Wageningen Academic Publishers

Lactobacillus fermentum NS9 restores the antibiotic induced physiological and psychological abnormalities in rats

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Received: 4 February 2015 / Accepted: 25 March 2015

RESEARCH ARTICLE

Abstract

Gut microbiota play a vital role in maintaining the health of the host. Many factors affect gut microbiota; application of broad range antibiotics disturb microbiota, while probiotic application protects the microbiota. To investigate how probiotics alter the physiological and psychological changes induced by antibiotics, we tested the performance of ampicillin-treated rats in the presence or absence of *Lactobacillus fermentum* strain NS9, in elevated plus maze and Morris water maze. The results showed that NS9 normalised the composition of gut microbiota and alleviated the ampicillin-induced inflammation in the colon. The levels of the mineralocorticoid and N-methyl-D-aspartate receptors were also elevated in the hippocampus of the ampillicin+NS9 treated group. NS9 administration also reduced the anxiety-like behaviour and alleviated the ampicillin-induced impairment in memory retention. These findings suggest that NS9 is beneficial to the host, because it restores the physiological and psychological abnormalities induced by ampicillin. Our results highlight how gut contents regulate the brain, and shed light on the clinical applications of probiotics to treat the side effect of antibiotics and mental disorders.

Keywords: gut microbiota, probiotic, inflammation, behaviour, hypothalamic-pituitary-adrenal axis

1. Introduction

Emerging evidence suggests that gut microbiota play an important role in human health. It is reported that almost 100 trillion microorganisms reside in the intestinal tract. Among these, most are bacteria but they may also include archaea and fungi. This microbiota has been selected through long-term co-evolution along with the host, and therefore they are involved in numerous physiological processes of the host. Dysbiosis of the microbiota is associated with the pathogenesis of several disorders, including inflammatory bowel disease (IBD), obesity and cardiovascular disease (Blumberg *et al.*, 2012; Hviid *et al.*, 2011; Ridaura *et al.*, 2013). Recent studies have suggested that the gut microbiota could affect the behaviour of hosts and implicated their involvement in depression, anxiety-like behaviours, and in modulating the hypothalamic-pituitary-adrenal axis (HPA-axis) (Barouei *et al.*, 2012; Collins *et al.*, 2012; Cryan and Dinan, 2012; Tillisch *et al.*, 2013).

The composition of gut microbiota is constantly affected by antibiotics, which are routinely administered to treat various infections (Cho *et al.*, 2010; Jernberg *et al.*, 2010; Pérez-Cobas *et al.*, 2012). Clinical research has shown that antibiotics induce diarrhoea (Alam and Mushtaq, 2009) in children and older patients, and could even cause IBD. These antibiotic-induced changes lead to immunity disorder, and increases susceptibility to infection and impair the host immune system (Sekirov *et al.*, 2008; Hviid *et al.*, 2011; Nutten *et al.*, 2007). Bercik *et al.* (2011a,b) reported that a mixture of three antibiotics, neomycin, bacitracin, and pimaricin, induced exploration behaviour in mice similar to the behaviour of germ-free mice (Heijtz *et al.*, 2011). However, these three antibiotics are not routinely used in clinics.

ISSN 1876-2833 print, ISSN 1876-2891 online, DOI 10.3920/BM2014.0177

When applied in adequate amounts probiotics are beneficial to the host; they improve the colonic barrier function impaired by early-life stress and have been used to treat many illnesses such as diarrhoea, IBD, and other gut disorders (Floch *et al.*, 2011). Administration of probiotics has been reported to modulate the gut microbiota (Lyra *et al.*, 2010) and regulate the HPA axis activity in the neonatal maternal separation model (Gareau *et al.*, 2007). In addition, various probiotic strains have been shown to reduce the anxiety-like behaviour induced by the intestinal inflammation in animal models and clinical trials (Bercik *et al.*, 2010; Bravo *et al.*, 2011; Gareau *et al.*, 2011; Messaoudi *et al.*, 2011). They also improve memory defects induced by bacterial infection and stress (Gareau *et al.*, 2011).

Although a number of studies have investigated the gut-microbiome-brain connection, the effects of antibiotics on learning and memory behaviour are not well understood. Therefore, in this study we determined whether clinical antibiotics can induce anxiety-like behaviour and hippocampal dependent spatial memory in rats, and whether the application of probiotics can provide protection. We treated rats with ampicillin in the presence or absence of *Lactobacillus fermentum* strain NS9, and analysed anxiety-like behaviour, spatial learning and memory behaviour using the elevated plus maze and Morris water maze. The gut microbiota composition, colon inflammation and brain biochemistry were also assessed. Our results suggest that *L. fermentum* NS9 can normalise the intestinal microbiota, as well as the anxiety-like behaviour and spatial memory defects induced by antibiotics. These findings shed light on the mechanism behind the clinical use of probiotics along with antibiotics during the treatment of an infection.

2. Materials and methods

Animal

All animal experimentation was performed according to the guidelines for the care and use of laboratory animals approved by Institute of Psychology, Chinese Academy of Sciences. Thirty weaned male Sprague-Dawley rats (*Rattus norvegicus*) were purchased from the Vital River Laboratories Co., Ltd. (Beijing, China P.R.). Rats were housed individually in wire-mesh cages in a special pathogen free environment, with 50% relative humidity, a temperature of 21±1 °C and on a 12 h light-dark cycle (light from 7:00 PM to 19:00 AM) with free access to food and water.

qPCR

Fresh faecal samples were collected from the rats on the day 0 and 41 day after initiating the antibiotic or probiotic treatment (Figure 1), and frozen at -20 °C. Bacterial DNA was isolated from the faeces using a stool kit (Tiangen Biotech Co. Ltd., Beijing, China P.R.) following the manufacturer's instructions. The bacterial 16S rRNA was amplified by qPCR using SYBR® Premix Ex Taq™ (Takara, Japan) on an ABI 7300 (Applied Biosystems, Carlsbad, CA, USA). Relative expression levels were calculated using the $2^{-\Delta\Delta}$CT method (Livak and Schmittgen, 2001). Relative levels of the gut microbiota including *Bacteroides*, *Clostridium coccoides, Firmicutes* and *Lactobacillus* to *Eubacteria* (all bacteria) were measured from the 16S rRNA expression levels using species specific primers (Table 1). The results are expressed as percentage expression of each species relative to all bacteria (Gareau *et al.*, 2011).

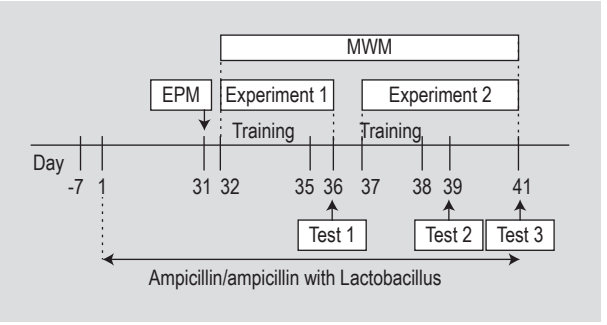

Figure 1. Schematic representation of the experimental design conducted in this study. After one week of acclimatisation, the rats were fed on water, ampicillin, or ampicillin with *Lactobacillus fermentum* NS9 for 41 days. From day 31, behavioural tests, including elevated plus maze (EPM) and Morris water maze (MWM), were carried out.

Probiotic treatment

L. fermentum strain NS9 (laboratory designated numbers) was isolated from naturally fermented dairy products collected from grasslands in Inner Mongolia, China P.R. The genome of this strain was sequenced (1,502 bp) and given the following unique GenBank accession no. JQ013298.1. Previous reports have shown that *L. fermentum* could modulate gut microbiota or prevent antibiotics induced side effects in gut (Axling *et al.*, 2012; Lönnermark *et al.*, 2010). The NS9 strain was incubated in De Man, Rogosa and Sharpe (MRS) broth at 37 °C overnight, collected by centrifugation at 3000 rpm for 10 min, and washed twice in saline (0.9% NaCl). The pellet was then re-suspended in sterile water and added to the drinking water for rats at a concentration of 10^9 cfu/ml. This water was provided to the rats and was changed every day.

Experimental design

After one week of acclimatisation, the rats were assigned to the following three groups. All groups were provided with identical food but with different drinking water as described below:

Table 1. Primer sequences used in qPCR to analyse the faecal microbiome.

Name of primer	Sequence (5'-3')	Target species	Annealing temp. (°C)	Reference
Lact-F	CGATGAGTGCTAGGTGTTGGA	*Lactobacillus*	61	Fu *et al.* (2006)
Lact-R	CAAGATGTCAAGACCTGGTAAG			
Bac-F	GAGAGGAAGGTCCCCCAC	*Bacteroides*	58	Gareau *et al.* (2011)
Bac-R	CGCTACTTGGCTGGTTCAG			
Fir-F	GGAGYATGTGGTTTAATTCGAAGCA	*Firmicutes*	60	Guo *et al.* (2008)
Fir-R	AGCTGACGACAACCATGCAC			
Clos-F	ACTCCTACGGGAGGCAGC	*Clostridium*	60	Gareau *et al.* (2011)
Clos-R	GCTTCTTAGTCAGGTACCGTCAT			
Eu-F	ACTCCTACGGGAGGCAGCAGT	Total bacteria	60	Gareau *et al.* (2011)
Eu-R	ATTACCGCGGCTGCTGGC			

1. Control group (n=10) was given regular drinking water;
2. Amp group (n=10) received ampicillin (Genview, Naples, FL, USA) at the concentration of 120 mg/kg body weight added to the drinking water;
3. Amp+NS9 group (n=10) received *L. fermentum* NS9 and ampicillin. NS9 and ampicillin was added to the drinking water at a concentration of 10^9 cfu/ml and 120 mg/kg body weight, respectively.

In order to avoid the adverse effects of ampicillin on NS9 we used the following approach. In the Amp+NS9 group, every day ampicillin was added to the drinking water at 7:00 AM and the water was changed at 19:00 PM with water containing NS9. Similarly, in the Amp group ampicillin was added to the drinking water at 7:00 AM and the water was changed at 19:00 PM with regular drinking water. This ensured that the ampicillin consumption was similar in the Amp and Amp+NS9 groups. The drinking water that had been removed from each group every day was analysed to calculate the amounts of ampicillin and NS9 based on the water consumption. The drinking water was changed every day. On average 8-10 ml water was consumed per 100 g rat body weight per day (Hu *et al.*, 2013). Treatment with probiotics and/or ampicillin was continued for 41 days after which the rats were sacrificed.

Rats were tested for behaviour 30 days after initiation of the antibiotic or probiotic treatment. For behavioural analysis, the rats were transferred to the test room to habituate to the new environment. On the day 31, an elevated plus maze test was performed followed by the Morris water maze on the next day. The water maze had two phases, experiment 1 was conducted from day 32 to 36 and experiment 2 was conducted from day 37 to 41. On the day 41 the rats were sacrificed.

Myeloperoxidase activity

Acute inflammation was assessed by myeloperoxidase (MPO) activity in the colon. About 1 cm long colon segments were dissected from the rats and gently cleaned with phosphate buffered saline (PBS), after which they were immediately frozen in liquid nitrogen and stored at -80 °C. The MPO activity assay was performed on frozen tissue as described by Boughton-Smith *et al.* (1988). MPO activity was expressed in units per mg of tissue, where one unit of MPO is defined as the quantity of enzyme able to convert 1 mol of hydrogen peroxide to water in 1 min at room temperature.

Behavioural tests

Two behavioural tests were used in this study, the elevated plus maze and the Morris water maze.

The elevated plus maze

The maze had two open arms (50×10 cm) and two closed arms of the same size and the closed arm had 25 cm high walls. The whole maze was at a height of 50 cm from the floor. All testing were conducted during the mid-portion of the light phase. The rats were placed individually in the central zone of the maze, facing an open arm, and allowed 5 min to explore freely. Animal behaviour was recorded by a video camera positioned at the top centre of the maze. The maze was thoroughly cleaned after each test using damp and dry cloths. All rats were tested once. The total distance travelled and number of arm entries was recorded as an index of general locomotor activity. The percentage of open arm entries (defined as four paws inside the maze arm) was quantified as a measure of anxiety-like behaviour (Anseloni *et al.*, 1999; Sharma *et al.*, in press).

The Morris water maze

The water maze pool (160 cm in diameter and 40 cm high) was filled with water (21±2° °C) to a height of 30 cm. Four distinctive landmarks approximately 20×20 cm in size were also placed at heights of 50 cm from the water surface. A movable circular platform (12 cm in diameter) placed 2 cm below the water surface served as an escape platform. A camera was mounted above the centre of the pool at a height of 2.1 m above the floor. The swimming speed, distance and latency to the escape platform were monitored through a computer based image analysis system (ANYmaze; Stoelting Co., Wood Dale, IL, USA). For analysis, the pool was subdivided into 4 equal quadrants (NE, SE, NW, and SW).

In Phase 1, the platform was placed in the SW quadrant. Rats were released from 4 symmetrical positions in a pseudo-randomised order so that the average distance of the starting position from the platform in 4 consecutive trials was same. Animals had four trials per day (separated by 15 min), for four consecutive days (day 32 to 35). Each trial lasted for 120 s. If a rat failed to find the platform during the time, it was guided to it. Rats were allowed to stay on the platform for 20 s. A probe trial was performed on the day 36. The platform was removed, and rats were allowed to swim for 120 s. The swimming speed, distance, latency to the platform and the time spent in the 4 quadrants were recorded.

Phase 2 started on day 37. The platform was placed in the NE quadrant, opposite to the previous position. The rats were trained for 2 days from day 37 to 38, with 3 trials per day with an interval of 15 min between the trials (Figure 1). On day 39 and 41, the platform was removed and the tests were carried out. The swimming speed, distance, latency to the platform and the time spent in each quadrant were recorded.

Corticosterone

HPA axis activity was assessed by measuring the corticosterone (CORT) level in rat serum (Oprica *et al.*, 2005). After the Morris water maze test, the rats were sacrificed and the trunk blood was collected. The blood samples were centrifuged at 2,000 rpm for 10 min at 4 °C and the isolated serum samples were frozen at -80 °C until further analysis. CORT level was measured using a commercial ELISA kit (IBL, Hamburg, Germany) and the results were recorded using a microplate reader (Labsystems Multiskan MS, Nelsirrki, Finland). The results are presented as ng/l.

Brain sample preparation

After sacrifice, the rat brains were removed immediately and the hippocampuses were isolated on ice. The samples were homogenised in PBS (pH 7.4) and the homogenate was centrifuged at 2,000 rpm for 20 min at 4 °C. The brain derived neurotrophic factor (BDNF), N-methyl-D-aspertate (NMDA), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) levels were measured with commercial ELISA kits (DRG, Springfield, NJ, USA) following the procedures recommended by the manufactures.

Statistical analysis

Results are expressed as mean ± standard error (SE). Non-parametric statistics (Mann Whitney U-test) was used to analyse the gut microbiota data. In other tests, groups were compared by analysis of variance (ANOVA) using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Post hoc analysis (Bonferroni or Student-Newman-Leuls) were performed, as indicated. *P*-values of <0.05 and <0.01 were considered significant and extremely significant, respectively.

3. Results

Effects of ***Lactobacillus fermentum*** on rat gut microbiota

Data obtained from qPCR indicated that similar levels of *Bacteroides*, *C. coccoides, Firmicutes,* and *Lactobacillus* (P>0.05) were observed in all three groups before ampicillin or *L. fermentum* NS9 administration (Figure 2A). Mann Whitney U-test analysis suggested that, compared to the control group, ampicillin administration led to a significant reduction in *Bacteroides* and *Lactobacillus* (P<0.01), and a remarkable increase in *C. coccoides* and *Firmicutes*, which were significantly higher than the control group (P<0.01, Figure 2B). In the Amp+NS9 group, *Bacteroides* was similar to the control rats (P>0.05). Compared to the ampicillin treated rats, the level of *Firmicutes* and *C. coccoides* was reduced significantly by NS9 application (P<0.01, Figure 2B). NS9 application also increased the level of *Lactobacillus* by 20%, which was significantly higher than the control rats and ampicillin treated rats (P<0.01, Figure 2B).

Effect on myeloperoxidase activity by *Lactobacillus fermentum*

Ampicillin treatment is known to induce colon inflammation. As shown in Figure 3, in contrast to the control group (4.3±0.9 U/mg), colon tissue in the ampicillin treated group had higher MPO activity (6.7±1.1 U/mg, P<0.05), which reduced to the normal value (5.0±1.1 U/mg, P<0.05) after *L. fermentum* NS9 treatment (P>0.05).

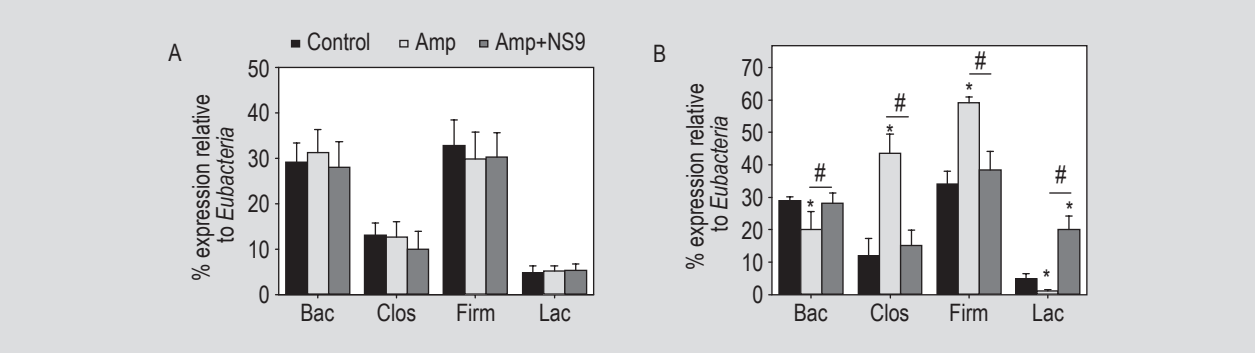

Figure 2. (A) The composition of gut microbiota in rat faeces before treatment with ampicillin (Amp) and *Lactobacillus fermentum* NS9 (NS9). (B) The composition of gut microbiota in rat faeces at the end of the experiment. Bac = *Bacteroides*; Clos = *Clostridium coccoides;* Fir = *Firmicutes;* Lac = *Lactobacillus.* * *P*<0.05, compared to the control; # *P*<0.05, significant differences between groups. Each group consisted of 10 rats.

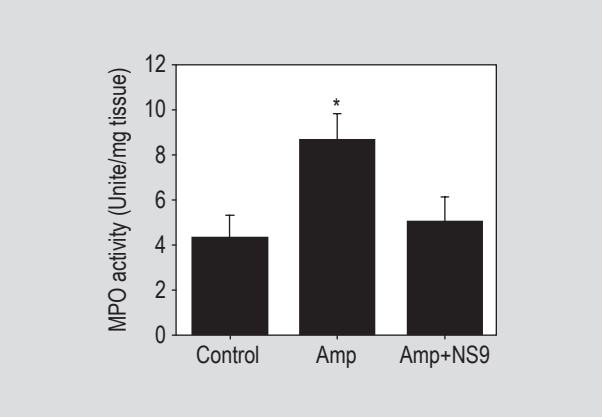

Figure 3. The effect of *Lactobacillus fermentum* NS9 and ampicillin (Amp) on myeloperoxidase (MPO) activity in the rat colon. Each group consisted of 10 rats. * *P*<0.05, compared to the control group.

Effect of *Lactobacillus fermentum* on ampicillin induced anxiety-like behaviour in rats

Ampicillin treatment did not change the locomotor activity, which was shown by the total distance travelled (Figure 4A) and total arm entries by the rats (Figure 4B). However, it induced a mild anxiety-like behaviour. Compared to control rats, ampicillin treated rats showed an obvious decrease in the percentage of open-arm entries and increase in the closed-arm entries. Treatment with *L. fermentum* NS9 alleviated this abnormal behaviour and elevated the percentage of open arm entries and reduced the percentage of close arm entries ($P<0.05$). However, there was no significant difference in the time spent in the open arm or closed arm between the Amp and control groups (data not shown).

Effect of *Lactobacillus fermentum* treatment on ampicillin induced spatial memory impairment

The Morris water maze test was carried out for 10 days in 2 phases to evaluate the effects of ampicillin and NS9 on spatial learning and memory. In phase 1 (day 32 to 36), the control group exhibited shorter latency than the Amp and Amp+NS9 groups (Figure 5A) on the first day of training. This demonstrated that ampicillin marginally impaired the learning behaviour, which was not alleviated by NS9 application. The difference between the three groups gradually diminished over time. Until the 4th day (treatment day 35), the three groups had similar latency to find the platform. On the 5th day (treatment day 36) when the platform was removed in test 1 all rats in the three groups spent a similar amount of time in the platform quadrant (Figure 5B) indicating that all rats remembered the location of the platform.

In phase 2, the platform was removed to the opposite quadrant (NE) to allow the rats to learn the new position of the platform. Figure 6A shows that the latency of control rats was evidently shorter than the ampicillin treated rats, and the NS9 application did not reduce the latency to the control level. This suggested that learning of rats fed on ampicillin was slower than the control rats. After 6 trials we observed that the rats in the three groups had a similar latency to the escape platform. On day 39, the platform was removed and the rats were subjected to the 2nd probe test. As shown in Figure 6B, control and Amp+NS9 treated rats spent most of their time in two quadrants; the quadrant where the platform was previously located and the new quadrant which contained the platform. This indicated that the rats in these two groups remembered the two locations of the escape platform (Figure 6B). But the ampicillin treated rats spent nearly equal times in all 4 quadrants suggesting that ampicillin treated rats did not only forget the new location but also forgot the previous location.

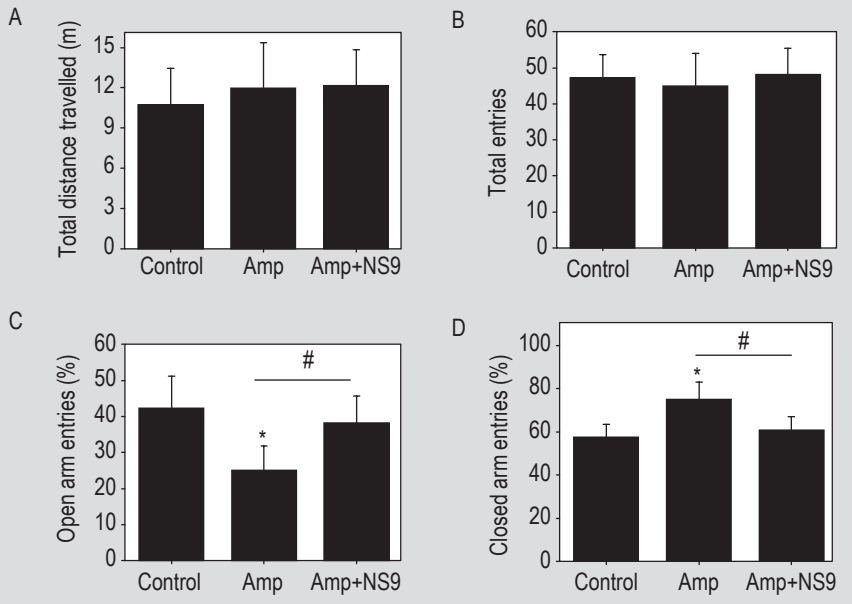

Figure 4. Anxiety-like behaviour of rats treated with ampicillin (Amp) and *Lactobacillus fermentum* NS9 in the elevated plus maze. (A) Total distance travelled; (B) number of total arm entries; (C) percentage open arms entries; and (D) percentage of closed arm entries. * *P*<0.05, compared to control group rats. # *P*<0.05, comparison between groups using one-way ANOVA followed by post-hoc Bonferroni test. n=10 rats/group.

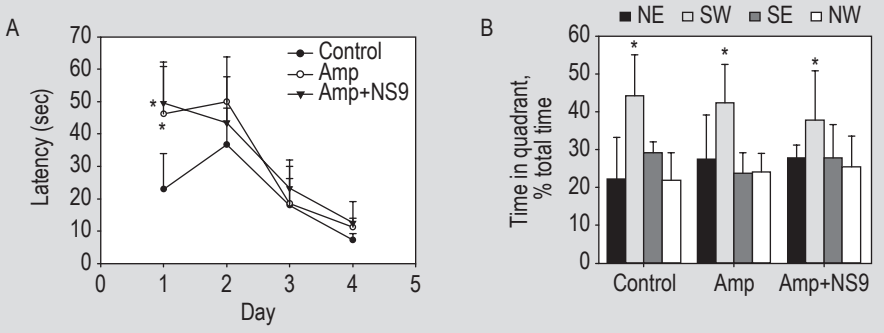

Figure 5. Rat performance in phase 1 of the Morris water maze test. (A) Escape latency to find platform in experiment 1. Each block represents mean of four trials. Values are means ± standard error. *P<0.05, compared to control group. (B) Percentage of time spent in the 4 quadrants. Values are means ± standard error. *P<0.05, the target quadrant compared to other quadrants. The platform was located in target quadrant SW. There were 10 rats in each group. Control = untreated; Amp = ampicillin treated; NS9 = *Lactobacillus fermentum* NS9 treated.

To investigate the ampicillin effects on long term memory, the probe test was repeated again after two days (test 3 in Figure 1). There were significant differences among the three groups. As shown in Figure 6C, the ampicillin treated rats still spent equal times (25%) in the 4 quadrants. However, the control and the NS9 treated rats spent (nearly 35 and 30%, respectively) significantly more time in quadrant SW, which previously contained the platform, than the ampicillin treated rats (P<0.05). This result shows that NS9 application significantly alleviated the memory defects induced by ampicillin. All rats in the three groups forgot the new location indicating that 6 trials were not enough to maintain the location memory for 72 h.

Effect of *Lactobacillus fermentum* on serum corticosterone

Ampicillin application markedly elevated serum corticosterone levels (from 550 to710 ng/l). Treatment with *L. fermentum* NS9 restored corticosterone levels to similar levels as the control rats (Figure 7).

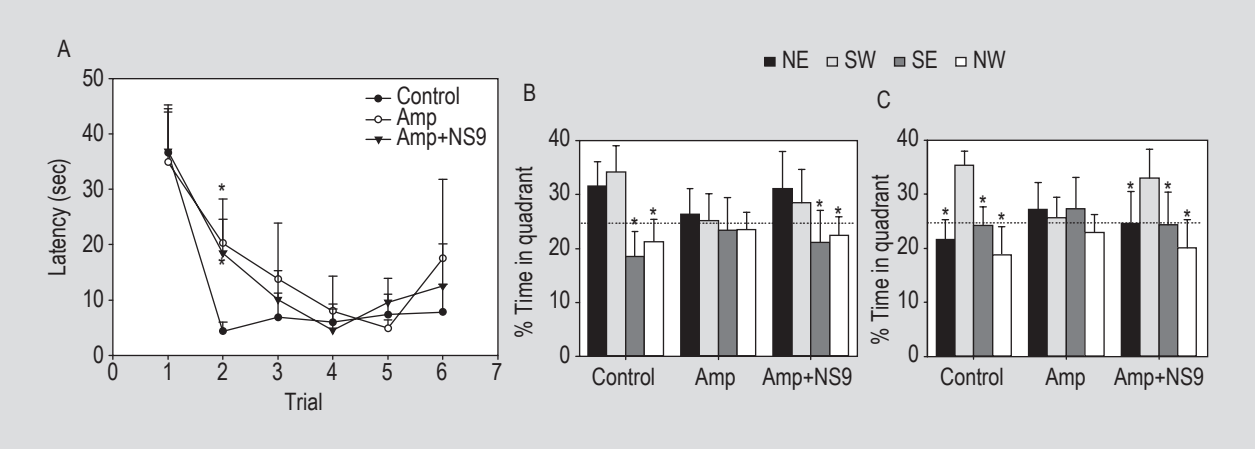

Figure 6. Rat performance in the second phase of Morris water maze. (A) Latency to find platform in every trial during training stage. Values are mean ± standard error. * *P*<0.05, compared to control group. (B) Percentage time spent in the 4 quadrants in probe test 2 after 24 h (day 39). Values are mean ± standard error. * *P*<0.05, other quadrants versus the target quadrant NE. (C) Percentage time spent in the 4 quadrants in probe test 3 after 72 h (day 41). Values are mean ± standard error. * *P*<0.05, other quadrants versus the target quadrant quadrant SW. Each group consisted of 10 rats.

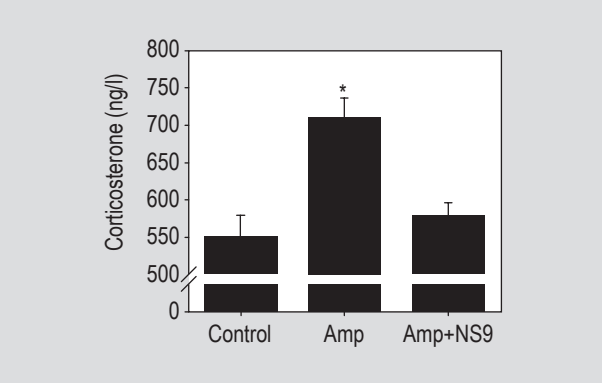

Figure 7. Serum corticosterone levels in rats treated with ampicillin (Amp) and *Lactobacillus fermentum* NS9. * *P*<0.05, compared to the control group.

Effect of *Lactobacillus fermentum* on BDNF, NMDA, MR and GR levels

The level of BDNF in the hippocampus was not different between the groups. As shown in Figure 8A, neither ampicillin application nor NS9 application changed the levels of BDNF in the hippocampus.

Two kinds of corticosterone receptors, MR and GR, have been reported in the hippocampus, which is the main target of corticosterone. Treatment with ampicillin and NS9 had no effect on GR levels (Figure 8B). However, ampicillin administration significantly reduced MR levels (P<0.05). Such a decrease was not observed in the NS9 treated group, which had remarkably increased levels of MR, when compared to control rats (P<0.05, Figure 8C). Ampicillin also significantly reduced the NMDA receptor levels from 1,249±299 to 835±127 ng/g tissue. Treatment with NS9 inhibited the ampicillin induced NMDA receptor reduction and maintained it at the normal level (1,192±176 ng/g tissue) (Figure 8D).

4. Discussion and conclusion

Gut microbiota are known to have a critical function in the overall health of an individual. Since intake of antibiotics is known to disturb microbiota and probiotics are routinely prescribed along with antibiotics to protect the microbiota, we performed this study to investigate how ampicillin alters the gut microbiome and whether probiotics can restore the antibiotic induced changes in or outside the gut. The results of our study suggested that treatment with ampicillin affected gut physiology, as well as emotional and spatial memory behaviour of male SD rats. NS9 application ameliorated the colon inflammation and normalised the emotional behaviour and spatial memory deficits induced by ampicillin. Furthermore, the efficiency of NS9 was associated in part, with the restoration of serum corticosterone level and the hippocampus MR and NMDA receptor levels in the normalised gut microbiota.

The microbiota-gut-brain axis, which is the line of interaction between the gut microbiota, gut and brain, plays an important role in maintaining the gut and brain function. Many factors that induce changes in the intestinal microbiota could also induce gut or brain disability, such as IBD (Hviid *et al.*, 2011), anxiety-like behaviour (Bercik *et al.*, 2010) or memory and learning alteration (Gareau *et al.*, 2011; Li *et al.*, 2009). In this study, treatment with ampicillin induced marked changes in the intestinal microbiota composition as evidenced by a decrease in *Lactobacillus* and *Bacteroides* levels and an increase in *C. coccoides* and *Firmicutes* in rat guts. NS9 application

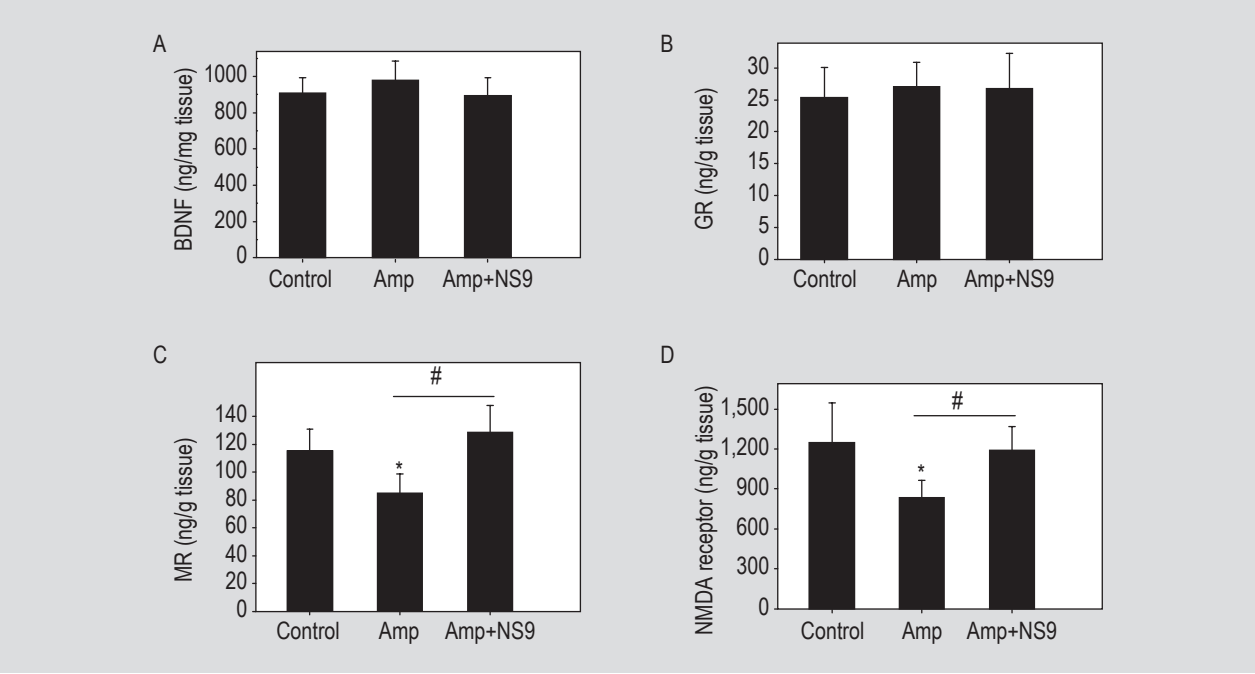

Figure 8. (A) Brain derived neurotrophic factor (BDNF), (B) glucocorticoid receptor (GR), (C) mineralocorticoid receptor (MR) and (D) N-methyl-D-aspertate (NMDA) protein levels in rat hippocampus treated by ampicillin (Amp) and *Lactobacillus fermentum* NS9. * *P*<0.05, compared to the control group. # *P*<0.05, differences between groups. Each group consisted of 10 rats.

improved the composition of the gut microbiota and alleviated the anxiety-like behaviour, and memory and learning behaviour. These findings highlight the role of the gut microbiota on behaviour.

Previous studies have shown that application of probiotics increase the gut immune response (Palomar *et al.*, 2013) and ameliorate colonic dysfunction induced by stress (Gareau *et al.*, 2007). In these reports, gut microbiota composition was also improved by probiotic treatment. The observed reduction in inflammation caused by NS9 application in our study may be associated with the restoration of gut microbiota by NS9. Emerging clinical and animal studies suggest that gut inflammation is associated with emotional behaviour (Bercik *et al.*, 2010; Stasi and Orlandlli, 2008). We used MPO activity as a measure to monitor mucosal inflammation induced by antibiotic treatment (Bercík *et al.*, 2004). The reduced MPO activity in NS9 treated rats could be at least in part, attributed to the alleviated anxiety-like behaviour in these rats.

Dysbiosis of the microbiota is known to affect the HPA axis activity (Sudo *et al.*, 2004). Dysfunction in the HPA axis in turn has been linked to the development of mood disorders. In this study, *L. fermentum* NS9 administration reduced higher serum corticosterone level and ameliorated the anxiety like behaviour induced by the treatment of ampicillin. These results are consistent with previous reports that showed elevated corticosterone levels associated with anxiety like behaviour (Kalinichev *et al.*, 2002; Kallen *et al.*, 2008; Mitra and Sapolsky, 2008). This result also suggested that the behavioural effect of *Lactobacillus* was at the physiological level.

Corticosterone levels and expression of GR and MR receptors in the brain are essential for learning and memory (Brinks *et al.*, 2007; Yau *et al.*, 1995). In the rat brain, hippocampus is the main target of corticosterone, with high expression levels of MR and GR (Reul and De Kloet, 1985). Genetic and pharmacological studies have implicated a key role for MR and GR in learning and memory. MR protects the neuron and is involved in the hippocampal neurogenesis (Gass *et al.*, 2000; Holsboer *et al.*, 1983). We observed that ampicillin administration did not impair learning seriously. All rats showed steady improvement across trials, as reflected by decreased latency to find the platform regardless of the treatment. Both central MR antagonism (Yau *et al.*, 1999) and loss of forebrain MR (Berger *et al.*, 2006) have been reported to impair memory, particularly in spatial memory retention tests. In addition, MR over-expression in the forebrain has been shown to enhance memory and improve spatial memory retention (Lai *et al.*, 2007). In this study, treatment with ampicillin reduced MR levels in the hippocampus and the rats failed to show quadrant preference similar to rats in the other two groups, suggesting that ampicillin impaired memory by reducing MR in the hippocampus. When rats were treated with NS9, the hippocampal MR levels increased along with the recovery of quadrant preference (Figure 6B). These results suggested that ampicillin treatment significantly

impaired memory retention in part, by reducing the MR levels in the hippocampus.

The NMDA receptor is a ligand-gated ion channel of glutamate essential for long-term potentiation and hippocampal-dependent spatial learning and memory (Villarreal *et al.*, 2002). It has been reported that NMDA receptor interacts with MR and GR to modulate memory (Maggio and Segal, 2012). NS9 administration elevated the expression of MR and NMDA receptors in our study. Animals fed on NS9 showed an enhanced memory of the first platform location compared to ampicillin treated rats. These findings are consistent with data generated by genetically blocking the NMDA receptor in the hippocampus dentate gyrus CA1 measured by radial maze (Niewoehner *et al.*, 2007; Tsien *et al.*, 1986). Our study highlights the importance of this receptor in the memory.

Previous reports have indicated that gut bacteria may also influence NMDA receptor and BDNF levels in the hippocampus (Sudo *et al.*, 2004). However, in the present study, NS9 administration elevated the NMDA receptor expression, but not the BDNF level, which is not consistent with previous reports (Gareau *et al.*, 2011). This implies that probiotic effects are strain dependent and could function in different ways. For example, a strain of *Bifidobacterium infantis* did not have anti-depressant properties but affected central neurochemical functions and crucial systems (Desbonnet *et al.*, 2008). These results suggest that the clinical use of probiotics must be performed with caution, and more animal and clinical research is needed to address this issue.

Previous studies have shown that administering a mixture of three antibiotics could change mice behaviour and central biochemical levels (Bercik *et al.*, 2011a,b). Our results in the present study suggested that even one antibiotic, which is considered as safe and have less adverse effects, could induce gut microbiota dysbiosis and behaviour abnormality. NS9, a strain of *Lactobacillus fermentum,* isolated in our laboratory alleviated these disabilities. We fed rats with probiotics and antibiotics almost simultaneously (rats were treated with antibiotic during the day and with probiotic at night respectively, which protected the probiotic from the antibiotic) similar to the study by Dai *et al.* (2013). This method varies from probiotic application prior to stress treatment (Gareau *et al.*, 2011) or after stress treatment (Bercik *et al.*, 2010), it protects the probiotic activity from destruction by the antibiotic and is more practical in clinical applications to reduce the side effect of antibiotic. However, a limitation of this study is the measurement of the total levels of NMDA receptor and MR in the hippocampus, as the levels of these receptors were not examined in the subfields of hippocampus.

In conclusion, NS9 is beneficial because it restores ampicillin induced physiological and psychological abnormalities in rats. Our data provide a basis to further investigate the utility of probiotics in the maintenance of brain health and adjunctive treatment of adverse effects induced by clinical antibiotic application.

Acknowledgements

This work was supported by a grant obtained from the NS Bio Japan and Beijing NS Health Biotechnology.

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