Original Article

Bifidobacterium longum and *Lactobacillus helveticus* Synergistically Suppress Stress-related Visceral Hypersensitivity Through Hypothalamic-Pituitary-Adrenal Axis Modulation

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Background/Aims

Visceral pain and hypothalamic-pituitary-adrenal axis (HPA) dysregulation is a common characteristic in irritable bowel syndrome (IBS) patients. Previously, we reported that a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) prevents chronic stress-mediated brain function abnormalities by attenuating the HPA axis response. Here, we compared the effect between different probiotic treatments on the perception of visceral pain during colorectal distension (CRD) following a chronic stress and the consequences to the activity of the HPA axis.

Methods

After a 2-week treatment with a combined probiotic formulation, or *L. helveticus* or *B. longum* alone in stressed mice, the visceral pain in response to CRD was recorded. The expression of glucocorticoid receptors was determined in the different brain areas involved in the stress response (hypothalamus, hippocampus, and prefrontal cortex). The plasma levels of stress hormones were also measured.

Results

A pretreatment using the combination of probiotic formulation significantly reduces the chronic stress-induced visceral hypersensitivity respectively at 0.06, 0.08, and 0.10 mL CRD volume. However, a single probiotic (*B. longum* or *L. helveticus*) administration is less effective in reducing visceral pain in stressed mice. Moreover, the expression of the glucocorticoid receptor mRNA was consistently up-regulated in several brain areas after pretreatment with a combined probiotic, which correlated with the normalization of stress response compared to the inconsistent effects of a single probiotic.

Conclusion

The combination of *L. helveticus* and *B. longum* is more effective in regulating glucocorticoid negative feedback on the HPA axis than probiotic alone and subsequently in treating stress-induced visceral pain.
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Key Words

Chronic stress; Glucocorticoid receptors; Probiotics; Visceral pain

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Introduction

Chronic or recurrent abdominal pain, bloating, and alteration of stool frequency and consistency, are frequent symptoms associated with the irritable bowel syndrome (IBS). In Western countries, 25% of medical consultations in gastroenterology are due to visceral hypersensitivity.[1] Although the IBS does not represent a life-threatening condition, it impacts the patient quality of life, and the management of this disorder represents an important economic burden.[2] The pathogenesis of visceral pain in association with clinical symptoms of IBS may occur within the peripheral and the central nervous systems. Evidence suggests that physical or psychological stress can aggravate IBS symptoms[3] and have a profound effect on visceral pain[4] via affecting the brain-gut interaction, central pain perception, abnormalities of hypothalamic-pituitary-adrenal (HPA) axis, and other brain structures.[5,6] In HPA axis activity, a central stress response is regulated by circulating glucocorticoid feedback[7] via binding to mineralocorticoid and glucocorticoid receptors (GR) at multiple sites in the brain including the hypothalamus, anterior pituitary, hippocampus, and prefrontal cortex.[8] It is well known that chronic stress attenuates the glucocorticoid negative feedback, disrupts GR expression in the brain-related areas,[9] and causes chronic abdominal pain.[10] At the intestinal level, exposure to chronic stress results in maladaptive changes at cellular, structural, functional, and microbial levels, the latter resulting in microbial dysbacteriosis.[11] Current research reports an altered composition of the intestinal microbiota in IBS patients,[12,13] and the lower microbial diversity in the intestine is correlated with the severity of IBS symptoms.[14] Moreover, an animal study has shown that the transplantation of the fecal microbiota from IBS patients into germ-free mice exhibits an increase of the abdominal contractions.[15] Thus, targeting intestinal microbiota to regulate the visceral pain seems to be an interesting therapeutic approach. Indeed, fecal transplants might be a highly effective treatment of chronic diseases[16,17] and reduce symptoms in IBS patients.[18] Dietary intervention by probiotics can also be used in the manipulation of intestinal microbiota. A growing body of clinical trials suggests that probiotic treatment may improve IBS symptoms[19,20] but their role in the criterion of visceral pain remains unknown. Interestingly, in IBS-like animal models, probiotics such as *bifidobacterium* or *lactobacillus* species are able to reduce visceral pain through the regulation of the neural function[21-23] and normalize the HPA function.[24]

In a previous study we have shown that the probiotic formulation (*Lactobacillus helveticus* and *Bifidobacterium longum*) at-tenuates the hyper-responsiveness of the HPA axis and facilitates neurogenesis in the hippocampus. These data suggest that the potential central regulation of stress response by the combination of the 2 probiotics may be due to the regulation of glucocorticoid negative feedback of the HPA axis.[25] In the current study, we aim to determine whether the attenuated effect of the stress response is due to a specific probiotic strain or to their combined actions. For this purpose, we evaluated the neuroendocrine response to stress and GR expression in specific brain areas. In addition, we evaluated the response of visceral pain to the nociceptive stimuli in mice treated with the probiotic strains, *B. longum* R0175 or *L. helveticus* R0052 alone or in combination.

Materials and Methods

Animals

C57BL/6J male mice (6-8 weeks old) (Janvier SA, Le Genest St Isle, France) weighing 21-23 g were used. Mice were housed under laboratory conditions ($25 \pm 2°C$, 12-hour light-dark cycle), with free access to standard diet and water. All experiments were approved by the local ethical board, Toulouse, France, following the European Union legislation (Council Directive 2010/63/EU).

Preparation of Probiotic

A combination of probiotic (*L. helveticus* R0052 and *B. longum* R0175), and individual probiotic strain *L. helveticus* R0052 or *B. longum* R0175 were provided by Lallemand Solutions Santé (Blagnac, France). Before administration, freeze-dried pure bacteria were rehydrated in 0.9% sodium chloride (NaCl) at a concentration of 10^9 colony-forming units (CFU) per milliliter. The bacterial solution was administered by oral gavage daily for 2 weeks before and during the experimental procedure.

Chronic Stress Protocol

Repeated exposure to water avoidance stress (WAS) was performed as described previously.[25] Briefly, the mice were placed individually during a 1-hour period on a plastic platform positioned in the middle of a basin filled with room temperature water. The procedure was repeated for 4 consecutive days. A control group was not exposed to the daily WAS but was placed into a basin without water. The WAS session has been shown to act a psychological stressor causing an increase of stress hormone,[26] visceral pain,[27] and intestinal dysbiosis.[28]

Plasma Stress Hormone Analysis

At the end of the chronic stress, blood samples were collected via the facial vein into heparinised tubes and the mice were sacrificed by cervical dislocation. Plasma was obtained after centrifugation and stored at -80°C until further analysis. Enzyme-linked immunosorbent assays (ELISA) were performed to quantify plasma corticosterone and adrenaline/noradrenaline levels (Immunodiagnostic system, Paris, France and LDN, Nordhorn, Germany, respectively).

Gene Expression Studies

Total RNA from the hypothalamus, hippocampus, and prefrontal cortex was extracted using the Qiazol reagent (Qiagen, Courtaboeuf, France). RNA integrity and quantification were confirmed using a NanoDrop ND2000 (Thermo Scientific, Illkirch, France). Total RNA samples (2 μg) were reverse-transcribed using high-capacity cDNA reverse transcription kit (Applied Biosystems, Courtaboeuf, France). Amplicons were performed with IQ SYBR green Supermix (Biorad, Marnes-la-Coquette, France) on the CFX 96 (Biorad). Quantitative polymerase chain reaction (qPCR) data was normalized by TATA-box binding protein (TBP) expression levels and analysed using $2^{-\Delta CT}$. The following primer sequences were: GR forward 5'-TCTCCTCAGTTCCTAAGGAAGGTC-3' and reverse 5'-CTGACTGGAGTTTCCTTCCCTTT-3'; TBP forward 5'-GGGAGAATCATGGACCAGAA-3' and reverse 5'-GATGGGAATTCCAGGAGTCA-3'.

Electromyography Recording

Animals were placed under anesthesia (xylasine-ketamine, 5 volume/volume) and electrodes were implanted in the abdominal muscle after an abdominal incision. Seven days later, the intensity of abdominal contractions was determined by recording the abdominal electromyography to graded colorectal distension (CRD), as a measure of visceral hypersensitivity.

Following an adaptation period of 1 hour in the small tunnel, a balloon catheter (Fogarty catheter for arterial embolectomy; Edwards Lifesciences, Nijmegen, The Netherlands) was inserted 2.5 cm into the colon and CRD was performed as previously described.[29]

Experimental Protocol

In the first set of experiments, 8 groups of mice (n = 8 mice per group) received orally, for 2 weeks, either the probiotic combination of the 2 bacterial strains (*B. longum* + *L. helveticus*) or single probiotic (*B. longum* or *L. helveticus*) at the concentration of

10^9 CFU/day. The control groups received NaCl 0.9% (vehicle). All the animals were submitted to WAS or sham stress during 1 hour per day for 4 days. During the WAS or sham stress session, the probiotic or vehicle treatments were maintained. At the end of the stress session, visceral sensitivity was recorded.

In the second set of experiments, the mice were divided into 8 groups (n = 8 mice per group) and received orally either the probiotic combination of 2 bacteria (*B. longum* + *L. helveticus*) or a single bacterium (*B. longum* or *L. helveticus*) (10^9 CFU/day) or vehicle (NaCl 0.9%) for 2 weeks. Following WAS or sham stress session, blood was collected through the facial vein puncture for measurement of the corticosterone, adrenaline, and noradrenaline. Immediately after blood drawing, the animals were decapitated and the brains removed from the skulls and cooled on ice. The hypothalamus, hippocampus, and prefrontal cortex were quickly dissected, frozen in liquid nitrogen and stored in -80°C until further qPCR analysis.

## Statistical Methods

All data, previously checked for normal distribution, are shown as means ± SEM. Analysis were evaluated using GraphPad Prism 4 software (GraphPad, San Diego, CA, USA). Abdominal contractions for a 5-minute period during CRD and plasma hormone concentration were analysed by ANOVA and Tukey tests for *post hoc* comparisons. Quantitative PCR data was analyzed using Student *t* test. A $P < 0.05$ was considered significant.

Results

Effect of the Combination of the Probiotics *Bifidobacterium longum* and *Lactobacillus helveticus* or Probiotic Alone on Stress-induced Visceral Pain Perception

In mice, the chronic stress (WAS, $P < 0.05$) increased significantly the abdominal pain in response to CRD compared with the control group (without chronic stress). The treatment with probiotic combination (*B. longum* and *L. helveticus*) significantly reversed chronic stress-induced abdominal pain in response to CRD at the volume of 0.06 mL (48.1 ± 9.1 vs 87.5 ± 14.3, $P < 0.05$), 0.08 mL (49.7 ± 15.7 vs 118.6 ± 12.3, $P < 0.05$), and 0.10 mL (69.5 ± 11.3 vs 120.4 ± 11.1, $P < 0.05$) (Fig. 1A). In contrast, the use of a probiotic alone (*L. helveticus* or *B. longum*) did not have any effect on the chronic stress induced-hypersensitivity at the volume of 0.08 mL and 0.10 mL of CRD (for example at 0.08 mL of CRD,
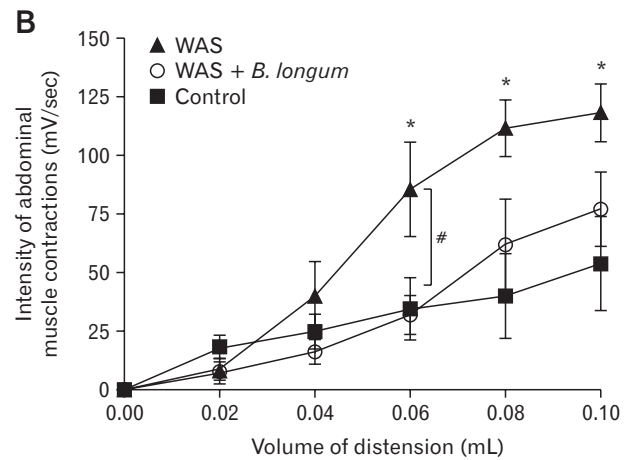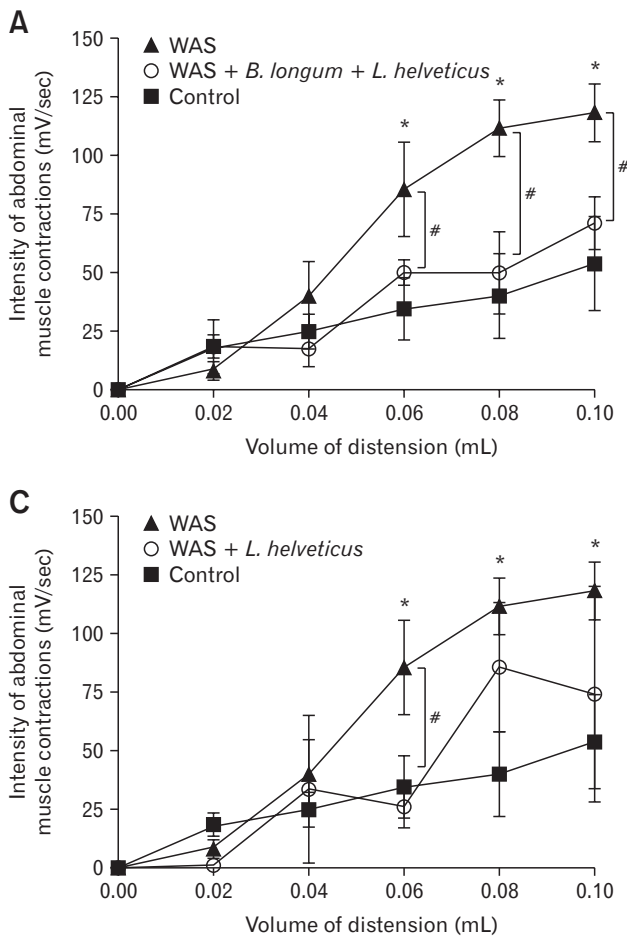
Figure 1. Prevention of abdominal pain induced by chronic stress (water avoidance stress [WAS]) depends on the strain probiotic. *$P < 0.05$ and #$P < 0.05$ are significantly different from control and WAS groups. *B. longum*, *Bifidobacterium longum*; *L. helveticus*, *Lactobacillus helveticus*.

L. helveticus effect: 85.5 ± 20.1 vs 118.6 ± 12.3 or *B. longum* effect: 67.4 ± 19.9 vs 118.6 ± 12.3). However, pretreatment with a single probiotic prevented the increase of abdominal pain induced by chronic stress at 0.06 mL CRD volume (*B. longum* and *L. helveticus* effect: respectively 37.7 ± 9.1 and 26.0 ± 9.0 vs 87.5 ± 14.3) (Fig. 1B and 1C).

Effect of the Probiotic Treatment on Stress-induced Increase of Neuroendocrine Hormone Levels

In mice, WAS induced a significant increase of the plasma levels of corticosterone and catecholamines. There was no effect on stress hormone levels in unstressed mice (control group), whatever probiotic treatment was used. The probiotic combination (*B. longum* and *L. helveticus*) and *B. longum* treatment alone prevented the increase of these plasmatic stress markers. However, the *L. helveticus* treatment had no effect on the increase of either corticosterone or catecholamine concentrations in stressed mice (Table and Fig. 2).

Table. Effect of Probiotic Treatment or Vehicle on Plasma Levels of Noradrenaline and Adrenaline in Water Avoidance Stress (Chronic Stress) or Control Groups

	Catecholamine concentration (ng/mL)	
	Nonadrenaline	Adrenaline
Control		
Vehicle	20 ± 2	19 ± 2
B. longum + *L. helveticus*	34 ± 3	13 ± 2
B. longum	50 ± 6	20 ± 3
L. helveticus	14 ± 2	14 ± 1
WAS		
Vehicle	71 ± 6[a]	33 ± 2[a]
B. longum + *L. helveticus*	46 ± 2[b]	24 ± 1[b]
B. longum	35 ± 5[b]	17 ± 1[b]
L. helveticus	52 ± 5	34 ± 4

[a]$P < 0.05$ and [b]$P < 0.05$ vs control + vehicle and WAS + vehicle group respectively.
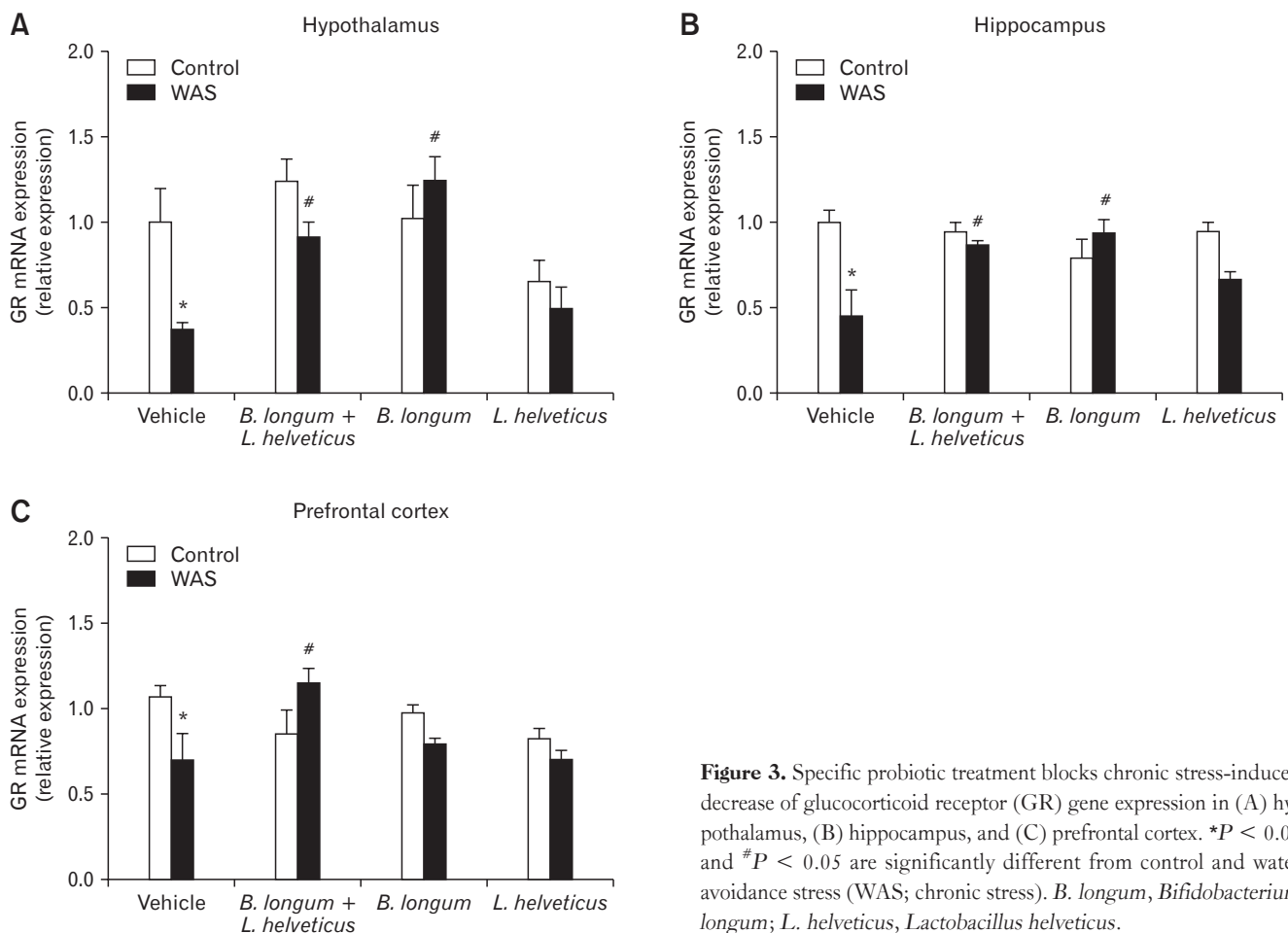WAS, water avoidance stress; *B. longum*, *Bifidobacterium longum*; *L. helveticus*, *Lactobacillus helveticus*.
Data are expressed as mean $\pm$ SEM.
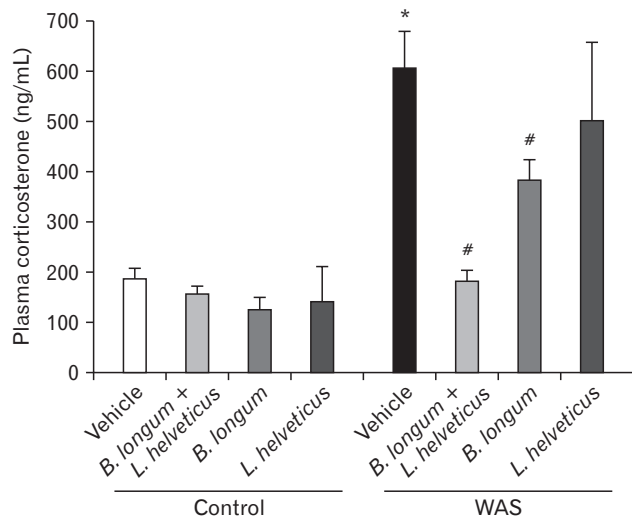
Figure 2. Probiotic treatments differentially prevent a plasma level of stress hormone markers. *$P < 0.05$ and #$P < 0.05$ are significantly different from control and water avoidance stress (WAS; chronic stress). *B. longum*, *Bifidobacterium longum*; *L. helveticus*, *Lactobacillus helveticus*.

Differential Effects of Probiotic Treatment on Glucocorticoid Receptor Gene Expression in Stress-involved Brain Areas

The statistical analysis of the GR gene expression revealed a significantly lower expression in the WAS group compared to the control group. The difference was observed in all three brain areas: hypothalamus, hippocampus, and prefrontal cortex. The pre-treatment with the combination of 2 probiotics (*B. longum* and *L. helveticus*) or probiotic *B. longum* alone prevented the WAS-induced decrease of the GR gene expression in the hypothalamus (Fig. 3A) and hippocampus (Fig. 3B). The combination of these 2 probiotics blocked the stress-induced decrease of the GR gene expression in the prefrontal cortex, while probiotic *B. longum* did not (Fig. 3C). There was no significant effect on GR gene expression after pre-treatment with *L. helveticus* alone in stressed mice.

Figure 3. Specific probiotic treatment blocks chronic stress-induced decrease of glucocorticoid receptor (GR) gene expression in (A) hypothalamus, (B) hippocampus, and (C) prefrontal cortex. *$P < 0.05$ and #$P < 0.05$ are significantly different from control and water avoidance stress (WAS; chronic stress). *B. longum*, *Bifidobacterium longum*; *L. helveticus*, *Lactobacillus helveticus*.

Discussion

In the present study, we have shown that a 2-week treatment with a combination of the 2 probiotics *B. longum* R0175 and *L. helveticus* R0052 decreases the chronic stress-induced visceral hypersensitivity in response to CRD. Even if the probiotics *B. longum* or *L. helveticus* alone decrease the visceral pain induced by chronic stress only at low volume of CRD, the data suggest that their association is more efficient. Furthermore, this study has shown that the combination of the 2 probiotics (*B. longum* and *L. helveticus*) or *B.longum* alone prevents the chronic stress-induced increase of plasma levels of stress hormones, whereas *L. helveticus* alone has no effect on these parameters. Interestingly, we have identified a decrease of GR gene expression in the hypothalamus and hippocampus after chronic stress, whereas the probiotic pre-treatment with the combination of the both probiotics (*B. longum* and *L. helveticus*) and *B. longum* alone normalised GR gene expression. These findings are supported by our previous study showing that the combination of the 2 probiotics attenuates the stress response by modulating neuronal plasticity in specific brain areas.[25] The effect of *B.longum* or *L. helveticus* probiotic treatment on the adverse stressful events, particularly on the visceral pain induced by chronic stress, depends on the glucocorticoid negative feedback regulation of the HPA axis. Finally, the combination of 2 probiotics (*B. longum* and *L. helveticus*) has a better antinociceptive effect than the probiotic alone (*B. longum* or *L. helveticus*). This effect is probably due to the prevention of the increase of the stress hormone associated with the GR expression normalized and consequently, the regulation of negative feedback of hormone released in HPA axis.

Evidence studies in preclinical and human research suggest that the bidirectional signals sent between the CNS and the gastrointestinal tract influence stress reactivity. The gut microbiota seems to be a critical mediator of information in this brain-gut dialogue.[30-32] Indeed, the microbiome may influence neuronal circuitry involved in stress response, neuronal development and plasticity, pain perception, neurochemistry, or blood brain barrier integrity.[33-35] Moreover, when gut microbiota in newborn rats was disrupted after antibiotic treatment, a visceral hypersensitivity was observed in adulthood and subsequently increased visceral pain signalling mechanism from the gastrointestinal tract.[36] Clinical data showed that IBS patients had altered microbial profiles.[11,12] Fecal microbiota transplanted from these patients into germ-free rats led to visceral hypersensitivity.[15] Therefore, restoring the balance of gut microbiota by using the probiotics combination could be an interesting alterna-

tive in the management of IBS symptoms. The physiological benefits of probiotics observed in IBS have highlighted the importance of using a cocktail of bacteria including the most effective probiotic strains. Some studies have shown that probiotic combination of *lactobacillus* and *bifidobacterium* species significantly decreases abdominal pain,[37] delays the colonic transit, and reduces flatulence.[38,39] In the current study, pre-treatment with *B. longum* or *L. helveticus* strains alone decreases visceral hypersensitivity induced by chronic stress in response to low noxious stimuli, but the combination of these 2 probiotics was more effective in stressed mice at all volumes of CRD. We have observed that the anti-nociceptive effect of probiotics depends on the probiotic strain used. The results have also shown a differential effect with respect to the influence of these different probiotic treatments on releasing plasma levels of stress hormones. Indeed, *B. longum* strain alone or in combination with *L. helveticus* strains decreased hypersecretion of corticosterone and catecholamine hormones under stressful conditions. However, the treatment with *L. helveticus* strain alone does not attenuate stress response in mice. The role of *L. helveticus* in the combination of probiotics can be explained by its anti-inflammatory properties. A recent study has shown that the *L. helveticus* strain effect depends on gut inflammatory state and elicits anti-inflammatory responses in intestinal inflammation-associated behaviour.[40] The anti-inflammatory properties of *L. helveticus* are probably due to its capacity to decrease pathogen adhesion to the epithelial cells.[41] On the other hand, *B. longum* probiotic strain enhances some positive effects on the cognition by decreasing anxiety in mice.[42,43] Taking into account the specificity of action of each strain at the peripheral and central level, all these data converge to show that CNS function is positively impacted by the combination of these 2 probiotics (*B. longum* and *L. helveticus*).

An exacerbated HPA axis in IBS patients after hormone challenge correlated with visceral pain and a reduction in GR protein expression in peripheral blood mononuclear cells.[44] Although, it has been shown in adult rats that an early life stress evokes a change of GR expression in the hippocampus and central amygdala,[45,46] which enhances an activation of somatosensory area and visceral hypersensitivity.[47] Moreover, in postnatal day 10, a single stress session for 4 hours induced an alteration of GR expression in the colon in pups, weakened the intestinal barrier integrity allowing bacterial translocation,[48] and subsequently provoked later visceral hypersensitivity.[49] The present study reveals that the treatment with the combination of 2 probiotics *L. helveticus* and *B. longum* or *B. longum* alone restores the chronic stress-induced decrease in hippocampal and hypothalamic GR expression. In addition to the

previous observations on the plasma level of stress hormones, the effect of probiotics on GR expression in several brain areas seems to participate in the regulation of visceral hypersensitivity. We hypothesize that the specific probiotic treatment counteracts the disrupted glucocorticoid signalling, impaired negative feedback, and pain. In fact, endogenous hormonal interaction with bacterial products released by probiotic and/or bacteria in the intestinal microenvironment can be suggested as a mechanism of action of the probiotic on the alleviation of visceral pain. Data from germ free rodents suggest that hormonal status plays an important role in the host-microbial interaction. Host hormonal signals are able to shape the behaviour of the gut microbiome, and in return, the microbiome residents signal the changes to their host and can affect physical and even mental well-being.[50] For example, endogenous hormones such as catecholamines enhance bacterial adhesion of pathogens to the host tissues[51] or downregulate resistance of pathogens of host microbial peptides.[52] Another study has shown that the human sex hormones decrease bacterial virulence by inhibiting quorum sensing, which is a specific communication between the bacterial and neuroendocrine systems.[53] Thereby, host neuroendocrine hormones sensing modifies indirectly or directly the gut environment. Thus, the observed effects of the probiotic strains on visceral hypersensitivity induced by chronic stress prove the importance of selecting a combination of "good" bacteria for endocrine-microbiome crosstalk. These findings highlight one of the mechanisms by which microbiome residents under probiotic influence can sense the emotional status of their host.

This study shows that the effect of probiotics on stress-induced hypersensitivity is strain specific. The treatment with the combination of the 2 probiotic strains (*B. longum* and *L. helveticus*) prevents the perception of visceral pain associated with an attenuation of stress hormones, and has a potential effect on GR expression. However, the probiotic treatment with only *L. helveticus* or *B. longum* is not effective to reduce chronic stress-induced hypersensitivity in response to all volumes of CRD. This differential effect correlated with the attenuation of stress hormone associated with a change of GR expression in specific areas involved in stress response.

We speculate that the combination of these probiotics may regulate the negative feedback of the HPA axis by reducing glucocorticoid signaling in the limbic system, particularly in the hippocampus. Finally, the prevention of increased stress hormone levels plays an important role in the attenuation of visceral pain induced by chronic stress. This study supports strongly the probiotic formulation Probio'Stick (*B. longum* R0175 and *L. helveticus* R0052)

as a therapeutic solution for treating chronic stress and associated visceral pain.

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