Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve

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Abstract
There is increasing, but largely indirect, evidence pointing to an effect of commensal gut microbiota on the central nervous system (CNS). However, it is unknown whether lactic acid bacteria such as *Lactobacillus rhamnosus* could have a direct effect on neurotransmitter receptors in the CNS in normal, healthy animals.
MGB axis
(microbiota–gut–brain axis)
• Alterations in central GABA receptor expression are implicated in the pathogenesis of anxiety and depression, which are highly comorbid with functional bowel disorders.
GABA

• GABA的簡介及作用機轉

GABA是γ-AminoButyricAcid的縮寫，在身體裡面GABA扮演一個相當重要的角色。GABA主要存在於人體的小腦皮質、脊髓和視網膜中，是脊髓動物中樞神經和神經系統結合點的抑制性傳導物，也是抑制人體神經訊息傳遞的最重要的物質。GABA是一種相當重要的神經傳導物質，如果身體缺少這樣的物質，得到一些精神方面疾病的機率就會增加。

• GABA可改進失眠的狀況

• GABA可促進腦活化

• GABA可以促進腦中的許多工作。

增加乙醯膽鹼，刺激副交感神經活潑，促進腦部機能正常運作。促進腦部組織中的氧或葡萄糖的代謝,增加腦的血流量，以改善頭痛、頭重、疲勞、頭昏眼花、耳鳴、記憶力衰退等現象,腦動脈硬化或頭部外傷所引起的後遺症。適度使用GABA可以適度消除壓力、防止宿醉。
GABA受器(GABA receptor)

- GABA也是一種中樞神經的神經傳導物質，其受器有三種，分別為GABA_A、GABA_B和GABA_C。GABA_A及GABAc是ligand-gated ion channels（亦稱ionotrophic受器），GABA_B則是G-protein coupled receptors（亦稱metabotropic受器），其中的GABA_A被認定對於GABA有快速的反應。GABA_A接受GABA後便會開啓本身之Cl⁻通道，讓Cl⁻進入神經細胞，降低細胞內電位。
  * GABA_A是ionotropic receptor，允許氯離子流進細胞

- 藥理作用
  barbiturates、benzodiazepines皆對GABA receptor具有allostERIC modulation，作用是加強GABA抑制中樞神經的作用，且不和GABA互相競爭結合位。因此在藥理上的作用就有鎮靜、麻醉、催眠、抗痙攣、肌肉鬆弛等等。
  由於barbiturates類的藥物在服用過量後比benzodiazepines有較多的危險性，現在已被benzodiazepines類藥物大量取代
GABA receptor
Lactobacillus rhamnosus (JB-1)

- One such organism is Lactobacillus rhamnosus (JB-1), which has been demonstrated to modulate the immune system because it prevents the induction of IL-8 by TNF-α in human colon epithelial cell lines (T84 and HT-29) (17) and modulates inflammation through the generation of regulatory T cells (18).

- Moreover, it inhibits the cardio–autonomic response to colorectal distension (CRD) in rats (19), reduces CRD-induced dorsal root ganglia excitability (20), and affects small intestine motility (21).
L. rhamnosus (JB-1)
Materials and Methods

Animals. Adult male BALB/c mice (n = 36)

All of the animals were allowed to acclimate for 7 days in the housing facility before the experiment.

Animals were group-housed (nine animals per cage) in standard conditions (room temperature of 21 °C, with a 12-h light–dark cycle, lights on at 07:00) with access to regular chow and water ad libitum. Cages were cleaned once weekly to avoid excessive handling.

Mice were of comparable weight (25–30 g) and age (10–11 wk) at the moment of sacrifice.
Treatments and Sacrifice

- Animals were orally gavaged with broth without bacteria (broth control group, n = 16) or with L.rhamnosus (JB-1) [109 cfu; L. rhamnosus (JB-1) group, n = 16].
- This procedure was carried out daily between 8:00 and 9:00 for a period of 28 continuous d.

Toward the end of the treatment,

- the animals underwent a series of behavioral testing including SIH, EPM, and fear conditioning.
- In addition, on the day samples were collected, half of the animals in each group were put in the FST [broth, n = 8; L. rhamnosus (JB-1), n = 8] to evaluate behavior and also to measure stress-induced levels of corticosterone.
- All of the animals were killed by cervical dislocation; the head was rapidly removed, and trunk blood was collected into EDTA-containing tubes for separating plasma.
- Animals that were not used for FST were killed between 8:00 and 9:00, while the FST stressed groups were killed 30 min after the end of the forced swim session (9:30–11:45). For each behavioral test, the experimenter was blinded to the treatment of each animal.
Bacterial Preparation

Fig. 56. AFLP analysis of *L. rhamnosus* (JB-1) and *L. rhamnosus* GG. A section of the detailed comparison of the E01/T11 and E01/T13 AFLP DNA fingerprints of *L. rhamnosus* (JB-1) and *L. rhamnosus* GG using the GeneMapper 4.0 software (Applera). There are clear differences in peak positions between strains (indicated by arrows).
Results
rhamnosus ( JB-1 ) had a larger number of entries to the open arms than broth-fed animals, suggesting anxiolytic effects (open arm entry defined as all four paws entering the arms of the EPM apparatus) ( \( t = 4.662, \text{ df } = 34; P < 0.001 \); Fig. 1 A ). This effect is also reflected in the percentage of time spent in the open arms, although this observation did not reach statistical significance [broth v. L. rhamnosus ( JB-1 ): 25.28 ± 6.67% vs. 38.36 ± 7.99%; \( t = 1.267, \text{ df } = 34; P = 0.2146 \)].
Effect of L. rhamnosus (JB-1) administration on behavior and stress-induced levels of corticosterone.

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Forced swim test (FST).
Elevated plus maze (EPM).
Effect of L. rhamnosus (JB-1) administration on central GABAB1b mRNA expression.

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Model of prelimbic (PL) and infralimbic (IL) interactions with the amygdala.

There was a significant interaction between acute stress and *L. rhamnosus ( JB-1 )* treatment \[ F (1, 28) = 7.425; P = 0.011 \], a significant effect of acute stress \[ F (1, 28) = 73.90; P < 0.0001 \] and *L. rhamnosus ( JB-1 )* treatment \[ F (1, 28) = 11.409; P = 0.0022 \] on corticosterone levels. Post hoc analysis showed that the levels of stress-induced corticosterone are significantly lower in stressed mice that received *L. rhamnosus ( JB-1 )* \( (P < 0.001) \) than the levels of the hormone in stressed broth-fed mice \( (\text{Fig. 1 C}) \).
Effects of *L. rhamnosus* (JB-1) on GABA Receptor Expression.

- **GABA$_{B1b}$ mRNA.**
- There was a differential expression of this transcript in the different studied areas. Higher levels of GABA$_{B1b}$ mRNA were found in cingulate cortex 1 (CG1) (Fig. 2A) and prelimbic (PrL) (Fig. 2B) cortical areas of *L. rhamnosus* (JB-1)-fed mice in comparison with broth-fed mice ($t = 3.485$, df = 10, $P < 0.01$; and $t = 2.965$, df = 10, $P < 0.05$, respectively), but no differences were observed in the infralimbic (IL) cortex ($t = 0.4558$, df = 10, $P = 0.658$; Fig. 2C). Conversely, *L. rhamnosus* (JB-1)-fed mice had lower levels of GABA$_{B1b}$ mRNA in the basolateral amygdala (BLA) ($t = 8.778$, df = 10, $P < 0.001$; Fig. 2D) and central amygdala (CeA) ($t = 3.372$, df = 10, $P < 0.01$; Fig. 2E), locus coeruleus (LC) ($t = 5.339$, df = 10, $P < 0.001$; Fig. 2F), hippocampal sub areas of the dentate gyrus (DG) ($t = 5.555$, df = 10, $P < 0.001$; Fig. 2G), cornus ammonis area 3 (CA3) ($t = 3.207$, df = 10, $P < 0.01$; Fig. 2H), and cornus ammonis area 1 (CA1) ($t = 3.826$, df = 10, $P < 0.01$; Fig. 2I) compared with broth-fed control mice.
Effect of L. rhamnosus (JB-1) administration on central GABA \alpha_2 mRNA expression.

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**GABA$_{A_2}$ mRNA.**
A differential expression of GABA$_{A_2}$ mRNA within the studied areas was also found (Fig. 3). In *L. rhamnosus (JB-1)*-fed animals, there were low levels of GABA$_{A_2}$ mRNA in CG1 ($t = 2.611$, df = 10, $P < 0.05$; Fig. 3A), PrL ($t = 2.267$, df = 10, $P < 0.05$; Fig. 3B), and IL ($t = 2.803$, df = 10, $P < 0.05$; Fig. 3C) cortical areas, as well as in the BLA ($t = 7.541$, df = 10, $P < 0.001$; Fig. 3D) and CeA ($t = 7.150$, df = 10, $P < 0.001$; Fig. 3E), in comparison with broth-fed mice. In addition, no differences in GABA$_{A_2}$ mRNA were found in the LC between the two groups of mice ($t = 1.190$, df = 10, $P = 0.2616$; Fig. 3F); however, higher levels of GABA$_{A_2}$ mRNA were found in the DG of *L. rhamnosus (JB-1)*-fed mice in comparison with broth-fed control animals ($t = 5.967$, df = 10, $P < 0.001$; Fig. 3G). No differences in GABA$_{A_2}$ mRNA were found in CA3 ($t = 0.403$, df = 10, $P = 0.6955$; Fig. 3H) and CA1 ($t = 2.161$, df = 10, $P = 0.0560$; Fig. 3I) neuronal layer of the hippocampus of *L. rhamnosus (JB-1)* compared with broth-fed mice.
Effect of vagotomy (Vx) on anxiety and depression-like behaviors and GABAA subunit expression of animals treated with L. rhamnosus (JB-1).

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迷走神經（vagus nerve）是第十對腦神經，故亦稱第十對腦神經（tenth cranial nerve），編號X。迷走神經屬混合性神經，是人的腦神經中最長和分布範圍最廣的一組神經，含有感覺、運動和副交感神經纖維。

迷走神經出延髓，從顱頂穿出後，沿著食道兩旁，縱貫頸部和胸腔，入腹部；支配呼吸系統、消化系統的絕大部分和心臟等器官的感覺、運動和腺體的分泌；因此迷走神經損傷會引起循環、呼吸、消化等功能失調。
Effects of *L. rhamnosus (JB-1)* Administration on the Behavior of Vagotomized Mice.

To further understand the role of the vagus nerve in communicating sensory information to the brain, subdiaphragmatic vagotomy (Vx) was carried out, and behavioral parameters were determined. As shown in Fig. 4A, two-way ANOVA revealed that there was an overall effect of Vx \[F(1, 36) = 8.91; P < 0.01\], an overall effect of *L. rhamnosus (JB-1)* treatment \[F(1, 36) = 5.80; P < 0.05\], and an interaction between Vx and *L. rhamnosus* (JB-1) \[F(1, 36) = 5.690; P < 0.05\]. In terms of time in the center of the open field arena, Vx prevented the anxiolytic effects of *L. rhamnosus* (JB-1) in mice, which is reflected in a reduction of the time spent in the center of the open field compared with sham surgery animals fed with *L. rhamnosus* (JB-1) \(P < 0.05\).

That Vx prevented the anxiolytic effect of *L. rhamnosus* (JB-1) is further verified because the analysis of the number entries to the central area of the open field reflects a similar profile as in the percentage of time spent in the central part of the arena [Fig. 6A; effect of Vx: \(F(1, 36) = 5.56, P < 0.05\); effect of *L. rhamnosus* (JB-1): \(F(1, 36) = 4.64, P < 0.05\); interaction between Vx and *L. rhamnosus* (JB-1): \(F(1, 36) = 7.66, P < 0.01\)]. This exploratory behavior seems to be related to an anxiolytic effect, because the total distance traveled by the mice in each experimental condition did not differ between them \[F(1, 36) = 0.44, P = 0.51; Fig. 4A\].
In addition, FST revealed that there was an overall effect of Vx \([F(1, 36) = 5.14, P < 0.05]\), an overall effect of \(L. \text{ rhamnosus (JB-1)}\) treatment \([F(1, 36) = 10.47, P = 0.01]\), and an interaction between Vx and \(L. \text{ rhamnosus (JB-1)}\) \([F(1, 36) = 6.22, P < 0.05]\) in terms of immobility time. Post hoc analysis showed that sham animals fed with \(L. \text{ rhamnosus (JB-1)}\) had significantly lower mobility time \((P < 0.05)\) compared with sham animals fed with broth \((\text{Fig. 4A})\). This effect was prevented by Vx, because immobility time of Vx animals fed with \(L. \text{ rhamnosus (JB-1)}\) was similar to the immobility time of control mice \((P > 0.05)\).
Discussion
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Fig. S1. Effect of vagotomy (Vx) on GABAₐₜ mRNA expression in the amygdala of animals treated with L. rhamnosus (JB-1). (A and B) Sham operated animals treated with L. rhamnosus (JB-1) (n = 6) had significantly lower levels of GABAₐₜ mRNA expression in the basolateral amygdala (BLA) (A) and central amygdala (CeA) (B) in comparison with sham operated mice fed with broth (n = 6; ***P < 0.001 and *P < 0.05, respectively). (C and D) In addition, Vx prevented the effect of L. rhamnosus (JB-1) on GABAₐₜ mRNA expression in the BLA (C) and CeA (D), as the levels of the transcript in broth-fed (n = 6) and L. rhamnosus (JB-1)-fed (n = 6) animals are not different from control sham operated animals. (E-H) Representative microphotographs of the analyzed areas of sham animal fed with broth (E); sham operated animal treated with L. rhamnosus (JB-1) (F); vagotomized animal fed with broth (G); and vagotomized animal treated with L. rhamnosus (JB-1) (H). (Scale bar: 1 mm.)
Moreover, we show that L. rhamnosus ( JB-1 ) can have a direct effect upon associated behavioral and physiological responses in a manner that is dependent on the vagus nerve. L. rhamnosus ( JB-1 ) consistently modulated GABA Aα2 , GABA Aα1 , and GABA B1b receptor mRNA expression—receptors implicated in anxiety behavior—in a regional-dependent manner.
• Early Life Stress Alters Behavior, Immunity, and Microbiota in Rats: Implications for Irritable Bowel Syndrome and Psychiatric Illnesses
Our data are in line with previous studies showing that subchronic or chronic treatment with antidepressants can prevent forced swim stress-induced increases in plasma corticosterone in both mice and rats (27).

cAMP Response Element-Binding Protein Is Essential for the Upregulation of Brain-Derived Neurotrophic Factor Transcription, But Not the Behavioral or Endocrine Responses to Antidepressant Drugs

it has been shown that alterations in HPA axis modulation can be reversed by treatment with Lactobacillus and Bifidobacterium (28, 29).

- 28.(2005) Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cyt
下丘腦-垂體-腎上腺軸
（HPA或HTPA軸）

• 下丘腦釋放CRH受到多種因素影響，包括緊張刺激——指神經衝動對於下丘腦的作用、血液中皮質醇含量和晝夜節律。

• 對於健康人來說，睡醒後皮質醇水平迅速升高，在30-45分鐘內就可以達到血濃度峰值。然後，在一天中皮質醇含量逐漸下降，在接近傍晚時又再次升高。到了晚上，皮質醇含量又再度下降，大約在午夜時到達最低值。

• 研究發現，不正常的皮質醇周期性波動與各種疾病有一定聯繫，比如：慢性疲勞綜合徵（chronic fatigue syndrome）(MacHale, 1998)，失眠（insomnia）(Backhaus, 2004)和倦怠（burnout）(Pruessner, 1999)。
STRESS (Psychological and Physical)

Hypothalamus

Pituitary

Adrenal Cortex

Cortisol

CRF
“下視丘- 腦下垂體- 腎上腺” 路徑

下視丘

腦下垂體

腎上腺皮質

皮質醇 (類固醇)

• It is important to note that the present neurochemical observations only represent changes at the mRNA level, and not protein, and they could only represent a more complex situation involving other neurotransmitter systems (48) and a variety of intracellular cascades that can affect the expression of these transcripts in the different studied areas.

• Nonetheless, our data conclusively demonstrate that a potential probiotic can robustly alter brain neurochemistry and behavior relevant to anxiety- and depression-related behavior in mice.
Fig. S2. Representative images of hippocampal expression of GABA<sub>A<sub>α2</sub> mRNA expression. (Upper, Left) Representative image of a sham vagotomised animal fed with broth. (Upper, Right) Representative image of a sham vagotomised animal fed with L. rhamnosus (JB-1). (Lower, Left) Representative image of a vagotomised animal fed with broth. (Lower, Right) Representative image of a vagotomised animal fed with L. rhamnosus (JB-1). (Scale bar: 1mm.)
Fig. 53. Effect of Vx on GABA<sub>A<sub>1</sub> mRNA expression in the amygdala of animals treated with *L. rhamnosus* (JB-1). (A and B) Sham operated animals treated with *L. rhamnosus* (JB-1) (*n* = 6) had significantly lower levels of GABA<sub>A<sub>1</sub> mRNA expression in the BLA (A) and CeA (B) in comparison with sham operated mice fed with broth (*n* = 6; ***P < 0.001 in both cases). (C and D) In addition, Vx prevented the effect of *L. rhamnosus* (JB-1) on GABA<sub>A<sub>1</sub> mRNA expression in the BLA (C) and CeA (D) as the levels of the transcript in broth-fed (*n* = 6) and *L. rhamnosus* (JB-1)-fed (*n* = 6) animals were not different from control sham operated animals. (E–H) Representative microphotographs of the analyzed areas of sham animal fed with broth (E); sham operated animal treated with *L. rhamnosus* (JB-1) (F); vagotomized animal fed with broth (G); and vagotomized animal treated with *L. rhamnosus* (JB-1) (H). (Scale bar: 1 mm.)
**Fig. S4.** Representative images of hippocampal expression of GABA$_{A\alpha1}$ mRNA expression. (Upper, Left) Representative image of a sham vagotomised animal fed with broth. (Upper, Right) Representative image of a sham vagotomised animal fed with *L. rhamnosus* (JB-1). (Lower, Left) Representative image of a vagotomised animal fed with broth. (Lower, Right) Representative image of a vagotomised animal fed with *L. rhamnosus* (JB-1). (Scale bar: 1mm.)
Fig. S5. In situ hybridization negative controls. Representative pictures for GABA_{B1b} (A), GABA_{A2} (B), and GABA_{A1} (C) negative controls are shown. DG, dentate gyrus; CA3, cornus ammonis region 3; CA1, cornus ammonis region 1. (Scale bar: 1 mm.) Negative controls were generated by using 100-fold excess of unlabeled oligodeoxynucleotide in the presence of 1x of the respectively labeled oligodeoxynucleotide, therefore displacing any specific binding that could be generated by the labeled oligodeoxynucleotide.
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<td>• <em>L. rhamnosus</em> (<em>JB-1</em>) treatment has an anxiolytic effect (Fig. 1A, Center) with no effect on motor activity (Fig. 1A)</td>
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<td>• GABA$_{B1b}$ receptor expression in frontal cortices is reduced in animal models of depression (1, 2)</td>
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<td>• GABA$_{B1b}$ is necessary for fear extinction (3)</td>
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<td>• The use of GABA$_{B}$ receptor antagonists mediates antidepressant-like behaviors (4)</td>
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<td>• Animals treated with <em>L. rhamnosus</em> (<em>JB-1</em>) have an enhanced emotional response towards an unconditioned stimulus, and also <em>L. rhamnosus</em> (<em>JB-1</em>) treatment allows fear extinction (Fig. 1B)</td>
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<td>• GABA$<em>{A_2}$ subunit mediates the anxiolytic effect of benzodiazepines while GABA$</em>{A_1}$ subunits mediate the amnesic and sedative effects of these compounds (5, 6)</td>
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<td>CA1</td>
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<td>• Genetic ablation of the GABA$_{A_1}$ subunit in mice enhances freezing behavior (6)</td>
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