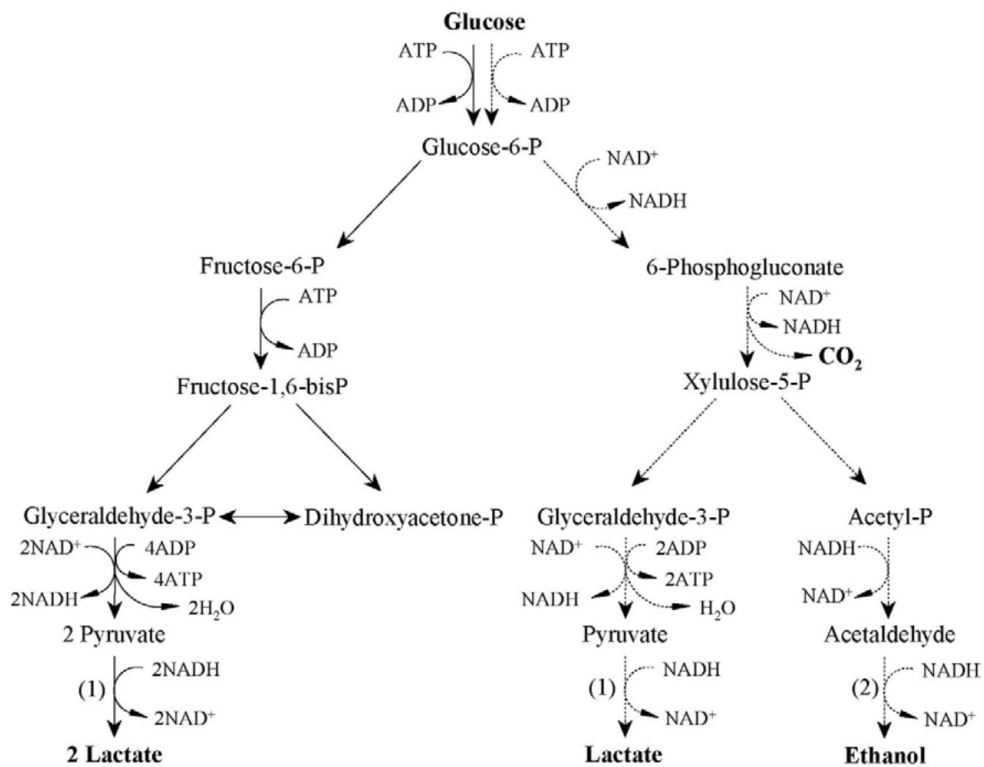


Figure 4. Metabolic pathways of homofermentative (solid line) and heterofermentative (dotted line) lactic acid bacteria (LAB). P, phosphate; ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; NAD⁺, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide (reduced form); (1), lactate dehydrogenase; (2), alcohol dehydrogenase. Adapted from⁸³.

Several factors have been recognized to influence the growth, fermentation, and GABA synthesis of bacteria, creating the ideal conditions for GABA-producing bacteria⁸⁵.

The primary and critical parameters include temperature, fermentation time, co-factors like enzymes, and the composition of the culture medium with various additives⁷⁸. The production of GABA is especially influenced by pH regulation during the manufacturing process⁸⁶. For example, the growth of LAB leads to acidification due to the production of organic acids. As the pH decreases, the enzyme GAD is activated, converting L-Glu to GABA as a mechanism to resist acidity. Adjusting the initial pH appropriately ensures an optimal effective pH for achieving the maximum GABA yield⁷⁸. Additionally, incubation temperature plays a crucial role in GABA biosynthesis during fermentation⁸⁷. The highest GABA synthesis in LAB strains is directly related to cell density and GAD activation, which is pH and temperature-dependent⁸⁸. Most LAB strains exhibit optimal GABA production at 37°C, where L-Glu is completely converted into GABA⁷⁸. Some studies have reported an optimal GABA metabolism within a fermenting temperature range of 35-40°C⁸⁹.

The duration of cultivation plays a crucial role in the fermentation process to achieve the highest level of GABA production. For instance, in one study, the peak GABA amount was reached after 30-35 hours of fermentation at pH 5.7 and 37°C⁸⁹. Similarly, adjusting time intervals along with the presence of L-Glu and PLP coenzyme has been shown to significantly enhance GABA production during fermentation⁷⁸. Moreover, the nutritional composition of the medium and the culture conditions during fermentation have a direct impact on the amount of GABA produced by bacteria⁷⁸. Nitrogen and carbon sources, such as L-Glu and ammonium sulfate, as well as PLP, are utilized as nutritive elements, substrates, and coenzyme supplements in the culture medium to enhance GAD activity and increase GABA yields in strains during fermentation⁶⁷.

The molecular mechanisms of GABA degradation have been elucidated in certain bacteria, such as *Escherichia coli*⁹⁰ and *Listeria monocytogenes*⁹¹. In these organisms, GABA is first degraded to succinic semialdehyde through the action of the major GABA-degradative

enzyme, GABA aminotransferase (GABA-AT, EC 2.6.1.19). Subsequently, succinic semialdehyde is further converted to succinic acid by succinate semialdehyde dehydrogenase (SSADH, EC 1.2.1.16), facilitating its entry into the tricarboxylic acid (TCA) cycle. In LAB, however, it is likely that GABA-AT transfers the GABA amino group to pyruvate, thus forming alanine⁹². Indeed, the gene encoding GABA-AT, *gadT*, has been identified in various species of LAB, including *Lactobacillus fermentum*, *Lactobacillus frumenti*, *Lactobacillus gastricus*, *Lactobacillus gorilla*, *Lactobacillus mucosae*, *Lactobacillus oris*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus reuteri*, *Lactobacillus similis*, *Lactobacillus vaginalis*, *Leuconostoc citreum*, *Leuconostoc mesenteroides*, and *Oenococcus oeni*, among others⁹³⁻⁹⁶. Recent findings indicate that the fermentation process of *Lb. plantarum* with L-Glu significantly enhances GABA production⁹⁷. Interestingly, during this fermentation, the expression of the *gadT* gene is down-regulated by approximately 2-fold rate, suggesting that the activity of GABA-AT is inhibited. These results demonstrate a direct relationship between GABA-AT activity and GABA production.

Therefore, achieving optimal microbial GABA production, considering species, fermentation conditions and GABA metabolism, assumes paramount significance in the development of commercial GABA products^{89,98,99}.

3. *Lactococcus lactis*: Promising health properties of GABA-producing strains

3.1. *L. lactis* relevance in industry and research

One group of LAB that has gained significant importance is *Lactococcus lactis* (*L. lactis*). It has a cocci shape and is homofermentative, producing 100% lactic acid as the fermentation product and is one of the most important bacterium among the LAB used commercially¹⁰⁰.

Despite the significant sequence similarity within *Streptococcaceae*, these organisms manage to inhabit a wide array of diverse environmental niches. Members of the *Lactococcus*

genus can be found in various locations including raw milk, raw-milk cheeses, and non-milk environments. In fact, strains of *L. lactis* have been isolated from sources as diverse as drain water and human vaginal samples^{101,102}. Although not a common resident of the GIT, *L. lactis* has demonstrated the ability to withstand gut passage, as indicated by studies^{103,104}. Although *L. lactis* exhibits the ability to inhabit a variety of environments naturally, it is most notably recognized for its close association with the milk environment and its role in dairy product production. Indeed, the significance of *L. lactis* for humanity becomes first evident when considering that approximately 107 tons of cheese were manufactured annually in the late 80's¹⁰⁵, resulting in the consumption of nearly 10¹⁸ lactococcal cells per year, largely due to its extensive use in industrial fermentation^{100,106}.

Based on multi-locus sequence typing and the creation of clonal complexes, Passerini et al. (2010) introduced the categorization of *L. lactis* strains into 'domesticated' and 'environmental' groups based on their origins¹⁰⁷. Accordingly, environmental strains consist of isolates derived from plants and animals¹⁰⁷. On the other hand, domesticated strains encompass those utilized as dairy starters for milk production and found in fermented products, thriving in a distinct environment defined by technological requirements, such as rapid growth, efficient lactic acid production in milk and aiding in the degradation of milk proteins during fermentation¹⁰⁸. This substantial contribution greatly impacts the final product's organoleptic characteristics and microbial quality¹⁰⁹. A select few strains of *L. lactis* are regularly employed in the production of fermented foods, aiming to achieve more consistent outcomes. These chosen strains are primarily valued for their acidification capabilities and their resilience against bacteriophage infections¹¹⁰. Nevertheless, the growing consumer preference for products with a wider range of flavors is pushing manufacturers of fermented dairy items to diversify and expand their culture systems¹⁰⁰.

The prevailing viewpoint suggests that the current industrial dairy strains employed in

production are thought to have originated from plant sources¹¹¹ and have subsequently evolved in a milk-based environment. It is conceivable that this species initially established a presence in milk through contact with grass or other plant materials, which might have been utilized as feed or bedding for cattle^{112,113}.

In 1985, the group known as "dairy streptococci" underwent reclassification, resulting in the division of the group into two *L. lactis* subspecies: *Lactococcus lactis* subsp. *lactis* (formerly known as *Streptococcus lactis*) and *L. lactis* subsp. *cremoris* (formerly known as *Streptococcus cremoris*). This distinction was made to differentiate them from the streptococci *sensu stricto* category, which includes several well-known human pathogens^{114,115}. *Lactococcus* species can be found in various ecological niches, including milk, plants, and the digestive tract. Within the dairy and nutraceutical industries, two particular species have gained significant attention, *L. lactis* (formerly *L. lactis* ssp. *lactis*) and *L. cremoris* (formerly *L. lactis* ssp. *cremoris*) following a recent taxonomic reclassification *cremoris*¹¹⁶. Traditionally, distinguishing between these species relied on a limited set of phenotypic characteristics. Nevertheless, thanks to modern molecular techniques^{107,116–118}, we now have more precise methods for differentiation based on parameters like average nucleotide identity and tetranucleotide frequency correlation coefficients¹¹⁹. It's worth noting that the *cremoris* species exhibits phenotypic diversity. Some strains display the typical *cremoris* phenotype, while others resemble the *lactis* phenotype^{120,121}. The *cremoris* phenotype is characterized by the inability to produce GABA, the incapacity to hydrolyze arginine, limited growth potential at 40 °C, and in the presence of 4% (w/v) NaCl^{32,122,123}.

L. lactis was the first LAB to have its genome completely sequenced, and it is easily genetically manipulated¹²⁴. It is gram-positive and does not produce endotoxins, LPS, or any other toxic metabolic products¹²⁵. Moreover, it holds the status of being generally regarded as safe (GRAS), a designation that recognizes the substance's safety when used as intended, in

accordance with the Food, Drug, and Cosmetic Act of 1958¹²⁶. This adaptability has led to exploring the use of these strains for probiotic properties, for delivering therapeutic drugs *in vivo*^{127–129}, and for investigating targeted vaccine delivery by this organism¹³⁰. Accordingly, in the last decades, the quantity of sequenced lactococcal strains from diverse environmental contexts has substantially increased (**Table 3**) as well as the number of publications concerning lactococci (**Figure 5A and 5B**).

In addition to its significant role in the food industry, *L. lactis* has emerged as the foremost LAB model for genetic engineering purposes. Its appeal as a model organism can be attributed to factors like its compact fully sequenced genome (2.3 Mbp) and the establishment of efficient genetic engineering tools, encompassing versatile cloning and expression systems. The attributes here mentioned have positioned it as a desirable LAB model¹³¹.

Table 3. List of available *L. lactis* and *L. cremoris* genomes. CDSs, coding sequences. Adapted from ¹¹⁹

Strain	Year	Genotype	Origin	Size (mb)	CDSs	Plasmid	G/ C content (%)	Reference
IL1403	2001	<i>lactis</i>	dairy	2.37	2, 277	0	35.3	132
KF147	2010	<i>lactis</i>	mung bean sprouts	2.60	2, 578	1	34.9	133
KF282	2010	<i>lactis</i>	mustard and cress	-	-	-	-	133
CV56	2011	<i>lactis</i>	vaginal flora	2.40	2, 301	5	35.2	134
CNCM I-1631	2011	<i>lactis</i>	fermented milk	2.51	2, 579	-	34.9	135
IO-1	2012	<i>lactis</i>	drain water	2.42	2, 224	-	35.1	102
YF11	2013	<i>lactis</i>	fermented corn	2.53	2, 531	0	34.8	136
TIFN2	2013	<i>lactis</i>	dairy- cheese	2.51	2, 521	-	35.1	137
TIFN4	2013	<i>lactis</i>	dairy- cheese	2.55	2, 598	-	35.0	137
KLDS 4.0325	2013	<i>lactis</i>	fermented horse milk	2.59	2, 587	3	35.4	138
LD61	2014	<i>lactis</i>	dairy-cheese	2.60	2, 601	6	36.4	139
NCDO2118	2014	<i>lactis</i>	plant	2.55	2, 386	0	34.9	140
SK11	2006	<i>cremoris</i>	dairy-cheese	2.44	2, 381	4	35.9	95
MG1363	2007	<i>cremoris</i>	dairy	2.53	2, 434	0	35.7	106
A76	2012	<i>cremoris</i>	dairy- cheese	2.45	2, 643	4	35.9	141
KW2	2013	<i>cremoris</i>	fermented corn	2.43	2,268	0	35.7	142
TIFN1	2013	<i>cremoris</i>	dairy- cheese	2.68	2, 754	-	35.5	137
TIFN3	2013	<i>cremoris</i>	dairy- cheese	2.73	2, 891	-	35.5	137
TIFN5	2013	<i>cremoris</i>	dairy- cheese	2.54	2, 232	-	35.5	137
TIFN7	2013	<i>cremoris</i>	dairy- cheese	2.63	2, 505	-	35.6	137
UC509.9	2013	<i>cremoris</i>	dairy	2.25	2, 208	8	35.9	143
HP ^T	2014	<i>cremoris</i>	Dairy	2.27	2, 374	7	36.7	144
S0	2015	<i>lactis</i>	Raw milk	2.48	2, 508	0	35.0	145
A12	2016	<i>lactis</i>	sourdough bread	2.60	2, 624	2	34.8	146
FM03	2017	<i>lactis</i>	Dairy-cheese	2.43	2, 304	7	35.5	147
G50	2018	<i>lactis</i>	Plant	2.34	2, 309	0	35.0	148
SLPE1-3	2018	<i>lactis</i>	Mushroom	2.52	2, 524	0	34.9	149
14B4	2018	<i>lactis</i>	Plant	2.57	2, 486	1	35.0	150
3107	2019	<i>cremoris</i>	dairy	2.40	2, 380	6	36.0	151

SD96	2020	<i>lactis</i>	Dairy-chees	2.42	2, 432	10	34.4	152
G121	2020	<i>lactis</i>	bovine	2.72	2, 783	4	35.1	153
K_LL005	2021	<i>lactis</i>	insect	2.37	2, 281	0	35.0	154
N8	2021	<i>Lactis</i>	Dairy	2.42	2, 435	2	35.1	155
JNU534	2022	<i>lactis</i>	kimchi	2.44	2, 468	1	35.2	156
AH1	2022	<i>lactis</i>	Dairy	2.42	2, 524	8	35.3	157
								158
7-1	2022	<i>lactis</i>	Dairy	2.57		2	35.7	
LL16	2023	<i>lactis</i>	Raw milk	2.58	2, 567	–	35.4	159

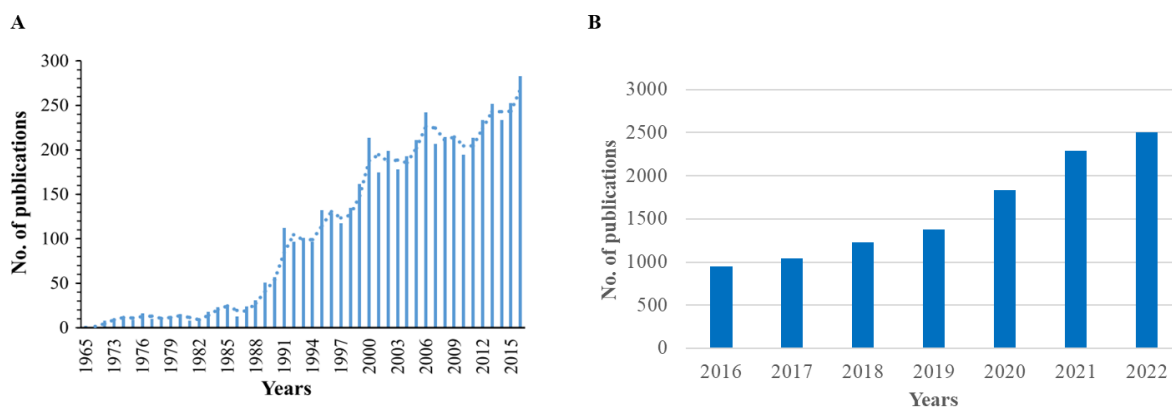


Figure 5. Graphs indicating an increasing trend of publications relating to lactococcal strains technological advancements and research. A) Adapted from ¹³¹. B) Pubmed database, accession on “*Lactococcus*” on publications search bar, September 21th 2023 (This Ph.D. study).

3.2. Health potential of *Lactococcus lactis*

In spite of the role played by *L. lactis* as a desirable LAB model, very little research has been performed on the health potential of lactococcal strains. Apart from its natural benefits such as supplying lactase in the digestive system of lactose-intolerant individuals ¹⁶⁰, *L. lactis* strains were recently shown to have powerful immunomodulative properties either in humans or rodents ¹⁶¹, exhibiting also stimulation of interleukin-18 expression in colorectal cancer cells ¹⁶². Moreover, it has been established that *L. lactis* containing interleukin can be utilized for the treatment and prevention of Inflammatory Bowel Disease (IBD) ¹⁶³. *L. lactis* exhibits remarkable effectiveness as a carrier for live antigens and enzymes ¹⁶⁴. There are documented instances of oral vaccination in mice against rodent malaria through the use of recombinant *L. lactis* ¹⁶⁵ as well as a live vaccine against brucellosis by targeting and producing the *Brucella abortus* antigen L7/L12 within the bacterium ¹⁶⁶. These qualities make *L. lactis* particularly valuable for oral administration, surpassing attenuated pathogens in this regard ¹⁶⁷. Even though most studies on the health potential of *L. lactis* concern genetically modified strains, two studies have recently reported probiotic-like effects of wild-type strain *L. lactis* NCDO2118. This strain

was reported to alleviate colitis symptoms in mice via its anti-inflammatory and immunomodulatory activities^{168,169} as well as to exert hypotensive properties in spontaneously hypertensive rats¹⁷⁰. Noteworthy, these studies considered that GABA production by NCDO2118 could be related to the *in vivo* effects detected, even though no experimental data is so far published for that matter. Overall, the studies presented here underscore the suitability of *L. lactis* as a safe candidate for biomedical applications, bolstered by its GRAS status.

3.3 GABA-producing *Lactococcus lactis*

Very limited studies have validated the GABA production of *L. lactis* strains (**Table 4**). Indeed, we were one of the first groups with our collaborators (Torino University, Italy) to give experimental data on GABA production by *L. lactis*, through the strain NCDO2118¹⁷¹. Additionally, we have shown environmental conditions affecting GABA production in *L. lactis* NCDO2118 *in vitro*, particularly demonstrating that chloride ions were the most significant factor influencing GABA yield in response to acidic stress, by not only enhancing the expression of the *gadBC* genes, but also increasing GAD synthesis^{172,173}.

Table 4. Reported GABA-producing *Lactococcus lactis* strains.

<i>L. lactis</i> strain	Source	Reference
A13	Canadian cheese	174
A23		
H02		
H12		
H13		
H15		
H20		
H24		
H24		
B	Cabbage kimchi	175
LEY 6	Raw camel milk	176
LEY 7		
LEY 8		
LEY 11		
LEY 12		
LEY 13		
LL16	Raw cow milk	177
ULAAC-A13	Canadian cheese	178
ULAAC-A23		
MK L37	Raw cow milk	179
MK L84		
NRRL B-50571	-	180
L-571	Chihuahua cheese	181
NCDO2118	Frozen peas	171

Our team has next screened through 132 lactococcal strains in microplate conditions, aiming to identify other possible GABA-producers¹²² (**Figure 6A and B**). This study encompassed various groups, including 88 *L. lactis* strains, 20 *L. cremoris* strains exhibiting the lactis phenotype, and 24 *L. cremoris* strains displaying the cremoris phenotype. A great diversity of GABA production was achieved with a highly strain-dependent GABA production in the presence and absence of NaCl (**Figure 6A and B**), in accordance with Sanders and colleagues (1998) who had formerly identified of a sodium chloride-regulated *gadCB* promoter in *L. lactis*³⁶. Two *L. lactis* strains (S642, EIP3I (also referred to as CNCM I-5388)) were identified as able to produce more GABA than the reference strain NCDO2118. Of note, this ability for S642 only occurs without NaCl, possibly because of a 1226 bp insertion sequence element (IS981) in the *gadCB* promoter region.

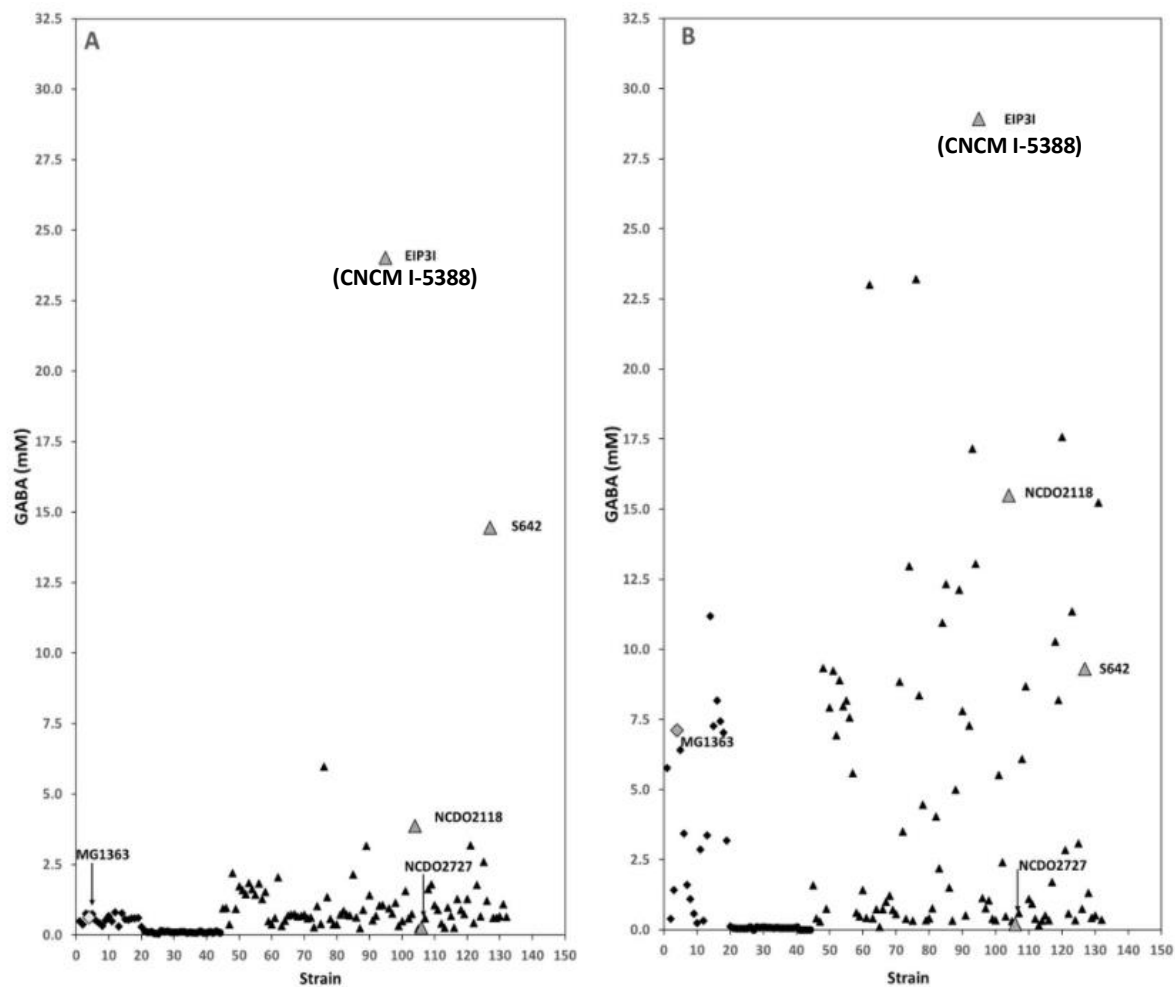


Figure 6. GABA production (mM) of 132 lactococci (▲ *L. lactis*, ◆ *L. cremoris* phenotype lactis, ● *L. cremoris* phenotype cremoris) after 24 h of growth on glucose-YE medium supplemented with 34 mM L-glutamic acid without NaCl (A) and with 0.3 M NaCl (B). Values represent the mean of duplicate experiments. The strains mentioned in the text are MG1363, a *L. cremoris* strain with a lactis phenotype ◆, and NCDO2118, NCDO2727, S642 and EIP3I (also known as CNCM I-5388), which are *L. lactis* strains ▲. Adapted from ¹²².

Table 5. GAD specific activity (mmol/min/g) in glucose-glutamate-YE supplemented medium with and without NaCl (0.3 M) for five lactococcal strains growth in a flask for 6 h. Adapted from ¹²².

NaCl	S642	EIP3I	NCDO2118	NCDO2727	MG1363
-	58.4±2.9	131.9±10.3	15.0±2.8	0.8±0.1	3.1±0.7
+	93.1±14.9	1464.3±58.2	643.7±104.4	0.5±0.0	134.9±29.5

In addition, some strains were shown not to produce GABA, notably NCDO2727, revealing the wide spectrum of GABA production levels in *L. lactis*. Finally, this work opens new avenues for GABA-producing strain candidates for human and domestic animals' health uses, for instance, in the context of visceral hypersensitivity (VH) in IBS and other disorders of the gut-brain interaction described in the first chapter of this manuscript. We then wonder whether GABA-producing *L. lactis* strains are capable of exerting anti-VH properties *in vivo* and through which mechanisms.

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THESIS OBJECTIVES

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In the context here presented in **section I**, this PhD work was dedicated to unravel for the first time the anti-visceral hypersensitivity (VH) properties of GABA-producing *Lactococcus lactis* strains by using complementary *in vitro* approaches to characterize their GAD activity and GABA-production, and an *in vivo* IBS-like model of stress-induced VH in rats.

The specific objectives of the Ph.D. work were as follows:

- (i) Characterize, through bioreactor fermentations and GAD enzyme activity assays, the GABA production of the three representative *L. lactis* strains (GABA-producer NCDO2118 considered as the reference strain, GABA low-producer NCDO2727 and GABA higher-producer CNCM I-5388)
- (ii) Evaluate the anti-VH properties in rat model of the three *L. lactis* strains, assessing the relevance of bacterial GAD enzyme, host's GABA_B receptors and gut microbiota.
- (iii) Gauge, through simplified static and advanced dynamic gastrointestinal models (*in vitro* upper gut and/or *in vivo* in rats) the impact of the gastric physico-chemical environment on GAD activity, GABA production and survival of *L. lactis*.

SECTION II – RESULTS

CHAPTER 1 - *Lactococcus lactis* NCDO2118 exerts visceral antinociceptive properties in rat via GABA production in the gastro-intestinal tract

As presented in **section I**, many lactic acid bacteria (LAB) have been long used in the past for their probiotic, prebiotic and/or psychobiotic properties, acting directly in the gut-brain axis or through interactions with gut microbiota. One of the many applications of LAB in human health has been the treatment of irritable bowel syndrome (IBS) symptoms, including visceral hypersensitivity (VH). Many mechanisms of action of LAB anti-VH properties have been proposed, however the properties of GABA-producing LAB have been weekly explored even though literature has strong evidence of the impact of GABA in pain perception and alleviation. Due to its extensive use in research as a model multi-faceted bacterium and its various and numerous applications in the food industry, *Lactococcus lactis* was the focus of our work. Based on this background, this study aimed to compare after growth under the same fermentation conditions, the glutamate decarboxylase (GAD) activity of two strains, NCDO2118 and NCDO2727, preliminarily identified as GABA-producer and GABA low-producer, respectively. Next, we evaluated their anti-VH properties *in vivo* in acutely stressed versus control rats, orally daily treated with *L. lactis* strains for 10 days. In addition, we studied the dependency of the *L. lactis* GAD activity for *in vivo* efficacy, the role of host's GABA_B receptors and gut microbiota in the analgesic effect. Finally, we finally proposed a hypothesis of a virtuous circle where GABA produced by bacterial GAD, activated by acidity in host's stomach, stimulates gastric secretion through activation of host's GABA_B receptors.

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Lactococcus lactis NCDO2118 exerts visceral antinociceptive properties in rat via GABA production in the gastro-intestinal tract

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Abstract Gut disorders associated to irritable bowel syndrome (IBS) are combined with anxiety and depression. Evidence suggests that microbially produced neuroactive molecules, like γ -aminobutyric acid (GABA), can modulate the gut-brain axis. Two natural strains of *Lactococcus lactis* and one mutant were characterized *in vitro* for their GABA production and tested *in vivo* in rat by oral gavage for their antinociceptive properties. *L. lactis* NCDO2118 significantly reduced visceral hypersensitivity induced by stress due to its glutamate decarboxylase (GAD) activity. *L. lactis* NCDO2727 with similar genes for GABA metabolism but no detectable GAD activity had no *in vivo* effect, as well as the NCDO2118 Δ gadB mutant. The antinociceptive effect observed for the NCDO2118 strain was mediated by the production of GABA in the gastro-intestinal tract and blocked by GABA_B receptor antagonist. Only minor changes in the faecal microbiota composition were observed after the *L. lactis* NCDO2118 treatment. These findings reveal the crucial role of the microbial GAD activity of *L. lactis* NCDO2118 to deliver GABA into the gastro-intestinal tract for exerting antinociceptive properties *in vivo* and open avenues for this GRAS (Generally Recognized As safe) bacterium in the management of visceral pain and anxious profile of IBS patients.

Editor's evaluation

Gut microbes produce neuromodulators including the neurotransmitter γ -aminobutyric acid (GABA), which could regulate pain and other neurological outcomes. This study examines how oral administration of the probiotic *Lactococcus lactis* affects visceral pain in rats, finding that a specific strain of *L. lactis* (NCD02118) suppresses stress induced pain in a manner dependent on bacterial GadB, the enzyme mediates GABA synthesis.

Introduction

As defined by the Rome IV criteria, irritable bowel syndrome (IBS) is a functional gastro-intestinal disorder affecting 3–5% of adults in industrial countries. IBS results in various symptoms that strongly affect the patients' quality of life (Dean et al., 2005) and a significant socioeconomic burden (Peery et al., 2012). Abdominal pain is a prevalent IBS symptom, associated with changes in bowel habits (diarrhoea and/or constipation; Camilleri et al., 2012). Even though IBS pathophysiology remains not completely understood, IBS symptoms may originate from peripheral and/or central mechanisms

resulting in a dysfunctional gut-brain axis (Fichna and Storr, 2012). Anxiety and stressful psychological life events lead to central nervous system (CNS) disorder triggering in turn gut motility dysfunctions (Mönnikes et al., 2001) and strengthen visceral sensitivity (Greenwood-Van Meerveld et al., 2016). For a better understanding of the IBS pathophysiology, stress animal models have been developed mimicking IBS features, such as changes in visceral sensitivity and gut transit time (Gué et al., 1997).

Related to the complex pathophysiology of IBS and the diversity of IBS patients' profiles, the existing therapeutic strategies promote solutions usually limited to treat symptoms (motor/sensory disturbances). In addition, in several cases with psychological comorbidity, psychopharmacological drugs are used (Dekel et al., 2013). As an effective treatment, alternative probiotics were used to modulate visceral pain in IBS patients (Moayyedi et al., 2010; Hungin et al., 2013; Ford et al., 2014; Didari et al., 2015). However, no consensus results were obtained from human clinical studies (Mazurak et al., 2015) and mechanisms by which microbes signal from the gut lumen to the CNS, thus influencing pain perception, remain to be elucidated.

In recent years, mounting evidence has suggested that microbially produced neuroactive molecules can modulate the gut-brain axis communication (Cryan and O'Mahony, 2011; Lyte, 2011; Reid, 2011; Cryan and Dinan, 2012). For example, ingestion of a *Lactobacillus* strain, i.e., *L. rhamnosus* (JB-1) is able to regulate emotional behaviour and central γ -aminobutyric acid (GABA)ergic system in mouse via vagus nerve pathway (Bravo et al., 2011). Recently, GABA-modulating bacteria of the human gut microbiota have been associated to brain signatures related to depression (Strandwitz et al., 2019). In fact, GABA is the main inhibitory CNS neurotransmitter in mammals (Wong et al., 2003), and several important physiological functions have been characterized, such as neurotransmission, relaxing, and tranquilizer effects (Hayakawa et al., 2007; Li and Cao, 2010). GABA exerts these major functional effects via two GABA receptor subtypes, i.e., GABA_A and GABA_B (Hyland and Cryan, 2010) doing these receptors important pharmacological targets for clinically relevant anti-anxiety agents (Foster and Kemp, 2006).

In the human diet, glutamate constitutes up to 8–10% of amino acids; it is involved in gut protein metabolism and is the precursor of different important molecules produced within the intestinal mucosa, like GABA. GABA is synthesized by a pyridoxal-5'-phosphate (PLP)-dependent enzyme glutamate decarboxylase (GAD; EC 4.1.1.15) by irreversible α -decarboxylation of L-glutamate and consumption of one cytoplasmic proton (Ueno, 2000). Prokaryotic and eukaryotic cells are able to synthesize GABA, through the decarboxylation of glutamate and genes encoding GAD are present in the gut microbiota (Mazzoli and Pessione, 2016). Glutamate import and GABA export generally occur in bacteria simultaneously via a specific glutamate/ γ -amino butyrate antiporter (Ma et al., 2012). The glutamate-dependent system is associated with acid resistance in many bacteria (Feehily and Karatzas, 2013).

Due to this large repertoire of beneficial effects, GABA has been classified as a health-promoting bioactive component in foods and pharmaceuticals (Li and Cao, 2010). Among bacteria, lactic acid bacteria (LAB) are generally recognized as safe sources to produce GABA at high levels and in an eco-friendly way (Dhakal et al., 2012). Numerous studies revealed that food-derived *Lactobacilli* and gut-derived *Lactobacillus* and *Bifidobacterium* species are able to produce GABA *in vitro* (Li and Cao, 2010; Yunes et al., 2016). Interestingly, Linares et al., 2016 reported the use of a novel *Streptococcus thermophilus* strain for the production of a naturally GABA-enriched yogurt. However, the link between bacterial GABA production *in vitro* and neuromodulatory activity *in vivo* remains poorly investigated. To date, only the human gut commensal GABA-producing *Bifidobacterium dentium* was shown *in vivo* to modulate sensory neuron activity in a rat faecal retention model of visceral hypersensitivity (Pokusaeva et al., 2017). Here, we focused on the transient food-borne LAB *Lactococcus lactis*. Frequently encountered in dairy products, *L. lactis* is one of the most ingested bacteria (Mills et al., 2010; Laroute et al., 2017). Although not considered as a commensal bacterium, *L. lactis* was found to persist transiently in the gut, depending on the strain under study (Wang et al., 2011; Radziwill-Bienkowska et al., 2016; Zhang et al., 2016). It was also demonstrated that *L. lactis* may exert *in vivo* a potent anti-inflammatory activity attenuating colitis (Nishitani et al., 2009; Luerce et al., 2014; Ballal et al., 2015; Berlec et al., 2017; Nomura et al., 1999). *L. lactis* is also able to produce GABA *in vitro* (Nomura et al., 1999) and the well-characterized NCDO2118 strain besides its probiotic properties (Cordeiro et al., 2021), is considered as an efficient GABA producer among

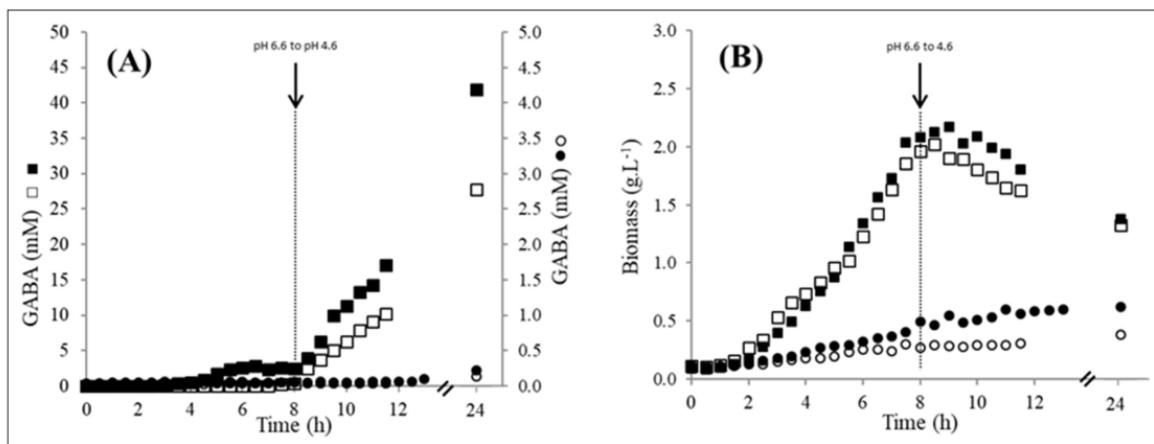


Figure 1. Growth and GABA production of the different *Lactococcus lactis* strains. (A) γ -Aminobutyric acid (GABA) production (mM); (B) evolution of biomass (g/L) during growth of *Lactococcus lactis* NCDO2118 (\square , \blacksquare) or NCDO2727 (\circ , \bullet) in M17 supplemented with glutamate (8 g/L), arginine (5 g/L), glucose (45 g/L), and NaCl (300 mM). Two independent duplicates were performed.

The online version of this article includes the following source data for figure 1:

Source data 1. Table of biomass and GABA concentrations for *L. lactis* NCDO2118 and NCDO2727 strains.

the species (Oliveira et al., 2017; Mazzoli et al., 2010; Oliveira et al., 2014; Laroute et al., 2016; Laroute et al., 2021).

Here, we determined whether *L. lactis* NCDO2118 is able to deliver GABA *in vivo* via glutamate decarboxylation (GAD activity) and to exert GABAergic signalling-dependent antinociceptive properties in a stress-induced visceral hypersensitivity rat model.

Results

High *in vitro* GABA production by *L. lactis* NCDO2118 is related to its GAD activity

The NCDO2118 strain previously demonstrated to produce GABA in chemically defined medium (Laroute et al., 2016), was grown in batch bioreactor with complex medium allowing high production of biomass and GABA (see Experimental section). The biomass and GABA concentrations were measured all along the culture (Figure 1A and B). In these conditions, the maximal concentrations of GABA and biomass reached, respectively, 40 mM at 24 hr and around 2.0 g/L at 8 hr, which are higher than the previously reported values in chemically defined medium (Laroute et al., 2016).

The NCDO2727 strain was demonstrated here to have the *gad* operon involved in GABA biosynthesis similar to NCDO2118, in terms of genetic organization (gene sequences and promoter). *gadR*, *gadC*, and *gadB*, the three genes of the *gad* operon are located between *kefA* and *rnhB* like in the NCDO2118 strain and the sequences of the corresponding proteins, GadR, C, and B, are highly similar with more than 99% of identity. Only two single nucleotide polymorphisms and a deletion of one bp were identified in the *gadR* and *gadCB* promoters, respectively, compared to NCDO2118. However, when tested for its GABA-producing performances under similar culture conditions, GABA production was very weak (GABA concentration <0.2 mM, Figure 1). The biomass production was lower (maximal biomass around 0.5 g/L at 24 hr) than for the NCDO2118 strain (Figure 1). The GABA concentration did not increase when the biomass production increased during growth of NCDO2727 strain in medium with yeast extract and glutamate (data not shown). For *in vivo* experiments, the bacterial amount to be orally administrated was adjusted to treat animals with similar viable bacterial cell number for the two strains (i.e. 10^9 CFU (Colony-Forming Units) per day; see below).

Interestingly, a high intracellular GAD activity was obtained at 7 hr in the NCDO2118 strain (45.3 ± 4.7 $\mu\text{mol}/\text{min}\cdot\text{mg}$) consistently with its high GABA production ability, while in the NCDO2727 strain,

no activity was detected. The difference of GAD activity between the two strains was confirmed at 24 hr of culture (*Supplementary file 1*). This comparison demonstrated that *in vitro* GABA production could not be solely associated to GABA genetic determinants in *L. lactis*.

Only GABA-producing *L. lactis* NCDO2118 prevents visceral hypersensitivity induced by stress in response to colorectal distension

The influence of a 10-day chronic treatment by *L. lactis* NCDO2118 or NCDO2727 on stress-induced visceral hypersensitivity to colorectal distension (CRD) is shown in *Figure 2*. Rats were given *L. lactis* strains by oral gavage once daily with washed bacterial cells at the same amount (10^9 CFU per day) whatever the strain under study. We first verified that GABA-producing *L. lactis* NCDO2118 in presence of glutamate had no impact on basal CRD sensitivity (*Figure 2—figure supplement 1*). Then, in vehicle-treated rats, a 2 hr of partial restraint stress (PRS) significantly increased the number of abdominal contractions compared to basal conditions for all the pressures of distention applied from 30 mmHg ($p < 0.001$, *Figure 2*). In presence of glutamate, oral administration of *L. lactis* NCDO2118 suppressed the PRS-induced enhancement of abdominal contractions ($p < 0.01$ for all CRD pressures applied, *Figure 2A*), restoring a quasi-basal sensitivity to CRD. However, in absence of glutamate, the NCDO2118 strain did not exert antinociceptive properties (*Figure 2A*). Glutamate alone, at the concentration used (0.2% [w/v]), had no impact on basal CRD sensitivity and on visceral hypersensitivity response (*Figure 2—figure supplement 2*). Oral administration of the low GABA-producing *L. lactis* NCDO2727 in presence of glutamate failed to reduce stress-induced visceral hypersensitivity (*Figure 2B*).

The antinociceptive effect of *L. lactis* NCDO2118 is due to its ability to deliver GABA *in vivo* and is mediated by the activation of GABA_B receptors

The growth of the NCDO2118 Δ gadB mutant strain, in which GABA pathway was interrupted (see Experimental section for strain construction details), was close to that of the NCDO2118 strain under same culture conditions (*Figure 3—figure supplement 1*). However, this strain did not produce GABA (GABA concentration in the bioreactor < 0.3 mM *Figure 3—figure supplement 1*) and had no detectable GAD activity. There was no impact of this strain on the visceral hypersensitivity response induced by stress in response to CRD in presence of glutamate (*Figure 3A*, $p < 0.05$ NCDO2118 Δ gadB- vs. NCDO2118-treated animals). The beneficial effect of *L. lactis* NCDO2118 treatment on stress-induced visceral hypersensitivity ($P < 0.05$) was completely abolished when animals received the GABA_B receptor antagonist SCH-50911 in presence of glutamate (*Figure 3B*).

GABA is produced by *L. lactis* NCDO2118 under 'stomach-like' conditions *in vitro* and *in vivo* in the stomach

Experiments were performed *in vivo* with rats fed *L. lactis* NCDO2118, vehicle or *L. lactis* NCDO2727, in the presence of glutamate for 10 days. For the NCDO2118 strain, the GABA level was high in the stomach and then tended to decrease in the other gut compartments ($p < 0.05$ in caecum vs stomach; *Figure 4A*). In contrast, in vehicle- or NCDO2727-treated animals, the GABA levels were similar all along the gastro-intestinal tract (*Figure 4B*). Consistently, in the stomach, the GABA production by *L. lactis* NCDO2118 was higher than that measured in vehicle or NCDO2727 strain (*Figure 4C*). To reinforce our findings on the potential role of the gastric region, we performed further experiments *in vitro* under 'stomach-like' conditions in the presence of glutamate (at pH = 4.6 in acetate buffer or gastric juice sampled from naive rats). In particular in rat gastric juice, we found that the rate of GABA production by the NCDO2727 strain was similar to the vehicle but the rate of GABA production by the NCDO2118 strain was fourfold increased (*Supplementary file 2*).

Oral treatment with *L. lactis* NCDO2118 has minor influence on faecal microbiota abundance, composition, and diversity but influences specific genera

The composition of the faecal microbiota was not globally impacted by the *L. lactis* NCDO2118 treatment (*Figure 5*). Particularly, no changes in α and β diversities were observed (*Figure 5A and*

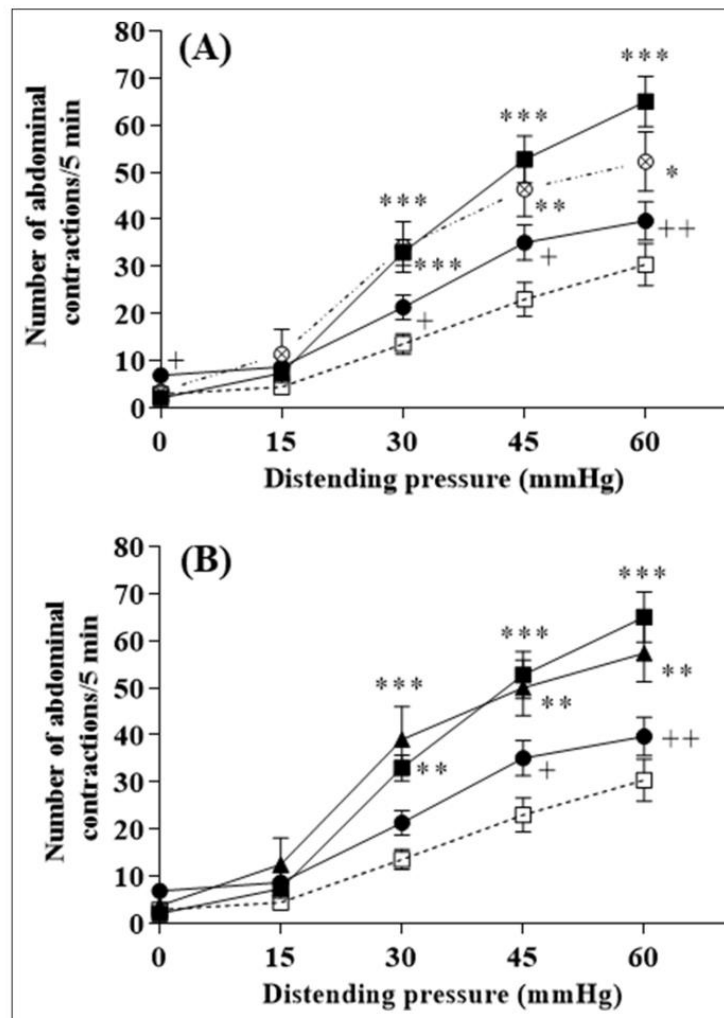


Figure 2. *L. lactis* effect on visceral hypersensitivity induced by stress in response to colorectal distension. (A) Effect of 10-day oral administration of γ -aminobutyric acid (GABA)-producing *Lactococcus lactis* NCDO2118 (10^9 CFU per day) in presence or absence of glutamate on PRS-induced visceral hypersensitivity at all the distension pressures of colorectal distension (CRD; from 15 to 60 mmHg). Data are expressed as means \pm SEM (n=9 for the 'vehicle' group [-□-] and 'PRS + vehicle' group [-■-]; n=7 for 'PRS + NCDO2118' group [-⊗-]; n=12 for the 'PRS + NCDO2118 + glutamate' group [-●-]); (B) Effect of 10-day oral administration of GABA-producing *L. lactis* NCDO2118 and low GABA-producing *L. lactis* NCDO2727 (10^9 CFU per day) in presence of glutamate on PRS-induced visceral hypersensitivity at all the distension pressures of CRD. Data are expressed as means \pm SEM (n=9 for the 'vehicle' group [-□-] and 'PRS + vehicle' group [-■-]; n=12 for the 'PRS + NCDO2118 + glutamate' group [-●-]; n=8 for the 'PRS + NCDO2727 + glutamate' group [-▲-]). *p<0.05, **p<0.01, ***p<0.001 vs. basal values for animals treated with vehicle. *p<0.05, **p<0.01 vs. values for stressed animals treated with vehicle.

The online version of this article includes the following source data and figure supplement(s) for figure 2:

Source data 1. Effect of 10-day oral administration of GABA-producing *L. lactis* NCDO2118 on PRS-induced visceral hypersensitivity.

Figure 2 continued on next page

Figure 2 continued

Source data 2. Effect of 10-day oral administration of GABA-producing *L. lactis* NCDO2118 and low GABA-producing *L. lactis* NCDO2727 on PRS-induced visceral hypersensitivity.

Source data 3. Incidence of 10-day oral administration of *L. lactis* NCDO2118 in presence of glutamate on basal visceral sensitivity.

Source data 4. Incidence of 10-day oral administration of glutamate on basal visceral sensitivity and PRS-induced visceral hypersensitivity.

Figure supplement 1. Incidence of 10-day oral administration of *L. lactis* NCDO2118 in presence of glutamate on basal visceral sensitivity at all the distension pressures of CRD (from 15 to 60 mmHg).

Figure supplement 2. Incidence of 10-day oral administration of glutamate on basal visceral sensitivity and PRS-induced visceral hypersensitivity at all the distension pressures of CRD (from 15 to 60 mmHg).

C). Besides, the non-parametric Kruskal-Wallis test on each of the 150 identified genera present in samples revealed few differences in taxon abundances in faecal microbiota, in particular significantly for three genera, *Clostridium* ($p=0.023$), *Frisingicoccus*, and *Papillibacter* ($p=0.041$), and nearly significantly for *Escherichia* genus ($p=0.069$), after *L. lactis* NCDO2118 treatment (**Supplementary file 3**).

Discussion

For the first time, we demonstrated that a transient food-borne LAB *L. lactis* (strain NCDO2118) exerts, in an animal model of acute stress, visceral anti-hypersensitivity effects via its ability to produce significant levels of GABA *in vivo*, due to an active GAD enzymatic potential and not solely to the presence of *gadB* gene. Indeed, the low GABA producer NCDO2727 strain and NCDO2118 Δ *gadB* mutant strain unable to produce GABA, fail to exert such antinociceptive effect. This effect is mediated by the metabolically active NCDO2118 which delivers GABA in the gastro-intestinal tract, that in turn influences host GABAergic host physiological system.

L. lactis is a very common bacterium widely used in food industry with a GRAS status. Bacteria can metabolize, via decarboxylation mechanisms, several amino acids to synthesize active amino compounds. In particular, GAD catalyses the irreversible α -decarboxylation of glutamate into GABA. Even though genes encoding GAD are found in numerous bacteria present in different environments and ecosystems, i.e., native soil, natural landscape as well as in the gastro-intestinal tract, the ability of these bacteria to produce GABA has not systematically been evidenced. Interestingly, among *L. lactis* bacteria studied here, NCDO2727 was qualified as a low producer of GABA and NCDO2118 as a high producer. Although these two strains harbour similar GAD encoding *gadB* gene, they differed significantly at the level of their GAD activity. Because the promoters of *gad* genes are nearly identical in the two strains, transcriptional regulations should not explain the observed differences. The growth defect of NCDO2727 should also not be involved since higher growth of the strain in another culture medium did not restore a high GABA production. Herein, we demonstrate *in vivo* that only a 10-day chronic oral administration of the high producer NCDO2118 strain displaying a high GAD activity is able to reduce visceral hypersensitivity induced by stress. This effect was accompanied by an increased GABA production in the gastric compartment. Taken together, all these observations suggest that transient NCDO2118 strain is metabolically active *in vivo* to deliver GABA in the gastro-intestinal tract lumen. We evaluated the impact of the NCDO2118 treatment on the faecal microbiota and observed no changes in α or β diversity. This result is consistent with observations in many other studies on IBS that have shown that probiotic treatment did not shift the overall diversity of the intestinal microbiota of mice, rats, or humans (Cussotto *et al.*, 2021; Grazul *et al.*, 2016; Jeffery *et al.*, 2020; Labus *et al.*, 2017; Tap *et al.*, 2017). Indeed, in 'healthy microbiota' the addition of a probiotic does not promptly impact the resident microbial populations but rather could have a significant impact on reshaping the microbiota in an already existing dysbiosis humans (Alander *et al.*, 1999; Eloe-Fadrosh *et al.*, 2015; Lee *et al.*, 2004). We next compared genera abundancies after treatment using non-parametric tests and found an enrichment of the *Clostridium* genus after *L. lactis* NCDO2118 treatment compared to the vehicle group. This genus was shown *in silico* as modulating GABA (i.e. GABA producer and consumer) in humans (Strandwitz *et al.*, 2019). An enrichment of the *Escherichia* genus was also observed. Interestingly, in the study on the *E. coli* Nissle 1917 strain, Pérez-Berezo and colleagues showed that GABA was not able to directly cross the epithelial barrier (Pérez-Berezo *et al.*, 2017),

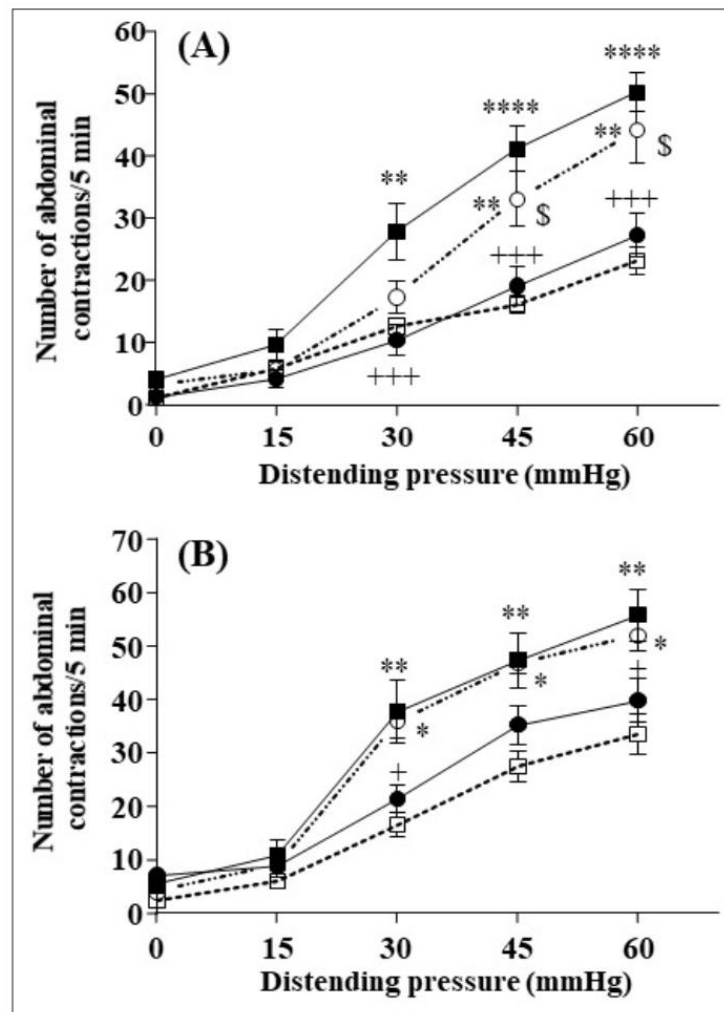


Figure 3. The anti-nociceptive effect of *L. lactis* NCDO2118. **(A)** Effect of 10-day oral administration of γ -aminobutyric acid (GABA)-producing *Lactococcus lactis* NCDO2118 and non-GABA producing NCDO2118 Δ *gadB* mutant (10^9 CFU per day) on PRS-induced visceral hypersensitivity at all the distension pressures of colorectal distension (CRD; from 15 to 60 mmHg). Data are expressed as means \pm SEM ($n=9$ for the 'vehicle' group [\square] and 'PRS + vehicle' group [\blacksquare]; $n=12$ for the 'PRS + NCDO2118 + glutamate' group [\bullet]; $n=13$ for the 'PRS + NCDO2118 Δ *gadB* + glutamate' group [\circ]); **(B)** Effect of 10-day oral administration of GABA-producing *L. lactis* NCDO2118 (10^9 CFU per day) on PRS-induced visceral hypersensitivity at all the distension pressures of CRD in presence or not of GABA_A receptor antagonist SCH-50911 (3 mg/kg bw, IP). Data are expressed as means \pm SEM ($n=9$ for the 'vehicle' group [\square] and 'PRS + vehicle' group [\blacksquare]; $n=12$ for the 'PRS + NCDO2118 + glutamate' group [\bullet]; $n=7$ for the 'PRS + NCDO2118 + glutamate + SCH-50911' group [\circ]). * $p<0.05$, ** $p<0.01$, **** $p<0.0001$ vs. basal values for animals treated with vehicle. * $p<0.05$, *** $p<0.001$ vs. values for stressed animals treated with vehicle. $\S p<0.05$ vs. for stressed animals treated with NCDO2118 + glutamate.

The online version of this article includes the following source data and figure supplement(s) for figure 3:

Source data 1. Effect of 10-day oral administration of GABA-producing *L. lactis* NCDO2118 and non-GABA producing NCDO2118 delta-gadB mutant on PRS-induced visceral hypersensitivity.

Figure 3 continued on next page

Figure 3 continued

Source data 2. Effect of 10-day oral administration of GABA-producing *L. lactis* NCDO2118 on PRS-induced visceral hypersensitivity in presence or not of GABA_B receptor antagonist SCH-50911.

Source data 3. Table of biomass and GABA concentrations for *L. lactis* NCDO2118 and its Δ gadB mutant.

Figure supplement 1. Evolution of biomass (g/L) during growth of *Lactococcus lactis* NCDO2118 Δ gadB mutant (◆) and evolution of γ -aminobutyric acid (GABA) production (▲) compared to *L. lactis* NCDO2118 (biomass □ and GABA Δ) in M17 supplemented with glutamate (8 g/L), arginine (5 g/L), glucose (45 g/L), and NaCl (300 mM).

requiring the C12AsnGABAOH lipopeptide for functionalization and subsequent translocation. It is then possible that the enrichment of the genus *Escherichia* in the faecal microbiota of *L. lactis*-treated animals could help reduce visceral pain via a lipopeptide-mediated GABA functionalization pathway. Of note, in our study, using the same method as previously described (Pérez-Berezo et al., 2017), we were not able to detect any C12AsnGABAOH lipopeptide in *L. lactis* NCDO2118 (results not shown). Other yet unidentified lipopeptides could be at play, with the possible contribution of other bacteria capable of producing such functional GABA. Altogether, further investigations are needed for a better understanding of the site(s) of interaction between luminal GABA delivery by *L. lactis* NCDO2118 and the gastro-intestinal barrier and its environment.

GABA is the major inhibitory neuromediator, mainly involved in regulating physiological and psychological responses. Today, indirect evidences point out the effect of gut microbiota as well as probiotic strains (Cryan and Dinan, 2012) on CNS by modulating the GABAergic system. Moreover, alterations of this GABAergic system have a key role in the development of stress-related psychiatric disorders (Schür et al., 2016). Converging evidence, using genetic and pharmacological approaches, illustrates the important role of GABA_B receptors in anxiety disorders. Using a specific antagonist (SCH-50911), we demonstrated the involvement of GABA_B receptor in the visceral antinociceptive effect mediated by the delivery of GABA produced by the NCDO2118 strain. GABA_B receptors are strongly expressed in the gastro-intestinal tract (Hyland and Cryan, 2010) and widely distributed from the stomach to the ileum in the enteric nervous system (ENS; Auteri et al., 2015). In rodents, previous studies established the presence of autocrine and paracrine GABA signalling mechanisms in gastro-intestinal epithelial cells via GABA receptor activation. In rat, GABA_B receptors have also been identified on the mucosal gland cells of the stomach (Castelli et al., 1999; Erdo and Bowery, 1986; Erdö et al., 1990; Krantis et al., 1994). Actually, the GABA_B metabotropic receptors in the gastro-intestinal tract are known to regulate several gut functions and gut-to-brain signalling pathway (Hyland and Cryan, 2010). In mice, selective stimulation of GABA_B receptors increases gastric acid secretion both through vagal cholinergic and gastrin-dependent mechanisms (Piqueras and Martinez, 2004). These combined central and peripheral mechanisms result in an increase in the luminal acidity, which provides a supportive environment for the GAD activity of *L. lactis* NCDO2118. Accordingly, and despite a limited number of animals, we observed herein a significant level of GABA production measured after 10-day administration of *L. lactis* NCDO2118 in the stomach, which was supported by an *in vitro* GABA production in gastric juice sampled from naive rats and supplemented with glutamate; in this way, a dynamic 'virtuous circle' would be generated between *L. lactis* NCDO2118 and the host. Furthermore, vagal and splanchnic afferents of GABA_B receptors are involved in the modulation of sensitivity (Hyland and Cryan, 2010). Probiotic strains have been described to modulate brain functions via a dependent vagus activation pathway (Bravo et al., 2011). This study was the first to demonstrate the ability of *Lactobacillus* to modify central levels of GABA through the stimulation of the vagus nerve. This central GABA release goes hand with beneficial effect on emotional behaviour impairments induced by stress. However, the primary molecular mechanisms underlying how *Lactobacillus* stimulated vagal afferents were not resolved. Here, we could firmly demonstrate that a neurotransmitter, i.e., GABA, directly produced by *L. lactis* NCDO2118 into the gastro-intestinal tract lumen, exerted beneficial effect on stress-induced gut visceral hypersensitivity via GABA_B receptor stimulation. Even though we do not investigate the activation of the ENS or the CNS herein, our results associated with previous published data bring strengths on the table in the functional communication between bacteria, gut, and brain.

In conclusion, our data strongly suggest that, according to its ability to deliver into the gastro-intestinal tract lumen neurometabolites like GABA in significant levels, *L. lactis* NCDO2118 could be considered as a helpful candidate in the management of functional gastro-intestinal disorders

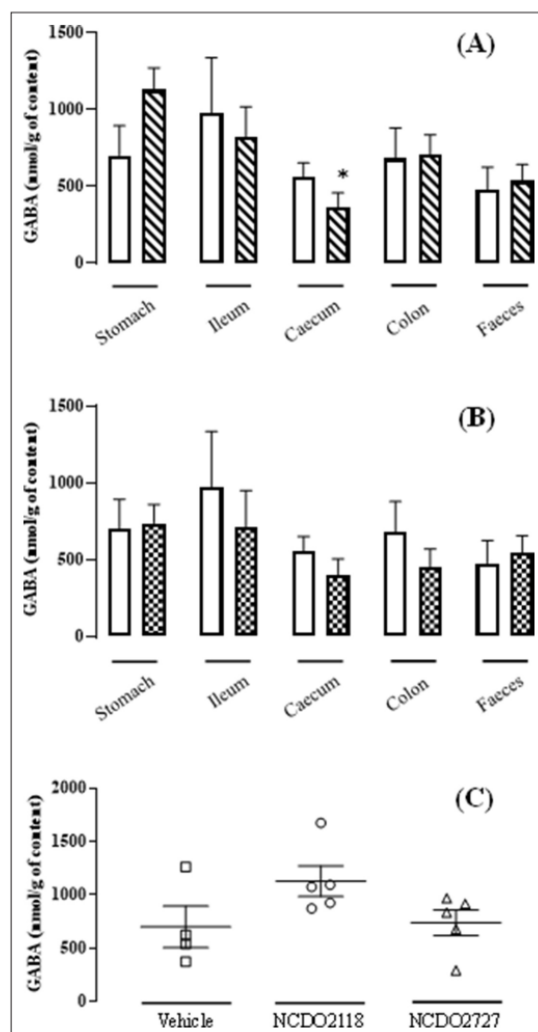


Figure 4. *L. lactis* effect on the GABA production all along the gastro-intestinal tract. (A) Variation of the γ -aminobutyric acid (GABA) concentration all along the gastro-intestinal tract for the group 'NCDO2118 + glutamate' (▨) vs. the group 'vehicle' (□) after a 10-day daily oral administration at 10^9 CFU per day. (B) Variation of the GABA concentration all along the gastro-intestinal tract for the group 'NCDO22727 + glutamate' (▩) vs. group 'vehicle' (□) after a 10-day daily oral administration at 10^9 CFU per day. (C) Variation of the GABA concentration in the gastric content for the group 'vehicle' (□), the group 'NCDO2118 + glutamate' (○), and the group 'NCDO2727 + glutamate' (△) after a 10-day daily oral administration at 10^9 CFU per day. Data are expressed as means \pm SEM. Non-parametric Kruskal-Wallis test, supplemented by Dunn's multiple comparison test, revealed no statistical differences between samples except caecum vs. stomach for the NCDO2118 strain ($p < 0.05$).

The online version of this article includes the following source data for figure 4:

Source data 1. Variation of the GABA concentration all along the gastro-intestinal tract for NCDO2118+glutamate.

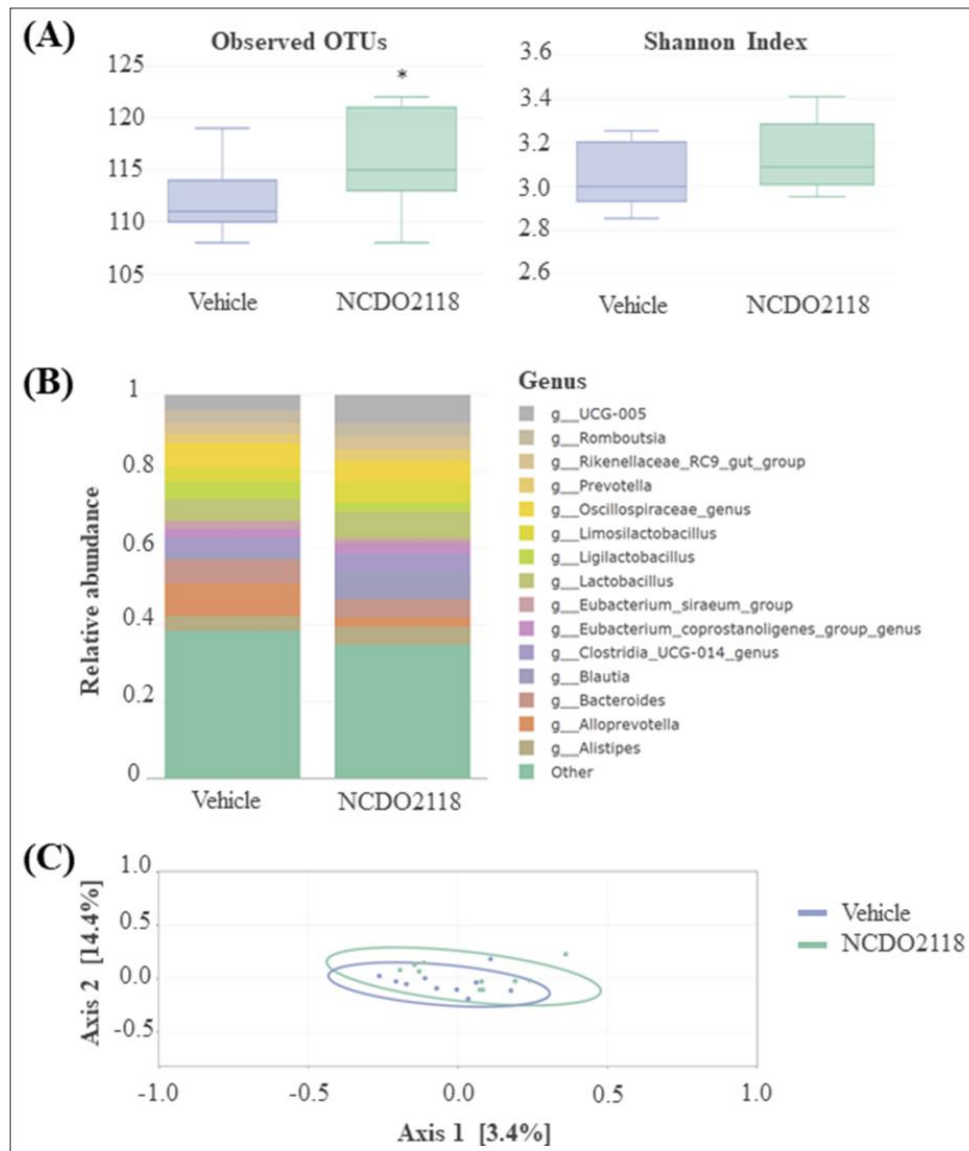


Figure 5. Overview of the faecal microbiota after a 10-day daily oral administration of γ -aminobutyric acid (GABA)-producing *L. lactis* NCDO2118 (10^7 CFU per day) in presence of glutamate vs. 'vehicle' group. **(A)** Alpha diversity within faecal samples. Two measures are shown: mean observed number of OTUs per sample, an estimate of richness (left panel), and Shannon Index, indicating the evenness of the sample (right panel). One-way ANOVA followed by the post hoc pairwise Tukey's test revealed no statistical differences between samples ($p > 0.05$). **(B)** Top 15 dominant bacterial genera in faecal samples; **(C)** MDS ordination plot of the Bray-Curtis distance between samples, a representation of phylogenetic similarity. PERMANOVA revealed no statistical differences between samples ($p > 0.05$).

associated with stressful situations. Regarding our results, GAD activity rather than genetic organization of *gad* genes appears as a key determinant for *in vivo* antinociceptive properties of *L. lactis*. Therefore, this work opens perspectives for GRAS *L. lactis* strains as future therapeutic agents for the management of visceral pain and the anxious profile of IBS patients.

Materials and methods

Bacterial strains, medium, and culture conditions

The strains NCDO2118 and NCDO2727, two *L. lactis* subsp. *lactis* from vegetable origin, were used throughout this study. *L. lactis* NCDO2118 Δ *gadB* mutant was constructed by double crossing over in the chromosome as previously described (Maguin *et al.*, 1996). 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 England Biolabs). Primers are listed in *Supplementary file 4*.

Bacterial cultures were performed in duplicate in 2 L Biostat B-plus bioreactor (Sartorius, Melsungen, Germany) in M17 supplemented with 55 mM (8 g/L) glutamate, 29 mM (5 g/L) arginine, 250 mM (45 g/L) glucose, and 300 mM NaCl. Cultures were incubated at 30°C. Fermentations were carried out under oxygen-limiting conditions. pH was maintained at 6.6 by KOH addition for 8 hr, then pH was dropped and regulated at 4.6. Culture was inoculated with cells from pre-cultures grown in Erlenmeyer flask on similar medium, harvested during the exponential phase, and concentrated in order to obtain an initial optical density at 580 nm of 0.25 in the fermenter. Bacterial growth was estimated by spectrophotometric measurements at 580 nm (Libra S11, Biochrom, BIOSERV, Massy, France; 1 Unity of absorbance is equivalent to 0.3 g dry weight/L). Samples were collected every 30 min for HPLC (High Performance Liquid Chromatography) measurement of GABA concentration in the growth medium. For *in vivo* assays and measurement of bacterial strain activity in the gastric juice *in vitro*, cells were harvested before the pH modification (i.e. at 7 hr for NCDO2118 and NCDO2727). The culture volume required for approximately 3×10^{11} CFU (colony-forming units) was centrifuged to pellet the bacterial cells. Then, cells were washed and suspended in 0.9% (m/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 hr and 24 hr of culture.

Sequence analysis of *gad* operon

In the NCDO2118 strain, the *gad* operon sequence was extracted from the chromosome sequence deposited in NCBI-GenBank database under the accession number CP009054. The *gad* operon in NCDO2727 strain was sequenced and deposited in NCBI-GenBank database under the accession number MK225577. Briefly, DNA was amplified using two primers, GadSeq_F (5' –TCCAGAAATAAC AGCTACATTGACATAATG –3') and GadSeq_R (5'– TAACAGCCCCATTATCTAAGATTACTCC –3') and the Q5 High-Fidelity DNA polymerase (NEB) and sequenced by Eurofins Genomics (primers listed in *Supplementary file 4*). Sequences of *gad* operon were compared by Blast alignment algorithm.

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 $22 \pm 2^\circ\text{C}$ and a 12 hr light/dark cycle) with free access to water and food (standard pellets 2016, Envigo RMS SARL, Gannat, France). All experiments were approved by the Local Animal Care and Use Committee (APAFiS#5577–201606061639777 v3, see Colorectal and Under PRS conditions sections, and APAFiS#14898–2018043016031426, see Without PRS) in compliance with European directive 2010/63/UE.

Colorectal distension procedure and acute stress procedure

Under general anaesthesia 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 EMG recording (Morteau *et al.*, 1994). Animals were then accustomed to be in polypropylene tunnels for several days before CRD. A 4-cm long latex balloon, fixed on 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 on an electroencephalograph machine (Mini VIII, Alvar, Paris, France).

PRS, a relatively mild non-ulcerogenic model of stress, was performed as previously described (Williams *et al.*, 1988). 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 prevent, body movements. Rats were then placed in their home cage for 2 hr.

Experimental protocol for in vivo assays

Under PRS conditions

Series of experiments, based on a 10-day treatment by oral gavage (as detailed below), were conducted using, for each series, three groups of 7–12 female rats equipped for EMG. For all oral treatments used, basal and post-PRS (20 min after the 2 h PRS session) abdominal responses to CRD were recorded on day 9 and day 10, respectively.

In the first series of experiments, groups of rats were orally treated for 10 days (1 mL/rat) with *L. lactis* NCDO2118 (10^9 CFU/day) plus glutamate (0.2% [w/v]) or not, *L. lactis* NCDO2727 plus glutamate (0.2% [w/v]) or vehicle (NaCl 0.9% [w/v] + glycerol 15% [v/v]). In the second series of experiments, rats were divided into three groups and orally treated for 10 days (1 mL/rat) with *L. lactis* NCDO2118 (10^9 CFU/day) plus glutamate (0.2% [w/v]), *L. lactis* NCDO2118 plus glutamate (0.2% [w/v]), and GABA_A receptor antagonist (2 S) (+)-5,5-dimethyl-2-morpholineacetic acid (SCH-50911, Sigma-Aldrich SML1040; 3 mg/kg body weight, IP 20 min before the PRS session) or vehicle (NaCl 0.9% [w/v] + glycerol 15% [v/v]). In the last series of experiments, rats were divided into three groups and orally treated for 10 days (1 mL/rat) with *L. lactis* NCDO2118 (10^9 CFU/day) plus glutamate (0.2% [w/v]), *L. lactis* NCDO2118 Δ gadB (10^9 CFU/day) plus glutamate (0.2% [w/v]) or vehicle (NaCl 0.9% [w/v] + glycerol 15% [v/v]).

Without PRS

In order to determine GABA levels at different locations of the gastro-intestinal tract, further experiments without PRS application were performed as follows: three groups of rats (n=5 per group) were orally treated for 10 days (1 mL/rat) with *L. lactis* NCDO2118 (10^9 CFU/day) plus glutamate (0.2% [w/v]), *L. lactis* NCDO2727 plus glutamate (0.2% [w/v]), or vehicle (NaCl 0.9% [w/v] + glycerol 15% [v/v]). After the last administration, deep inhalation anaesthesia with Isoflurane, followed by terminal aortic blood sampling, was performed. After the sacrifice, gastric, ileal, caecal, and colonic contents, as well as faeces, were collected for GABA measurements (see GABA extraction and quantification).

GAD activity and GABA production by *L. lactis* cells

For GAD activity measurement, 150 mg bacterial 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 MgCl₂, 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 six cycles of 30 s at 6.5 m/s interrupted by 1 min incubation on ice. Cell debris was 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. Enzyme assay was realized with 0.5 mL of substrate solution, consisting of 20 mM sodium glutamate, 2 mM PLP incubated at 30°C then mixed with 0.5 mL supernatant. Every 30 min until 4 hr, 100 μ L were sampled and inactivated by boiling for 5 min to stop the decarboxylation reaction. Reaction mixtures were subsequently analysed for the presence of GABA using HPLC.

The kinetics of GABA production by *L. lactis* NCDO2118 and *L. lactis* NCDO2727 was monitored *in vitro* at 37°C as follows: 0.5 mL of each strain (10^9 CFU/mL) or the NaCl/glycerol vehicle was added to 1 mL of 100 mM acetate buffer pH = 4.6 or gastric content from naive rat and 0.5 mL glutamate 0.4% (w/v). 100 μ L of assay mixture were sampled at regular time intervals (until 60 min) and inactivated by boiling to stop the decarboxylation reaction. Reaction mixtures were subsequently analysed

using HPLC. The initial rate was determined as the amount glutamate converted into GABA (μmol) per minute.

GABA extraction and quantification

GABA concentration in culture supernatant or in reaction mixtures, associated to assays for GAD activity and GABA production, was measured by HPLC (Agilent Technologies 1200 Series, Waldbronn, Germany) as previously described (Laroute *et al.*, 2016).

GABA measurements were realised in samples from different digestive tract locations (gastric, ileal, caecal, and colonic contents and faeces). GABA was extracted from 200 mg of contents with methanol (three times with 3 mL) at room temperature. The mixture was centrifuged and the supernatant was transferred into tube and concentrated to dryness. Dried samples were resuspended in 100 μL methanol then analysed by HPLC as described above.

Faecal microbiota composition using 16S rRNA gene sequencing

Faeces were collected at the end of the 10-day oral treatment, just before the 2 hr PRS session (see Under PRS conditions). Faecal samples were stored at -80°C until DNA was extracted using the ZymoBIOMICS DNA Miniprep Kit (D4300, Zymo Research) following manufacturer's instructions. The 314 F/805 R primers (5' GACTACHVGGGTATCTAATCC-Forward primer), 5' GACTACHVGGGTATCT AATCC-Reverse primer) were used to amplify the V3-V4 variable regions of the 16 S rRNA gene. Forward primer and reverse primer carried overhang adapters (5' CTTCCCTACACGACGCTCTCCG ATCT-Forward primer, (5' GGAGTTCAGACGTGTGCTCTCCGATCT-Reverse primer) for Illumina index and sequencing adapters. First round of PCR was carried in a single-step 30 cycles 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 treatments of all samples were performed with the Find Rapidly OTUs with Galaxy Solution pipeline (Escudié *et al.*, 2018). Briefly, reads were contigued with the VSEARCH software and sequences with sizes inferior to 300 bp or superior to 700 bp were eliminated. A clustering at 97% similarity was used to define OTUs with the SWARM algorithm 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 (Bokulich *et al.*, 2013). The R package ANOMALY (Fisch *et al.*, 2022) 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 16 S and GTDB bac120_arc22 were used (Parks *et al.*, 2020; Quast *et al.*, 2013). Alpha (α) and beta (β) diversities and differential abundancies analyses were performed in R using the Phyloseq package (McMurdie *et al.*, 2013). Alpha diversity metrics (i.e. observed OTUs and Shannon Index) were calculated based on the genus level. One-way ANOVA was used to test α diversity metric dissimilarities with post hoc Tukey's test. The Bray-Curtis distance was used to measure β diversity metrics. We explored the community structure of the samples with PERMANOVA (Kelly *et al.*, 2015).

Statistical methods

The software GraphPad Prism 9.1 (GraphPad, San Diego, CA) was used for other statistical analyses. For animal experiments, data are reported as the means \pm SEM. One-way ANOVA, followed by Tukey's Multiple Comparison test, was performed to compare data between the different groups of animals. For GABA measurements, data are reported as the means \pm SEM and the non-parametric Kruskal-Wallis test, supplemented by Dunn's multiple comparison test, was used. Statistical significance was accepted at $p < 0.05$.

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Author contributions

Valérie Laroute, Catherine Beaufrand, Data curation, Formal analysis, Investigation, Methodology; Pedro Gomes, Data curation, Formal analysis, Methodology; Sébastien Nouaille, Valérie Tondereau, Marie-Line Daveran-Mingot, Formal analysis, Investigation, Methodology; Vassilia Theodorou, Conceptualization, Supervision, Validation; Hélène Eutamene, Muriel Mercier-Bonin, Muriel Coccain-Bousquet, Conceptualization, Funding acquisition, Supervision, Validation, Writing – original draft, Writing – review and editing

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Ethics

Human Subjects: No Animal Subjects: Yes Ethics Statement: All experiments were approved by the Local Animal Care and Use Committee (APAFIS#5577-201606061639777v3, see Colorectal and Under PRS conditions sections and APAFIS#14898-2018043016031426, see Without PRS) in compliance with European directive 2010/63/UE.

Decision letter and Author response

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Additional files

Supplementary files

- Transparent reporting form
- Supplementary file 1. Specific glutamate decarboxylase activity ($\mu\text{mol}/\text{min}\cdot\text{mg}$) of the two *Lactococcus lactis* strains after 7 or 24 hr of growth in M17 supplemented with glutamate (8 g/L), arginine (5 g/L), and NaCl (300 mM; $n=3$ for each of the two cultures replicates in bioreactor).
- Supplementary file 2. *In vitro* kinetics of γ -aminobutyric acid (GABA) production by *Lactococcus lactis* NCDO2118 and *L. lactis* NCDO2727. GABA production rates ($\mu\text{mol}/\text{min}$) were estimated when bacteria or control vehicle, were in presence of 0.2% (w/v) glutamate at 37°C and pH=4.4, either in 100 mM acetate buffer or with gastric content of naive rat. Two independent replicates were performed.
- Supplementary file 3. Non-parametric comparison of taxa relative abundancies between conditions. Highlighted lines show conditions with significant differences in relative abundancies after Kruskal-Wallis test ($p<0.07$). Purple highlight indicates that the taxon is more abundant in the faecal microbiota of vehicle-treated animals, green highlight indicates that the taxon is more abundant in the faecal microbiota of NCDO2118-treated animals.
- Supplementary file 4. List of primers. Primers used for the inactivation of *gadB* in *Lactococcus lactis* NCDO2118.

Data availability

Relevant additional data files are provided as source data files. For figures 1A 1B and S3: Excel file. For figures 2A 2B, 3A 3B, 4, S1 and S2: txt format. For figure 5: All data (raw and treated) can be found in this link: <https://forgemia.inra.fr/umrf/exploremetabar>.

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Supplementary file 1. Specific glutamate decarboxylase activity ($\mu\text{mol}/\text{min}\cdot\text{mg}$) of the two *Lactococcus lactis* strains after 7 or 24 hr of growth in M17 supplemented with glutamate (8 g/L), arginine (5 g/L), and NaCl (300 mM; n=3 for each of the two cultures replicates in bioreactor).

	GAD 7 h	GAD 24 h
NCDO2118	45.3 \pm 4.7	80.2 \pm 15.0
NCDO2727	0.0 \pm 0.0	0.1 \pm 0.1

Supplementary file 2. *In vitro* kinetics of γ -aminobutyric acid (GABA) production by *Lactococcus lactis* NCDO2118 and *L. lactis* NCDO2727. GABA production rates ($\mu\text{mol}/\text{min}$) were estimated when bacteria or control vehicle, were in presence of 0.2% (w/v) glutamate at 37°C and pH=4.4, either in 100 mM acetate buffer or with gastric content of naive rat. Two independent replicates were performed.

	Acetate buffer	Gastric content
NCDO2118	2.9 \pm 1.4	3.0 \pm 1.7
NCDO2727	Not detected	0.8 \pm 0.4
Vehicle	Not detected	0.9 \pm 0.2

Supplementary file 3. Non-parametric comparison of taxa relative abundancies between conditions. Highlighted lines show conditions with significant differences in relative abundancies after Kruskal-Wallis test ($p < 0.07$). Purple highlight indicates that the taxon is more abundant in the faecal microbiota of vehicle-treated animals, green highlight indicates that the taxon is more abundant in the faecal microbiota of NCDO2118-treated animals.

Taxa	p-value
1 g__Prevotella_Cluster_2	0.496291702231095
2 g__Ligilactobacillus_Cluster_3	0.545349668011121
3 g__Bacteroides_Cluster_4	0.545349668011121
4 g__Lactobacillus_Cluster_5	0.70545698611127
5 g__Limosilactobacillus_Cluster_6	0.496291702231095
6 g__Rikenellaceae_RC9_gut_group_Cluster_7	0.545349668011121
7 g__Muribaculaceae_genus_Cluster_8	0.0283655056052102
8 g__Blautia_Cluster_9	0.289918453942572
9 g__Lachnospiraceae_NK4A136_group_Cluster_10	0.256839257957856
10 g__UCG-005_Cluster_11	0.289918453942572
11 g__Odoribacter_Cluster_13	0.496291702231095
12 g__Romboutsia_Cluster_17	0.364346126633552
13 g__Clostridium_Cluster_22	0.0233422020128909
14 g__Desulfovibrionaceae_genus_Cluster_26	0.130570018115735
15 g__Turicibacter_Cluster_28	0.325751354478715
16 g__Ruminococcus_gauvreauii_group_Cluster_30	0.762368818469847
17 g__Oscillospiraceae_genus_Cluster_31	0.256839257957856
18 g__Phascolarctobacterium_Cluster_32	0.545349668011121
19 g__Paramuribaculum_Cluster_37	0.256839257957856
20 g__CAG-485_Cluster_41	0.173617334424944
21 g__Ruminococcus_Cluster_42	0.596701216729351
22 g__Acholeplasmataceae_genus_Cluster_44	0.256839257957856
23 g__Bifidobacterium_Cluster_46	0.173617334424944
24 g__Alistipes_Cluster_50	0.496291702231095
25 g__Akkermansia_Cluster_57	0.112410584655363
26 g__Escherichia_Cluster_59	0.0696424047983281
27 g__Anaerostipes_Cluster_63	0.130570018115735
28 g__Eubacterium_F_Cluster_64	0.596701216729351
29 g__Lachnospiraceae_genus_Cluster_74	0.289918453942572
30 g__Gastranaerophilales_genus_Cluster_75	0.130570018115735
31 g__UMGS1872_Cluster_77	0.939742989577085
32 g__Bacteroidales_genus_Cluster_81	0.364346126633552
33 g__UBA1394_Cluster_84	0.325751354478715
34 g__Prevotellaceae_NK3B31_group_Cluster_85	0.364346126633552

35	g__Muribaculum_Cluster_92	0.65014744409486
36	g__TF01-11_Cluster_96	0.879829160011815
37	g__Erysipelatoclostridium_Cluster_100	0.449691797968892
38	g__Eubacterium_coprostanoligenes_group_genus_Cluster_105	0.762368818469847
39	g__Schaedlerella_Cluster_107	0.226476066043488
40	g__UBA3282_Cluster_115	0.0233422020128909
41	g__Peptococcaceae_genus_Cluster_129	0.00319709944793456
42	g__Lachnospiraceae_NK4B4_group_Cluster_134	0.879829160011815
43	g__Marvinbryantia_Cluster_135	0.820595839755438
44	g__UBA7160_Cluster_139	0.130570018115735
45	g__Blautia_A_Cluster_140	0.762368818469847
46	g__Turicimonas_Cluster_144	0.820595839755438
47	g__Colidextribacter_Cluster_148	0.449691797968892
48	g__ASF356_Cluster_153	0.173617334424944
49	g__Eubacterium_siraeum_group_Cluster_154	0.150926950066717
50	g__Eubacterium_G_Cluster_155	0.364346126633552
51	g__Lachnospiraceae_NC2004_group_Cluster_159	0.879829160011815
52	g__Campylobacter_Cluster_164	0.596701216729351
53	g__1XD42-69_Cluster_177	0.879829160011815
54	g__UBA9502_Cluster_179	0.545349668011121
55	g__Enterorhabdus_Cluster_182	0.096303692028687
56	g__Bacilli_genus_Cluster_185	0.939742989577085
57	g__Lawsonibacter_Cluster_189	0.0155644113866339
58	g__Rhodospirillales_genus_Cluster_192	0.70545698611127
59	g__Defluviitaleaceae_UCG-011_Cluster_194	0.173617334424944
60	g__Fusicatenibacter_Cluster_197	0.596701216729351
61	g__Eubacterium_xylanophilum_group_Cluster_199	0.70545698611127
62	g__Kineothrix_Cluster_203	0.325751354478715
63	g__COE1_Cluster_208	0.545349668011121
64	g__Phocaeicola_Cluster_209	0.449691797968892
65	g__Oscillibacter_Cluster_214	0.256839257957856
66	g__Lachnospiraceae_UCG-006_Cluster_216	0.879829160011815
67	g__Eubacterium_fissicatena_group_Cluster_218	0.226476066043488
68	g__Ruminococcus_torques_group_Cluster_226	0.545349668011121
69	g__Eubacterium_Cluster_229	0.325751354478715
70	g__CAG-95_Cluster_234	0.596701216729351
71	g__Roseburia_Cluster_248	0.879829160011815
72	g__UMGS1994_Cluster_255	0.70545698611127
73	g__UCG-003_Cluster_258	0.289918453942572
74	g__Frisingicoccus_Cluster_269	0.0412500165939395
75	g__Duncaniella_Cluster_278	0.820595839755438
76	g__Emergencia_Cluster_280	0.939742989577085
77	g__Erysipelotrichaceae_UCG-003_Cluster_285	0.173617334424944
78	g__AM07-15_Cluster_296	0.65014744409486

79	g_Papillibacter_Cluster_308	0.0412500165939395
80	g_Rothia_Cluster_313	0.70545698611127
81	g_Streptococcus_Cluster_312	0.596701216729351
82	g_Acutalibacter_Cluster_320	0.65014744409486
83	g_Lachnospiraceae_UCG-001_Cluster_323	0.496291702231095
84	g_Lachnospiraceae_FCS020_group_Cluster_336	0.112410584655363
85	g_Lachnoclostridium_Cluster_339	0.65014744409486
86	g_Anaerotruncus_Cluster_341	0.545349668011121
87	g_Murimonas_Cluster_346	0.939742989577085
88	g_NK4A214_group_Cluster_353	0.496291702231095
89	g_A2_Cluster_361	0.65014744409486
90	g_Clostridia_vadinBB60_group_genus_Cluster_363	1
91	g_UMGS1815_Cluster_369	0.762368818469847
92	g_Anaerosacchariphilus_Cluster_373	0.325751354478715
93	g_14-2_Cluster_429	0.289918453942572
94	g_Clostridia_UCG-014_genus_Cluster_435	0.226476066043488
95	g_Paludicola_Cluster_440	0.939742989577085
96	g_Ruthenibacterium_Cluster_460	0.112410584655363
97	g_Christensenellaceae_R-7_group_Cluster_481	0.173617334424944
98	g_Tuzzerella_Cluster_486	0.112410584655363
99	g_Eubacterium_brachy_group_Cluster_493	0.496291702231095
100	g_Family_XIII_UCG-001_Cluster_510	0.00319709944793456
101	g_Parabacteroides_Cluster_509	0.762368818469847
102	g_OEMS01_Cluster_516	0.939742989577085
103	g_Enterococcus_Cluster_527	0.226476066043488
104	g_Ruminococcaceae_genus_Cluster_539	0.096303692028687
105	g_CAG-41_Cluster_540	0.65014744409486
106	g_GCA-900066575_Cluster_548	0.325751354478715
107	g_Eubacterium_nodatum_group_Cluster_546	0.939742989577085
108	g_Butyricoccus_Cluster_565	0.289918453942572
109	g_Candidatus_Stoquefichus_Cluster_619	0.879829160011815
110	g_Lachnospirales_genus_Cluster_670	0.150926950066717
111	g_Clostridia_genus_Cluster_693	0.596701216729351
112	g_Prevotellamassilia_Cluster_699	0.939742989577085
113	g_Eisenbergiella_Cluster_707	0.820595839755438
114	g_Stoquefichus_Cluster_717	0.364346126633552
115	g_Peptococcus_Cluster_722	0.496291702231095
116	g_CAG-56_Cluster_749	0.545349668011121
117	g_Candidatus_Soleaferrea_Cluster_747	1
118	g_Eubacterium_J_Cluster_775	0.325751354478715

119	g_Adlercreutzia_Cluster_789	1
120	g_CAG-508_genus_Cluster_799	0.939742989577085
121	g_Prevotellaceae_UCG-001_Cluster_803	0.879829160011815
122	g_Bacteria_genus_Cluster_817	0.545349668011121
123	g_Candidatus_Saccharimonas_Cluster_840	0.939742989577085
124	g_CAG-110_Cluster_871	0.325751354478715
125	g_Coprococcus_Cluster_875	0.879829160011815
126	g_Flavonifractor_Cluster_893	0.130570018115735
127	g_Staphylococcus_Cluster_902	0.496291702231095
128	g_Family_XIII_AD3011_group_Cluster_948	0.596701216729351
129	g_QXXE01_Cluster_954	0.405678895285055
130	g_Christensenellaceae_genus_Cluster_978	0.150926950066717
131	g_CAG-81_Cluster_999	0.496291702231095
132	g_MD308_Cluster_1054	0.596701216729351
133	g_Anaerovorax_Cluster_1050	0.449691797968892
134	g_An92_Cluster_1053	0.939742989577085
135	g_Parasutterella_Cluster_1044	0.820595839755438
136	g_Dysosmobacter_Cluster_1099	0.173617334424944
137	g_Oscillospirales_genus_Cluster_1076	0.545349668011121
138	g_D16-34_Cluster_1121	0.405678895285055
139	g_Eubacterium_hallii_group_Cluster_1206	0.879829160011815
140	g_Angelakisella_Cluster_1290	0.545349668011121
141	g_Erysipelatoclostridiaceae_genus_Cluster_1268	0.130570018115735
142	g_Desulfovibrio_Cluster_1285	0.939742989577085
143	g_Mediterraneibacter_Cluster_1304	0.198764606373234
144	g_UCG-010_genus_Cluster_1319	0.150926950066717
145	g_Bilophila_Cluster_1307	0.70545698611127
146	g_Acetatifactor_Cluster_1361	0.130570018115735
147	g_Intestinimonas_Cluster_1355	0.364346126633552
148	g_Defluviitaleaceae_genus_Cluster_1422	0.939742989577085
149	g_Allobaculum_Cluster_1858	0.762368818469847

Supplementary file 4. List of primers. Primers used for the inactivation of *gadB* in *Lactococcus lactis* NCDO2118.

Primer name	5'-3' sequence	
821-GBgadCR	GGAATTCGATTTAGATGCCATAGGAGGATTTTC	Amplification of <i>gadB</i> upstream sequence
894-GBgadC2F	GATGAATATCGTACATCCTCCAATTTTTTAATG	
892-GBkefA2F	AAGCTTGATAAAACAAGAAAATATTCATGAAATTC AG	Amplification of <i>gadB</i> downstream sequence
893-GBkefA2R	GGAGGATGTACGATATTCATCTTAAGAAAAATCAA AAGC	
822-GBpGhost9EVF	CATCTAAATCGAATTCCTGCAGCCCCG	Amplification of pGhost9 backbone
891-GBpGh9EV2R	TCTTGTTTTATCAAGCTTATCGATACCGTC	
898-amt GadB(DCO)	TTGGATTAGCTGCGCATATTTTATCG	Verification of <i>gadB</i> deletion onto the chromosome
899-avl GadB(DCO)	CCTTGTTGACCATAATGCAAAGCAGGT	

Primer name	5'-3' sequence
GabSeq_2_F	AAAATATAGAAGGAGACTATTGCAAATAGC
GabSeq_2_R	AAAAATTAATGGCCATCGTTGGTAGTTCTC
GabSeq_3_F	TCTGTGCAGCAGAAATGGCGACGGTTGAA
GabSeq_3_R	TCCCATAAATTTTTCTTTTCACTCGCAT
GabSeq_4_F	CGGTTATTCCTCAAAAAGACTTATCATTA

CHAPTER 2 - *Lactococcus lactis* CNCM I-5388 versus NCDO2118 by its GABA**hyperproduction ability, counteracts faster stress-induced****intestinal hypersensitivity in rats**

In the first chapter of section II, we have unraveled the GAD/GABA anti-VH properties of GABA-producer *L. lactis* NCDO2118 supplemented with L-glutamic acid (L-Glu) after 10-day oral treatment. Here, we contrast the anti-VH properties of NCDO2118 and higher GABA-producing strain CNCM I-5388. We firstly aimed to understand, through GAD activity assays and *in silico* comparison of GAD enzymes, why these two strains, belonging to the same species, present such differences in GABA production. We have also compared the GAD activity of the two strains, facing different scales of HCl-induced acidic stress *in vitro* under static conditions, as a first attempt to better comprehend the GABA production of *L. lactis* in host's acidic stomach. Finally, we expanded the analyze of the anti-VH properties in order to reveal if higher GABA-producer CNCMI-5388 has faster analgesic effects than NCDO2118 (e.g. treatment duration reduced to five days, single-dose treatment) while sharing the same mechanisms of action (dependency of GAD/GABA, action on host's GABA_B receptors and independency of the gut microbiota composition assessed via alpha and beta diversities).

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RESEARCH ARTICLE

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

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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.

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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 CO₂ 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 NH₃.¹¹ 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 vitro¹³ 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 England 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 55mM (8g/L) L-Glu, 29mM (5g/L) arginine, 250mM (45g/L) glucose, and 300mM. Fermentations were carried out under oxygen-limiting conditions at 30°C. pH was maintained at 6.6 by KOH addition for 8h, then pH was dropped and regulated at 4.6. Culture was inoculated with cells from pre-cultures grown in Erlenmeyer flask on similar medium, harvested during the exponential phase and concentrated to obtain an initial optical density (OD) at 580nm of 0.25 in the fermenter. Bacterial growth was monitored by spectrophotometric measurements at 580nm (Libra S11, Biochrom, BIOSERV, Massy, France; 1 unit of optical density is equivalent to 0.3g dry weight/L). Samples were collected every 30min 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 7h). 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, 150mg of bacterial cells were harvested at 7 and 24h of culture.

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

For cytoplasmic GAD activity measurement, 150mg cells were washed twice with 0.2% KCl [w/v] and suspended in 3mL sodium acetate buffer (100mM, pH4.6) containing 4.5mM MgCl₂, 22% [v/v] glycerol and 1.5mM dithiothreitol. This mixture was distributed into three tubes containing 6mg of glass beads. Then, cells were disrupted in a FastPrep-24 homogenizer (MP Biomedicals, Illkirch, France) using 6cycles of 30s at 6.5m/s interrupted by 1min incubation on ice. Cell debris were removed by centrifugation for 15min at 10000g 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, 240mM NaCl was added to the sodium acetate buffer and pH was controlled with 100mM 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 $22 \pm 2^\circ\text{C}$ 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#14898-20180430160331426v2 (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 *ΔgadB* (10⁹ 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 into two groups and orally treated for 5 days (1 mL/rat) (1 mL/rat) with: (a) *L. lactis* CNCM I-5388 (10⁹ 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 (10⁹ 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 ANOMALY²² 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 \pm 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

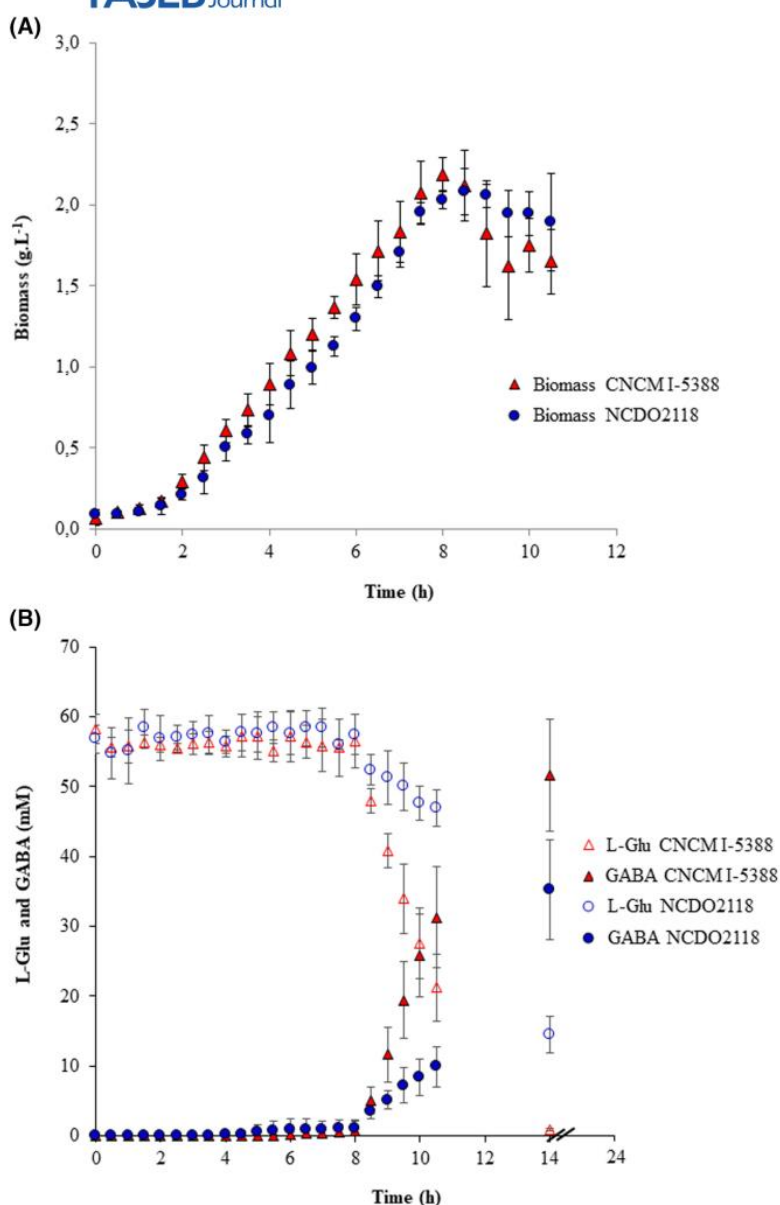


FIGURE 1 (A) Evolution of biomass (g/L) and (B) GABA production and L-Glu consumption (mM) during 10.5-h growth of *L. lactis* CNCM I-5388 and NCDO2118 in bioreactor.

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 $\Delta gadB$, 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.

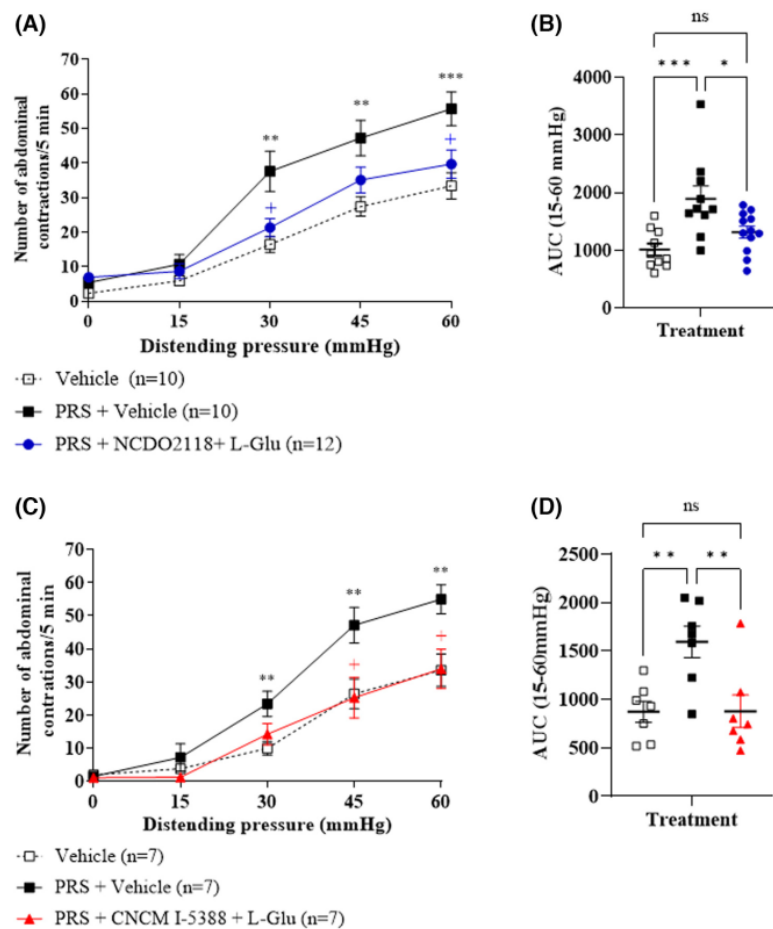
<i>L. lactis</i> strain	CNCM I-5388	NCDO2118
μ_{max} (h^{-1})	0.86 ± 0.06	0.81 ± 0.05
$q_{glucose}$ (mmol/g.h)	18.5 ± 2.6	15.1 ± 1.1
$\nu_{lactate}$ (mmol/g.h)	36.4 ± 4.5	30.3 ± 1.4
q_{L-Glu} (mmol/g.h)	7.9 ± 1.8	1.6 ± 0.4*
ν_{GABA} (mmol/g.h)	7.2 ± 2.1	1.8 ± 0.7*
[L-Glu] _{10.5 h} (mM)	21.3 ± 4.8	46.9 ± 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 Δ gadB 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 ($813.0 \pm 183.7 \mu\text{mol}/\text{min}\cdot\text{mg}$), consistently with its GABA hyperproduction ability, compared with NCDO2118 ($48.7 \pm 10.7 \mu\text{mol}/\text{min}\cdot\text{mg}$) ($p < .05$) (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 \pm 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 ($p > .05$).

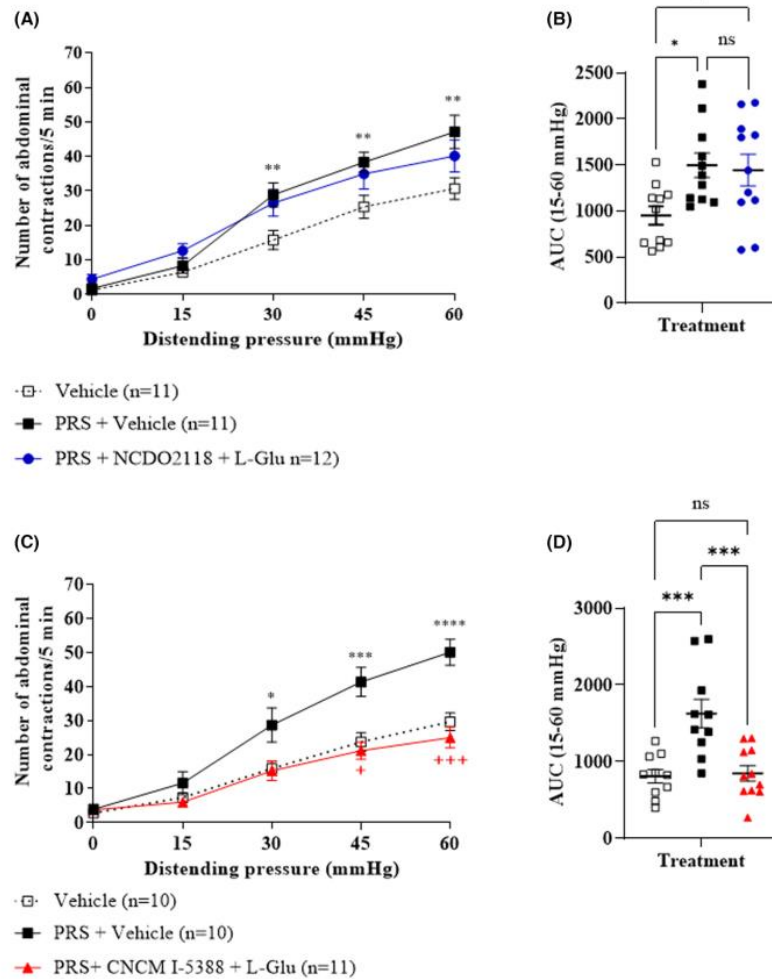
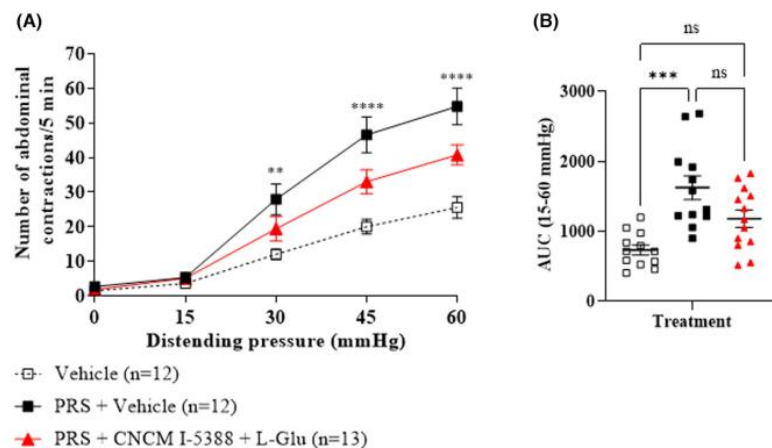


FIGURE 4 Effect of a single oral administration of *L. lactis* CNCM I-5388 in the presence of L-Glu monosodium salt hydrate on PRS-induced VH induced by stress in response to colorectal distension. (A) Treatment response to all colorectal distension pressures (15 to 60 mmHg) and respective (B) area under the curve. Data are expressed as mean \pm 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

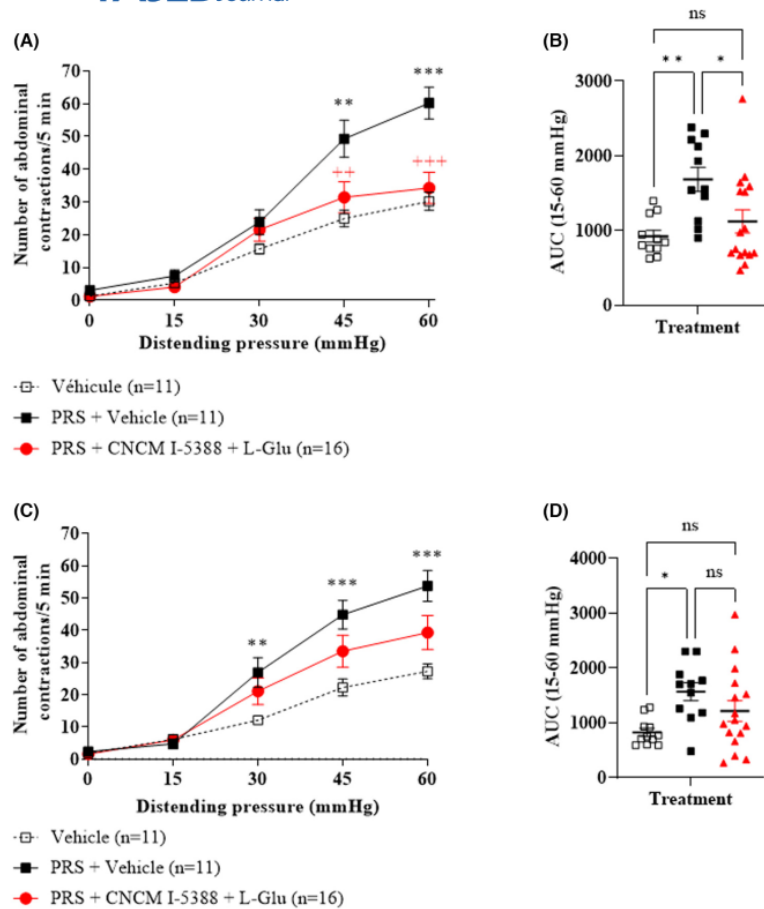


FIGURE 5 Effect of 5-day oral administration followed by 5-day treatment interruption of *L. lactis* CNCM I-5388 in the presence of L-Glu monosodium salt hydrate on VH induced by PRS in response to colorectal distension. (A) 5-day treatment response to all colorectal distension pressures (15–60 mmHg) 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 \pm 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.

<i>L. lactis</i> strain	GAD 7h ($\mu\text{mol}/\text{min}\cdot\text{mg}$)	q arginine (mmol/g/h)	q ornithine (mmol/g/h)	v citrulline (mmol/g/h)
CNCM I-5388	813.0 \pm 183.7	3.6 \pm 0.5	3.5 \pm 0.7	0.5 \pm 0.3
NCDO2118	48.7 \pm 10.7*	9.2 \pm 0.1*	5.3 \pm 0.5*	2.8 \pm 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.

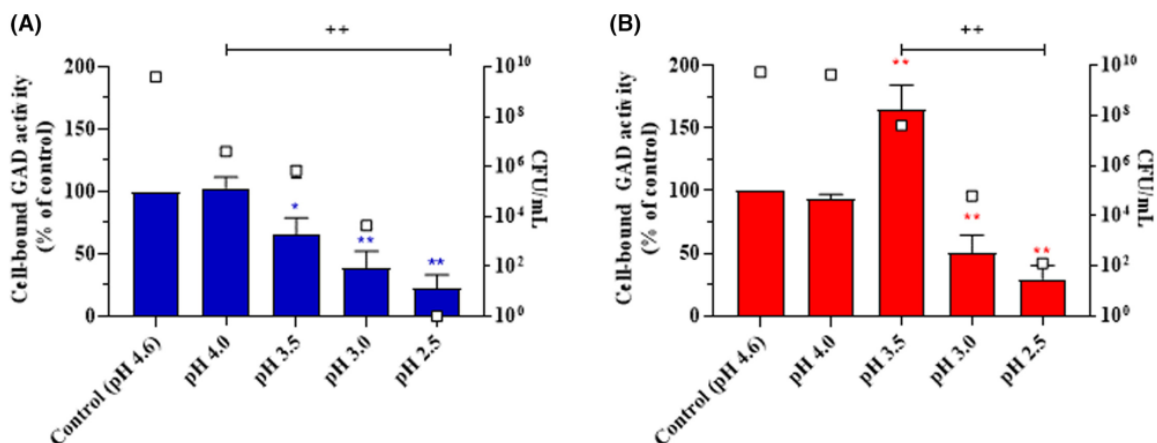
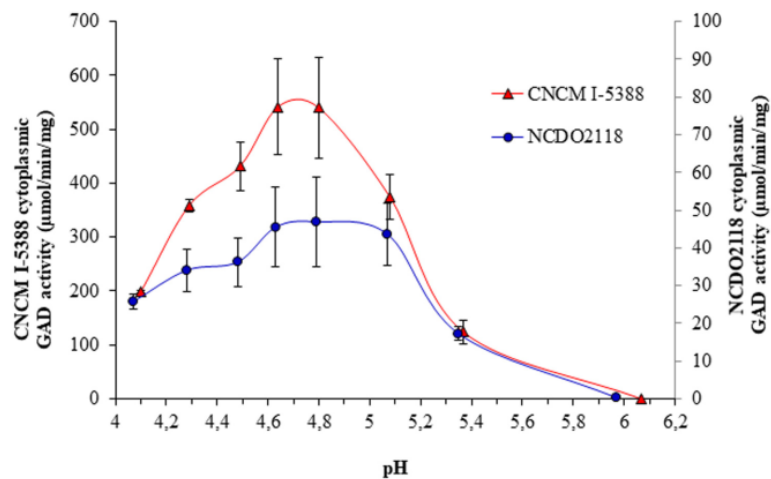


FIGURE 7 *L. lactis* cell-bound GAD activity and bacterial viable counts after HCl-induced acidic stress. (A) NCDO2118. (B) CNCM I-5388. Bars represent percentage values for cell-bound GAD activity (% of control (pH 4.6)) and squares represent values for CFU/mL. Data are expressed as mean \pm SEM. * $p < .05$, ** $p < .01$ 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 Δ pH (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 lumen¹⁴; 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 CNS³⁴. 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.

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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.

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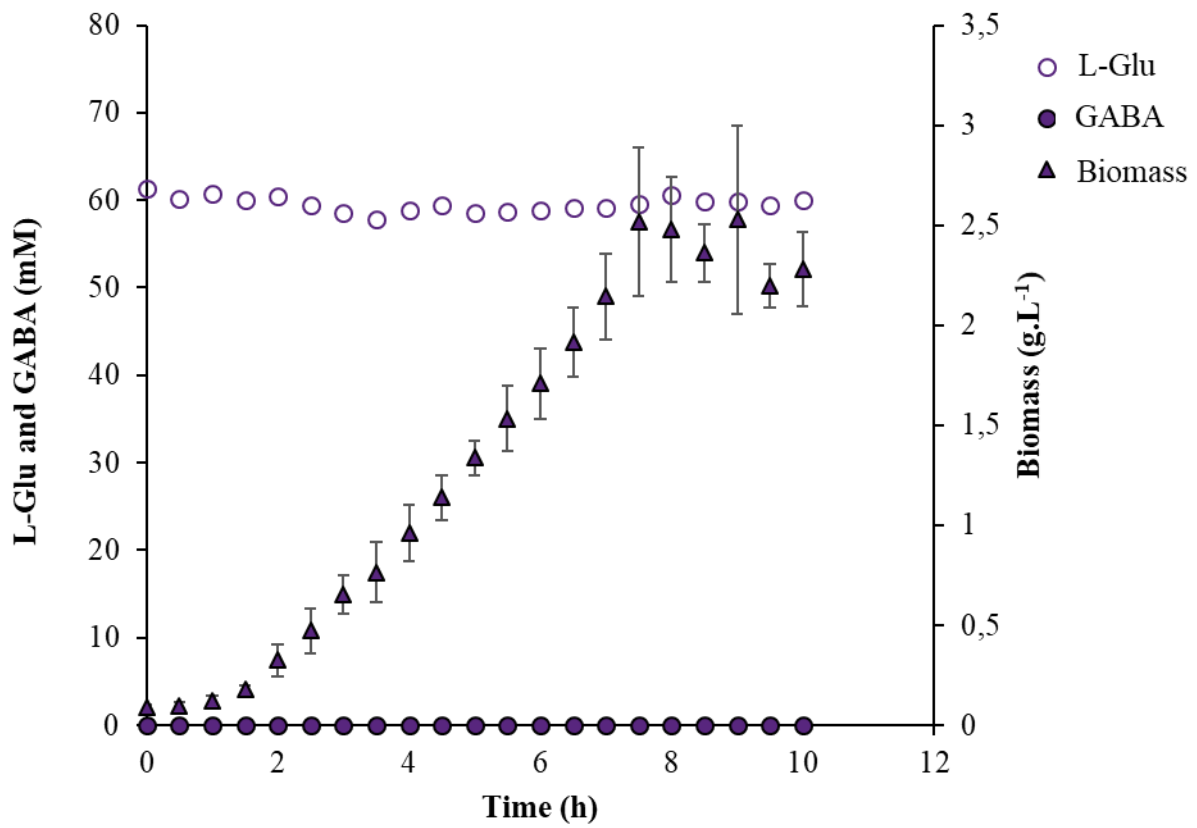
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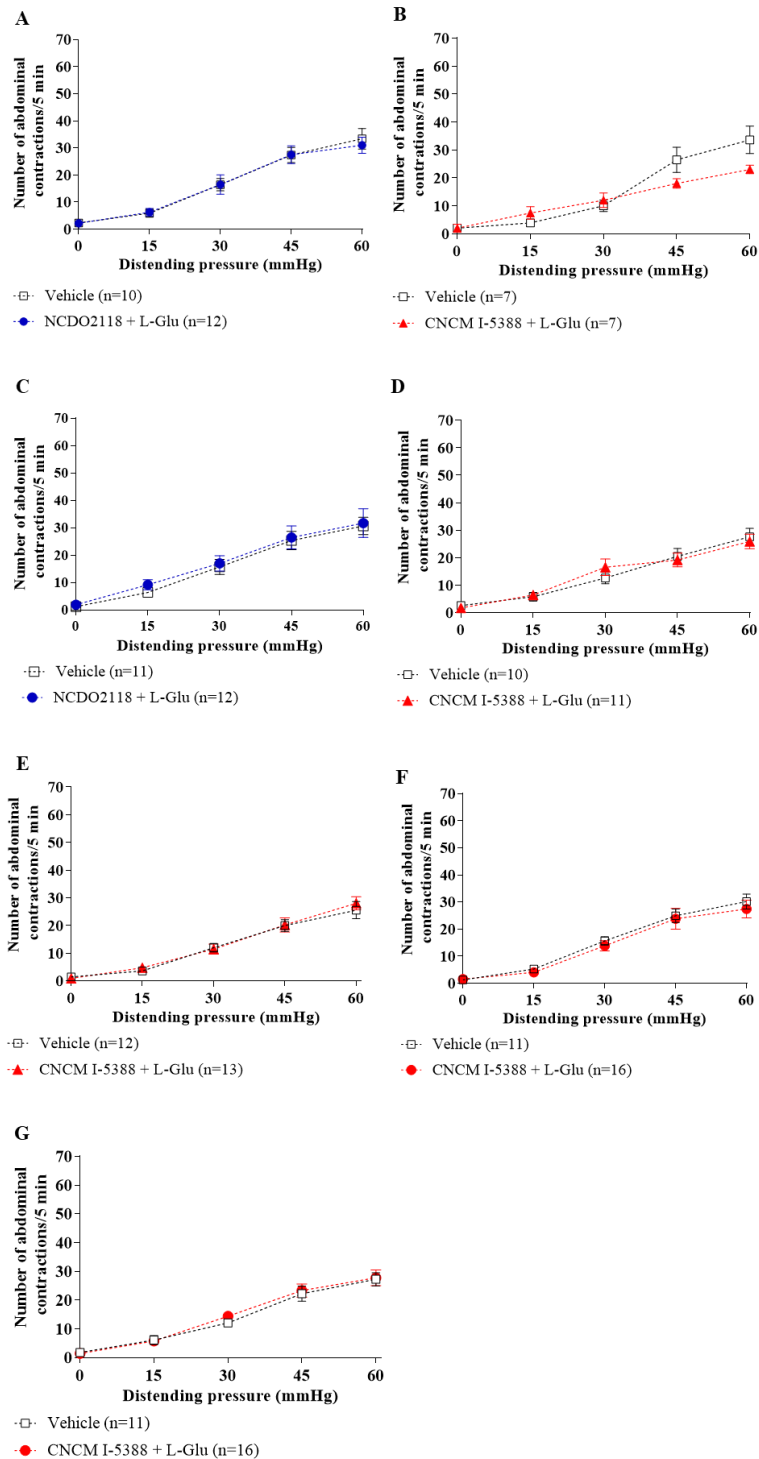
SUPPORTING INFORMATION

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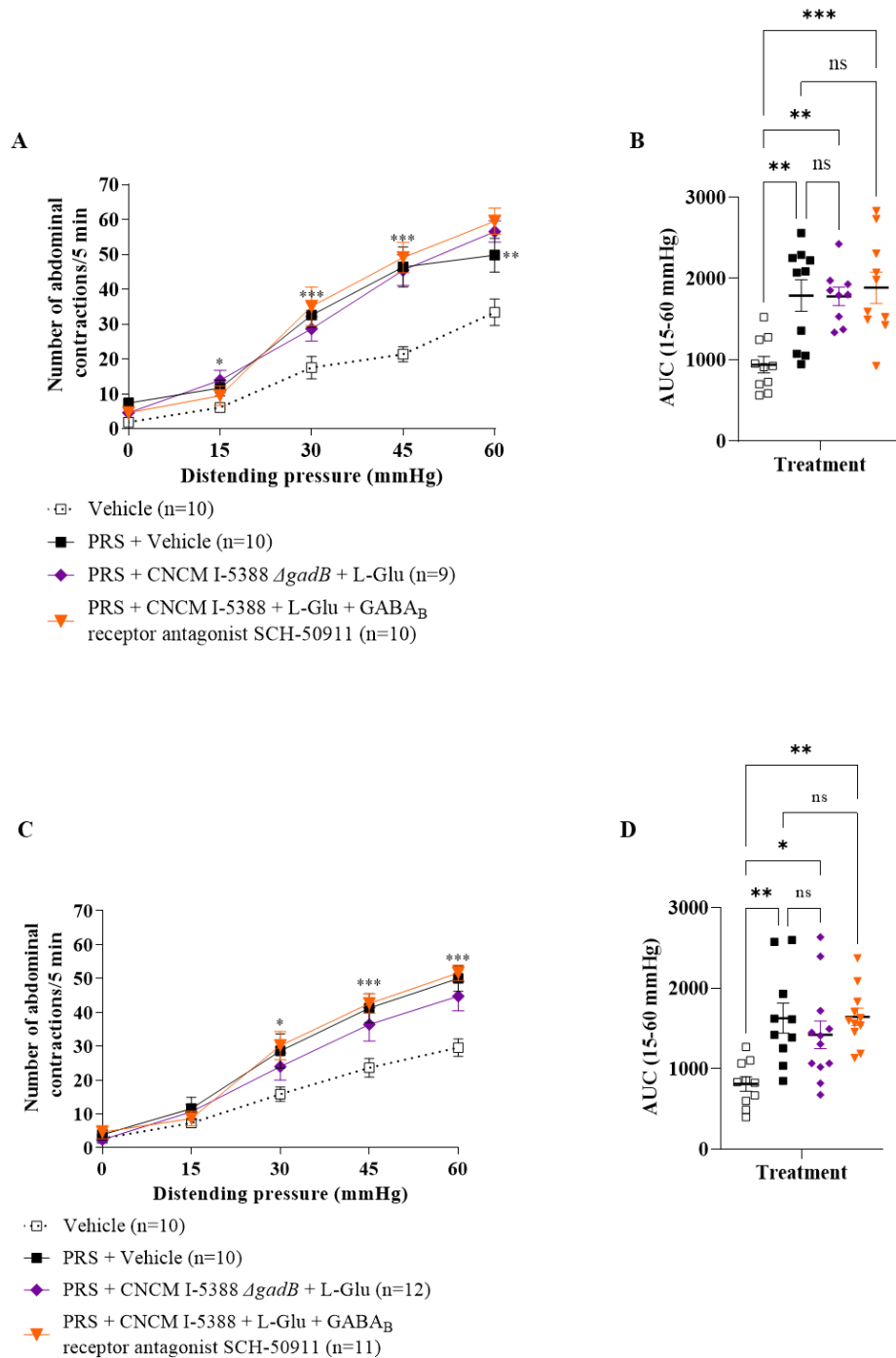
How to cite this article: Gomes P, Laroute V, Beaufrand C, et al. *Lactococcus lactis* CNCM I-5388 versus NCDO2118 by its GABA hyperproduction ability, counteracts faster stress-induced intestinal hypersensitivity in rats. *The FASEB Journal*. 2023;37:e23264. doi:[10.1096/fj.202301588R](https://doi.org/10.1096/fj.202301588R)



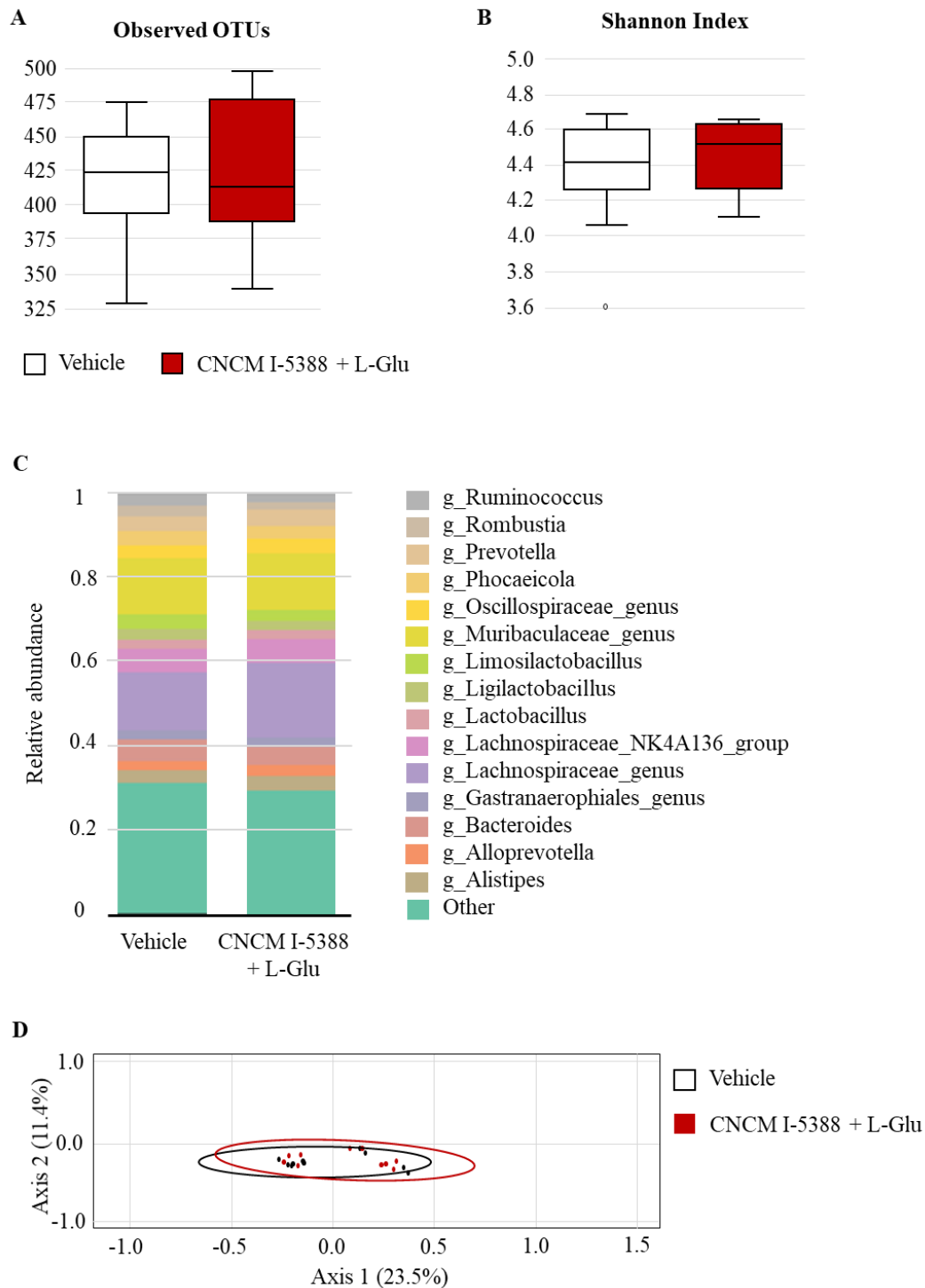
Supplementary figure 1. GABA production (filled circles) and L-Glu consumption (mM) and evolution of biomass (g/L) during 10.5-hour growth of *L. lactis* CNCM I-5388 Δ gadB.



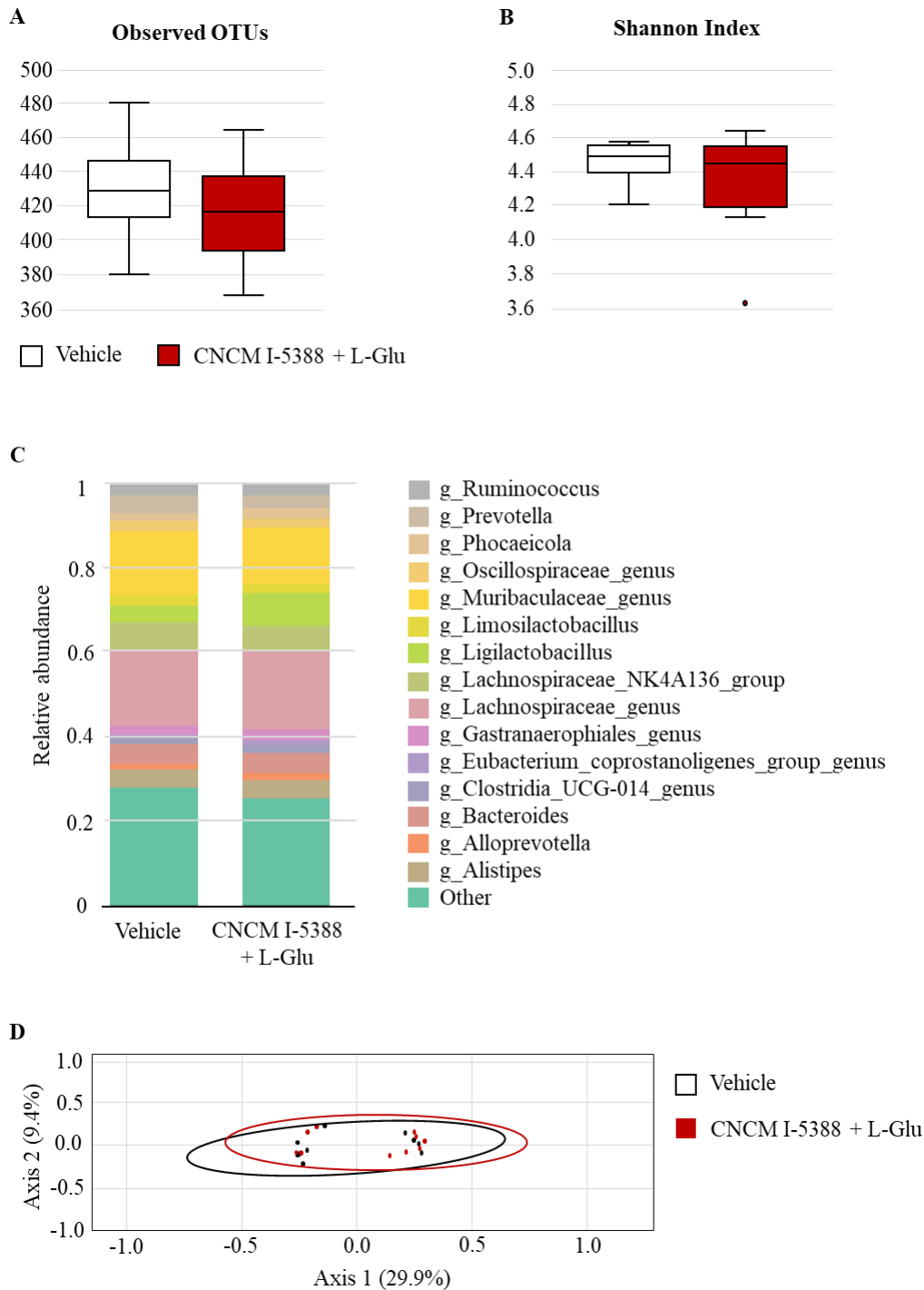
Supplementary figure 2. Effect of oral administration of *L. lactis* supplemented with L-Glu monosodium salt hydrate on non-stressed animals to all colorectal distension pressures (15 to 60 mmHg). 10-day oral administration of (A) NCDO2118 and (B) CNCM I-5388 and 5-day oral administration of (C) NCDO2118 and (D) CNCM I-5388. (E) 1-day oral administration of CNCM I-5388 supplemented with L-Glu monosodium salt hydrate. (F) 5-day oral administration of CNCM I-5388 followed by (G) 5-day treatment interruption. Data are expressed as mean \pm SEM. No significant statistical differences were detected ($p > 0.05$).



Supplementary figure 3. Effect of oral administration of *L. lactis* CNCM I-5388 $\Delta gadB$ and CNCM I-5388 in the presence of GABA_B receptor antagonist SCH-50911 on VH induced by PRS in response to colorectal distension. (A) 10-day oral administration for all colorectal distension pressures (from 15 to 60 mmHg) and respective (B) area under the curve. (C) 5-day oral administration for all colorectal distension pressures (from 15 to 60 mmHg) and respective (D) area under the curve. Data are expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ stressed animals vs. basal values for animals treated with vehicle. No statistical differences ($p > 0.05$) were detected for stressed animals treated with either CNCM I-5388 $\Delta gadB$ or CNCM I-5388 in the presence of SCH-50911 vs. values for stressed animals treated with vehicle.



Supplementary figure 4. Overview of the fecal bacterial microbiota after 10-day daily oral administration of *L. lactis* CNCM I-5388 supplemented with L-Glu monosodium salt hydrate. (A) Alpha diversity within fecal samples. Two representations are displayed: (A) mean observed number of OTUs per sample, an estimate of richness and (B) Shannon Index, indicating the evenness of the sample. One-way ANOVA followed by the *post hoc* pairwise Tukey's test revealed no statistical differences between samples ($p > 0.05$). (C) Top 15 dominant bacterial genera in fecal samples. (D) Multidimensional scaling (MDS) plot of the Bray-Curtis distance between samples, a representation of the phylogenetic similarity. PERMANOVA revealed no statistical differences between samples ($p > 0.05$).



Supplementary figure 5. Overview of the fecal bacterial microbiota after 5-day daily oral administration of *L. lactis* CNCM I-5388 supplemented with L-Glu monosodium salt hydrate. (A) Alpha diversity within fecal samples. Two representations are displayed: (A) mean observed number of OTUs per sample and (B) Shannon Index. One-way ANOVA followed by the *post hoc* pairwise Tukey's test revealed no statistical differences between samples ($p > 0.05$). (C) Top 15 dominant bacterial genera in fecal samples. (D) MDS ordination plot of the Bray-Curtis distance between samples. PERMANOVA revealed no statistical differences between samples ($p > 0.05$).

A

NCDO2118	MNQKLSLFGFFALTASMLTVYVEYPTFATSKLHLVFFLLGGLLWFLPVALCAAEMATV	60
CNCM I-5388	MNQKLSLFGFFALTASMLTVYVEYPTFATSKLHLVFFLLGGLLWFLPVALCAAEMATV *****	60
NCDO2118	EGWKNGGIFSWVSQTLGERFGFAAIFQWFQITVGFVTMIYFILGALSIVLNFQALNTDP	120
CNCM I-5388	EGWKNGGIFSWVSQTLGERFGFAAIFQWFQITVGFVTMIYFILGALSIVLNFQALNTDP *****	120
NCDO2118	LIKFIGLLIIFWGLTFSQLGGTQRTAKLVKAGFVVGIVIPSIILFGLAAAYFIGGNPIEI	180
CNCM I-5388	LIKFIGLLIIFWGLTFSQLGGTQRTAKLVKAGFVVGIVIPSIILFGLAAAYFIGGNPIEI *****	180
NCDO2118	PINKRAFVPDFSQVSTLVVFSFILAYMGVEASASHINELENPKRNYPLAMILLVILAIS	240
CNCM I-5388	PINKHAFVPDFSQVSTLVVFSFILAYMGVEASASHINELENPKRNYPLAMILLVILAIS ***.:*****	240
NCDO2118	LDAIGGFSVAAVIPQKDLSSAGVIQTFQTLILHFNHHLGWLKVIKIALMIAFGVMGEVSS	300
CNCM I-5388	LDAIGGFSVAAVIPQKDLSSAGVIQTFQTLILHFNHHLGWLKVIKIALMIAFGVMGEVSS *****	300
NCDO2118	WVVGPSRGMFAAAQRGLLPKFLRKTNTHEVPVPLVMIQGIIVTLWGAVLTFGGGGNNLSF	360
CNCM I-5388	WVVGPSRGMFAAAQRGLLPKFLRKTNTHEVPVPLVMIQGIIVTLWGAVLTFGGGGNNLSF *****	360
NCDO2118	LVAISLTVVIYLVGYLLFFIGYFVLIYKKNLKRNTYNVPGKRVGKTIAGIGFLLSIFAL	420
CNCM I-5388	LVAISLTVVIYLVGYLLFFIGYFVLIYKKNLKRNTYNVPGKRVGKTIAGIGFLLSIFAL *****	420
NCDO2118	FISFVPPASIAKNETHYQMILLISFVVTAILPFIVYELHDKRGHDTIEEPRHFKARDVN	480
CNCM I-5388	FISFVPPASIAKNETHYQMILLISFVVTAILPFIVYELHDKRGHDTIEEPRHFKARDVN *****	480
NCDO2118	PAIYPAARGEHHIIKKEEHILKH	503
CNCM I-5388	PAIYPAARGEHHIIKKEEHILKH *****	503

B

NCDO2118	MLYGKENRDEAEFLEPIFGSESEQVDLPKYKLAQKSIEPRVAYQLVQDEMLDEGNARLNL	60
CNCM I-5388	MLYGKENRDEAEFLEPIFGSESEQVDLPKYKLAQKSIEPRVAYQLVQDEMLDEGNARLNL *****	60
NCDO2118	ATFCQTYMEPEALKLMSQTLKNAIDKSEYPRTEIENRCVNMIADLWNAEKEKFMGTS	120
CNCM I-5388	ATFCQTYMEPEAVKLMSTLEKNAIDKSEYPRTEIENRCVNMIADLWNAEKEKFMGTS *****:*****	120
NCDO2118	TIGSSEACMLGGMAMKFSWRKRAEKLGLDINAKKPNLVISSGYQVCWEKFCVYWDIEMRE	180
CNCM I-5388	TIGSSEACMLGGMAMKFSWRKRAEKLGLDINAKKPNLVISSGYQVCWEKFCVYWDIEMRE *****	180
NCDO2118	VPMDKEHMSINLDKVMYDVEYTIQVVGIMGITYTGRYDDIKALDNLIBEYNKQTDYKVY	240
CNCM I-5388	VPMDKEHMSINLDKVMYDVEYTIQVVGIMGITYTGRYDDIKALDNLIBEYNKQTDYKVY *****	240
NCDO2118	IHVDAASGGLYAPFVEPELEWDFRLKNVISINTSGHKYGLVYPGVGWLWRDKKYLPEEL	300
CNCM I-5388	IHVDAASGGLYAPFVEPELEWDFRLKNVISINTSGHKYGLVYPGVGWLWRDKKYLPEEL *****	300
NCDO2118	IFKVSYLGGELPTMAINFSHSASQLIGQYYNFVRYGFDGYKAIHERTHKVAMFLAKEIEK	360
CNCM I-5388	IFKVSYLGGELPTMAINFSHSASQLIGQYYNFVRYGFDGYKAIHERTHKVAMFLAKEIEK *****	360
NCDO2118	TGMFEIMNDGSQLPVICYKLENSNRGWNLYDLADRLMKGWQVPAYPLPKNLENEIIQR	420
CNCM I-5388	TGMFEIMNDGSQLPVICYKLENSNRGWNLYDLADRLMKGWQVPAYPLPKNLENEIIQR *****:*****	420
NCDO2118	LVIRADFGMNAFNQVDMQEAIEALNKAHILYHEEPENKTYGFTH	466
CNCM I-5388	LVIRADFGMNAFNQVDMQEAIEALNKAHILYHEEPENKTYGFTH *****	466

Supplementary figure 6. *L. lactis* NCDO2118 and CNCM I-5388 GAD and GadC amino acid conservation. (A) GAD amino acid residue alignments. (B) GadC amino acid residue alignment. Asterisks = positions with a single, fully conserved residue. Colons = positions with conservation between amino acid groups of similar properties. Dots

CHAPTER 3**Is the optimal visceral antinociceptive effect of *Lactococcus lactis* CNCM I-5388 in rat dependent of its viability?**

In the second chapter of section II, we have shown that higher GABA-producer *L. lactis* CNCM I-5388 has faster antinociceptive properties than the NCDO2118 reference strain. Also, applying a HCl-induced acidic stress *in vitro*, we have shown an enhanced cell-bound GAD activity at pH 3.5 for this specific strain while a loss in bacterial viable counts began to be detected. In the following, we focused only in *L. lactis* CNCM I-5388 and its higher anti-visceral hypersensitivity (VH) properties. Here, we wondered if inactive CNCM I-5388 cells would have similar *in vitro* GAD activity and *in vivo* anti-VH properties, including under single-dose and wash-out treatments, compared to the viable strain. We also wondered if both forms would differ in their GABA content *in vivo* in host's stomach but also other gastrointestinal compartments. Finally, we compared GABA production of viable vs inactive CNCM I-5388 in a dynamic gastrointestinal human-like model (TIM-1), focusing in the stomach compartment for supporting our findings on the animal model.

This work is in preparation for publishing in 2024. I am the first author of this article.

***Lactococcus lactis* CNCM I-5388 as a carrier of an active GAD enzyme: a novel approach for alleviating visceral hypersensitivity through bacterial GABA production in the stomach**

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Abbreviations: **CFU**, colony-forming units; **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, **EtOH**, Ethanol

Keywords: *Lactococcus lactis*, GABA, GAD activity, psychological acute stress, visceral hypersensitivity, bacterial viability, TIM-1

ABSTRACT

Irritable Bowel Syndrome (IBS) is a common multifactorial disorder of the gut-brain axis, often associated with stress, characterized by visceral hypersensitivity (VH) as the cardinal symptom, associated with altered bowel movement and often bloating in the absence of morphological changes. Patients with such functional gastro-intestinal disorders also have high rates of psychological comorbidity and treatments focusing on reducing stress and anxiety (e.g., hypnotherapy, cognitive behavioral therapy, exercise, and antidepressants) appear to be good strategies. Research highlights the potential therapeutic impact of probiotics and more recently postbiotics, particularly those with psychobiotics properties, on IBS. Through their ability to synthesize GABA, the main central nervous system inhibitory neurotransmitter in mammals, psychobiotics are able to alleviate gastrointestinal discomfort associated with VH. The use of postbiotics is promising as they eliminate the necessity of live microorganisms and the potential acquisition of antibiotic resistance genes or virulence factors. We have previously shown the potential of GABA-producing *Lactococcus lactis* CNCM I-5388 through its glutamate decarboxylase (GAD) enzyme to mitigate VH induced by acute stress in a rat model in a GAD-dependent manner. Here we demonstrate the anti-VH properties of CNCM I-5388 in its postbiotic “active GAD-bag” form. By inactivating CNCM I-5388 with ethanol treatment, our findings revealed that after a daily oral treatment, nonviable bacterial cells exhibited analgesic properties after 10 or 5-day oral treatment, similarly to their viable counterparts. However, they failed to exert anti-VH properties in rats at the first day of oral treatment and after 5-day treatment followed by 5-day washout. Furthermore, our investigation unveiled a crucial factor contributing to this analgesic effect: the production of GABA within the rat stomach, driven by its acidity, was observed in the host exclusively under stress conditions. Finally, the use of TIM-1 dynamic *in vitro* model allowed us to validate the GABA-production of viable and nonviable CNCM I-5388 in human-like gastric conditions. These results underscore the probiotic and

postbiotic anti-VH properties of CNCM I-5388 as an innovative approach for managing IBS symptoms, shedding light on the intriguing interplay between transient bacterial metabolism, gut physiology, and stress-induced VH.

INTRODUCTION

Irritable Bowel Syndrome (IBS) is a chronic disorder of the gut-brain interaction that affects 3-5% of the global population, following the Rome IV criteria, with incidence 2-fold higher for women than men. It has not only a considerable impact on quality of life and healthcare costs, but also making it the largest group of gastrointestinal disorders seen in gastroenterology clinics ¹. Central to the multifaceted nature of IBS are the alteration of bowel habits (either diarrhea or constipation) and visceral hypersensitivity (VH), which is characterized by heightened perception of abdominal pain in the absence of organic causes ². The factors believed to play a role in the development of such symptoms are diverse and they encompass a range of environmental and host-related characteristics, including not only changes in pain perception and gut-brain interaction, enteric infections and microbial dysbiosis, but also psychosocial stress ³. Existing literature indicates that stress, anxiety and depression are prevalent in individuals with IBS ^{2,3}. Among some IBS patients, augmented intestinal permeability, linked with heightened VH, is displayed. This feature can contribute to more severe IBS symptoms, altered pain perception, and increased sensitivity to both somatic and visceral stimuli ⁴.

The brain-gut interplay, characterized by reciprocal interactions between the central nervous system and the gastrointestinal tract (GIT), including gut microbiota, actively influences and regulates various physiological functions involved in IBS ⁵. Undeniably, gut microbiota and orally-administered bacteria play a crucial role, facilitating neural, endocrine, immune, and humoral connections ⁶. Studies concerning ingested bacteria have shown that several probiotic bacterial genera (e.g. *Lactobacillus*, *Bifidobacterium*, *Bacillus*) promote beneficial effects alleviating IBS symptoms in patients ⁷. Moreover, postbiotics have been used more recently for IBS symptom management ⁸. While probiotics are described by the World Gastroenterology Organization as "live microorganisms that, when administered in sufficient

quantities, confer health benefits on the host" ⁹, postbiotics are defined as “a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host” ⁸. From safety, downstream processing and formulation perspectives, postbiotics offer advantages such as extended shelf life, simplified storage and transportation requirements, and decreased reliance on maintaining low temperatures when contrasted with probiotics, since it does not contain any more live microorganisms. This is a particularly critical factor in children with developing immune systems and compromised intestinal barriers ¹⁰. When considering therapeutic advantages, it's crucial to emphasize the superior safety profile of postbiotics in comparison to probiotics. A clear advantage of postbiotics lies in their ability to circumvent the potential acquisition of antibiotic resistance genes and virulence factors, a concern that may arise with the use of probiotics *in vivo* ¹¹.

A novel category within the realm of ingested bacteria with health properties is referred to as psychobiotics. These are bacterial strains capable of exerting influence over brain function by impacting immune responses, hormonal signaling, and modulation of neurotransmitter levels and availability ¹². Furthermore, such synthesized neurotransmitters, for instance serotonin, dopamine and gamma (γ)-amino butyric acid (GABA), might act locally in the GIT on the peripheral nervous system or enter the bloodstream and be transported to various parts of the body (Chen et al., 2021). GABA is produced by many bacteria, gut commensals or food-borne transient ones, especially lactic acid bacteria (LAB) ¹⁴. Within the GIT, GABA produced by bacteria primarily exerts its effects by binding to GABA_A and GABA_B receptors. Metabotropic GABA_B receptors are mainly found in the fibers of the vagal origin within the enteric nervous system and in the glandular portion of the gastric mucosa. Activation of these GABA-dependent receptors enables the modulation of intestinal motility, gastric emptying, transient relaxation of the lower esophageal sphincter, and visceral sensitivity to painful colonic stimuli ¹⁵. Therefore, the use of GABA-producing bacteria might represent a potential treatment

against IBS symptoms, especially VH.

Within these GABA-producing bacteria, the glutamate decarboxylase (GAD, EC 4.1.1.15) system, comprising the GAD enzyme (encoded by the gene *gadB*) and the glutamate/GABA antiporter GadC, plays a crucial role. GadC facilitates the transportation of L-glutamic acid (L-Glu) into the microorganism. Once inside the cytosol, L-Glu undergoes decarboxylation by the pyridoxal-5'-phosphate-dependent GAD enzyme, resulting in the formation of GABA while consuming cytoplasmic H⁺ and releasing CO₂ as a byproduct. Finally, GABA is transported outside the cytoplasm by GadC¹⁶. Such a system is common in LAB, particularly adapted to grow under acidic conditions (Yogeswara et al., 2020). As proposed elsewhere, a core function of bacteria GAD might be to aid them in transit and survival by enabling adaptation to the harsh acidic conditions of the stomach^{16,18}. Accordingly, orally administered pathogenic bacterium *Brucella microti* single and double mutants of *gadB* and *gadC* failed to survive under acidic stress (pH 2.5)¹⁹.

Our previous findings demonstrate that the food-borne LAB *L. lactis* NCDO2118 was able to reduce VH in an acute-stress IBS-like rat model and to produce GABA under *in vivo* and stomach-like *in vitro* conditions. These observations raised the possibility of a dynamic "virtuous circle" forming between GABA-producing *L. lactis* and the host²⁰. Within this framework, the GAD enzyme could become active in the acidic environment of the host's stomach, thereby stimulating GABA_B receptors that are abundantly distributed in the GIT, with a decreasing number from the mouth to the anus¹⁵. Consequently, this activation would lead to an increased gastric secretion through vagal cholinergic and gastrin-dependent mechanisms, thereby maintaining acidic conditions in the stomach^{15,20}. Additionally, we have formerly demonstrated that *L. lactis* CNCM I-5388 is a higher GABA producer *in vitro* and exerts faster anti-VH properties in the same acute-stress rat model than NCDO2118²¹. Using both mutant *L. lactis* Δ *gadB* strains and GABA_B receptor antagonist, we have demonstrated that these

antinociceptive properties were dependent on the GAD activity of *L. lactis* and mediated by host's GABA_B receptors. Moreover, we have shown that CNCM I-5388 displayed not only a higher cytoplasmic GAD activity, while sharing the same optimal pH as NCDO2118 GAD, but also higher cell-bound GAD activity and survival *in vitro* under HCl-mediated acidic stress. In line with the relevance of bacterial viability for GAD activity (and vice-versa) and GABA production under acidic conditions, we first wonder whether it is necessary for the strain CNCM I-5388 to be viable or not in order to produce GABA *in vitro* or *in vivo* and to exert its anti-VH properties in our acute-stress rat model. Since it has been observed that acute stresses in rats might enhance gastric acidity^{22–24}, we inquire as well if our acute stress exerts any influence on GABA production by viable or nonviable *L. lactis* in rat's GIT. Finally, we ask ourselves how human-like gastric physico-chemical conditions (e.g. pH kinetics, electrolyte and digestive enzyme concentrations) affect survival and GABA production of CNCM I-5388 in its viable or nonviable form, monitoring these parameters in real-time within an *in vitro* dynamic gastrointestinal model (TIM-1), simulating the human gastric compartment.

MATERIALS AND METHODS

Bacterial strain and culture in bioreactor

Lactococcus lactis strain CNCM I-5388 bacterial cultures were conducted as previously described^{20,21}. The cultures were performed in a 2-L Biostat B-plus bioreactor (Sartorius, Melsungen, Germany) filled with M17 medium (37.5 g/L) supplemented with the following concentrations: 55 mM (8 g/L) L-glutamate (L-Glu), 29 mM (5 g/L) arginine, 250 mM (45 g/L) glucose, and 300 mM. The fermentations took place under conditions with limited oxygen supply at a temperature of 30°C. The pH of the medium was initially maintained at 6.6 by adding KOH for the first 8 h, after which it was adjusted and regulated at 4.6.

To initiate the cultures, cells from pre-cultures grown in Erlenmeyer flasks with a similar medium were inoculated into the bioreactor. The cells used for inoculation were harvested during the exponential growth phase and concentrated to achieve an initial optical density (OD) of 0.25 at 580 nm in the fermenter. The growth of bacteria was monitored by measuring the optical density at 580 nm using a spectrophotometer (Libra S11, Biochrom, BIOSERV, Massy, France). Each unit of optical density corresponded to 0.3 g of dry weight per liter.

Samples were collected at 30-min intervals for high-performance liquid chromatography (HPLC) analysis to measure the concentration of gamma-aminobutyric acid (GABA) 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 to obtain approximately 3×10^{11} colony-forming units (CFU) was centrifuged to pellet the cells, followed by washing and suspension in 0.9% NaCl (w/v) solution containing 15% glycerol (w/v) to achieve a final concentration of 10^{10} CFU/mL for TIM-1 experiments (see below) and 10^9 CFU/mL for all other experiments. For bacterial cell inactivation, CNCM I-5388 cells were next exposed to ethanol (EtOH) 70%

(v/v) for 15 min, as proposed elsewhere ²⁵, followed by washing and resuspension in NaCl 0.9% (w/v) + Glycerol 15% (v/v) at the bacterial concentrations mentioned above.

***L. lactis* CNCM I-5388 inactivation and morphology maintenance validation after EtOH-treatment**

Viable bacterial counts were determined by plating samples of untreated or EtOH-treated (see above) CNCM I-5388 on M17 broth medium (37.5 g/L) supplemented with glucose (20 g/L). Heat-killed CNCM I-5388 (95°C heat-treatment in a dry block heater at 95°C for 15 min) was used as positive control for cell lysis and inactivation. Plates were incubated for 48 h at 30°C to determine the colony-forming units (CFU).

The intracellular ATP levels, another indicator of cellular viability ²⁶, were used to validate the inactivation of EtOH-treated bacteria by using the BacTiter-Glo™ Microbial Cell Viability Assay Kit (Promega) based on the luciferin/luciferase system. Briefly, this system utilizes luciferase, which converts luciferin into oxyluciferin by consuming ATP, thereby generating luminescence in proportion to the ATP concentration. Samples of 75 µL of untreated, EtOH-treated and heat-killed bacteria were transferred to a black 96-well plate containing 125 µL of BacTiter Glo reagent. The plate was then shaken for 10 s and incubated at room temperature for 5 min. Luminescence was measured using a Synergy H1 Microplate reader from BioTek with the following settings: integration time of 1 s, a plate height of 1 mm, and a gain of 180. Controls for medium luminescence (background) were included in the measurements. To establish a standard curve, 100 µL of ATP standards mixed with 100 µL of BacTiter Glo reagent were incubated for 1 min before measuring the luminescence using the same parameters as the sample readings.

Resazurin/resorufin assay was also used for validation of bacterial metabolism inactivation. Briefly, resazurin is a hydrophobic dye used in redox-based colorimetric assays to

determine cellular metabolic activity with minimal cellular toxicity. Living cells maintain a reducing environment in their cytoplasm and mitochondria. This reducing environment induces changes in the resazurin indicator from the oxidized form (violet) to the reduced form or resorufin (pink) through the action of NADH^{26,27}. High purity resazurin sodium salt (R12204, ThermoFisher Scientific) was dissolved in phosphate buffered saline (1x, pH 7.4) to 0.15 mg/mL and the solution was filter-sterilized through a 0.2 µm filter. Untreated, EtOH-treated or heat-killed CNCM I-5388 (initial concentration of 10⁹ CFU/mL) were centrifuged, resuspended in M17 broth medium (37.5 g/L) supplemented with glucose (20 g/L) and inoculated in different dilutions rates (from 10⁷ to 10⁵ CFU/mL) in 96-well plate containing a final volume of 100 µL with addition of 20 µL resazurin per well and incubated 1 h at 37°C, according to a previous protocol²⁶. Resazurin reduction was recorded at 600 nm with a Spectramax Plus spectrophotometer (Molecular Devices, Wokingham, United Kingdom) during 15 h at 30°C. A set of wells was prepared with medium only for background subtraction and instrument gain adjustment. Since the wavelength of 580 nm is also used for measuring *L. lactis* cell growth, another set of wells was prepared with the exact same conditions as mentioned above, but without resazurin added.

For extracellular DNA detection, a measurement of cellular morphology integrity²⁵, untreated, EtOH-treated and heat-killed CNCM I-5388 cells were centrifuged and the top fraction of the supernatant was recovered for DNA detection at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Rockland, DE). In this case, heat-killed CNCM I-5388 served as positive control.

Cell-bound glutamate decarboxylase (GAD) activities by *L. lactis* cells

The cell-bound GAD activities for untreated, EtOH-treated or heat-killed *L. lactis* CNCM I-5388 were determined by measuring the amount of GABA produced. The experiment

was conducted at 37°C using a reaction mixture consisting of 0.5 mL of each bacterial condition (10^9 CFU), 1 mL of 75 mM sodium acetate buffer (pH 4.6), and 0.5 mL of 67 mM L-glutamate (L-Glu). Cell-bound GAD activity assay was performed over a 2-h period, with 100 µL samples collected at regular time intervals (0, 10, 20, 30, 60, 90, and 120 min). The samples were then incubated at 95°C for 5 min to deactivate the enzyme. Protein precipitation was achieved by adding four volumes of methanol, followed by incubation on ice. After centrifugation, the supernatants were recovered for high-performance liquid chromatography (HPLC) analysis. The cell-bound GAD activity was defined as the production of 1 µmol of GABA per minute under the specified conditions.

Animals

Adult female Wistar rats weighing between 200-225 g were obtained from Janvier Labs (Le Genest St Isle, France). They were housed individually in polypropylene cages under standard conditions, including a controlled temperature of $22\pm 2^\circ\text{C}$ and a 12-h light/dark cycle. Animals were provided with *ad libitum* access to water and standard pellets (2016, Envigo RMS SARL, Gannat, France) as their food source.

The experimental procedures involving the animals were conducted in accordance with the guidelines outlined in the European directive 2010/63/EU on the protection of animals used for scientific purposes. The study protocols and procedures were reviewed and approved by the local ethics committee of Toulouse Midi-Pyrénées. Additionally, the experiments were authorized by the French Ministry of Higher Education and Research, with the reference APAFIS#14898-20180430160331426v2.

Surgical procedure, colorectal distension (CRD) and acute stress procedures

Under the administration of general anesthesia using intraperitoneal injections of 0.6 mg/kg acepromazine (calmivet, Vetoquinol, Lure, France) and 120 mg/kg ketamine (Imalgene 1000, Merial, Lyon, France), rats in the experiment underwent a surgical procedure. NiCr wire electrodes were implanted in the abdominal striated muscle to enable electromyography (EMG) recording, following a method formerly described²⁸.

Animals were first habituated to polypropylene tunnels for several days before the commencement of colorectal distension (CRD). For CRD, a 4-cm long latex balloon attached to a rigid catheter was inserted into the rectum, positioned 1 cm from the anus. The catheter was secured at the base of the tail. Isobaric distensions of the colon were performed using a Distender Series IIR Barostat (G&J Electronics Inc, Toronto, Canada), gradually increasing the pressure from 0 to 60 mmHg. Each distension step lasted for 5 min. The electromyographic (EMG) recordings using the Mini VIII system (Alvar, Paris, France) captured the spike bursts of the striated muscle, which corresponded to abdominal cramps.

For the partial restraint stress (PRS) experiment, a mild non-ulcerogenic stress model, rats were sedated with diethyl-ether. Their fore shoulders, upper forelimbs, and thoracic trunk were confined using a paper tape harness to restrict, but not completely restrict, their body movements. Subsequently, rats were placed in their home cages for a duration of 2 h, following the protocol previously described by our team²⁰.

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 to 13 female rats equipped for EMG as previously described²⁰.

In the first series of experiments, rats were divided into three groups and orally treated for 10 or 5 days (1 mL/rat) with *L. lactis* CNCM I-5388 in its viable (10^9 CFU/day) or nonviable

(3×10^9 CFU/day) form, plus L-glutamic acid (L-Glu) in its monosodium salt hydrate form (0.2% [w/v]), or vehicle (NaCl 0.9% [w/v] + glycerol 15% [v/v]).

In the second series, rats were orally treated for 5 days (1 mL/rat) with viable (10^9 CFU/day) or nonviable (3×10^9 CFU/day) CNCM I-5388, plus L-Glu in its monosodium salt hydrate form (0.2% [w/v]), or vehicle (NaCl 0.9% [w/v] + glycerol 15% [v/v]), followed by 5 days of treatment interruption.

In the third series, rats were orally treated (1 mL/rat) with a single dose of viable (10^9 CFU/day) or nonviable (3×10^9 CFU/day), plus L-Glu monosodium salt (0.2% [w/v]), or 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.

In a final series of experiments, rats were orally treated (1 mL/rat) for 5 days with viable (10^9 CFU/day) or nonviable (3×10^9 CFU/day) CNCM I-5388, plus L-Glu monosodium salt (0.2% [w/v]), or vehicle (NaCl 0.9% [w/v] + glycerol 15% [v/v]). One hour after the last oral treatment, rats were divided into two groups: one stressed group (2-h PRS) and another non-stressed (or sham-stressed) one. Next, rats received intraperitoneal injection with Rompun (8 mg/kg). 20 min after injection, isoflurane was used for deep inhalation anesthesia, and subsequently, terminal aortic procedure was conducted. Once animals were sacrificed, samples of gastric, duodenal, jejunal, ileal and colonic contents were gathered for GABA analysis (see GABA extraction and quantification methods).

Gastric *L. lactis* survival and GABA production in the TIM-1 GIT model

To study the untreated and EtOH-treated CNCM I-5388 GABA production and survival (for untreated cells, only) during human gastric simulated digestion, the TIM-1 GIT model (TIM-Company, NL), with validated fed state probiotic survival protocol, was used as previously described^{29–34}, with small modifications. Typically, the TIM-1 model consists of four serial connected compartments, mimicking different parts of the upper GIT. The focus of the current study was on the gastric phase, and therefore the stomach vessel was disconnected from the duodenal vessel, where the gastric residues were collected on ice at intake (0h), 15 min, 30 min, 60 min, 90 min and 120 min. At the given timepoints, part of the sample was treated for 10 min at 95°C to inactivate enzyme activity and the other subsample passed serial dilutions followed by CFU plate counting on solid M17 broth medium (37.5 g/L) supplemented with glucose (20 g/L). Plates were incubated at 30°C for 48 h. The inactivated subsamples were stored at -20°C until GABA extraction and quantification.

GABA extraction and quantification

GABA measurements were conducted on animal GIT samples as well on samples recovered from TIM-1 GIT model (see above). To extract GABA from these samples, 200 mg of contents were mixed with methanol (three times with 3 mL) at room temperature. The mixture was then subjected to centrifugation, and the resulting supernatant was carefully transferred to another tube and concentrated until dry. The dried samples were subsequently resuspended in 100 µL of methanol and analyzed using high-performance liquid chromatography (HPLC), following the same procedure as described above.

Statistical methods

The software GraphPad Prism 9.1 (GraphPad, San Diego, CA) was used for statistical analyses. For the results of untreated, EtOH-treated and heat-killed CNCM I-5388 CFU/mL, cell-bound GAD activity and extracellular DNA concentration (at least three biological replicates for each), Kruskal-Wallis test and *post hoc* Dunn's test were performed. Mann-Whitney U test was performed to compare the cell-bound GAD activity of untreated (10^9 CFU/mL) and EtOH-treated (3×10^9 CFU/mL) bacteria (at least three biological replicates for each). For all *in vivo* experiments, including measurements of abdominal contractions in response to CRD and GABA concentration in rats' GIT contents, one-way ANOVA, followed by Tukey's multiple comparison test, was performed (at least eight biological replicates for each). Untreated (10^9 CFU/mL) and EtOH-treated (3×10^9 CFU/mL) CNCM I-5388 in TIM-1 gastric survival and GABA content analyses were performed in biological triplicates. For survival of untreated *L. lactis*, Kruskal-Wallis test and *post hoc* Dunn's test were performed comparing each sampling time (15, 30, 60, 90 and 120 min) to the intake (0 min). For TIM-1 gastric GABA content, 2-way ANOVA was performed to compare samples from untreated and EtOH-treated *L. lactis*. Data are reported as the means \pm SEM and p-values lower than 0.05 were considered as significant.

RESULTS

EtOH 70% treatment inactivates *L. lactis* CNCM I-5388 metabolism while maintaining a third of its cell-bound GAD activity

In order to further evaluate the antinociceptive properties of nonviable *L. lactis* CNCM I-5388, we expected here to develop an “active GAD-bag”, i.e. inactivated bacteria that maintain an active GAD enzyme (essential for anti-VH properties in rats) within cells, in spite

of the lack of bacterial viability. For *L. lactis* CNCM I-5388 inactivation, we exposed bacterial cells to ethanol (EtOH) 70% (v/v) based on a previous study on the inactivation of bacteria while maintaining their morphology (see materials and methods section). We confirmed that EtOH-treated CNCM I-5388 cells were unable to form colonies in solid growth medium (**Figure 1A**). No statistical differences with heat-killed CNCM I-5388 (positive control for non-viability) ($p>0.05$) were indeed found in contrast with untreated CNCM I-5388 ($p<0.001$). The inactivation of EtOH-treated bacteria was also confirmed since no intracellular ATP was detected, as for heat-killed CNCM I-5388 (**Figure 2A**). In addition to the lack of detectable intracellular ATP, the resazurin/resorufin test indicated undetectable levels of NADH within EtOH-treated CNCM I-5388 (**Supplementary Figure 1A, B and C**). Altogether, these results revealed that EtOH-treated cells were not only non-culturable but also nonviable. Importantly, EtOH-treated cells displayed a low concentration of extracellular DNA, statistically indifferent from untreated cells ($p>0.05$) while the heat-killed bacteria (positive control for bacterial lysis) exhibited high extracellular DNA concentrations, as expected (**Supplementary figure 2**). These findings indicate that the EtOH treatment successfully rendered CNCM I-5388 cells inactive while maintaining their cellular morphology intact.

Next, we evaluated if EtOH inactivation treatment would also affect CNCM I-5388 cell-bound GAD activity. **Figure 1C** shows that EtOH-treated cells had their cell-bound GAD activity reduced by a factor of approximately three compared to untreated CNCM I-5388 ($p<0.001$); however, their activity was still statistically higher than that of the heat-killed CNCM I-5388 control ($p<0.05$), where GAD enzyme was completely inactivated. Indeed, cell-bound GAD activity comparison between viable untreated CNCM I-5388 at 10^9 CFU/mL and EtOH-treated CNCM I-5388 at 3×10^9 CFU/mL revealed no statistical differences ($p>0.05$) (**Supplementary figure 3**), indicating that a three-fold bacterial concentration enhancement of EtOH-inactivated cells is able to maintain a cell-bound GAD activity comparable to that of

untreated cells. Therefore, EtOH treatment was able to render nonviable CNCM I-5388 cells, preserving not only their cellular integrity, but also their GAD activity, creating a fit-for-purpose “active GAD-bag” for further *in vivo* and *in vitro* experimentation.

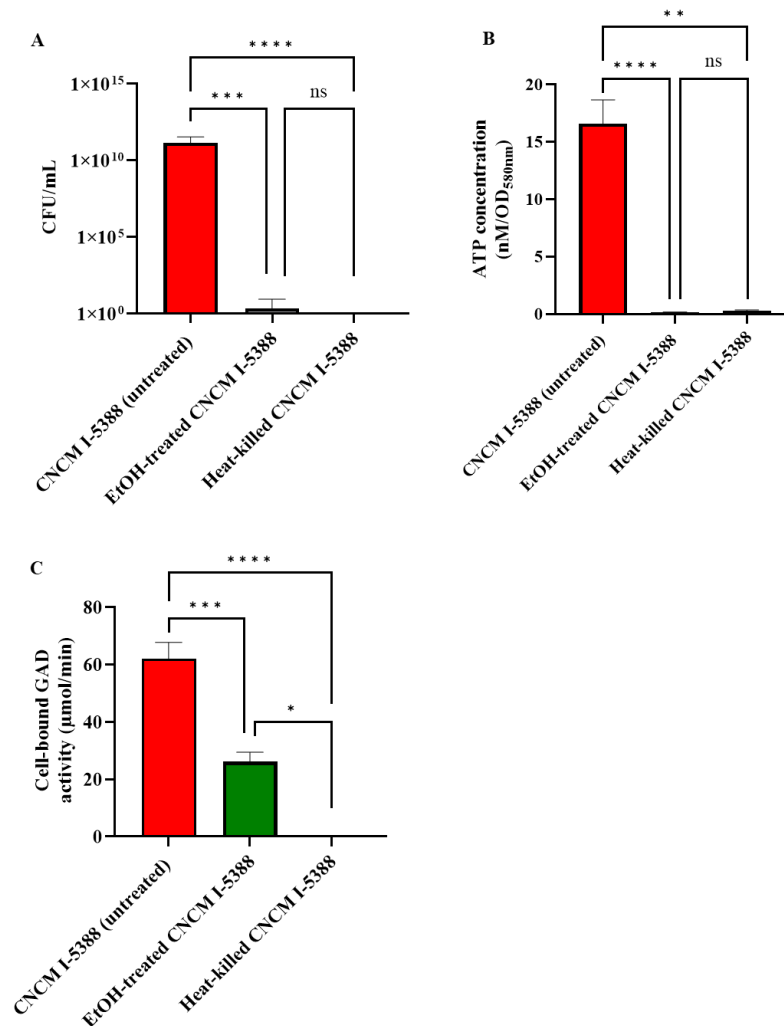


Figure 1. Effect of EtOH treatment on bacterial inactivation and cell-bound GAD activity of *L. lactis* CNCM I-5388.

(A) Colony-forming units (CFU) per mL, (B) Intracellular ATP concentration (OD: optical density at 580nm) and C) cell-bound GAD activity of untreated, EtOH-treated and heat-killed CNCM I-5388 (positive control for cell lysis and non-viability).

Data are expressed as means ± SEM. * p < 0.05, *** p < 0.001, **** p < 0.0001. ns = non-significant (p > 0.05).

EtOH-treated *L. lactis* CNCM I-5388 has anti-VH properties after 10 or 5-day daily oral treatment

To compare the *in vivo* anti-VH properties of untreated and EtOH-treated *L. lactis* CNCM I-5388, rats were orally administered each bacterial suspension once daily (10^9 and 3×10^9 CFU per day, respectively) for 5 or 10 days, and the administration included supplementation with L-Glu monosodium salt hydrate. After 10 or 5 days of treatment, the response to colorectal distension (CRD) under partial restraint stress (PRS) was measured. Firstly, we confirmed that the oral treatment with untreated or EtOH-treated CNCM I-5388 for either 9 days or 4 days did not affect the basal sensitivity to CRD, as previously described ²¹ (**Supplementary figures 4A and B**, respectively).

Furthermore, we successfully demonstrated that a 10-day oral administration of EtOH-treated CNCM I-5388 restored near-basal sensitivity to CRD in stressed rats. The treatment resulted in a significant reduction in the number of abdominal contractions at 60 mmHg ($p < 0.05$), as compared to the group of stressed rats treated with the vehicle alone (**Figure 2A**), a result further validated when we calculated the area under the curve (AUC) for all distension pressures ($p < 0.05$ for stressed rats that received vehicle *vs* EtOH-treated CNCMI-5388) (**Figure 2B**). Interestingly, a 5-day oral administration of EtOH-treated bacteria exhibited a similar restorative effect, significantly reducing the number of abdominal contractions in stressed rats at 45 mmHg ($p < 0.05$) and 60 mmHg ($p < 0.01$) compared to the vehicle-treated stressed group (**Figure 2C**), also validated by AUC for all distension pressures ($p < 0.05$ for stressed rats that received vehicle *vs* EtOH-treated CNCMI-5388) (**Figure 2D**). As already shown in our previous study ²¹, viable CNCM I-5388 restored quasi-basal sensitivity to CRD either at 10 (**Figure 2A and B**) or 5-day treatment (**Figure 2C and D**).

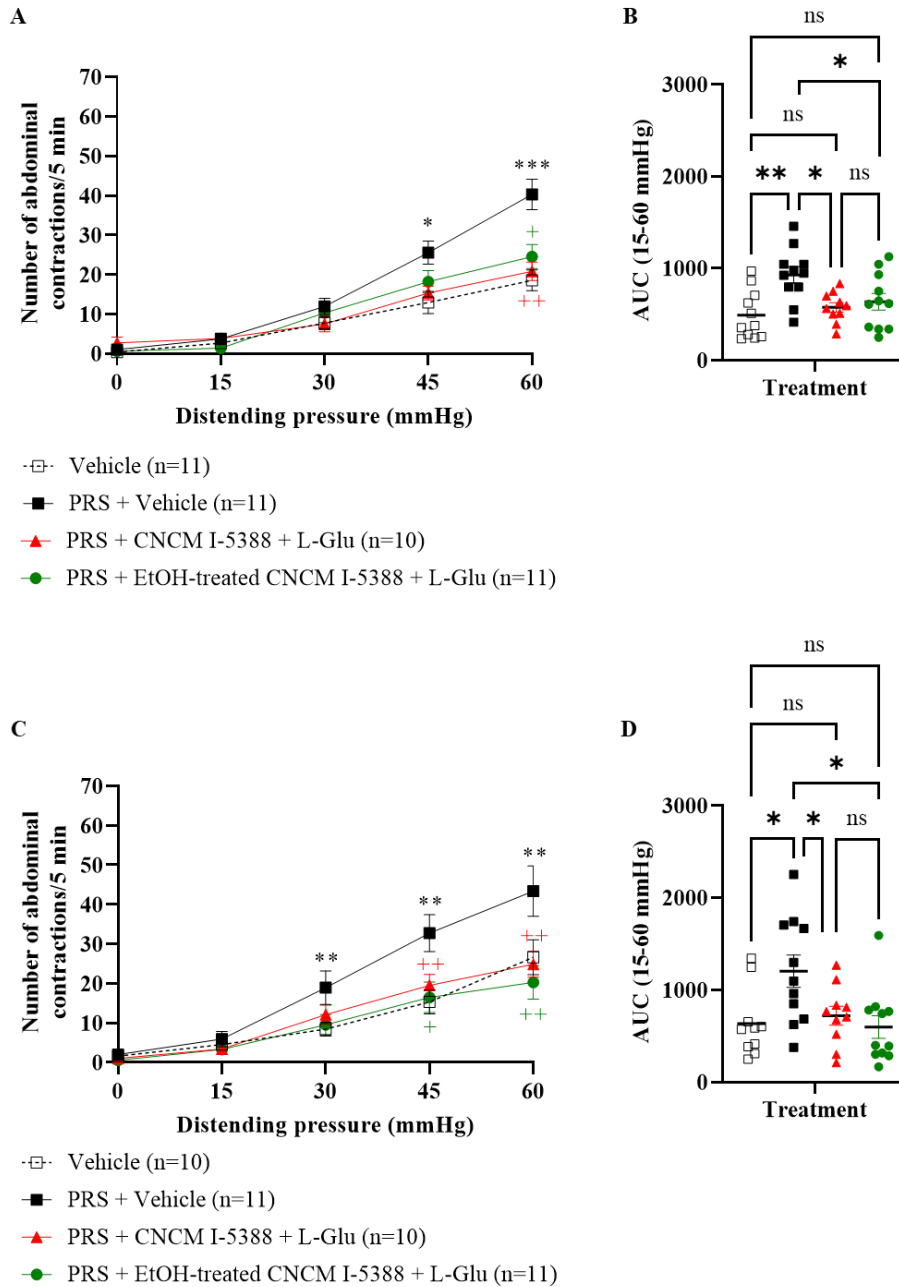


Figure 2. Effect of oral daily administration of EtOH-treated *L. lactis* CNCM I-5388 in the presence of L-Glu monosodium salt hydrate on VH-induced by PRS in response to colorectal distension. (A) Effect of 10-day oral administration on PRS-induced VH to all colorectal distension pressures (15 to 60 mmHg) and respective (B) area under the curve. (C) Effect of 5-day oral administration on PRS-induced VH to all colorectal distension pressures (15 to 60 mmHg) and respective (D) area under the curve. Results for untreated CNCM I-5388 were adapted from Gomes et al. (2023). Data are expressed as means \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs basal values for animals treated with vehicle. + $p < 0.05$, ++ $p < 0.01$ vs values for stressed animals treated with vehicle. ns = non-significant ($p > 0.05$).

No anti-VH properties are observed in EtOH-treated *L. lactis* CNCM I-5388 following 1-day treatment or 5-day washout

Since our previous work has shown that *L. lactis* CNCM I-5388 tended to promote antinociceptive properties in rats after a single-day oral administration and also after 5-day treatment followed by 5-day washout²¹, we wondered if that would be also the case for EtOH-treated CNCM I-5388. We thus studied the impact of the single-dose treatment or 5-day treatment followed by 5-day washout considering untreated or EtOH-treated CNCM I-5388 (10^9 and 3×10^9 CFU, respectively) supplemented with L-Glu monosodium salt hydrate. We first checked that there were no observed effects on the basal sensitivity (**Supplementary Figure 4C, D and E**, respectively). We detected no statistical differences between the number of abdominal contractions of stressed rats that received a single-day treatment with EtOH-treated CNCM I-5388 compared to stressed rats that received the vehicle, for any distension pressure ($p > 0.05$) (**Figures 3A**), also confirmed by AUC for all distension pressures ($p > 0.05$ for stressed rats that received vehicle vs EtOH-treated CNCM I-5388) (**Figure 3B**). As observed above, a 5-day oral administration of EtOH-treated bacteria reduced the number of abdominal contractions in stressed rats at 45 mmHg ($p < 0.001$) and 60 mmHg ($p < 0.001$) compared to the vehicle-treated stressed group (**Figure 4A**), also validated by AUC for all distension pressures (**Figure 4B**) ($p < 0.05$ for stressed rats that received vehicle vs EtOH-treated CNCM I-5388). However, this effect was completely lost after 5-day treatment interruption since, for all distension pressures, we detected no statistical differences ($p > 0.05$) between stressed rats that received EtOH-treated CNCM I-5388 and the ones that were only treated with the vehicle (**Figure 4C and D**). Altogether, these results indicate that neither single-dose treatment nor 5-day washout after 5-day oral administration of EtOH-treated CNCM I-5388 are efficient in the reduction of VH in our rat model.

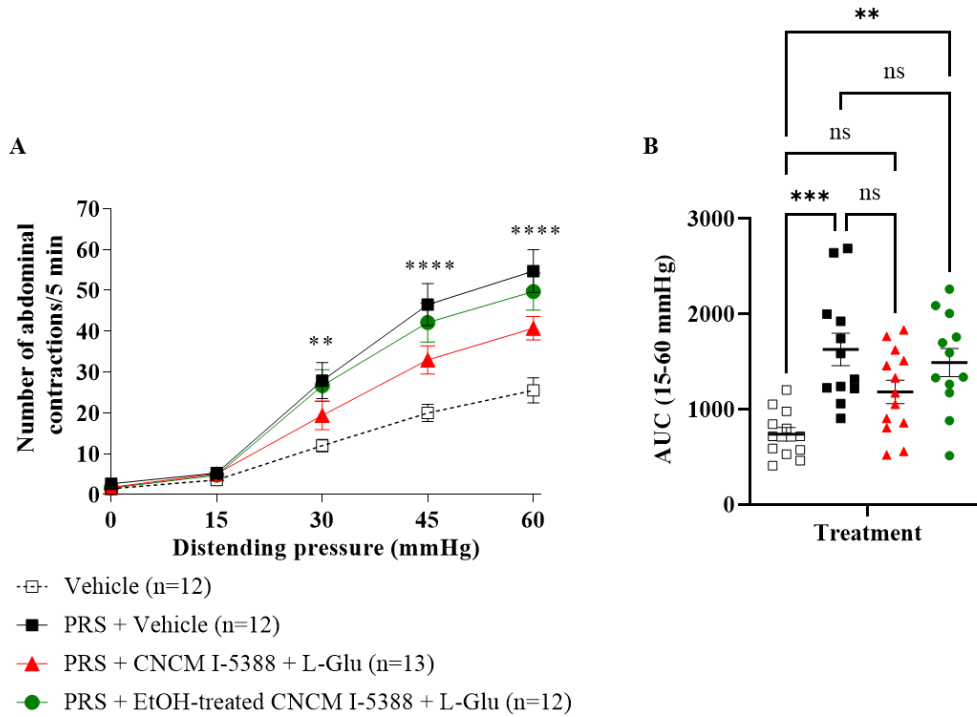


Figure 3. Effect of a single oral administration of EtOH-treated *L. lactis* CNCM I-5388 in the presence of L-Glu monosodium salt hydrate on PRS-induced VH induced by stress in response to colorectal distension. (A) Treatment response to all colorectal distension pressures (15 to 60 mmHg) and respective (B) area under the curve. Results for untreated CNCM I-5388 were adapted from Gomes et al. (2023). Data are expressed as means \pm SEM. **p < 0.01, ***p < 0.001, ****p < 0.0001 vs basal values for animals treated with vehicle. ns = non-significant (p > 0.05).

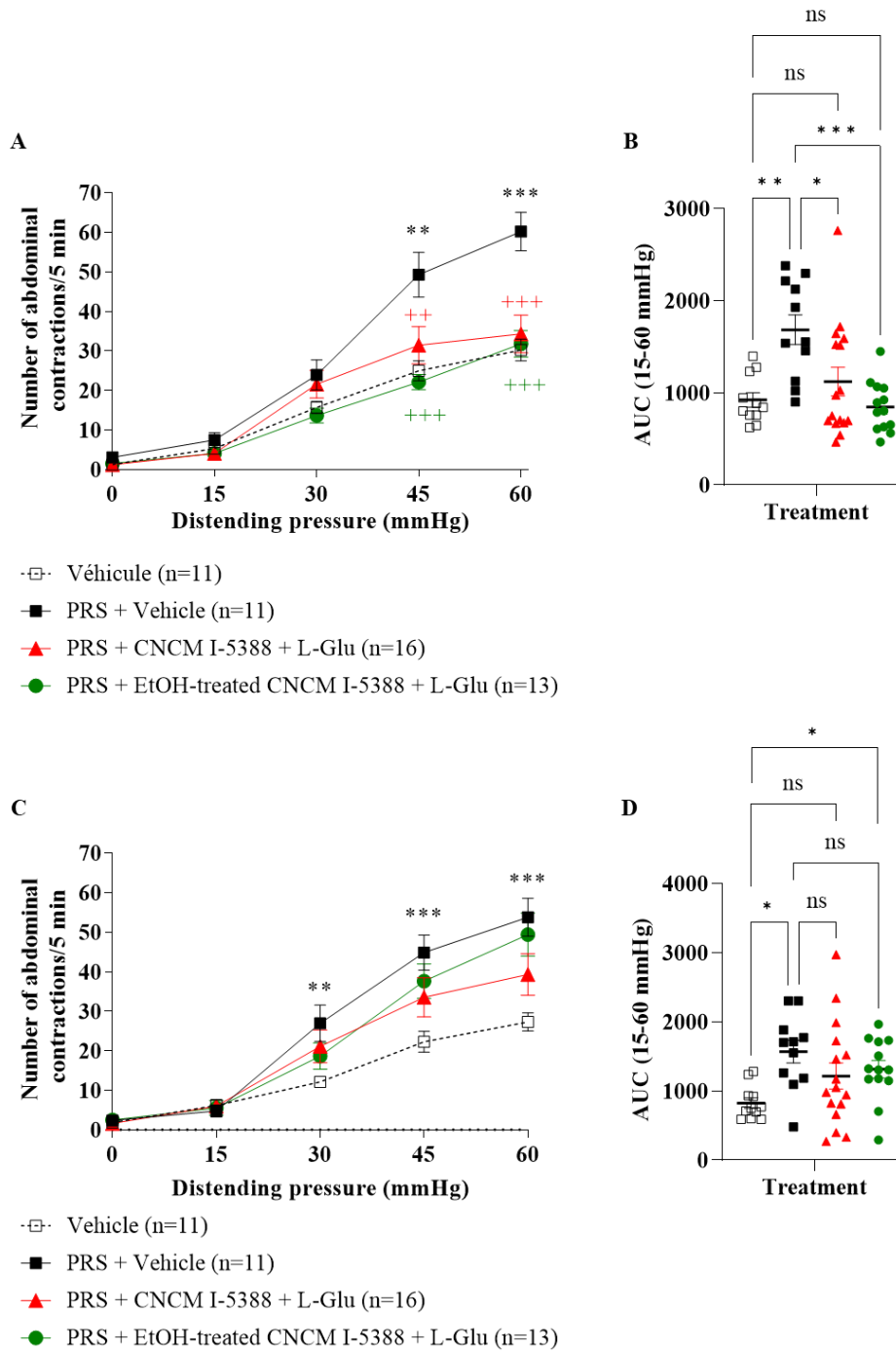


Figure 4. Effect of 5-day oral administration followed by 5-day treatment interruption of EtOH-treated *L. lactis* CNCM I-5388 in the presence of L-Glu monosodium salt hydrate on VH induced by PRS in response to colorectal distension. (A) 5-day treatment response to all colorectal distension pressures (15 to 60 mmHg) and respective (B) area under the curve. (C) 5-day treatment interruption response to all colorectal distension pressures (15 to 60 mmHg) and respective (D) area under the curve. Results for untreated CNCM I-5388 were adapted from Gomes et al. (2023). Data are expressed as means \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs basal values for animals treated with vehicle. ++ $p < 0.01$, +++ $p < 0.001$ vs values for stressed animals treated with vehicle. ns = non-significant ($p > 0.05$).

Increase in GABA production of untreated and EtOH-treated *L. lactis* CNCM I-5388 in the rat stomach exclusively occurs under stress conditions

In order to evaluate the GABA production of *L. lactis in vivo*, sham-stressed or PRS-stressed rats were treated for five days with untreated or EtOH-treated CNCM I-5388 supplemented with L-Glu monosodium salt hydrate. We detected no statistical differences ($p>0.05$) in the gastric GABA concentrations of sham-stressed rats that received either the vehicle, untreated or EtOH-treated CNCM I-5388 (**Figure 5**). Nevertheless, this was not the case for stressed rats, since gastric GABA content of animals that received either untreated or EtOH-treated CNCM I-5388 was significantly higher ($p<0.05$) than for vehicle-treated animals (**Figure 5**). Worthy of notice, even though no statistical differences were detected, gastric GABA mean concentration for untreated CNCM I-5388 was approximately 1.6 times higher than for the EtOH-treated strain (**Figure 5**). Moreover, stress only significantly heightened gastric GABA content of animals that received untreated or EtOH-treated CNCM I-5388 ($p<0.05$), contrary to the vehicle ($p>0.05$) (**Figure 5**). No statistical differences ($p>0.05$) were detected, for vehicle, untreated or EtOH-treated CNCM I-5388, concerning the GABA concentration in the duodenum (**Supplementary Figure 5**), jejunum (**Supplementary Figure 6**), ileum (**Supplementary Figure 7**) and colon (**Supplementary Figure 8**) of sham-stressed or PRS-stressed animals. Collectively, these findings suggest that GABA production by *L. lactis* CNCM I-5388, in its viable and nonviable forms, occurs in the host's stomach and is intensified by stress.

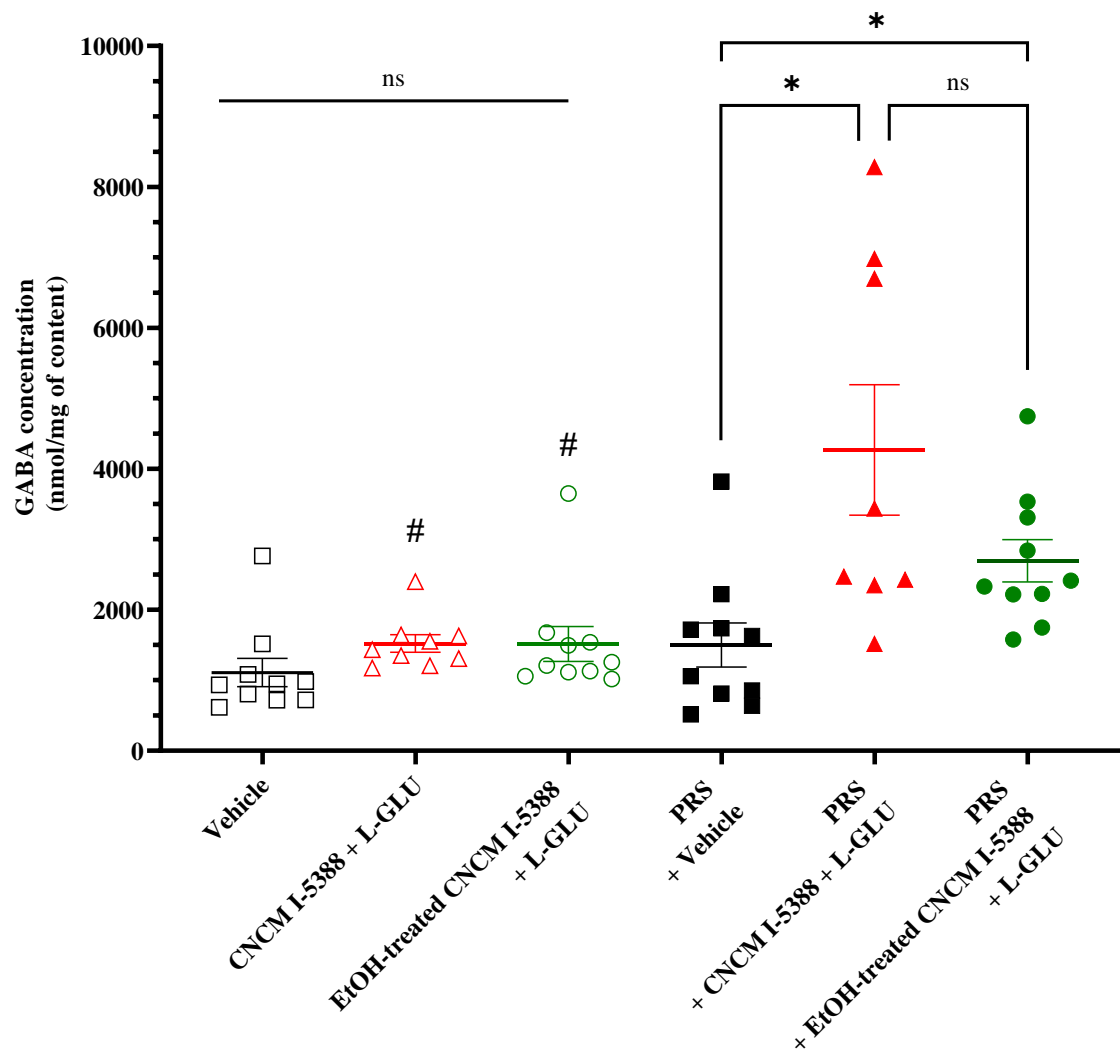


Figure 5. GABA concentration in gastric content after 5-day daily oral administration of untreated (10^9 CFU per day) or EtOH-treated CNCM I-5388 (3×10^9 CFU per day) supplemented with L-Glu monosodium glutamate salt. Data are expressed as mean \pm SEM. * $p < 0.05$ comparing different types of treatment within the same stress condition. # $p < 0.05$ comparing different stress conditions within the same type of treatment. ns = non-significant ($p > 0.05$).

Untreated and EtOH-treated *L. lactis* GABA-production initiates in acidic pH in the *in vitro* human TIM-1 GIT model.

In our previous work, we have shown how GABA was produced by *L. lactis in vitro* under stomach-like conditions, at pH 4.6 in acetate buffer or gastric juice sampled from naive rats²⁰. In the present work, we have demonstrated an enhanced GABA production by untreated and EtOH-treated CNCM I-5388 only detectable in rats' stomach. We then reinforced the focus on the potential role of the gastric compartment and anticipated the extrapolation to humans by using the *in vitro* dynamic TIM-1 GIT model (see the materials and methods section).

Therefore, we evaluated the mean GABA production by untreated and EtOH-treated CNCM I-5388 in the gastric compartment over time, while viable counts of CNCM I-5388 were determined in parallel. **Figure 6A** shows that from 0 (pH 6.2) to 30 min (pH 5.4) of simulated digestion, the mean GABA concentration was close to 70 μM for both untreated and EtOH-treated CNCM I-53885. The highest GABA production rate for both strains occurred between 30 (pH 5.4) and 60 min (pH 4.3). At 60 min (pH 4.3), the mean GABA concentration for untreated and EtOH-treated CNCM I-5388 reached 516 and 418 μM , respectively. Between 60 and 90 min (pH 3.2), it continued to increase to 666 μM for untreated CNCM I-5388 while it tended to stabilize for the EtOH-treated condition (410 μM). Finally, at 120 min (pH 2.4), GABA levels for untreated and EtOH-treated *L. lactis* decreased to 359 and 266 μM , respectively, indicating that GABA concentration reduced because its production did not surpass its dilution within TIM-1 gastric solution addition. Overall, for all digestion timepoints, we observed no statistical differences ($p>0.05$) between GABA concentrations produced by untreated or EtOH-treated CNCM I-5388 (**Figure 6A**). **Figure 6B** shows that bacterial viable counts of untreated CNCM I-5388 tended to decrease slightly but was only at 120 min (10^7 CFU/mL, pH 2.4) statistically different ($p<0.05$) from the initial value at the start of the simulated digestion (10^9 CFU/mL, pH 6.2).

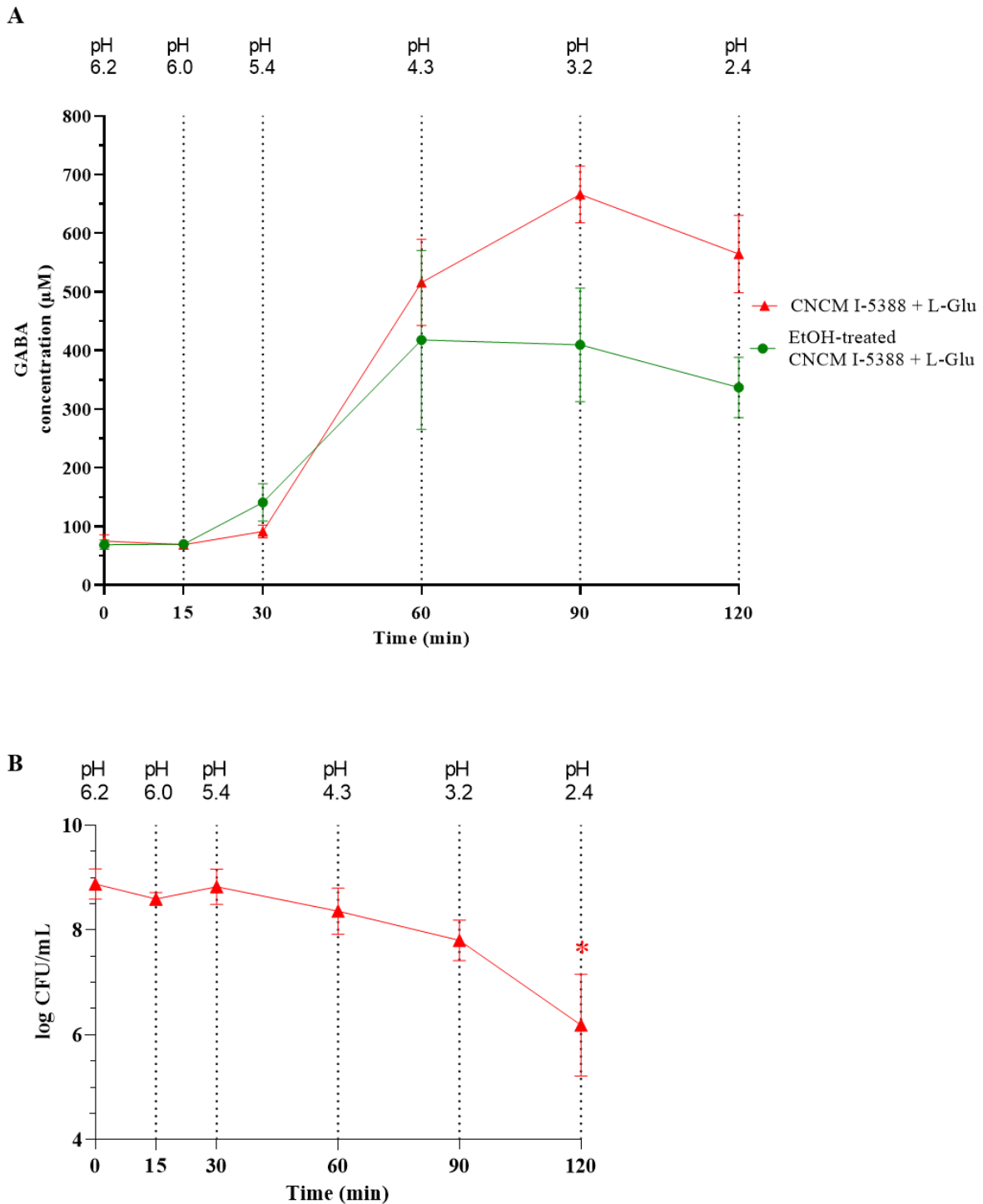


Figure 6. Untreated and EtOH-treated *L. lactis* CNCM I-5388 in the gastric compartment of TIM-1 GIT model. A) GABA concentration time variation for untreated and EtOH treated-CNCM I-5388. **B)** Viable counts time variation for untreated CNCM I-5388. * $p < 0.05$ for viable counts at 120 min vs viable counts at 0 min. No statistical differences in viable counts were detected for all other sampling times vs viable counts at 0 min. Data are expressed as means \pm SEM.

Altogether, our results suggest that, under fed-state condition, untreated and EtOH-treated CNCM I-5388 are capable to produce GABA in gastric human-like *in vitro* model.

DISCUSSION

There has been a growing body of evidence underscoring the beneficial impact of probiotics and, more recently, postbiotics with psychobiotics properties in relieving stress-related gastrointestinal disorders among patients, especially those suffering from IBS. We have previously shown that GABA-producer *L. lactis* CNCM I-5388 exerts anti-nociceptive properties after 10 or 5-day daily oral treatment in an IBS-like rat model ²¹. Here, we were interested in evaluating GABA production and anti-VH properties of nonviable EtOH-treated CNCM I-5388 in the same rat model exploring its postbiotic potential via an “active-GAD bag” innovative concept.

Our results have shown that either 10 or 5-day oral treatment with EtOH-treated *L. lactis* CNCM I-5388 in rats counteracts the stress-induced VH to quasi-basal sensitivity levels. This is not the first time a nonviable bacterial strain is shown to exert therapeutic properties in an IBS context. Heat-inactivated *Lactobacillus casei* DKG7 and *Bifidobacterium bifidum* MIMBb75 were shown to ameliorate IBS symptoms in rats (diarrhea) and humans (abdominal pain), respectively ^{35,36}. Kamiya and colleagues demonstrated that a 9-day oral treatment with heat-killed or gamma-irradiated *Lactobacillus reuteri* had inhibitory effects on VH in rats ³⁷. Noteworthy, these studies focused on bacterial inactivation protocols that enable not only to disrupt cells, but also to inactivate the activity of bacterial enzymes ³⁸. We are thus the first group to present an enzymatic activity-dependency in the anti-VH properties in the postbiotic use of a bacterial strain. This represents a new approach for the treatment of IBS symptoms, using nonviable bacteria as a delivery tool for GAD enzyme.

Consistent with our previous results concerning *L. lactis* NCDO2118²⁰, GABA produced by CNCM I-5388 (EtOH-treated or not) was enhanced in the host's stomach, also in agreement with its optimal cell-bound and specific GAD activities in the pH range between 3 and 5²¹. Interestingly, gastric GABA levels of stressed rats treated with EtOH-treated CNCM I-5388 (with a three-fold cell concentration increase to maintain a cell-bound GAD activity comparable to that of untreated cells) for 5 days were approximately 1.6 times lower than that achieved with untreated CNCM I-5388. This implies that inactivated *L. lactis* has a tendency to present reduced cell-bound GAD activity *in vivo* when compared to the viable strain. This was also observed in the TIM-1 experiments, where we detected a maximum in the mean GABA concentration at 666 μ M for untreated CNCM I-5388 at 90 min of simulated digestion (pH 3.2) and 416 μ M for EtOH-treated CNCM I-5388 at 60 min (pH 4.3), also representing a difference factor of roughly 1.6. Ethanol has been for long known in the literature for its bactericidal effectiveness due to multiple factors, including the disruption of membrane structure or function³⁹⁻⁴², changes in fatty acid composition and protein synthesis⁴³ modification of membrane pH, membrane potential and reduction in intracellular pH⁴⁴⁻⁴⁶. All these factors are intrinsically related to cell-bound and specific GAD activities, either at the level of the optimal pH for the enzyme activity or L-Glu/GABA transport across bacterial cells. Therefore, it is likely that specific and cell-bound GAD activities of EtOH-treated *L. lactis* are more vulnerable to gastric physico-chemical variables (e.g. pH kinetics, electrolyte and digestive enzyme concentrations) than those of the untreated strain.

Another interesting observation is that rats treated with either inactivated or active CNCM I-5388 had GABA levels superior to vehicle-treated animals exclusively under stress conditions. The boosted GABA production by *L. lactis* in rats' stomach might be explained by differences in gastric secretion and emptying between stressed and sham-stressed animals (i.e. considered under basal conditions). While formerly studies indicated restraint stresses reduced

gastric secretion⁴⁷⁻⁴⁹, more recent works have pointed out the opposite. For instance, restraint stress was shown to heighten gastric secretion in mice⁵⁰. Correspondingly, previous research work demonstrated that restrained rats exhibited a high gastric acid content while it was low for unrestrained animals²². More recently, Fatemeh and contributors conducted a study in rats, in which both physical and psychological stresses could potentially elevate gastric acid secretion, an effect attributed to an elevation in the levels of nitric oxide within the gastric tissue²³. Additionally, the activation of GABA_B receptors in rats' stomach might lead to an increased gastric secretion through vagal cholinergic and gastrin-dependent mechanisms, thereby also maintaining acidic conditions in the stomach in a “virtuous circle” manner as previously pointed out by our group and others^{15,20}. Reinforcing this hypothesis, lipophilic GABA mimetic was also found to enhance gastric secretion in rats through the vagal nerve⁵¹. Moreover, GABA_B agonists were shown to stimulate gastric acid secretion in mice⁵². Considering our results and the literature, it seems flagrant that gastric acidity plays a pivotal role in activating and maintaining *L. lactis* GAD activity inside the host's stomach, necessary for GABA production to exert anti-VH properties.

Concerning gastric emptying, acute restraint stress was shown by Zheng and colleagues (2009) to reduce gastric clearance in rats⁵³, which was also observed more recently elsewhere⁵⁴. When GABA_B receptor agonist baclofen was administered intracerebroventricularly to Wistar rats, it delayed the gastric emptying of the solid meal⁵⁵. More recently, a study has shown that GABA microinjected into the motor vagus dorsal nucleus of rats reduced gastric motility⁵⁶. It is not unreasonable to figure that an augmented exposition of *L. lactis* GAD enzyme to the host's acidic stomach content, due to a delayed gastric emptying, might sustain GAD activity and GABA production. Noteworthy, the enhanced GABA production by CNCM I-5388 in the host's stomach under PRS conditions is also consistent with the fact that, throughout this work and our previous studies^{20,21}, animals considered under basal conditions

and treated with *L. lactis* never presented diminished abdominal contractions compared to vehicle-treated ones. Hence, it is possible in our study model that stressed animals, contrarily to their non-stressed counterparts, present an optimal acidic environment for the activation of CNCM I-5388 (viable or not) GAD enzyme and/or delayed gastric emptying sustaining GAD activity.

We have previously proposed that a daily oral administration over a short period of CNCM I-5388, due to its capacity to deliver elevated GABA levels in acidic environments, plays a role in initiating and maintaining the activation of GABA_B receptors on gastric epithelial cells, within the enteric plexus, and possibly on the vagus nerve²¹. The stability and movement of GABA_B receptors on the cell surface play a pivotal role in determining the intensity and duration of synaptic inhibition following GABA_B receptor activation. While earlier research suggested that GABA_B receptors exhibit a high stability on the cell surface^{57,58}, more recent studies have introduced contrasting findings, indicating that GABA_B receptors possess some mobility characteristics. GABA_B receptors are observed to undergo a rapid and constitutive internalization in the absence of agonists, both in neurons^{59,60} or when heterologously expressed in human embryonic kidney cell line HEK 293⁶¹. This, together with the gastric levels of GABA detected *in vivo* and *in vitro* in the TIM-1 GIT model, might explain why EtOH-treated CNCM I-5388 shows a lower tendency to reduce VH in the single-dose and washout treatment scenario as shown in **Figure 7**. We suggest that GABA levels produced *in vivo* by the nonviable *L. lactis* might not be sufficient to activate GABA_B receptors already distributed along the GIT and exert anti-VH properties in our rat model. However, a 10 or 5-day daily oral treatment with this quantity of GABA produced in the host's stomach might be sufficiently efficient to enhance stabilization and number of GABA_B receptors through time at the cell level so that GABA delivered by *L. lactis in situ* exerts anti-nociceptive properties.

Finally, during washout (lacking *L. lactis* and L-Glu supplementation), GABA produced in host's GIT would not be sufficient to activate GABA_B for anti-nociception (**Figure 7**).

In conclusion, our results highlight how GABA-producing CNCM I-5388 in its viable or nonviable postbiotic form acts as an efficient carrier of GAD enzyme for *in situ* GABA production in the stomach and activation of GABA_B receptors distributed in the GIT. This “active GAD-bag” concept offers a new therapeutic strategy for treating VH in IBS patients.

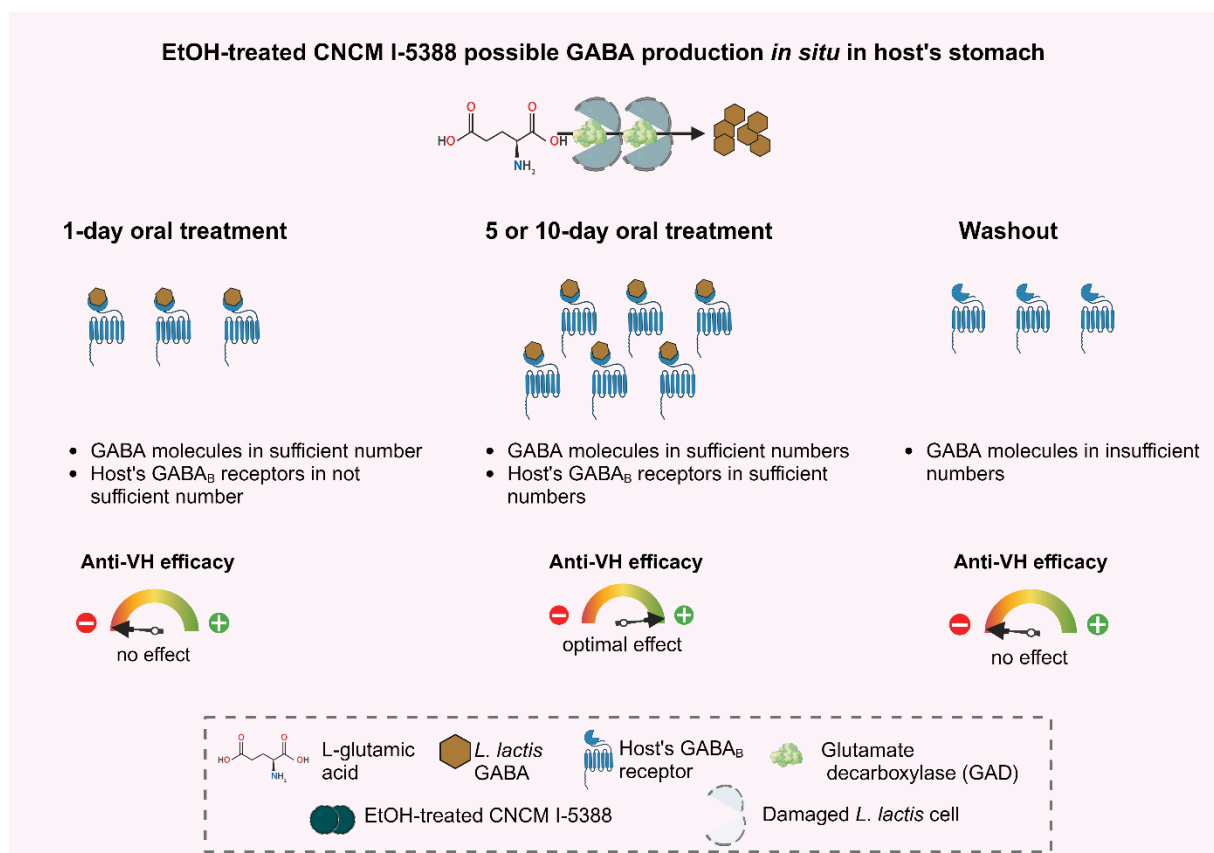


Figure 7. Possible mechanisms involved in EtOH-treated *L. lactis* CNCM I-5388 anti-visceral hypersensitivity (VH) properties.

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DISCLOSURE STATEMENT

Jonna Koper, Elyse Parent, Nabil Bosco, Nathalie Ballet and Sophie Legrain-Raspaud are full-time employees of Lesaffre. The other authors report no conflict of interest.

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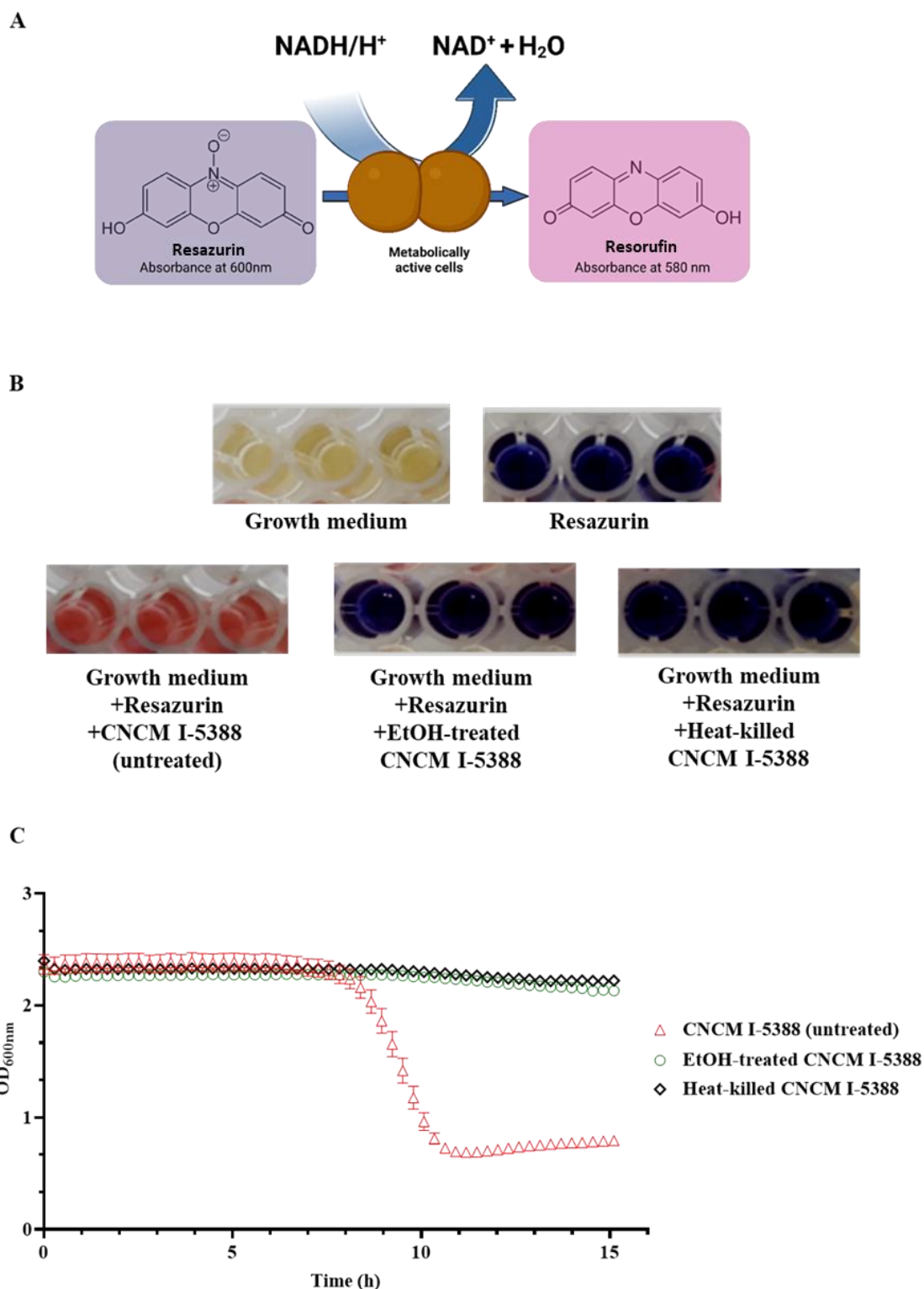
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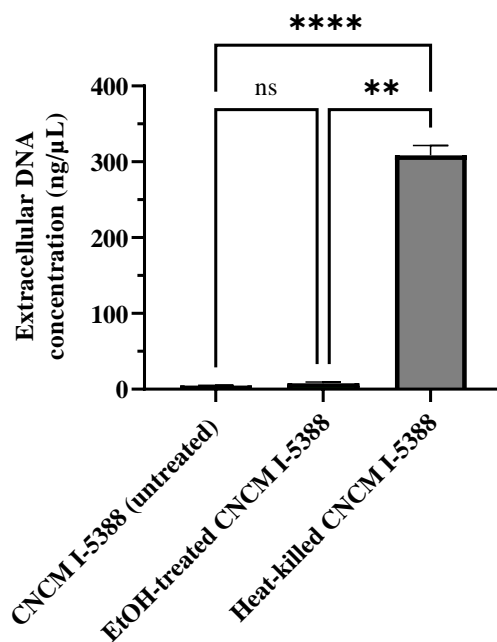
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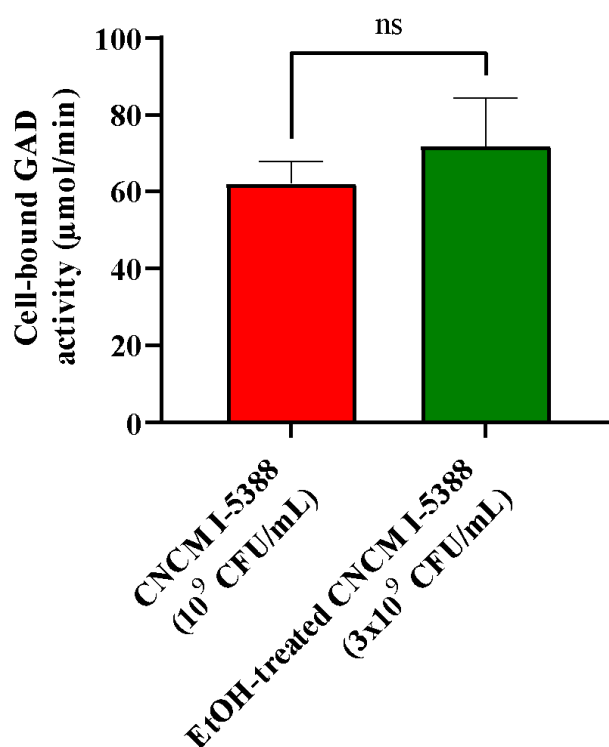
SUPPLEMENTARY INFORMATION



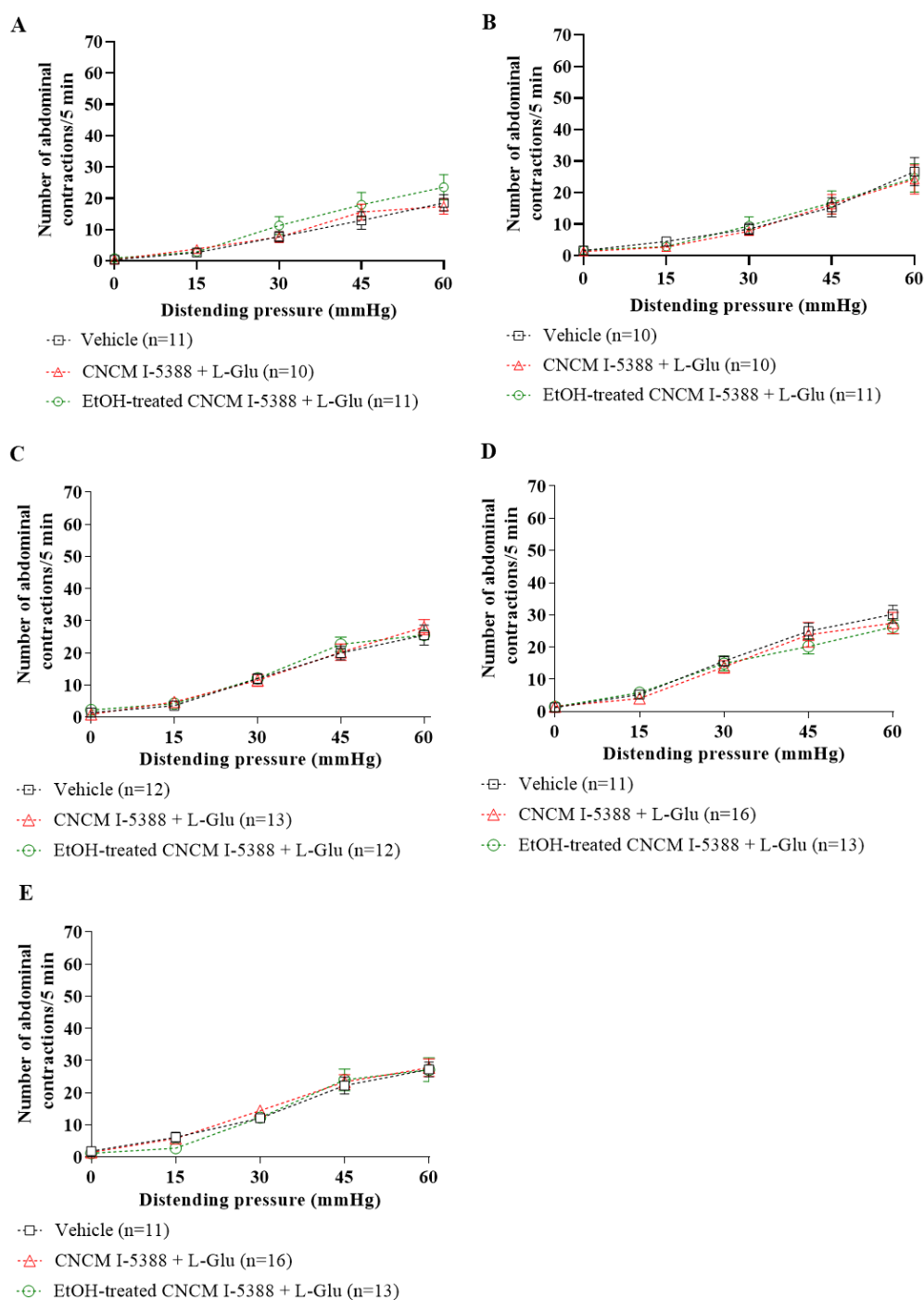
Supplementary Figure 1. Untreated and EtOH-treated *L. lactis* CNCM I-5388 resazurin conversion after 15 h of growth in microplate. (A) Schematic representation of reduction of resazurin and formation of resorufin (B) Visual colorimetric confirmation. (C) Optical density (600 nm) of bacterial cells during growth, a measurement allowing to monitor resazurin reduction (see materials and methods section).



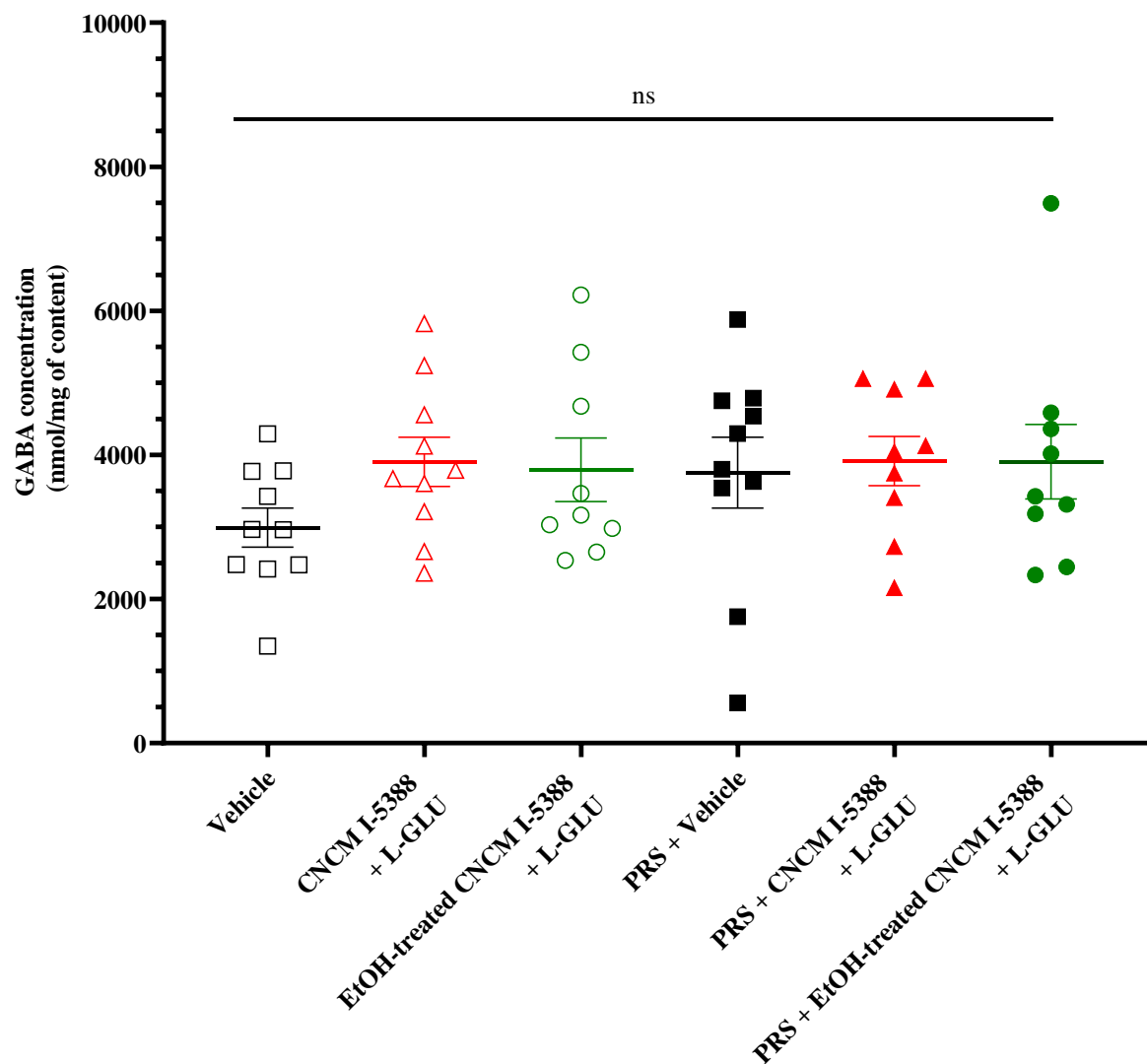
Supplementary Figure 2. EtOH-treated *L. lactis* CNCM I-5388 extracellular DNA concentration. Data are expressed as mean \pm SEM. **p < 0.01, ****p < 0.0001. ns = non-significant (p > 0.05).



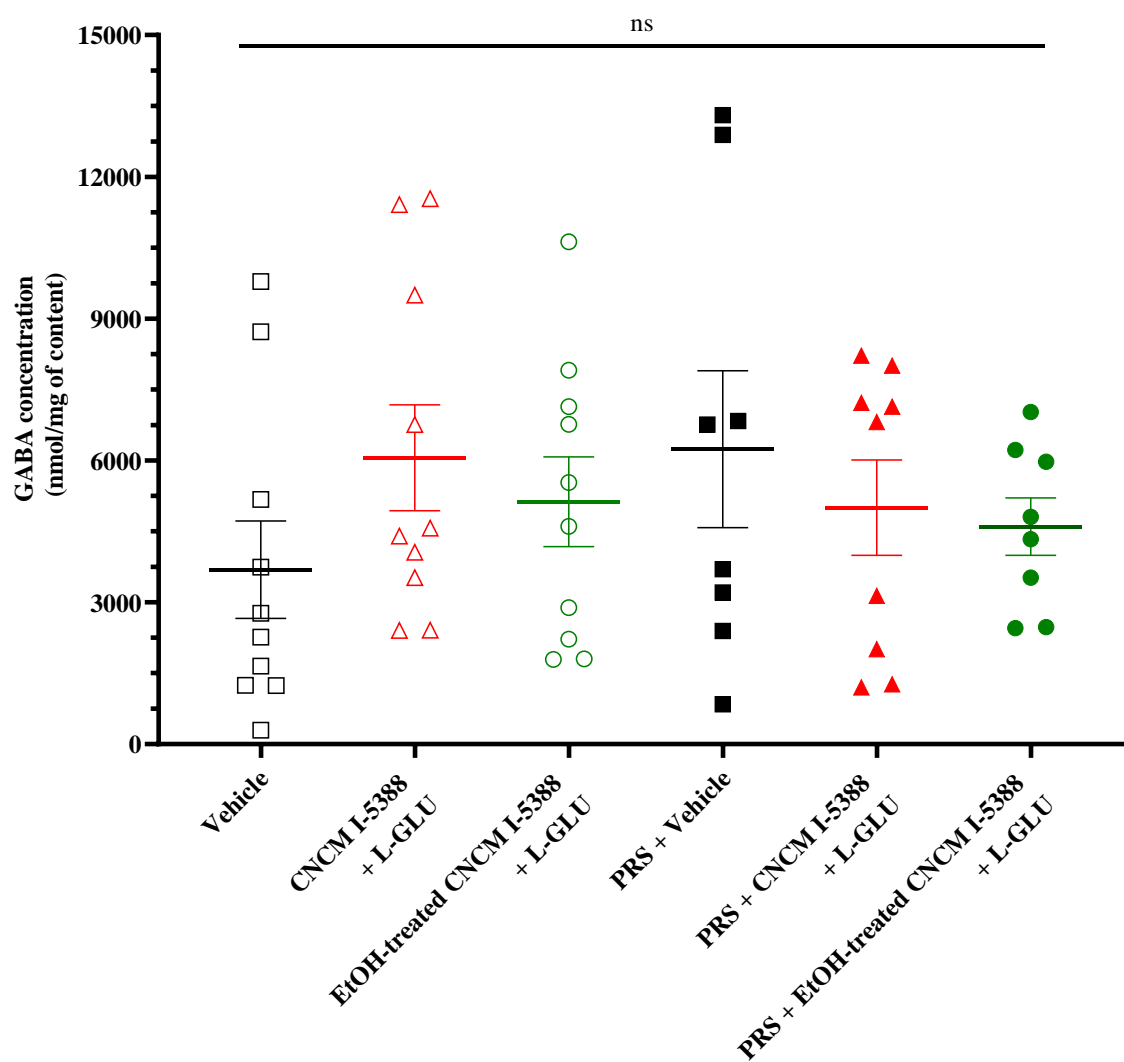
Supplementary figure 3. Cell-bound GAD activity of untreated (10⁹ CFU/mL) or EtOH-treated (3x10⁹ CFU/mL) *L. lactis* CNCM I-5388. Data are expressed as mean \pm SEM. ns = non-significant (p > 0.05).



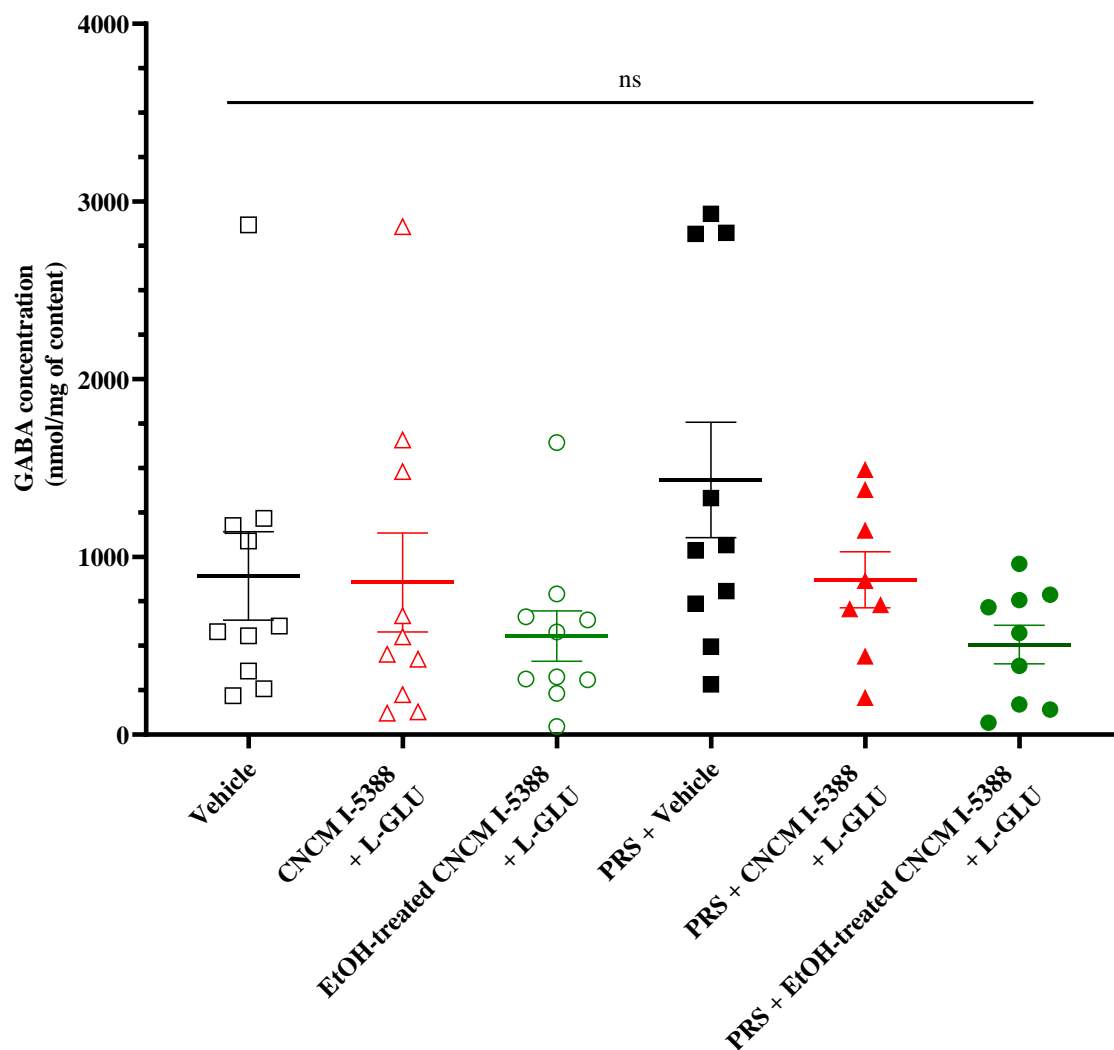
Supplementary Figure 4. Effect of oral administration of EtOH-treated *L. lactis* CNCM I-5388 supplemented with L-Glu monosodium salt hydrate on animals under basal conditions (i.e. one day before the PRS session) in response to all colorectal distension pressures (15 to 60 mmHg). (A) 10-day oral administration. (B) 5-day oral administration. (C) 1-day oral administration (D) 5-day oral administration followed by (G) 5-day treatment interruption. Results for untreated CNCM I-5388 were adapted from Gomes et al. (2023). Data are expressed as mean \pm SEM. No significant statistical differences were detected ($p > 0.05$).



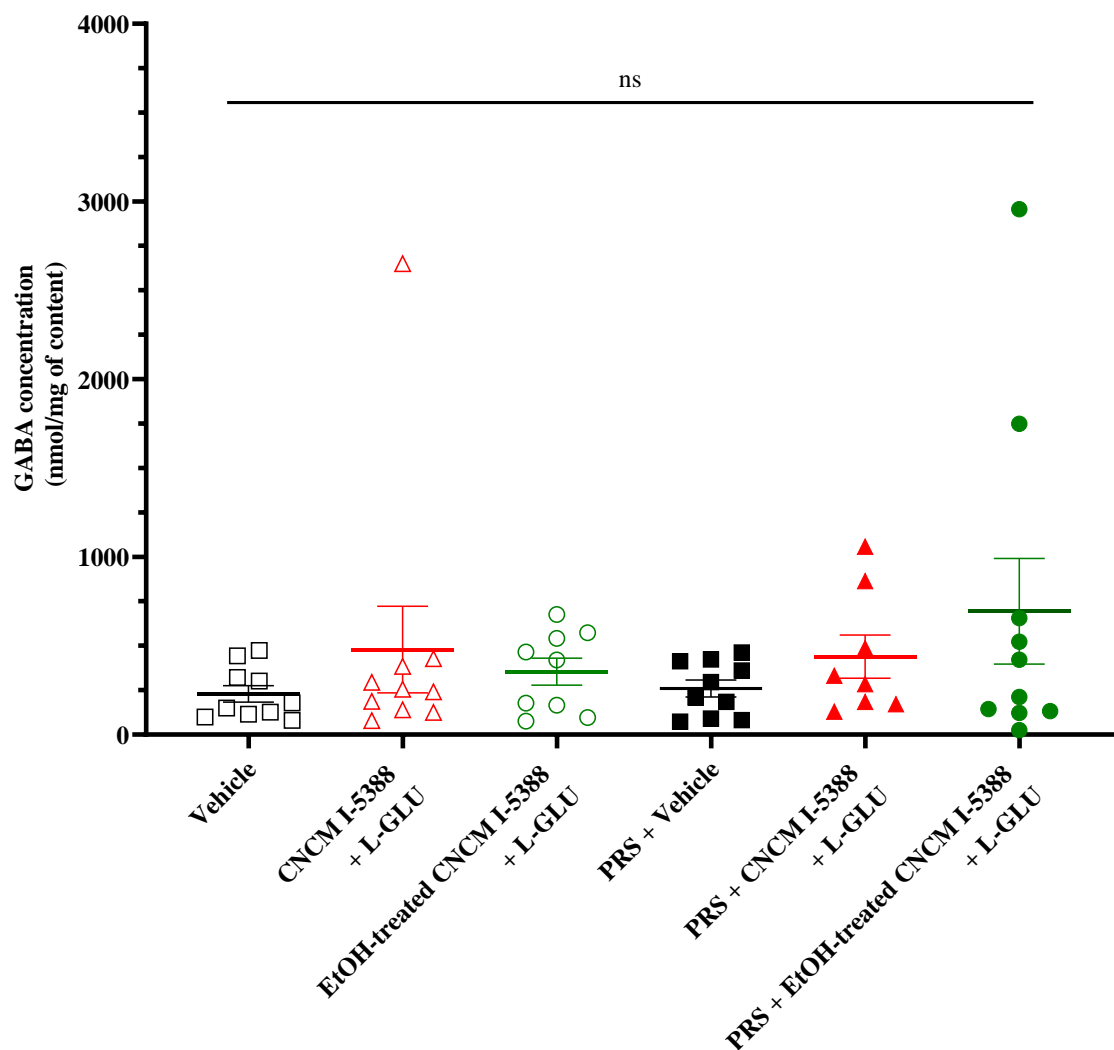
Supplementary Figure 5. Duodenal GABA concentration in gastric content after 5-day daily oral administration of untreated (10^9 CFU per day) or EtOH-treated CNCM I-5388 (3×10^9 CFU per day) supplemented with L-Glu monosodium glutamate salt. Data are expressed as mean \pm SEM. ns = non-significant ($p > 0.05$).



Supplementary Figure 6. Jejunal GABA concentration in gastric content after 5-day daily oral administration of untreated (10^9 CFU per day) or EtOH-treated CNCM I-5388 (3×10^9 CFU per day) supplemented with L-Glu monosodium glutamate salt. Data are expressed as mean \pm SEM. ns = non-significant ($p > 0.05$).



Supplementary Figure 7. Ileal GABA concentration in gastric content after 5-day daily oral administration of untreated (10^9 CFU per day) or EtOH-treated CNCM I-5388 (3×10^9 CFU per day) supplemented with L-Glu monosodium glutamate salt. Data are expressed as mean \pm SEM. ns = non-significant ($p > 0.05$).



Supplementary Figure 8. Colonic GABA concentration in gastric content after 5-day daily oral administration of untreated (10^9 CFU per day) or EtOH-treated CNCM I-5388 (3×10^9 CFU per day) supplemented with L-Glu monosodium glutamate salt. Data are expressed as mean \pm SEM. ns = non-significant ($p > 0.05$).

**SECTION III – GENERAL
DISCUSSION, PERSPECTIVES
AND CONCLUSION**

GENERAL DISCUSSION, PERSPECTIVES AND CONCLUSION

This section represents the culmination of extensive research and analysis presented throughout this doctoral dissertation. In the preceding sections, we have delved into the intricacies of irritable bowel syndrome (IBS) and its main symptom, visceral hypersensitivity (VH), and introduced probiotics and postbiotics with psychobiotic properties as potential therapeutic solutions for VH. We have next introduced different *Lactococcus lactis* strains and characterized their GABA production and glutamate decarboxylase (GAD) enzyme activity. Further, we have demonstrated the anti-VH properties (in an IBS-like stress-induced rat model) of GABA-producing *L. lactis* strains (viable or not) through the activation of host's GABA_B receptors, and we associated this effect to the GAD activity of these strains. Furthermore, through metabarcoding analyses, we have shown evidence that these anti-VH properties are independent of fecal microbiota alpha and beta diversities. Finally, through *in vitro* and *in vivo* investigations, we have observed that stomach acidic pH is the major variable coordinating GAD activity and GABA production and we extended our observations to a dynamic *in vitro* digestion model (TIM-1) mimicking the human upper gut, with here a focus on the gastric compartment. In this concluding section, we bring together the threads of our research findings, synthesize the implications of our discoveries, and explore the broader significance of our work within the context of VH treatment based on GABA-producing bacteria. By critically evaluating the data, methodologies, and outcomes, this chapter aims to provide a comprehensive perspective on the contributions, limitations, and future directions of our research. Additionally, we address the implications of our findings that extend beyond the scope of this dissertation, ultimately contributing to the ongoing discourse in microbiology and neurogastroenterology. This chapter underscores the significance of our research, places it within a broader academic and industrial context, and paves the way for future investigations that build upon the foundation laid out herein.

GENERAL DISCUSSION AND PERSPECTIVES

1. The GABA production diversity of *L. lactis* strains *in vitro*

In this study, we have validated the GABA production of wild-type *L. lactis* strains, under the same fermentative conditions in bioreactor, as well as mutants lacking the gene coding for the GAD enzyme (*gadB*) (**Figure 1**). Moreover, we extended this characterization to the GAD enzyme, at the level of the cytoplasmic activity (based on the enzyme activity itself and normalized by bacterial cell mass) and cell-bound activity (measured within whole cells). The analyses, here performed, open up new perspectives on the GABA production diversity of *L. lactis* strains, as also recently published by our team ¹, and integrate their potential for food and health applications. Based on the reference GABA-producing strain NCDO2118 (cytoplasmic GAD activity = 48.7 ± 10.7 $\mu\text{mol}/\text{min}.\text{mg}$), we categorized the strains used in this study as lower GABA-producing (NCDO2727, undetectable cytoplasmic GAD activity) and higher GABA-producing (CNCM I-5388, cytoplasmic GAD activity = 813.0 ± 183.7 $\mu\text{mol}/\text{min}.\text{mg}$). Worthy of notice, we have also previously published the main environmental conditions affecting GABA production in NCDO2118, for instance the presence of chloride ions, free L-glutamate (L-Glu) and L-arginine ^{2,3}. For standardization purposes, fermentation conditions used throughout our experiments were the same as used for NCDO2118; however, those parameters are not necessarily optimal for GABA production of all *L. lactis* strains in the fine balance context between biomass and GABA production. Future studies should consider refining the parameters affecting GABA production in *L. lactis*. This can be extended to the evaluation of the parameters (other than chloride ions, free L-Glu and arginine) that might affect GABA production, focusing on the production of the GAD enzyme through the expression of the *gadCB* genes. As observed in **chapters 1 and 2 of section 2**, *in vitro* GABA production could not be solely associated to GABA genetic determinants in *L. lactis*. Additionally, *in silico*

Section III– General Discussion, Perspectives and Conclusion

analysis of the GAD amino-acid sequence does not reveal major differences between NCDO2118 and CNCM I-5388 (**chapter 2 of section 2**) and NCDO2727 (**Figure 2**) strains. Given that the differences in cytoplasmic GAD activities *in vitro* we observe within the different strains under study can possibly lie in differences of strains' *gadCB* operon expression, our group is currently working on the study of the control of this operon within *L. lactis* strains. The latest research on this matter was published 25 years ago by Sanders and colleagues, where *gadCB* operon expression was found to be induced by chloride⁴. Therefore, our current study will enlighten other environmental factors in regency of *gadCB* expression.

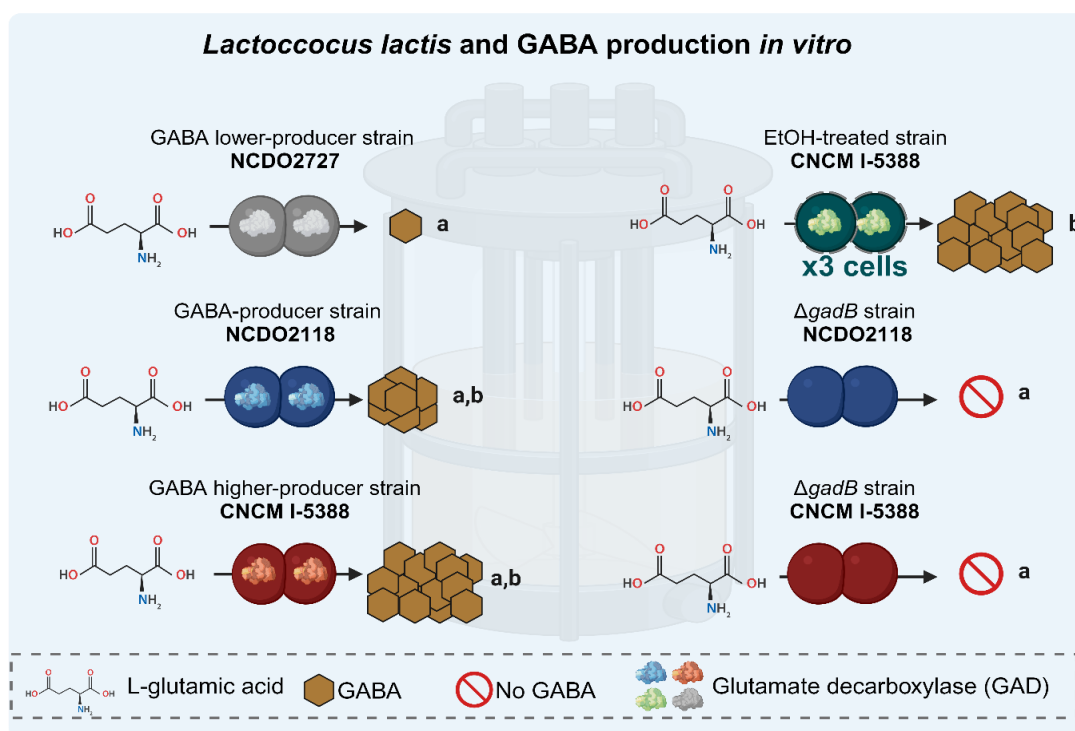


Figure 1. Main findings concerning *L. lactis* and GABA production *in vitro*. (a) Based on fermentation in bioreactor and *in vitro* cytoplasmic GAD activity results. (b) Based on *in vitro* cell-bound GAD activity results (pH 4.6). Note that EtOH-treated strain had to be concentrated three times in order to have the same GABA production as the untreated strain.

1.1. EtOH treatment and its impact in GABA production by *L. lactis*

Based on an EtOH-treatment protocol, we have developed a *L. lactis* “GAD bag”, consisting of inactivated bacteria with a minimally altered cell morphology and carrying the GAD enzyme (**chapter 3 of section 3**). This “GAD bag” kept a third of cell-bound GAD activity of untreated *L. lactis* at the same bacterial concentration, and as expected the same activity once bacterial concentration of EtOH-treated cells was enhanced in a 3-fold factor (**Figure 1**). The EtOH treatment was first described by Tadesse and colleagues (2018) as a tool to inactivate bacterial metabolism while maintaining cell morphology in *L. lactis*, but also other gram positive and negative bacteria. The validation of an unaffected cell morphology was confirmed not only via extracellular DNA measurements, as here performed (**chapter 3 of section 3**), but also with scanning electron microscopy ⁵. Consistent with the “GAD bag” concept is the observation that, once we sonicated untreated (10^9 CFU/mL) and EtOH-treated (3×10^9 CFU/mL) CNCM I-5388 in order to completely disrupt cells, cell-bound GAD activity was roughly 3-fold enhanced (**data not shown**). This suggests that GAD enzyme, once free of bacterial cell-related limitations (i.e. cell membrane, cell wall and glutamate/GABA antiporter GadC), “finds” more easily the L-Glu supplemented in medium and enhances GABA production. Additionally, our results pointed that cell-bound GAD activity of EtOH-treated CNCM I-5388 (3×10^9 CFU/mL) after sonication was lower than that of untreated cells (10^9 CFU/mL), indicating that EtOH treatment directly affects GAD enzyme, weakening its activity in our control pH of 4.6 once cells were disrupted (**data not shown**).

2. Acidic pH: the main parameter for *L. lactis* GAD activation and GABA production *in vitro* and *in situ* in host's stomach.

As described in **chapter 2 of section 1**, many authors have experimentally demonstrated that optimum pH window for most bacterial cytoplasmic GAD enzyme activities lies between 4.5 and 5⁶⁻¹³. Not surprisingly, we demonstrated this was the case for *L. lactis* strains NCDO2118 and CNCM I-5388 (**Figure 3**). However, cell-bound GAD activity did not exhibit the same variation profile in the acidic pH spectrum (pH 2.5, 3.0, 3.5, 4.0 and 4.6). As seen in **chapter 2 of section 2**, cell-bound GAD activity of CNCM I-5388 was enhanced at pH 3.5 while bacterial viable counts were reduced. In the meantime, both parameters for NCDO2118 were reduced at the same pH. We propose the existence of a balance between different optimal pH values for bacterial cell integrity, cytoplasmic and cell-bound GAD activities. For NCDO2118, considering its higher loss of viability than CNCM I-5388 at the same pH, it is possible that cells are damaged to a higher extent than CNCM I-5388, exposing GAD to a more acidic environment and reducing its activity. It has been experimentally evidenced in many bacteria that in acidic extracellular pH (3.5-5), intracellular pH was found to be approximately one unity more alkaline (pH 4.5-6.5)¹⁴⁻¹⁶. For CNCM I-5388, given its enhanced GAD activity and reduced bacterial viability at pH 3.5, it is likely that cells are sufficiently damaged so that intracellular pH can be closer to 4.6 and optimal for enhanced cytoplasmic GAD activity. At pH 3.0, both NCDO2118 and CNCM I-5388 strains would be sufficiently damaged so differences in extracellular and intracellular pH were lost, reducing drastically GAD enzyme activity until total inactivation. It is for the same reason we suppose that EtOH-treated cells might be more easily damaged by acidic pH, affecting its cell-bound GAD activity (**Figure 3**). Indeed, there's extended literature, which supports our hypothesis on the damage of EtOH 70% treatment in bacterial membrane, fatty acid composition, protein synthesis¹⁷⁻²¹ and reduction in intracellular pH²²⁻²⁴.

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Conversely, in neither analysis in rat stomach, nor in TIM-1 human-like stomach model in fed-state conditions (**chapter 3 of section 2**), we detected any statistical differences between GABA levels from EtOH-treated and untreated CNCM I-5388. It is relevant to highlight, yet, that in rat stomach the mean GABA content from untreated CNCM I-5388 was approximately 1.5 times higher than that observed for EtOH-treated bacteria and, worthy of notice, the same factor was found in TIM-1 stomach compartment analyses. In order to give more precise information on the link between bacterial integrity and cell-bound GAD activity in the pH spectrum, it would be interesting to: (i) evaluate the morphology and cell structures of *L. lactis* cells through electronic microscopy, as proposed by Taddese and colleagues (2018) ⁵; (ii) evaluate GABA production of EtOH-treated *L. lactis* throughout pH variations in a static HCl-based model, as performed for NCDO2118 and CNCM I-5388 strains in **chapter 2 of section 2**.

Moreover, we performed the same run in the TIM-1 model under fasted-state conditions, where initial pH of 3.0 rapidly drops to 1.7 throughout 2-h gastric digestion. In these conditions, GABA produced by untreated CNCM I-5388 was only detectable at the first 15 min of digestion (pH 2.0), while it was undetectable for EtOH-treated strain (**data not shown**). Noteworthy, untreated CNCM I-5388 viable counts descended from 10^9 CFU/mL (0 min, pH 3.0) to approximately 10^3 CFU/mL (**data not shown**). Altogether, these results imply that EtOH-treated *L. lactis* are indeed more sensitive to acidic pH at the level of cell-bound GAD activity. Considering the observations in the literature concerning human gastric pH, our results also imply that GABA production by *L. lactis*, as well as its survival in human stomach, would be higher in postprandial (pH>4) rather than fasting conditions (pH<3) ^{25,26}. This is an interesting observation to be considered in future clinical studies. Additionally, our observations reinforces previous studies that suggested that GAD acid resistance system's main use in ingested bacteria, would be to enhance bacterial survival when exposed to stomach acidic environment in order

to transit through downstream compartments^{27–29}.

Overall, our results indicate that pH is the major variable influencing the *L. lactis* GABA production *in vitro* and *in situ* in the host's stomach through acid pH-dependent activation of the GAD enzyme. In order to enlighten this consideration, future studies should evaluate *L. lactis* anti-VH properties either when: (i) the host presents an inhibited gastric acid secretion, which can be achieved, for instance, with proton-pump inhibitor omeprazole; (ii) the host exhibits a stimulated gastric secretion, which can be attained with the pentapeptide pentagastrin³⁰.

3. GABA-producing *L. lactis* and the anti-VH properties in rat: towards an innovative approach in IBS patients

Throughout the three chapters in the second section of this work, we have evaluated more than dozen oral treatment combinations (e.g. different strains, treatment durations) in the context of stress-induced visceral hypersensitivity in rats (IBS-like model), and the main findings can be seen in **Figure 4**.

3.1. The impact of gut microbiota on the GABA-dependent anti-VH properties

As decrypted by 16S metabarcoding analyses in **chapters 1 and 2 of section 2**, no oral treatment used in this study in rats exerted any influence in fecal microbiota at the level of alpha and beta diversities. This finding aligns with observations from numerous other studies on IBS, which have indicated that probiotic-based treatments do not lead to a significant shift in the overall diversity of the intestinal microbiota in mice, rats, or humans^{31–35}. In fact, when it comes to an already balanced "healthy microbiota", the introduction of a probiotic doesn't alter the existing microbial populations, however, it may have a noteworthy impact on restructuring the microbiota in individuals who already present dysbiosis^{31–35}. In agreement with these observations, a recent study has shown that *L. lactis* was capable of improving beta diversity of

gut microbiota of piglets, when associated with gut microbiota dysbiosis caused by the pathogenic bacterium *Clostridium difficile*³⁶. Moreover, a study on rats 10-day orally treated with a fermented milk product with five lactic acid bacteria revealed that *L. lactis* (present in the microbial consortium) was detectable in feces by RT-PCR after no more than two days of treatment interruption³⁷. This result indicated that *L. lactis* is rapidly washed out from rats' GIT, being therefore unlikely to induce compositional changes in host's autochthonous bacteria³⁸.

We cannot discard, however, the possibility of *L. lactis* affecting gut microbiota at the functional level. As extensively observed in the literature, ingested bacteria might affect individuals favoring metabolic rather than compositional changes in microbiota, for instance enhancing or diminishing short-chain fatty acid and branched-chain amino acid production³⁹. In our context and in order to undoubtedly confirm if gut microbiota impacts on *L. lactis* anti-VH properties, future studies could be performed on germ-free animals^{40,41} or animals treated with antibiotic cocktail⁴¹⁻⁴³ prior to *L. lactis* oral treatment.

3.2. The factors involved in anti-VH properties

Of all *L. lactis* daily oral treatment combinations evaluated, three of them successfully reduced abdominal contractions of stressed rats to quasi-basal levels: NCDO2118 (10-day treatment, 10⁹ CFU/day), untreated CNCM I-5388 (10 or 5-day treatment 10⁹ CFU/day) and EtOH-treated CNCM I-5388 (10 or 5-day treatment, 3x10⁹ CFU/day), all supplemented with L-Glu. Based on all *in vivo* results, we verified that the following factors are related to the analgesic efficiency of *L. lactis* oral treatment: (i) free L-Glu supplementation in *L. lactis* oral treatment; (ii) *L. lactis* GABA production through the activation of the GAD enzyme in host's stomach acidic pH; (iii) the activation of host's GABA_B receptors at the level of the central and/or peripheral nervous systems; (iv) the duration of the treatment.

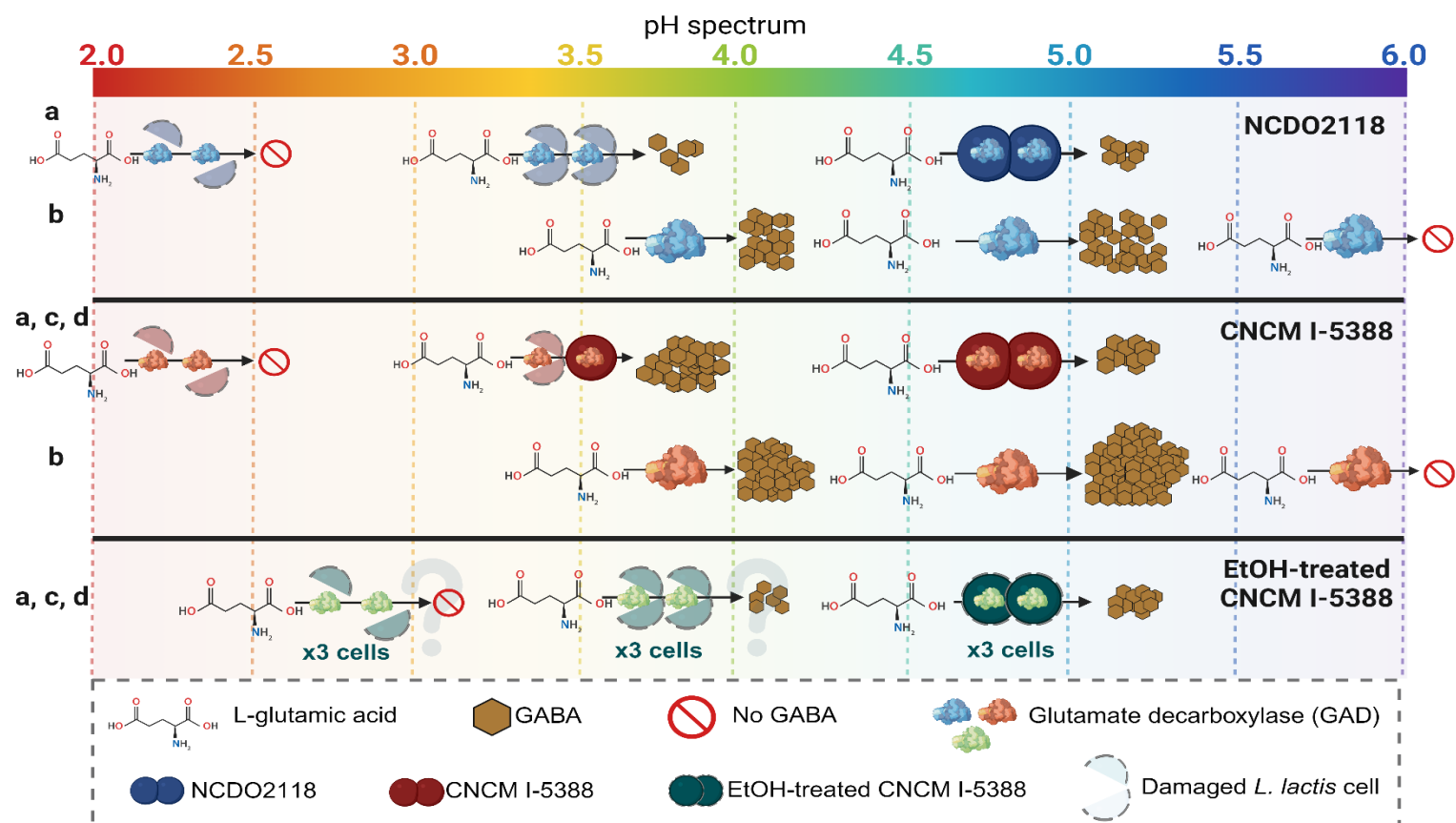


Figure 3. Main findings in *L. lactis* and GABA production as a function of environmental pH. (a) Based on *in vitro* cell-bound GAD activity results. (b) Based on *in vitro* cytoplasmic GAD activity results. (c) Based on measurements of GABA concentration in rat stomach. (d) Based on TIM-1 results in human-like gastric compartment. Interrogation marks indicate that validation is further needed through *in vitro* cell-bound GAD activity within the pH spectrum described.

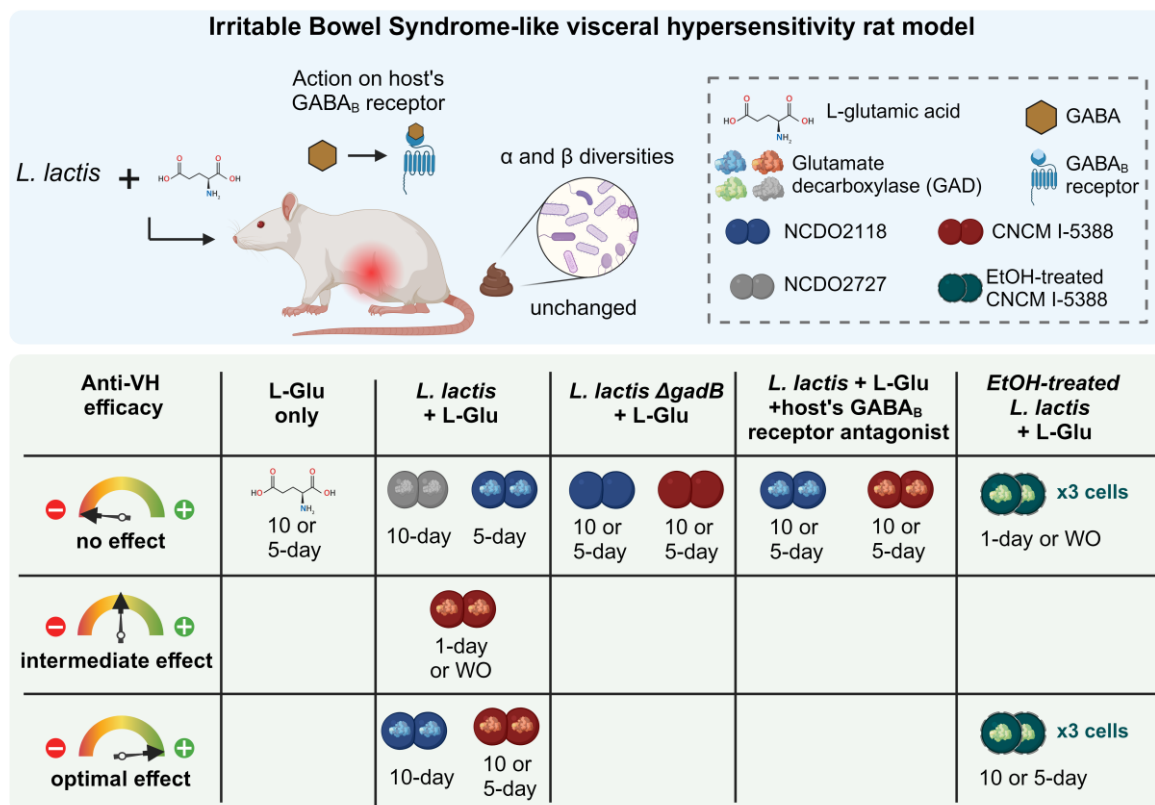


Figure 4. Main findings in *L. lactis* anti-VH properties. Figure shows oral treatments that are efficient (+) or inefficient (-) in IBS-like rat model with VH induced by acute stress. Note that efficacy is also dependent on the time of treatment: 1-day or 5-day or 10-day or 5-day treatment followed by 5-day treatment interruption (wash-out, WO).

3.2.1. L-Glu supplementation in *L. lactis* oral treatment

As described in **chapters 1 and 2 of section 2**, abolishment of anti-VH properties of either *L. lactis* NCDO2118 or CNCMI-5388 was observed once free L-Glu supplementation was no longer present in oral treatments (**Figure 4**). Our group has previously submitted *L. lactis* cultures to varying glutamate concentrations, ranging from 0 to 136 mM². These different concentrations did not have an impact on either the specific growth rate or the final biomass, however, there were slight deviations in the final pH levels, and more notably, discernible shifts in the accumulated GABA levels were observed across the L-Glu-supplemented cultures. Indeed, with increased L-Glu supplementation, there was a corresponding rise in both final pH and the accumulated GABA levels. For instance, cultures without free L-Glu supplementation

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exhibited a pH of 4.1 and 0.3 mM of GABA, while cultures supplemented with 136 mM glutamate showed a pH of 4.5 and 3.8 mM of GABA mM². Based on this result, it seems clear that *L. lactis* GABA production in the GIT is not sufficient to exert anti-VH properties if it is not enhanced by an external free L-Glu source, supplemented in oral treatment.

Interestingly, in our study, L-Glu administration itself neither diminished nor enhanced VH in rats (**Figure 4**). In the context of neurotransmitters, L-Glu is the antithesis of GABA. This amino acid serves as the primary excitatory neurotransmitter in the nervous system of mammals, playing a prominent role transmitting pain signals from the periphery to the brain⁴⁴. Indeed, there is clinical evidence indicating that a specific subgroup of IBS patients may have heightened sensitivity to L-Glu⁴⁵. A recent study has shown that dietary L-Glu increases VH in a mouse model of visceral pain⁴⁶. Another study has shown that metabotropic glutamate-5 receptor antagonists reduced visceral nociception in rats through an effect related to peripheral afferent endings⁴⁷. These studies are contrasting with ours, since VH in rats was unaffected by L-Glu itself. However, it is important to highlight that studies evaluating the impact of L-Glu in visceral nociception differ concerning the host (e.g. human, mouse, rat), the type of treatment (e.g. oral, intraluminal, intravenous) and respective concentration, the type and duration of application of induced-stress model to achieve VH (acute or chronic stress) and respective stimulus employed (e.g. mechanical, electrical or chemical⁴⁵⁻⁴⁹).

3.2.2. *L. lactis* GABA production through the activation of the GAD enzyme in host's stomach acidic pH

We have also demonstrated in **chapters 1 and 2 of section 2** that mutant *L. lactis* NCDO2118 and CNCM I-5388 strains lacking the gene coding for the GAD enzyme (*gadB*), failed to prevent VH in rats (**Figure 4**). Indeed, Δ *gadB* strains were shown to not produce detectable amounts of GABA (**Figure 1**), implicating that the anti-VH properties of NCDO2118 and CNCM I-5388 are GAD-dependent. Moreover, this effect seems to be directly correlated to the levels of GABA produced by the strain in host's stomach. This assumption is enforced by the observation that GABA-lower producer NCDO2727 exerts no anti-VH properties (**Figure 4**), even though it counts with a *gadCB* operon with similar genetic organization (*gadCB* genes and promoter and *gadR*, coding for a transcriptional activator of *gadCB* operon) with more than 99% of identity with NCDO2118 (**chapter 2, section 2**) and CNCM I-5388 (**Figure 5**). Therefore, not only the presence of *gadCB* operon and positive regulator *gadR*, but also GAD activity levels, are the main variables impacting *L. lactis* GABA production *in vitro* and *in vivo* in host's GIT. As earlier mentioned, our group is currently working on elucidating if the differences in GAD activity among strains we observe *in vitro* are rather due to differences in the expression of *gadCB*. If we consider GABA lower-producing *L. lactis* strains, their native *gadCB* and *gadR* genes and operons could be replaced by those of a higher GABA producer, in order to evaluate if GAD activity is enhanced *in vitro* with enhanced production of GABA. Moreover, this mutant strain could be tested for its anti-VH properties *in vivo* compared to the wild-type lower and higher GABA-producing strains. These experimentations would clarify the existence of a GABA-production threshold by *L. lactis in vivo* as the main factor in order to exert anti-VH properties, further detailed in the next paragraphs.

<i>gadR2</i> (1401 bp)				<i>gadC</i> (1512 bp)				<i>gadB</i> (831 bp)			
Strain	NCDO 2118	NCDO 2727	CNCM I-5388	Strain	NCDO 2118	NCDO 2727	CNCM I-5388	gadR2 (831bp)	NCDO 2118	NCDO 2727	CNCM I-5388
NCDO 2118	---	---	---	NCDO 2118	---	---	---	NCDO 2118	---	---	---
NCDO 2727	98.22%	---	---	NCDO 2727	99.14%	---	---	NCDO 2727	98.8%	---	---
CNCM I-5388	98.5%	98.86%	---	CNCM I-5388	99.27%	99.47%	---	CNCM I-5388	100%	98.8%	---

Figure 5. Percentage of identity between *gadR*, *gadC* and *gadB* genes between *L. lactis* NCDO2118, NCDO2727 and CNCM I-5388.

Analysis performed as described on chapter 1 of section 2.

3.2.3. The activation of host's GABA_B receptors at the level of the central and/or peripheral nervous systems

We have demonstrated in **chapters 1 and 2 of section 2** that, once rats were treated with GABA_B receptor antagonist SCH 50911, NCDO2118 and CNCM I-5388 lost their antinociceptive properties independently of treatment duration (**Figure 4**). We concluded that the anti-VH effect is dependent on the activation of the host's GABA_B receptors. Supporting our conclusions is the fact that administration of GABA_B agonists have been for long known to produce analgesia in acute and chronic pain models⁵⁰. Moreover, analogs of GABA, such as gabapentin and pregabalin, have demonstrated effectiveness in experimental models of VH^{51–57}. Finally, the most commonly employed GABA_B receptor agonist, baclofen, has demonstrated its ability to elicit anti-nociceptive effects in various rat models of VH^{58–63}.

Nevertheless, it is not clear if the activation of the host's GABA_B receptors, as we observed in our study, occurs locally in the GIT at the level of the peripheral or central nervous system (PNS and CNS, respectively). Indeed, GABA_B receptors are widely distributed in the GIT PNS with decreasing numbers from the mouth to the anus⁶⁴ and also in the CNS⁶⁵. Peripherally restricted analgesics are desirable to avoid CNS side effects. For instance, a study in a mouse model has shown that GABA_B receptor agonist baclofen effectively penetrates the CNS and has been observed to induce depression and hypothermia when administered in

analgesic doses ⁶⁶.

The capacity of GABA to cross the epithelial barrier is still debated in the literature. Pérez-Berezo and colleagues (2017) have shown that GABA produced by the strain *E. coli* Nissle 1917 was not able to directly cross the epithelial barrier, requiring functionalization by a lipopeptide and subsequent translocation ⁶⁷. Nonetheless, studies have shown that H⁺/GABA symport occurs through the apical membrane of human intestinal epithelial Caco-2 cells, which is believed to facilitate the absorption of luminal GABA, from the stomach to the descending colon, with the small intestine expressing it to its fullest extent ^{68,69}. Moreover, GABA transporters have been found in the basolateral membrane of Caco-2 cells ⁷⁰, implying that luminal GABA should be able to cross the intestinal barrier and perhaps reach sites outside the digestive tract. In fact, a single oral GABA administration to rats raised blood levels of the neurotransmitter, with a peak concentration occurring between 30 and 60 minutes ⁷¹. In our study on stressed or sham-stressed rats, treated for 5 days with vehicle, untreated or EtOH-treated CNCM I-5388 (**chapter 3 of section 3**), we have measured GABA concentrations in blood samples, however we did not detect any differences between treatments (**data not shown**). Nevertheless, blood sampling was performed only 3h after oral treatments, so it is possible that GABA concentration peak was lost before we collected samples. Future studies might consider performing a similar analysis, but with regular blood sampling over time, in order to evaluate the variation of GABA levels in the blood.

Moreover, through electrophysiological assessments, it has been observed that GABA_B receptor agonists can influence the responses of vagal mucosal and muscle afferents that innervate specific regions of the GIT, specifically the esophagus and proximal stomach ^{72–74}. More recently, it has been established that baclofen exhibits a dose-dependent reduction in the responses of mechanosensitive pelvic nerve afferents to noxious colorectal distension ^{75,76}. Another study has shown that ingestion of *Lactobacillus* strain regulates emotional behavior

and GABA receptor expression in a mouse via the vagus nerve ⁷⁷. These observations provide support for the idea that GABA_B receptor agonists may exert their beneficial effects by acting mainly on peripheral sites. In order to confirm this assumption in our context, it would be interesting to evaluate the impact of GABA-producing *L. lactis* in rats submitted to vagotomy, as proposed elsewhere ⁷⁷.

3.2.4. The duration of the oral treatment

Within our investigations, daily oral treatments were based on different durations, varying between 1-day, 5-day, 10-day and 5-day treatment followed by 5-day treatment interruption (wash-out, WO) (**Figure 4**). Noteworthy, not all durations were applied to all treatments. For instance, since 10-day treatment with GABA lower-producer NCDO2727 had already demonstrated no anti-VH properties, we inferred it would be the case for 5-day treatment. Similarly, GABA-producer NCDO2118 demonstrated no antinociceptive potential after 5-day treatment, therefore we did not test it after 1-day treatment. The differences in oral treatments durations for optimal anti-VH properties in rats seem to be consistently related to the levels of GABA produced by *L. lactis* strains, *in vitro* and *in situ* in the host's stomach. In other words, our results might indicate that, the higher GABA production by a *L. lactis* strain, the faster it will exert anti-VH properties. The treatment exerting the fastest antinociceptive properties in rats is undoubtedly achieved with higher GABA producer CNCM I-5388, with optimal effects after only 5-day oral treatment, which was also the case for the EtOH-treated nonviable strain (**Figure 4**). This result is very interesting because it reveals the potential of GABA-producing psychobiotics, either in their probiotic or postbiotic form, in VH treatment of IBS patients. As described in **chapter 3 of section 2**, a clear practical and economical advantage of postbiotics is that they offer extended shelf life, simplified storage, and reduced temperature maintenance compared to probiotics due to their lack of live microorganisms ⁷⁸.

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Therapeutically, postbiotics are safer than probiotics, as they prevent the acquisition of antibiotic resistance genes and virulence factors, especially crucial for children with developing immune systems and compromised intestinal barriers ⁷⁹. Studies have already indicated that treatments with dead bacterial cells presented antinociceptive properties in rats and humans ^{80–82}. For the first time, we introduced a new concept, based on the “GAD bag”. Nevertheless, it is crucial to emphasize that in the context of our study, an active *L. lactis* GAD enzyme is necessary for anti-VH properties, therefore storage should be proven not to affect its activity. As an illustration, we have observed that more than 3-month sample freezing at -20°C, either for untreated EtOH-treated CNCMI-5388 is sufficient to significantly reduce cell-bound and cytoplasmic GAD activities (**data not shown**). In order to better clarify the implications of *in vitro* and *in vivo* variabilities in GAD activity and GABA production in the antinociceptive effect of *L. lactis*, future studies should consider evaluating how to better preserve bacteria through time, avoiding a potential loss of GAD activity.

4. GABA production threshold hypothesis: host's GABA_B receptors activation and/or expression through time in the GIT

Interestingly, we showed an intermediate anti-VH effect in rats treated with higher GABA producer CNCM I-5388 at the first day of treatment and at the fifth day of wash-out, which was not the case for its EtOH-treated counterpart (**Figure 4**). **Figure 6** decrypts our main hypotheses for the observed phenomena. Note that 1-day treatment might be sufficient to activate the existing GABA_B receptors in the host's stomach and/or downstream GIT compartments (with decreasing numbers) ⁶⁴. This would lead to an intermediate anti-VH efficacy for the untreated CNCM I-5388, given its high GABA production *in vitro* and *in situ* in the stomach (**chapters 2 and 3 of section 2**). As earlier explained, we suppose that EtOH treatment turns not only CNCM I-5388 cells but also their GAD enzyme more fragile to pH variations, thus reducing GABA production *in situ*. After 5-day treatment, both untreated and EtOH-treated CNCM I-5388 exert optimal anti-VH properties. We postulate that, through time, GABA-producing *L. lactis* strains are capable of enhancing GABA_B receptor expression and/or stabilizing them at the cell surface. In various studies, it has been noted that GABA_B receptors exhibit swift and intrinsic internalization even in the absence of agonists ^{83–85}. Additionally, in a mouse study, it was demonstrated that the administration of *Lactobacillus rhamnosus* (JB-1) consistently influenced the mRNA expression of GABA_B receptor subunits in the central nervous system (CNS) ⁷⁷. There are currently no studies concerning a bacterial-based oral treatment-dependent enhancement of GABA_B receptor subunits mRNA expression in the PNS. However, this possibility is enforced by *Lactobacillus acidophilus* administration to mice, which induced expression of cannabinoid and opioid receptors in intestinal cells ⁸⁶. Therefore, even having less GABA production *in situ* than CNCM I-5388 strain, the EtOH-treated bacteria might compensate for the anti-VH properties with higher numbers of accessible GABA_B receptors in the host, facilitating their activation. In both treatments, after 5-day wash-out (WO),

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we deduce the number of GABA_B receptors at the host's cell surface could be reduced. However, in the case of the untreated strain, the numbers of GABA_B receptors would be still superior to those of the EtOH-treated strain, facilitating their activation from another source than *L. lactis*. In fact, 3.2% of the amino-acid composition of the rodent diet used in this study is composed by L-Glu (**Annex 1**), it is not unreasonable to postulate that GABA-producing bacteria colonizing the stomach (e.g. *Clostridium*, *Lactobacillus*)^{87,88}, even at small amounts, could produce sufficient GABA to exert intermediate anti-VH properties if the host's GABA_B receptors are sufficiently numerous, which would be the case for untreated but not EtOH-treated *L. lactis*. Moreover, GABA levels are not described in the rodent diet used in this study, however, the company's notice indicates that wheat and corn seeds as well as brewer's dry yeast (irradiated *Saccharomyces cerevisiae*) are part of the ingredient's composition (**Annex 1**). Since accumulated GABA was detected in wheat and corn seeds⁸⁹⁻⁹¹ and *S. cerevisiae* has been described as a GABA-producer⁹², it is reasonable to infer that a rodent diet might be a non-negligible source of GABA for activation of GABA_B receptors, if they are sufficiently numerous in the host.

In agreement with these observations, it is crucial to emphasize that the GABA-producer NCDO2118 exerts no anti-VH properties after 5-day treatment, contrarily to 10-day treatment (**Figure 4**). Once again, this exposes the possible existence of a threshold of GABA production by *L. lactis* in the host's stomach capable of: (i) rapidly triggering the activation of the host's GABA_B receptors and maintenance of gastric secretion (ii) enhancing the number of GABA_B receptors (or subunits) in the host's GIT through time.

5. Implications of stress in visceral hypersensitivity

Due to the heightened sensitivity to stress commonly observed in patients with IBS and the significant impact of IBS on their quality of life, many studies aimed to investigate the physiological factors associated with perceived stress in IBS patients, exploring the links between perceived stress levels and the overall quality of life in this specific patient group. Studies examining the stress response often consider two markers of the HPA axis: cortisol and adrenocorticotrophic hormone (ACTH). Research has revealed that women with IBS typically have lower baseline levels of ACTH compared to healthy controls and often exhibit higher cortisol levels both at baseline and in response to physical stressors⁹³. When subjected to the Trier Social Stress Test (TSST), a psychosocial stressor, women with IBS showed reduced cortisol reactivity compared to healthy control and scored higher on stress-related psychological assessments⁹⁴. Another study employing the TSST found that female IBS patients had a sustained cortisol output in response to the stressor, unlike healthy controls⁹⁵. Some authors reported a diminished stress response in IBS patients, as evidenced by lower levels of ACTH and cortisol following an acute physical stressor compared to healthy controls⁹⁶. Therefore, in our study, we cannot discard the possibility that anti-VH properties exerted by GABA-producing *L. lactis* are actually a consequence of reduced psychological stress. Accordingly, literature have extensively shown the positive effects of GABA receptor agonists on psychological stresses⁹⁷. In this scenario, GABA produced by bacteria could act on the psychophysiological responses of rats after partial restraint stress, for instance modulating the markers of the HPA axis and reducing the perception visceral hypersensitivity. Future studies might consider evaluating if GABA-producing bacteria impact on the perception of psychological stress, since it has implications for clinical interventions. If a connection, or conversely, a disconnection, is identified between patients' self-reported stress levels and objective physiological markers, treatments can be customized to enhance patient outcomes.

6. Applications of GABA-producing *L. lactis* in health

The study presented here was performed in the single context of VH in an IBS-like animal model for proposing a new therapeutic strategy in such a pathophysiological context. However, as reviewed by Diez-Gutiérrez and colleagues (2020), GABA-producing bacteria have health potential uses in many disease contexts, including: other bowel disorders (e.g. Crohn's disease), cardiovascular disorders, nervous system disorders (e.g. epilepsy, anxiety and depression), diabetes and asthma ⁹⁸. Therefore, the human health applications of GABA-producing *L. lactis* could be extended to all these mentioned contexts.

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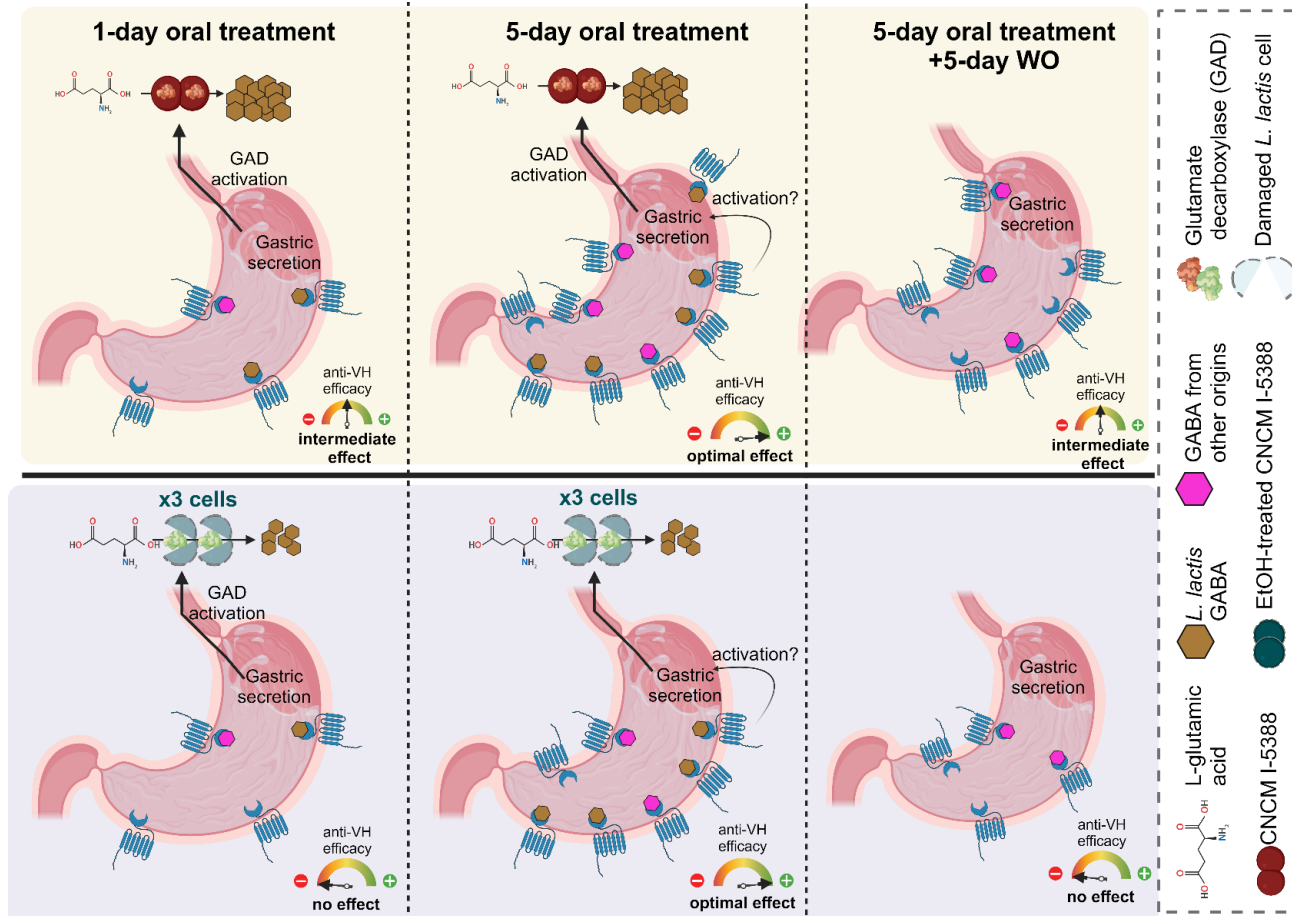


Figure 6. Main hypotheses concerning *L. lactis* anti-VH properties. Figure focuses on untreated and EtOH-treated *L. lactis* CNCM I-5388 supplemented with L-Glu, the two treatments exhibiting the fastest anti-VH properties in the IBS-like rat model with VH induced by stress. (WO) wash-out.

CONCLUSION

In conclusion, the research presented in this dissertation has explored the potential of GABA-producing *Lactococcus lactis* strains as a novel treatment strategy for alleviating visceral hypersensitivity (VH), a primary symptom of irritable bowel syndrome (IBS). Through a comprehensive investigation using stress-induced VH in an IBS-like rat model, we have made significant strides in elucidating the therapeutic potential of these interventions relying on their probiotic or postbiotic properties. Our findings have unequivocally demonstrated that the administration of GABA-producing *L. lactis* can effectively reduce VH in rats, offering promise for IBS management. This effect was accompanied by a marked reduction in the high nociceptive responses observed in our model, underscoring the potential of this treatment to mitigate the discomfort and pain associated with IBS. Furthermore, our research delved into the mechanistic underpinnings of this therapeutic approach, shedding light on the pivotal role of the host's gastric acidity in GABA production by *L. lactis*. By elucidating the intricate pathways through which these strains exert their beneficial effects, we provide a foundation for further studies aimed at refining and optimizing this treatment strategy. While our findings hold great promise, it is essential to acknowledge the complexities of translating these results to clinical applications. Human trials and extensive safety assessments will be pivotal in determining the full scope of this therapeutic approach and its applicability to the diverse IBS patient population. In sum, this research offers a new avenue for therapeutic intervention in IBS patients. The potential of GABA-producing lactic acid bacteria to alleviate VH represents a significant step toward improving the quality of life for individuals afflicted by this debilitating condition. As we move forward, further research and clinical trials will be necessary to fully harness the promise of this innovative treatment strategy for IBS.

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ANNEXES

ANNEX 1

2016 Teklad Global 16% Protein Rodent Diet

Product Description—2016 Teklad Global 16% Protein Rodent Diet is designed and manufactured with high quality ingredients. 2016 is a **fixed formula**, nutritionally balanced, non-autoclavable diet containing a minimum of 16% protein and 3.5% fat which supports growth and maintenance. 2016 does not contain alfalfa or soybean meal, thus minimizing the occurrence of natural phytoestrogens. Absence of animal protein and fish meal eliminates the presence of nitrosamines. For autoclavable diet please refer to 2016S Teklad Global 16% Protein Rodent Diet (Sterilizable). **Diet 2016 is available certified (2016C) and irradiated (2916).**

Ingredients—Ground wheat, ground corn, wheat middlings, corn gluten meal, calcium carbonate, soybean oil, brewers dried yeast, dicalcium phosphate, iodized salt, L-lysine, DL-methionine, choline chloride, niacin, vitamin A acetate, biotin, pyridoxine hydrochloride, thiamine mononitrate, vitamin D₃ supplement, folic acid, menadione sodium bisulfite complex (source of vitamin K activity), vitamin E supplement, vitamin B₁₂ supplement, riboflavin, calcium pantothenate, ferrous sulfate, magnesium oxide, manganese oxide, zinc oxide, copper sulfate, calcium iodate, cobalt carbonate, chromium potassium sulfate.

Macronutrients*

Crude Protein	%	16.7
Crude Oil (Fat)	%	4.2
Crude Fiber	%	3.9
Ash	%	5.6
Carbohydrate (available)	%	56.0
Starch	%	52.0
Sugar	%	4.0
Digestible Energy [†]	Kcal/g (MJ/kg)	3.3(13.7)
Metabolizable Energy [†]	Kcal/g (MJ/kg)	3.2(13.3)
Calories from Protein*	%	20
Calories from Fat*	%	11
Calories from Carbohydrate*	%	69

Minerals*

Calcium	%	0.98
Phosphorus	%	0.65
Sodium	%	0.25
Potassium	%	0.53
Chloride	%	0.42
Magnesium	%	0.21
Zinc	mg/kg	80.0
Manganese	mg/kg	124.0
Copper	mg/kg	15.0
Iodine (added)	mg/kg	12.0
Iron	mg/kg	240.0
Selenium	ug/kg	200
Cobalt	ug/kg	600
Chromium	ug/kg	500

Amino Acids*

Aspartic Acid	%	1.04
Glutamic Acid	%	3.40
Alanine	%	1.03
Glycine	%	0.69
Threonine	%	0.57
Proline	%	1.57
Serine	%	0.81
Leucine	%	1.89
Isoleucine	%	0.74
Valine	%	0.83
Phenylalanine	%	0.89
Tyrosine	%	0.48
Phe + Tyr	%	1.37
Methionine	%	0.33
Cystine	%	0.31
Met + Cyst	%	0.64
Lysine	%	0.75
Histidine	%	0.41
Arginine	%	0.82
Tryptophan	%	0.15
Available Lysine	%	0.65

Vitamins*

Vitamin A	iu/g	15.8
Retinol	mg/kg	4.8
Vitamin D ₃	iu/g	1.5
Cholecalciferol	ug/kg	38
Vitamin E (a-tocopherol)	mg/kg	100.0
Vitamin K ₃ (menadione)	mg/kg	51.3
Vitamin B ₁ (thiamine)	mg/kg	17.2
Vitamin B ₂ (riboflavin)	mg/kg	14.5
Avail. Niacin (nicotinic acid)	mg/kg	64.5
Vitamin B ₆ (pyridoxine)	mg/kg	18.5
Pantothenic Acid	mg/kg	32.2
Vitamin B ₁₂ (cyanocobalamin)	ug/kg	80
Avail. Biotin	mg/kg	0.4
Folate	mg/kg	3.4
Vitamin C	mg/kg	—
Choline	mg/kg	1095.0
B Carotene	mg/kg	3.1
Inositol	mg/kg	1630.0

Fatty Acids*

C16:0 Palmitic	g/kg	6.1
C18:0 Stearic	g/kg	0.9
C18:1ω9 Oleic	g/kg	8.9
C18:2ω6 Linoleic	g/kg	23.1
C18:3ω3 Linolenic	g/kg	1.5
Total Saturated	g/kg	7.3
Total Monounsaturated	g/kg	9.1
Total Polyunsaturated	g/kg	24.6

Cholesterol	mg/kg	—
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*Calculations performed using values of 4 kcal/g for protein and carbohydrate and 9 kcal/g for oil (fat).

[†]**Digestible Energy (DE)** is the total (gross) amount of energy in the diet minus the energy eliminated in the feces. It accounts for energy contained in the indigestible portion of the diet. Of the digestible energy, some is lost as a result of various metabolic processes in the urine and sometimes in gases from the digestive tract. The remaining energy in the body is available to support metabolism and is known as the **Metabolizable Energy (ME)**. This value more accurately reflects the usable energy contained in the diet.

*Nutrient levels are calculated from raw material data and are adjusted to 10% moisture level in the diet. Nutrient values may vary due to the inherent variability in the natural ingredients and from laboratory analysis.

Standard Product Form: **PELLET**

APPENDIX

SCIENTIFIC CURRICULUM VITAE

■ Jan 2021 - Dec 2023

Ph.D. Student: Infectology, Physiopathology, Toxicology, Genetics and Nutrition

Ph.D. School: Sciences Ecologiques, Vétérinaires, Agronomiques & Bioingénieries (SEVAB)

Supervisors: Hélène EUTAMENE, Muriel COCAIGN-BOUSQUET et Muriel MERCIER-BONIN

Host laboratories: INRAE & Toxalim (Research Centre in Food Toxicology), Université de Toulouse, INRAE, ENVT, INP-Purpan, UPS, Toulouse; INSA-Toulouse & TBI (Toulouse Biotechnology Institute); Toulouse Biotechnology Institute (TBI), Université de Toulouse, CNRS, INRAE, INSA, Toulouse, France; Lesaffre International, Marcq-en-Barœul, France.

Subject: Exploring the health potential of GABA-producing *Lactococcus lactis* strains: towards novel therapeutic strategies in visceral hypersensitivity management.

■ Jul 2022

Post graduate diploma : Ecole National Vétérinaire de Toulouse (ENVT)

General surgery of laboratory animals.

■ Oct 2020

Post graduate diploma : Ecole National Vétérinaire de Toulouse (ENVT)

Care and use of laboratory animals, function B

■ Jan 2020 – Dec 2020

Scientific researcher

Same supervisors, host laboratories and subject as above.

■ Jan 2019-Jul 2019

Intern

Supervisor: Brice ENJALBERT

Host laboratory: Toulouse Biotechnology Institute (TBI), Université de Toulouse, CNRS, INRAE, INSA, Toulouse, France ;.

Subject: Genetic control of the glucose-xylose transition in *Escherichia coli*.

SCIENTIFIC COMMUNICATION

Publications

Gomes P, Laroute V, Beaufrand C, Daveran-Mingot ML, Aubry N, Liebgott C, Ballet N, Legrain-Raspaud S, Theodorou V, Mercier-Bonin M, Eutamene H. 2023. *Lactococcus lactis* CNCM I-5388 versus NCDO2118 by its GABA hyperproduction ability, counteracts faster stress-induced intestinal hypersensitivity in rats. The FASEB Journal. Accepted on October 4th 2023.

Laroute V, Beaufrand C, **Gomes P**, Nouaille S, Tondereau V, Daveran-Mingot ML, Theodorou V, Eutamene H, Mercier-Bonin M, Cocaign-Bousquet M. 2022. *Lactococcus lactis* NCDO2118 exerts visceral antinociceptive properties in rat via GABA production in the gastro-intestinal tract. Elife. 2022 Jun 21;11:e77100. doi: 10.7554/eLife.77100.

Conferences and Posters

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APPENDIX

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