

***Lactiplantibacillus plantarum* LAB12 Induced Neuroprotection Through the Gut-Brain Axis**

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ABSTRACT

It is increasingly recognized that probiotic lactic acid bacteria (LAB) could potentially reverse dysbiosis and suppress neuroinflammation via the Gut-Brain Axis. The present study investigated *Lactiplantibacillus plantarum* LAB12-mediated crosstalk between the Enteric Nervous System (ENS) and the Central Nervous System (CNS) by assessing the beneficial effects on memory, modulation of major neurotransmitters, neuropeptides, and gut hormones as well as mitochondrial functions *in vivo*. To this end, Sprague Dawley rats (male, three months old) were administered with an antibiotic cocktail (i.e., imipenem, vancomycin, ampicillin, ciprofloxacin, and metronidazole) before being challenged with 0.25mg/kg LPS to mimic germ-free gut and neuroinflammation. The rats were divided into groups (n=8/group) of wild-type, lipopolysaccharide (LPS) control, LAB12+LPS, antibiotics+LPS (ABX), and ABX+LAB12 (ABXL). The rodents were then subjected to the Morris Water Maze (MWM) Test. Rat hippocampi and colons were harvested and subjected to biochemical analyses and metabolism assays. It was found that ABXL spent relatively more time in the platform zone (+16%) as opposed to their ABX counterparts. In terms of mitochondrial enzymes, the ABXL group was presented with increased Complex III enzyme activities in their cortices (+57 %; $p<0.05$) and hippocampi (+33 %; $p<0.01$) when compared to the ABX group. In terms of neurotransmitters, the ABXL group significantly increased the 5-hydroxytryptamine (5-HT) level (+32%, $p<0.05$) in the hippocampi when compared to the ABX group. The ABXL group also significantly increased ghrelin (GHRL) level (+85%, $p<0.05$), a gut hormone, in the hippocampi, when compared to ABXL. Nevertheless, ABXL did not bring about significant changes against neuropeptides [neurotensin (NT), neuropeptide Y (NPY), and vasoactive intestinal peptide (VIP)] in both the hippocampi and colons. The present findings implied that LAB12 could potentially improve memory impairment via the modulation of signaling pathways between the ENS and the CNS.

Keywords: *Lactiplantibacillus plantarum* LAB12, neurotransmitters, neuropeptides, gut hormones, mitochondrial function

INTRODUCTION

Increasing evidence supports the link between the gut and the brain. Changes in gut microbiota composition may lead to increased permeability of the gut epithelial barrier, resulting in the invasion of different bacteria, viruses, and their neuroactive products that would promote neuroinflammatory reactions in the brain [1]. Growing evidence suggested that probiotics may exert neuroprotection by using the Gut-Brain Axis as the basis. A recent study by Bonfili *et al.* [2], for example, found a probiotic formulation (SLAB51) that consists of nine live bacterial strains [i.e., *Streptococcus thermophilus*, bifidobacteria (*Bifidobacterium longum*, *B. breve*, and *B. infantis*), lactobacilli (*Lactobacillus acidophilus*, *L. plantarum*, *L. paracasei*, *L. delbrueckii* subsp. *Bulgaricus*, and *L. brevis*)] to markedly reduce oxidative stress in Alzheimer's disease (AD) mice brain by activating Sirtuin 1 (SIRT1)-dependent mechanisms. However, in another study by Huang [3], *L. paracasei* PS23 (LPPS23) was found to have lower scores of senescence and less severe anxiety-like behaviors as well as memory impairment in senescence-accelerated mouse prone 8 (SAMP8) mice model when compared to their control counterparts. The mechanisms underlying probiotic-induced neuroprotection have yet to be fully understood. It appears that the probiotic-induced neuroprotective effect is strain-dependent. Orally administered *L. plantarum* C29, for instance, attenuated 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced memory impairment in mice. This was mediated, in part, via increased TNBS-suppressed hippocampal brain-derived neurotrophic factor (BDNF) expression and inhibited TNBS-induced hippocampal NF- κ B activation as well as blood lipopolysaccharide (LPS) levels [4]. On the other hand, *L. plantarum* MTCC1325 (60-day supplementation) restored acetylcholine and ameliorated cognition deficits of D-galactose-induced AD rats [5].

It was also reported that repeated administration of antibiotics to juvenile BALB/c mice affected both the microbiota status and behaviour at the adult age [6]. Despite their beneficial effect against infectious diseases, antibiotics could also negatively impact patients physiologically and psychologically [7]. Several studies showed that different antibiotic treatments result in short- and/ or long-term changes in the intestinal microbiota in humans and animals [8]. In addition, both animal and clinical studies have demonstrated that antibiotics and concomitant dysbiosis are associated with changes in behaviour and brain chemistry [9]. A study of the role of the host microbiome in regulating amyloidosis in the APP_{SWE}/PS1_{ΔE9} mouse model of AD has found that long-term broad-spectrum combinatorial antibiotic treatment showed prolonged shifts in gut microbial composition and diversity as well as decreases in Amyloid- β (A β) plaque deposition and levels of soluble A β are elevated [10]. However, another study of physiological and psychological abnormalities induced by ampicillin in rats had shown that administration of ampicillin in rats produced an elevation of serum corticosterone and increased anxiety-like behaviour, impairment of spatial memory as well as elevated glucocorticoids which were associated with memory dysfunctions and reduction of hippocampal BDNF, two common features of AD pathology [11].

Based on the notion that probiotics could improve deficits in neurogenesis caused by antibiotics [12], the present study investigated the neuroprotective potential of *Lactiplantibacillus plantarum* LAB12 against antibiotic-LPS-challenged rats characterized by closed to germ-free gut and neuroinflammation. To this end, Sprague Dawley rats (male, three months old) were administered with an antibiotic cocktail (i.e., imipenem, vancomycin, ampicillin, ciprofloxacin, and metronidazole) before being challenged with 0.25 mg/kg LPS to mimic closed to germ-free gut and neuroinflammation. Ampicillin is a broad-spectrum antibiotic that inhibits bacterial cell wall production. While imipenem inhibits the cell wall synthesis of Gram-positive and Gram-negative bacteria, vancomycin only inhibits the cell wall synthesis of Gram-

positive bacteria. Ciprofloxacin is a broad-spectrum antibiotic that inhibits the cell division of Gram-positive and Gram-negative bacteria. Metronidazole, on the other hand, inhibits nucleic acid synthesis by disrupting the DNA of microbial cells. The impact of the crosstalk between the Central Nervous System (CNS) and the Enteric Nervous System (ENS) on LAB12-induced neuroprotection was expanded to additional major neurotransmitters (serotonin [5HT] and γ -Aminobutyric acid [GABA]), neuropeptides (neurotensin [NT], neuropeptide Y [NPY] and vasoactive intestinal peptide [VIP]) and gut hormones (Ghrelin and glucagon-like peptide 1 [GLP-1]).

EXPERIMENTAL

Figure 1 shows the experimental overview of the present study. Briefly, Sprague-Dawley rats were divided into five groups and fed either a normal diet only or a normal diet supplemented with LAB 12. The animals were then exposed to LPS and subjected to the behavioural test. The rodents were sacrificed after which the whole brains and colons were harvested and processed for subsequent bioanalyses.

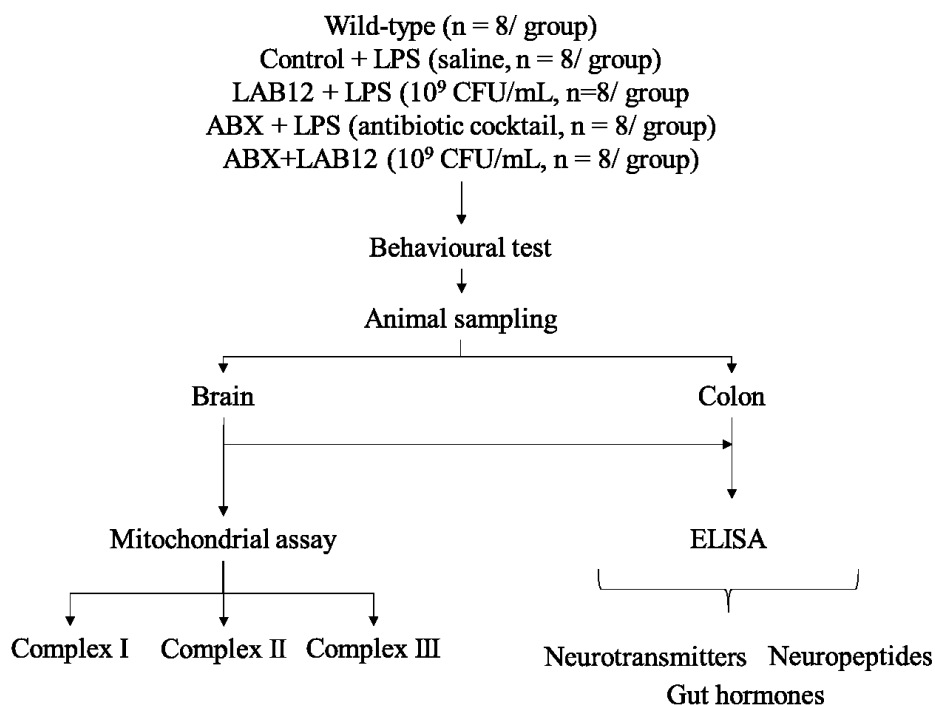


Figure 1: Experimental overview of the effect of LAB12 pre-treatment on antibiotic-induced germ-free and LPS-induced memory-impaired rats.

Freeze-Dried LAB12 Preparation

In this study, the *L. plantarum* LAB12 was obtained from the culture collection of CDDR, Faculty of Pharmacy, UiTM Puncak Alam. LAB12 was initially isolated from local fermented food, *tempeh* [13]. LAB12 was subcultured three times in MRS broth and incubated at 37 °C for 24 h. The bacteria were harvested by centrifugation (Jouan BR4i Multifunction Centrifuge, Thermo Electron Corporation, Massachusetts, United States) at 13,000 x g and 4 °C for 10 min. The bacteria pellet was washed with PBS, frozen at -20 °C (Sanyo, Osaka, Japan), and freeze-dried using Freeze-dryer Freezone 12-Plus (Labconco, MO, USA). Freeze-dried LAB12 was then dissolved in saline before feeding. The viability test was conducted weekly using the Spread Plate Technique to ensure that the amount of viable LAB was equivalent to 10⁹ CFU/mL.

Ethics Approval

The present study was approved by the Committee on Animal Research and Ethics (CARE) of the UiTM (Reference No: 600-FF PT.5/2, 15 /2012; dated 26th April 2012).

Animals

Male Sprague-Dawley rats of three months of age (250-300 g) were obtained from the Laboratory and Facilities Animal Management (LAFAM), Faculty of Pharmacy, UiTM. The animals were allowed to acclimate to laboratory conditions and access food pellets and water *ad libitum* before experiments. The care of laboratory animals was performed by the Guide for the Care and Use of Laboratory Animal [14] adopted by LAFAM.

Groupings

In Figure 2, male Sprague Dawley rats were divided into five groups; Wild-type (n = 8/ group), control (LPS, n = 8/ group), LAB12 (10⁹ CFU/mL, n = 8/ group), antibiotic cocktail (ABX: ampicillin (250 mg/mL), ciprofloxacin (50 mg/mL), imipenem (62.5 mg/mL), metronidazole (62.5 mg/mL) and vancomycin (50 mg/mL), n = 8/ group), antibiotics + LAB12 (ABXL, n = 8/ group). All groups had access to food and water *ad libitum* for 32 days. LAB12 and ABX groups were supplemented orally with LAB12 and antibiotic cocktail, respectively, from Day 1 until Day 32. For the ABXL group, the antibiotic cocktail was given to the rats from day 1 until day 21, followed by a washout period (2 days), and continued with supplementation of LAB12 from day 24 until day 32. On day 26, Morris Water Maze (MWM) was performed, which included training, actual test, and probe test. Except for the wild type, rats in all groups were injected with 0.25 mg/kg LPS intraperitoneally [15] for three consecutive days starting from day 29 (Figure 2). Rats were then sacrificed on day 33.

Behavioural Model: MWM

Morris water maze was used in this study to test the rats' spatial memory. The behavioural test requires the animals to learn, search and memorize the location of a hidden platform from opaque water. The time taken from the initial point to the platform [escape latency (sec) and the distance traveled (m)] was recorded to

evaluate memory. A probe test (the platform was removed) reflecting memory consolidation was performed on day 31 [16].

Generally, it consists of a circular water pool with four equal quadrants, 1, 2, 3, and 4, a transparent platform, and was located 2 cm below the water surface, which is fixed in the centre of one of the quadrants. The size of the tank was 210 cm in diameter. Both have sides that are 51 cm in height with non-reflective interior surfaces. For Sprague Dawley rats, dark brown food colouring was used to darken the water. After each test, the water was maintained at room temperature and cleansed from the rat's faeces.

The experiment began with habituation trials (twice per day) performed on days 26, 27, and 28 before the actual tests. The actual MWM Test was performed for the next four consecutive days (28, 29, 30, and 31). The time taken from the initial point to the platform [escape latency (sec) and the distance travelled (m)] was recorded. A probe test (the platform was removed) was performed on day 32.

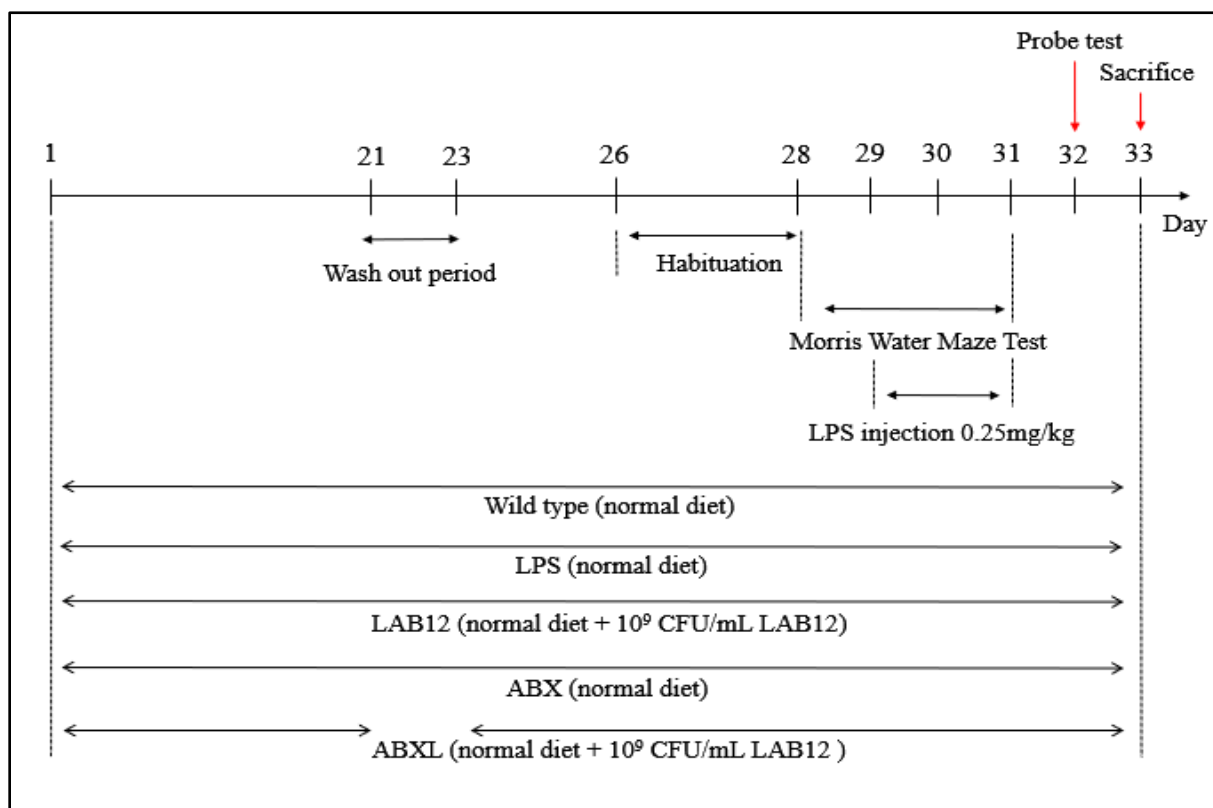


Figure 2: Schematic representation of pre-treatment and behavioural test sequences of antibiotic-induced germ-free and LPS-induced memory-impaired rats. Note: Except for the wild type, rats in all groups were injected with 0.25 mg/kg LPS intraperitoneally.

Sample Collection and Processing

The rats were anaesthetized (100 mg/kg ketamine and 10 mg/kg xylazine) and decapitated. The whole brains were harvested, and the hippocampus was collected and homogenized using a TissuRuptor Homogeniser (Qiagen, Hilden, Germany). The colon was harvested, with colon content and fat tissues removed before cutting the colons into 5 cm segments. The rat hippocampi and colons were stored in a lysis buffer at -80°C until further biochemical analysis, Enzyme Link Immunosorbent Assay (ELISA).

Mitochondrial Assays

The hippocampi and cortices were homogenized in isolation buffer with ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (215 mM Mannitol, 75 mM sucrose, 0.1 % BSA, 20 mM HEPES, 1 mM EGTA, pH 7.2). Homogenates were centrifuged at 13,000 x g and 4 °C for 5 min. Pellet was resuspended in an isolation buffer with EGTA and spun again at 13,000 x g for 5 min. The resulting supernatant was transferred to new tubes, topped off with isolation buffer with EGTA, and spun again at 13,000 x g for 10 min. A pellet containing pure mitochondria was resuspended in an isolation buffer without EGTA.

Complex-I (NADH dehydrogenase activity) was measured spectrophotometrically by the method of Prakash *et al.* [17]. The reaction mixture contained 0.2 M glycyl glycine buffer pH 8.5, 6 mM NADH in 2 mM glycyl glycine buffer, and 10.5 mM cytochrome c. For each sample, the reaction was initiated by the addition of 10 µL solubilized mitochondrial sample in a mixture of 350 µL glycylglycine buffer, 100 µL cytochrome c, 100 µL NADH, 2.5 mL distilled water, 20 µL sodium bicarbonate in 2 mL cuvette. Absorbance change was quickly measured after the start of the reaction at 550 nm for 2 minutes using a Genesys 20 spectrophotometer (Thermo Spectronic, New York, USA). Equation 1 describes Complex I enzyme activity as follows:

$$\frac{180\text{ s} - 0\text{ s}}{2.5\text{ min}} = \frac{x \times 0.262 \times 3 \times 1000}{\text{protein concentration}(\frac{\text{mg}}{\text{mL}})} \quad \text{Equation 1}$$

$x = \text{mole of NADH oxidised/ min/ mg Protein}$

Complex-II (succinate dehydrogenase (SDH) activity) was measured spectrophotometrically according to Prakash *et al.* [17]. The reaction mixture contained 0.2 M phosphate buffer pH 7.8, 1% BSA, 0.6 M succinic acid, and 0.03 M potassium ferricyanide. For each sample, the reaction was initiated by the addition of 25 µL solubilized mitochondrial sample in a mixture of 200 µL succinic acid, 300 µL BSA, 100 µL NADH, 1.75 mL distilled water, and 1.5 mL phosphate buffer in 2 mL cuvette. Absorbance changes were quickly measured after the reaction at 550 nm for 2 minutes using a spectrophotometer (Genesys 20, Thermo Spectronic, New York, USA). Equation 2 describes Complex II enzyme activity as follows:

$$\frac{180\text{ s} - 0\text{ s}}{2.5\text{ min}} = \frac{x \times 3.8 \times 0.435 \times 1\,000\,000}{\text{protein concentration}(\frac{\text{mg}}{\text{mL}})} \quad \text{Equation 2}$$

$x = \text{mole of SDH oxidised/ min/ mg Protein}$

Complex-III (MTT ability) assay was used to assess the activity of the mitochondrial respiratory chain in isolated mitochondria by the method of Prakash et al. [17]. Briefly, 100 µL mitochondrial samples were incubated with 10 µL MTT at 37 °C for 3 hours. The blue formazan crystals were solubilized with dimethylsulfoxide and measured at 580 nm using the Infinite M200 Plate Reader (Tecan, Mannedorf, Switzerland). Equation 3 describes Complex III enzyme activity as follows:

$$\text{Complex III activity (x)} = \text{Absorbance} - \text{Blank} \quad \text{Equation 3}$$

ELISA

5-HT, VIP, NT, Ghrelin and GLP-1

5-HT, VIP, NT, Ghrelin, and GLP-1 concentration in supernatant derived from either brain tissue lysates or intestinal tissue homogenates was measured using the Rat (5HT, VIP, NT, Ghrelin, and GLP-1) ELISA Kit (Finestest, Wuhan, China) in accordance with the manufacturer's instructions respectively. The principle of this assay is based on competitive ELISA as the plate was pre-coated with 5-HT, VIP, NT, Ghrelin, and GLP-1, respectively [18 - 22].

Briefly, 50 µL sample and standard were added to each well. The biotin-detection antibody (50 µL) was then added immediately. The plate was incubated at 37 °C for 45 min. The solution was aspirated, and the plate was washed three times. SABC (100 µL) was added to each well, and the plate was incubated at 37 °C for 30 min. The solution was aspirated, and the plate was washed five times. 3,3',5,5'-tetramethylbenzidine (TMB) substrate (90 µL) was added and incubated at 37 °C for 15 min (protected from light). Finally, 50 µL stop solution was added to each well, and the plate was read at 450 nm using the Infinite M200 Plate Reader (Tecan, Mannedorf, Switzerland).

GABA and NPY

GABA and NPY concentrations in supernatant derived from either brain tissue lysates or intestinal tissue homogenates were measured using the Rat (GABA and NPY) ELISA Kit (Sunlong Biotech, Zhejiang, China) by the manufacturer's instructions, respectively [23, 24].

Briefly, 50 µL sample and standard were added to each well. The plate was gently shaken before being incubated at 37 °C for 30 min (sealed with closure plate membrane). The solution was aspirated, and the plate was washed five times. Horseradish peroxidase (HRP)-conjugate reagent (50 µL) was added to each well except the blank control wells. The plate was incubated at 37 °C for 30 min. The solution was aspirated, and the plate was washed five times. Chromogen Solution A (50 µL) and Chromogen Solution B (50 µL) were added to each well. The solution was mixed and incubated at 37 °C for 15 min. Stop solution (50 µL) was added to each well to terminate the reaction. Absorbance was read at 450nm using the Infinite M200 Plate Reader (Tecan, Mannedorf, Switzerland).

Statistical Analysis

Statistical significance was analyzed using GraphPad Prism version 6.01 (GraphPad Software Incorporated, USA). All results were expressed as Mean \pm Standard Error (SEM). Data were analyzed using the One-way ANOVA followed by Tukey's multiple comparison test. p values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The Effects of LAB12 Pre-Treatment on Spatial Memory and Motor Function of Antibiotic-LPS-Challenged Rats

The MWM Test examined the cognitive functions of antibiotic-LPS-challenged rats supplemented with LAB12 in terms of their escape latency (i.e., time spent to reach the platform), escape distance (i.e., searching distance) and probe test (i.e., time spent in platform quadrant). In addition, the present study also assessed the motor function of these rats by considering the average swimming speed. When compared to the wild-type group, it was found that LPS-challenged rats took a significantly longer time (Figure 3A; +49 %; 32 s vs. 14 s; $p < 0.001$) and travelled significantly further (Figure 3B; +69 %; 9 s vs. 4 s; $p < 0.05$) before locating the escape platform. LPS, however, did not result in significant changes in swimming speed compared to their wild-type counterparts (Figure 3C). Illustrative images of rat trajectories in the MWM (Figure 3D) indicated very different patterns of swimming tracks between wild-type and LPS-challenged groups. The direct swim to the platform reflected the development of spatial memory in the wild-type group. LPS-challenged rats, on the other hand, searched the whole surface area of the pool, first randomly and later selectively, scanning the inner area of the pool containing the escape platform.

Pre-treatment of LPS-challenged rats with 10^9 CFU/ mL LAB12 for 31 days attenuated the memory impairment effect of the neurotoxin. In fact, LAB12 restored escape latency, escape distance, and time spent in the platform quadrant of LPS-challenged rats compared to those of the baseline (i.e., wild-type group). In terms of escape latency (Figure 3A), LAB12 significantly ($p < 0.05$) reduced the escape latency of LPS-challenged rats by 38 % (18 s vs. 32 s), as opposed to their LPS control counterparts. In terms of escape distance (Figure 3B), LAB12 significantly ($p < 0.01$) reduced the escape distance of LPS-challenged rats to reach the platform by 36 % (5 s vs. 9 s) when compared to their LPS control rats. No changes were observed in average speed between the LAB12-treated rats compared to the LPS counterparts (Figure 3C). Illustrative images of rat trajectories in the MWM (Figure 3D) indicated LAB12 groups to be thigmotaxic initially (i.e., the rats swam almost next to the walls). However, they later exhibited an incursion pattern towards the platform.

An antibiotic cocktail (ABX) was administered orally to LPS-challenged rats to mimic closed to germ-free conditions. Regarding escape latency, ABX (Figure 3A) did not worsen the spatial memory of LPS-challenged rats further, as this parameter was not significantly different compared to the LPS-challenged rats. In terms of escape distance (Figure 3B), there was also no significant difference between ABX and LPS control groups. Illustrative images of rat trajectories in the MWM (Figure 3D) indicated a similar pattern of swimming track between ABX and LPS control groups. Regarding average speed, no significant differences (mean Days 1-3) were observed between the groups. Supplementation of LAB12 to

antibiotic-LPS challenged rats (ABXL) improved neither escape latency nor distance (Figures 3A and 3B). Illustrative images of rat trajectories in the MWM (Figure 3D) indicated a similar pattern of swimming track between ABXL and ABX groups. In terms of average speed, no significant differences (mean Days 1-3) were observed between the groups (Figure 3C).

In the probe test (Figure 3E), LPS-challenged rats were presented with reduced time spent in the platform zone by 13 % (15.67 s vs 18.3s) when compared to the wild-type group. Pre-treatment with LAB12 slightly increased this parameter by 23 % (21.66 s vs. 15.7 s) compared to its LPS counterparts, but the difference was insignificant. No changes were observed between ABX and LPS control groups. ABXL, on the other hand, showed a modest increment of time spent in the platform zone by 16 % (18.78 s vs. 16.2 s) as opposed to their ABX counterparts. Illustrative images of rat trajectories in the MWM (Figure 3F) indicated that the LPS-control group displayed more circular and wider swims that often covered the neighbouring quadrant areas, confirming the diminished spatial learning abilities and memory of LPS-challenged rats. However, LAB12, ABX, and ABXL groups exhibited a chaining response strategy whereby the rats performed a focal search in the correct pool area containing the escape platform.

Pre-treatment of LPS-challenged rats with LAB12 only (without ABX) gave rise to enhanced and consolidated memory of locating the escape platform. Antibiotics, on the other hand, though remarkably useful, can also cause certain adverse effects. Antibiotics are normally used to remove or prevent bacterial colonization in the human body without targeting specific types of bacteria. As a result, broad-spectrum antibiotics can significantly affect the composition of the gut microbiota, reduce its biodiversity, and delay colonization for an extended period after administration [25]. The present study acknowledges the limitation regarding the lack of information on gut microbiota dysbiosis. Future studies may analyze the gut microbiota using 16s rRNA or shotgun gene sequencing. Shotgun gene sequencing, in particular, would allow the sequencing of the entire genomes of all the organisms present in the samples [26]. Möhle et al. [12] reported that a mixture of Abx compounds [ampicillin plus sulbactam (1.5 g/l; Pfizer), vancomycin (500 mg/l; Cell Pharm), ciprofloxacin (200 mg/l; Bayer Vital), imipenem plus cilastatin (250 mg/l; MSD), and metronidazole (1 g/l; Fresenius)] applied via drinking water for seven weeks in adult mice decreased hippocampal neurogenesis and memory retention. Results from the present study showed a significant difference in escape latency, escape distance, and average speed between rats challenged with a mixture of antibiotics (ABX) and wild type but not with LPS control rats. This could be attributed to the fact that LPS and antibiotics can cause dysbiosis independently. Most of the previous studies used either LPS or antibiotics but not both. In AD, it has been shown that using a cocktail of antibiotics in APP/PS1 transgenic mice increased the neuroinflammatory state and cytokine levels and, therefore, the disease itself [10]. On the other hand, Fröhlich et al. [27] reported that antibiotic-induced dysbiosis in an adult mouse model revealed that only novel object recognition memory was impaired in antibiotic-treated mice, not the spatial memory in support of the present study.

Reconstitution with probiotics did not reverse the memory deficits in the present study. This is in contrast to previous studies where the administration of probiotics (*L. fermentum* strain NS9) reversed memory dysfunctions and increased hippocampal BDNF [11]. In another study, a mixture of probiotics (lactobacilli and bifidobacteria) also improved the memory impairment caused by a mixture of antibiotics in mice [12]. The differences observed from the previous study compared to the present study could be attributed to the duration and the study design. The use of a concoction of antibiotics may have resulted in the loss of the core gut microbiota.

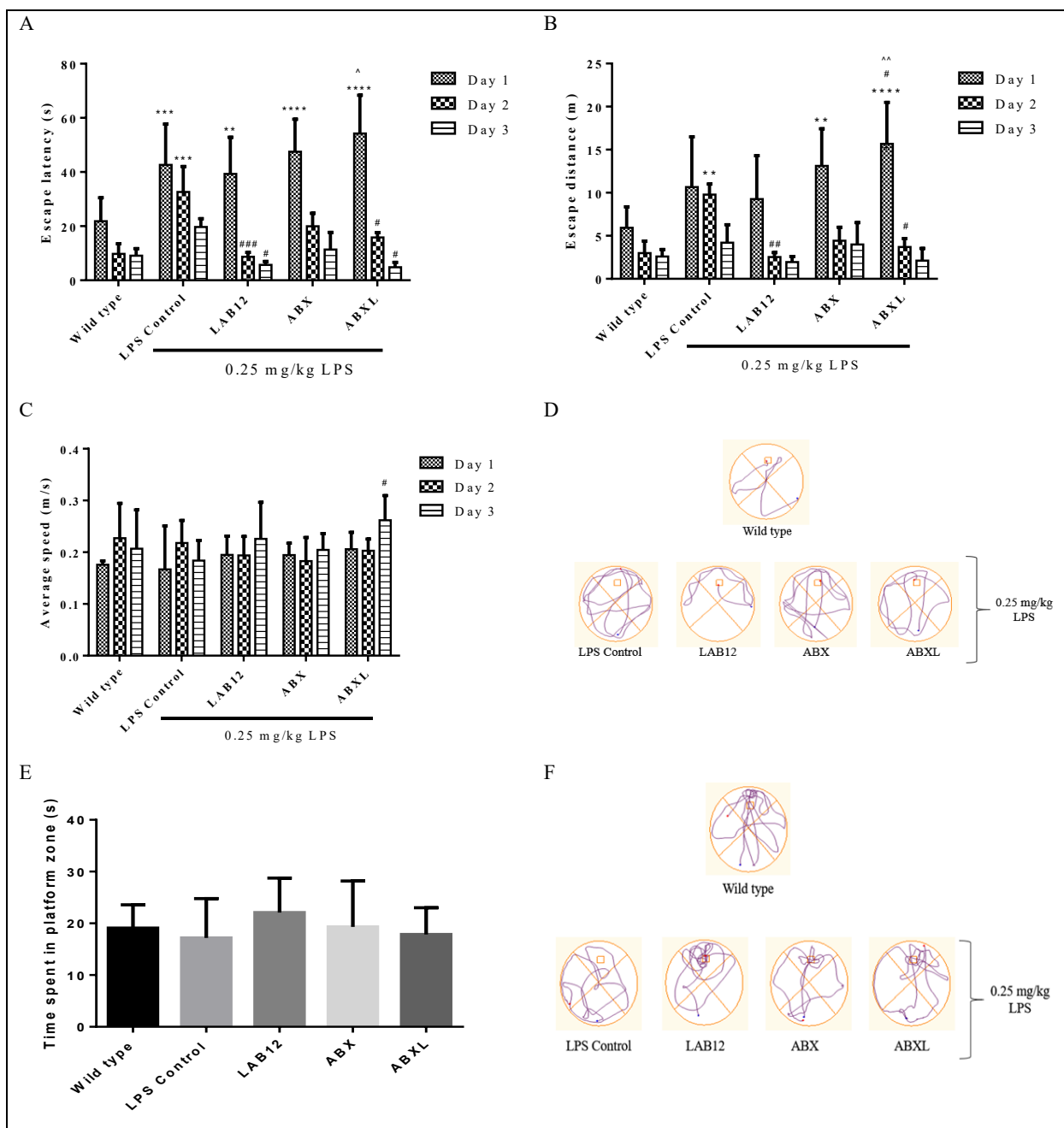


Figure 3: Effect of pre-treatment with LAB on spatial memory of antibiotic-LPS-challenged rats. Antibiotic-LPS-challenged rats pre-treated with LAB12 were subjected to behavioural study using the MWM Test. The rodents were assessed for A) escape latency, B) escape distance, and C) average speed taken to reach the escape platform. The rat trajectories in the MWM were recorded using ANY-Maze software (D). LPS control, LAB12, ABX, and ABXL rats were subjected to a behavioural study using the MWM Test. The rodents were assessed for the time spent in platform zone (E). The rat trajectories of the Probe test in the MWM were recoded using ANY-Maze software (F). Each bar represents mean \pm SEM of $n=8$. * $p<0.05$, ** $p<0.01$, *** $p<0.005$, **** $p<0.001$ when compared to wild type group; # $p<0.05$, ## $p<0.01$, ### $p<0.005$, #### $p<0.001$ when compared to LPS control group; + $p<0.05$, ++ $p<0.01$, +++ $p<0.005$, ++++ $p<0.001$ when compared to LAB12; ^ $p<0.05$, ^^ $p<0.01$ when compared to ABX treatment group.

Furthermore, the delay in colonization for an extended period after the administration of antibiotics would not have allowed the administration of probiotics to be as effective. The LAB 12 probiotics should have been fed longer to allow colonization and restoration of gut microbiota. Moreover, the study design of including LPS and ABX would have caused severe dysbiosis that probiotics could not improve. Previous studies used only ABX and not ABX and LPS [11, 12]. Besides, the present studies utilized only one behavioral study, the Morris Water Maze test, to assess spatial memory. Future studies may include a few other behavioural tests, like the object recognition test, used to study cognitive impairment in animal models of AD [28]. Elsewhere, the combination of the Y-maze test, object recognition test, and Morris Water Maze test were used to assess the effects of hippocampus reticulatum on memory, spatial learning, and object recognition in a scopolamine-induced animal model of AD [29].

The Effects of LAB12 Pre-Treatment on Mitochondrial Enzymes in The Cortex and Hippocampus of Antibiotic-LPS-Challenged Rats

Figure 4 shows the effects of LAB12 pre-treatment on mitochondrial enzymes in the cortex and hippocampus of antibiotic-LPS-challenged rats. It was found that LPS-challenged rats were presented with decreased Complex I enzyme activity by 72 % (7.3 nmol/min/mg vs. 25.43 nmol/min/mg) in the cortices (Figure 4A) and by 74 % (29.7 nmol/min/mg vs. 112.8 nmol/min/mg) in the hippocampi (Figure 4B) when compared to their wild type counterparts. Pre-treatment of LPS-challenged rats with LAB12 showed an increase of Complex I enzyme activity by 74 % (28.7 nmol/min/mg vs. 7.3 nmol/min/mg) in the cortices (Figure 4A) and by 61 % (76.8 nmol/min/mg vs. 29.7 nmol/min/mg) in the hippocampi (Figure 4B) when compared to the LPS control group. The increased enzyme activities were, however, statistically insignificant. Neither groups of ABX nor ABXL showed significant changes in Complex I enzyme activity in their cortices and hippocampi compared to the LPS control group.

On the other hand, no significant changes were observed for Complex II enzyme activities in cortices (Figure 4C) and hippocampi (Figure 4D) across all groups. Regarding Complex III enzyme activities, no significant changes were observed in the cortices (Figure 4E) and hippocampi (Figure 4F) of the LPS control group compared to their wild-type counterparts. Pre-treatment of LPS-challenged rats with LAB12 showed significantly higher enzyme activity in cortices when compared to LPS control (+64 %; 213.6 % vs. 76.57 %; $p < 0.01$) and wild type (+53 %; 213.6 % vs. 100 %; $p < 0.01$) groups. ABX did not result in further changes to Complex III enzyme activities in the brain compared to the LPS control group. ABXL group were, however, presented with increased Complex III enzyme activities in their cortices (+57 %; 155.6 % vs. 68.12 %; $p < 0.05$; Figure 4E) and hippocampi (+33 %; 113.1 % vs. 75.45 %; $p < 0.01$; Figure 4F) when compared to ABX group.

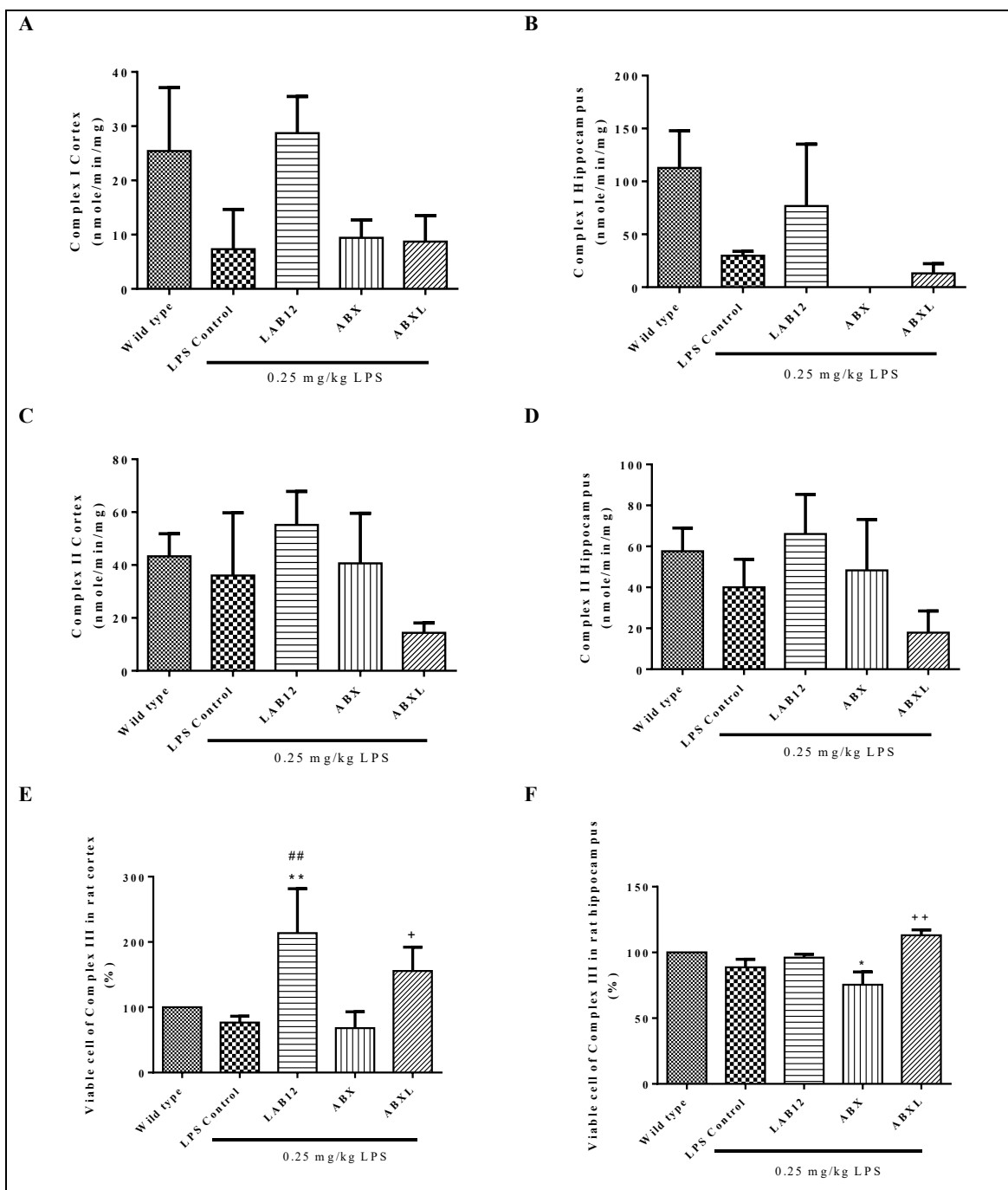


Figure 4: Effect of LAB12 pre-treatment on mitochondrial enzymes in cortices and hippocampi of antibiotic-LPS-challenged rats. Cortices and hippocampi of wild type, LPS challenged, LAB12, ABX, and ABXL groups were evaluated for enzyme activities of Complex I (A, B), Complex II (C, D), and Complex III (E, F). Each bar represents mean \pm SEM of $n=4$; $*p<0.05$, $**p<0.01$ when compared to wild type group; $^{##}p<0.01$ when compared to LPS control group; $^{+}p<0.05$, $^{++}p<0.01$ when compared to ABX group.

In recent years, oxidative stress and mitochondrial dysfunction have been proposed to be the major causative factors of cognitive impairment in the aging brain. Many studies have shown that the generation of adenosine triphosphate (ATP) is significantly affected due to the impairment of multiple mitochondrial enzymes in neurodegenerative disorders, suggesting an impaired energy metabolism. Our study indicated that the impact of LAB12 was not very significant in restoring the altered activity of mitochondrial respiratory enzyme complexes (NADH dehydrogenase, succinate dehydrogenase activity, cytochrome C oxidase) of LPS-challenged rats these enzymes. The administration of LAB12 pre-treatment significantly restored only the mitochondrial respiratory enzyme complex III activity in the cortex of LPS-challenged rats. There need to be more studies on the effect of probiotics on mitochondrial dysfunction of enzyme complexes. However, one study on D-galactose (D-gal)-induced mitochondrial dysfunction in rat cerebral cortex had found that *L. plantarum* NDC 75017 significantly improved the learning and memory, mitochondrial ultrastructure and functions, including the mitochondrial respiratory chain, mitochondrial membrane potential and mitochondrial permeability transition [30].

The Effect of LAB12 Pre-Treatment on Neurotransmitters, Neuropeptides, and Gut Hormone Levels in Hippocampi and Colons of Antibiotic-LPS-Challenged Rats

Figure 5 (A and B) shows the effect of LAB12 pre-treatment on the 5-HT level in hippocampi and colons of antibiotic-LPS-challenged rats. No significant changes were observed between the LPS control and wild-type groups. Neither LAB12 nor ABX brings about significant changes in LPS-challenged rats. ABXL group was, however, presented with significantly increased levels of 5-HT (+32 %; 18.8 ng/ml vs. 13 ng/ml; $p < 0.05$) in hippocampi when compared to their ABX counterpart. On the other hand, no significant changes in 5-HT level were observed in colons across all groups. Figure 5 (C and D) shows the effect of LAB12 pre-treatment on GABA levels in rat hippocampi and colons of antibiotic-LPS-challenged rats. No significant changes in GABA levels were observed in hippocampi and colons across all groups.

Figure 6 (A and B) shows the effect of LAB12 pre-treatment on NT levels in hippocampi and colons of antibiotic-LPS-challenged rats. No significant changes in NT level were observed in hippocampi and colons across all groups. Figure 6 (C and D) shows the effect of LAB12 pre-treatment on NPY level in hippocampi and colons of antibiotic-LPS-challenged rats. No significant changes in NPY level were observed in hippocampi and colons across all groups. Figure 6 (E and F) shows the effect of LAB12 pre-treatment on VIP level in hippocampi and colons of antibiotic-LPS-challenged rats. No significant changes in VIP level were observed in hippocampi across all groups. On the other hand, no significant changes in VIP level were observed in colons between LPS control and wild-type groups. LAB12 did not bring about significant changes to the VIP level when compared to LPS-challenged rats. However, the ABX group presented with low VIP level (-58 %; 25 pg/ml/mg vs. 58 pg/ml/mg; $p < 0.05$) in the colons compared to the LPS control group. ABXL, however, did not bring about significant changes to the VIP levels compared to ABX rats.

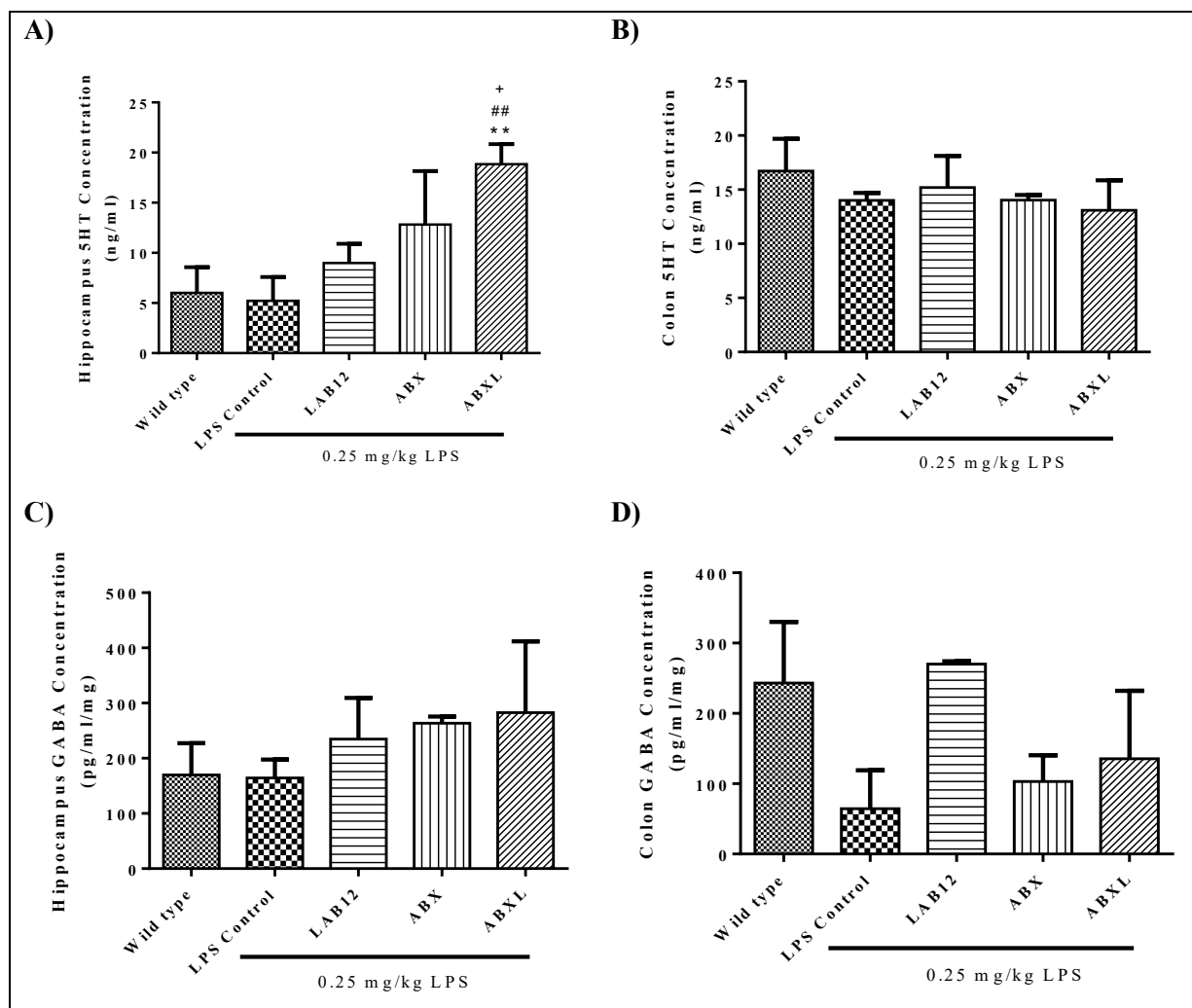


Figure 5: Effect of LAB12 pre-treatment on protein levels of neurotransmitter in hippocampi and colons of antibiotic-LPS-challenged rats. Cortices and hippocampi of wild type, LPS challenged, LAB12, ABX, and ABXL groups were evaluated for 5-HT (A, B) protein expression and GABA (C, D). Each bar represents mean \pm SEM of $n=4$; $**p<0.01$ when compared to wild type group; $##p<0.01$ when compared to the LPS control group; $+p<0.05$ when compared to the LAB12 group.

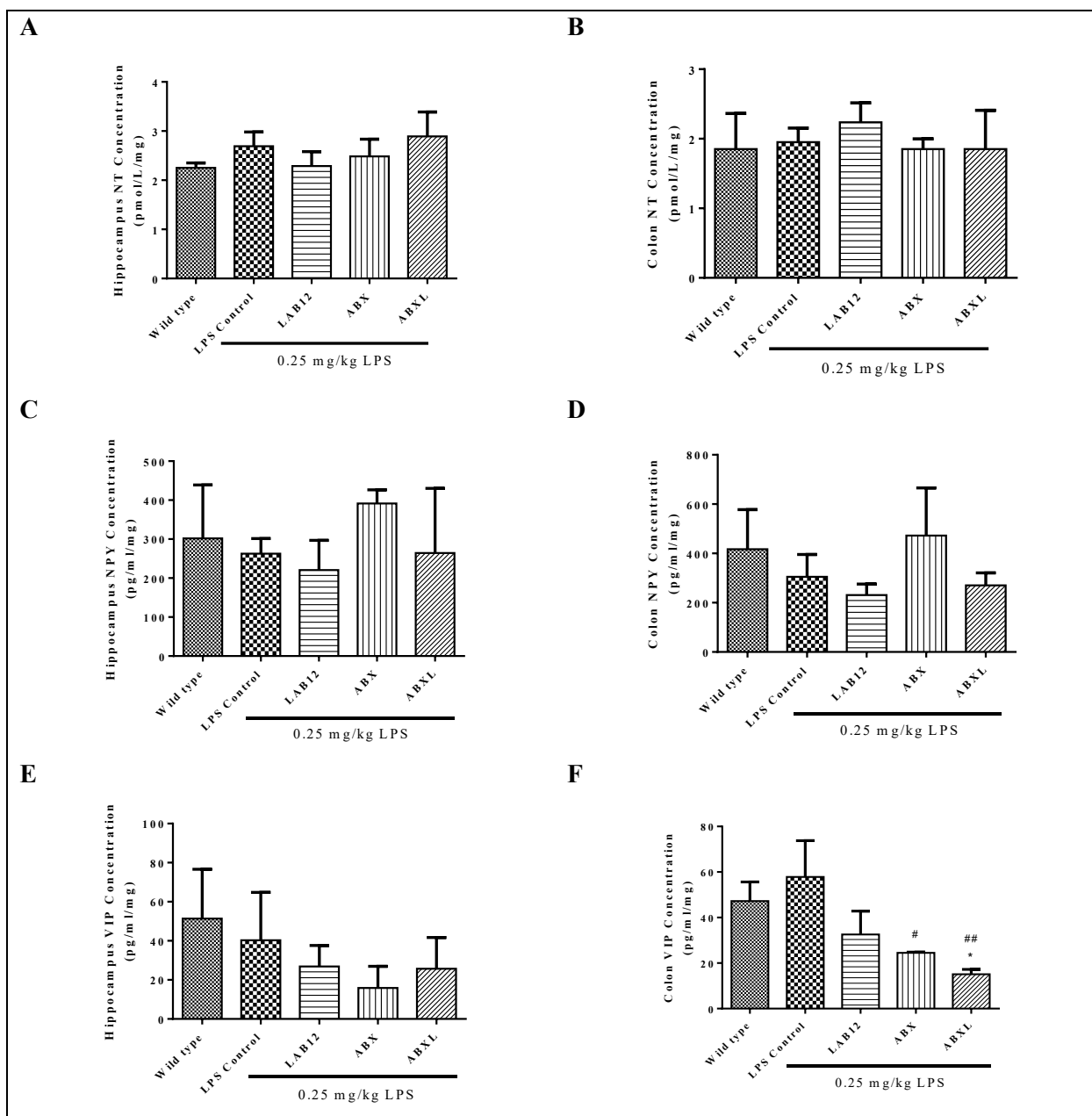


Figure 6: Effect of LAB12 pre-treatment on neuropeptide protein level in hippocampi and colons of antibiotic-LPS-challenged rats. Cortices and hippocampi of wild type, LPS challenged, LAB12, ABX, and ABXL groups were evaluated for protein expression of NT (A, B), NPY (C, D), and VIP (E, F). Each bar represents mean ± SEM of n=4; * $p < 0.05$ compared to wild type group; # $p < 0.05$, ## $p < 0.01$ compared to the LPS control group.

Figure 7 (A and B) shows the effect of LAB12 pre-treatment on GHRL level in hippocampi and colons of antibiotic-LPS-challenged rats. No significant changes in GHRL level were observed in hippocampi between LPS control and wild-type groups. LAB12 did not bring about significant changes to the GHRL level when compared to LPS-challenged rats. Likewise, ABX did not bring about significant changes to GHRL levels when compared to LPS-challenged rats. ABXL group was, however, presented with high GHRL levels (+85 %; 129 pg/ml/mg vs. 19 pg/ml/mg; $p<0.05$) in the hippocampi when compared to the ABX group. On the other hand, no significant changes in GHRL level were observed in colons across all groups. Figure 7 (C and D) shows the effect of LAB12 pre-treatment on GLP-1 level in hippocampi and colons of antibiotic-LPS-challenged rats. No significant changes in GLP-1 level were observed in hippocampi between LPS control and wild-type groups. LAB12 group was, however, presented with low GLP-1 level (-40 %; 27 pg/ml/mg vs. 43 pg/ml/mg; $p<0.05$) in the hippocampi compared to the LPS control group. No significant changes in GLP-1 level were observed in hippocampi between ABX and LPS control groups. ABXL did not significantly change the GLP-1 level compared to ABX rats. On the other hand, no significant changes in GLP-1 level were observed in colons across all groups.

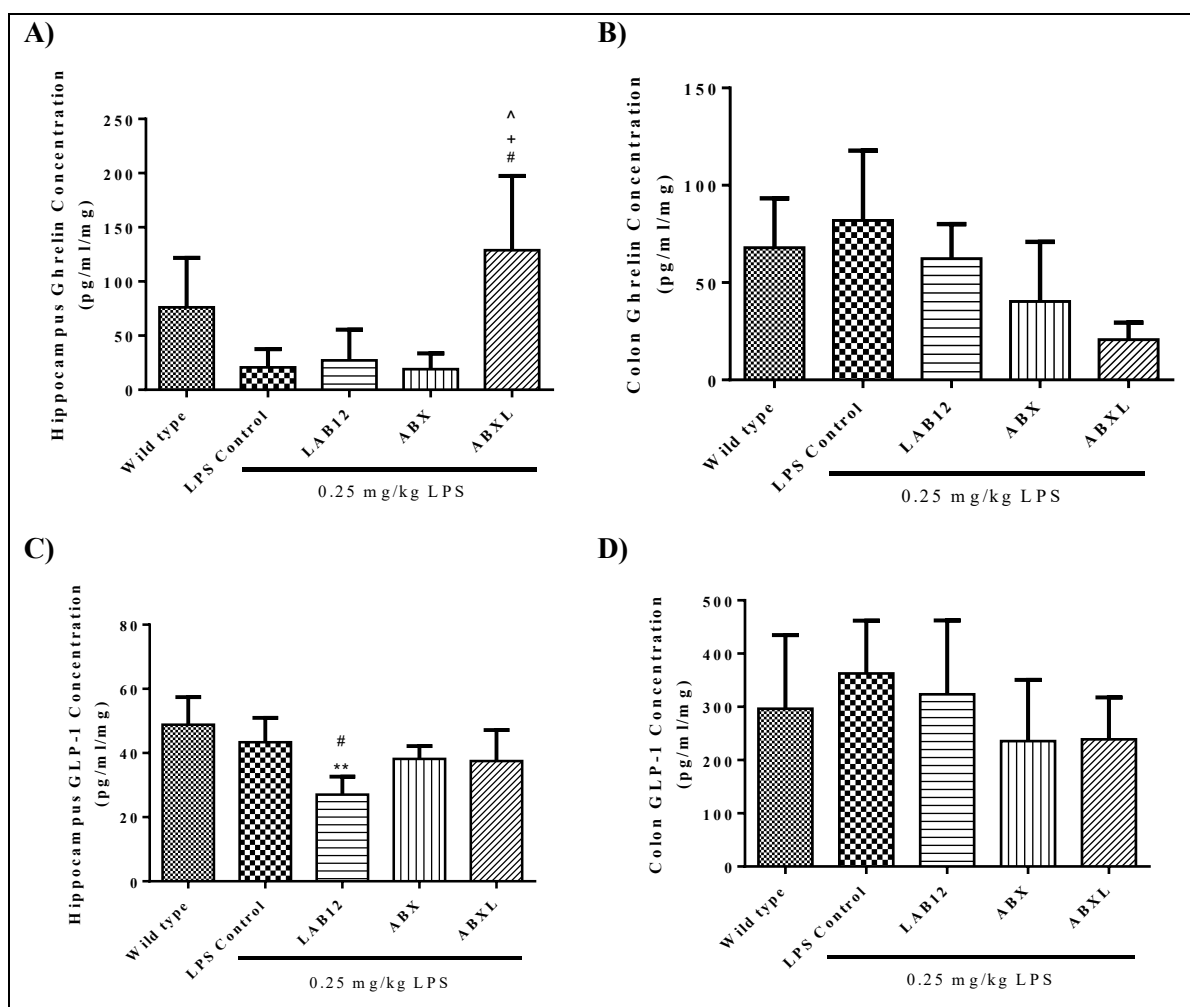


Figure 7: Effect of LAB12 pre-treatment on gut hormone level in hippocampi and colons of antibiotic-LPS-challenged rats.

Cortices and hippocampi of wild type, LPS challenged, LAB12, ABX, and ABXL groups were evaluated for protein expression of GHRL (A, B) and GLP-1 (C, D). Each bar represents mean \pm SEM of $n=4$; ** $p<0.01$ when compared to wild type group; # $p<0.05$ when compared to LPS control group; + $p<0.05$ when compare to LAB12 control; ^ $p<0.05$ when compared to ABX group

In this study, ELISA subjected hippocampus and colon tissues to 5HT, GABA, NT, VIP, NPY, Ghrelin, and GLP-1 protein expression quantification. The results were generally insignificant in most parameters due to the number of animals used in the study. As the standard deviation was very high, more animals may be required to measure these parameters.

In the brain, especially the hippocampus, 5-HT significantly regulates mood and cognition [31]. In the gastrointestinal tract (GIT), 5-HT promotes intestinal peristalsis, initiation and propagation of intrinsic enteric reflexes, and communication between the intestine and the brain (Johnston et al. 2014). The present study showed that a significant increase in serotonin from the hippocampus region of rats was observed in the LPS-ABXL group compared to the LPS-ABX group. Consistent results were found in previous studies. Chronic administration of live *L. plantarum* PS128 significantly increased the levels of both serotonin and dopamine in the striatum, but not in the prefrontal cortex or hippocampus [32]. *L. helveticus* NS8 also restored rat hippocampal serotonin (5-HT) [33]. Recently, *B. longum* and *L. rhamnosus* intervention in chronic unpredictable mild stress (CUMS) rat model, reduced colonic 5-HT and increased 5-HT in the frontal cortex and hippocampus [34].

GABA and glutamate are the two most important neurotransmitters of the brain. The most abundant synapses in the CNS of vertebrates are inhibitory synapses that use GABA [35]. Once again, only the LPS-ABXL group of rats showed significantly higher expression of GABA in the hippocampus when compared to the ABX group. Previously, *L. reuteri* was found to increase the GABA receptor gene expression and protein levels in multiple brain regions [36]. A study to investigate the therapeutic potency of probiotics (bifidobacteria and lactobacilli) on glutamate excitotoxicity as a neurotoxic effect that was induced by clindamycin and propionic acid (PPA) in juvenile hamsters revealed that probiotics can be used safely to ameliorate glutamate excitotoxicity mostly through increasing GABA [37].

NT is an endogenous tridecapeptide in the central and peripheral nervous systems [38]. Under normal physiological conditions, neurotensin is involved in regulating pain, body temperature, physical activity, appetite, learning, and memory. In addition, it plays a vital role in fat metabolism. Previous studies have demonstrated that neurotensin level alterations were associated with neuropathological conditions such as Alzheimer's disease, mood disorders, and obesity-associated eating disorders. Obesity is associated with low-grade systemic inflammation, brain inflammation, and cognitive decline [39]. Microinjection of NT into the entorhinal cortex (EC), a structure that is crucial for learning and memory (EC), increased spatial learning as assessed by the Barnes Maze Test and significantly improved the memory status in APP/PS1 mice, an animal model of AD [40]. However, the current study showed no significant differences in the level of NT among the groups. There have been no previous studies on the effect of probiotics on neurotensin.

NPY is a 36 amino acid involved in various physiological and homeostatic processes in the central and peripheral nervous systems. NPY and its receptors (especially Y1, Y2, and Y5) are highly expressed in brain regions involved in learning and memory processes. Intracerebroventricular (iv) infusion of NPY was found to have memory-enhancing effects in a non-social context [41]. In the present study, no significant differences in NPY of rat hippocampus and colon were observed among the various groups.

Most of the previous studies on probiotics or antibiotics did not evaluate the role of NPY on memory function.

VIP is a peptide of 28 amino acids produced in many tissues of vertebrates, including the gut, pancreas, and hypothalamus in the brain. VIP is neuroprotective and plays a vital role in acquiring learning and memory. VIP, when microinjected into the hippocampal area, improved rats' learning and memory [42]. A potent lipophilic analog to VIP exhibited neuroprotection in model systems related to Alzheimer's disease [43]. In a recent study, VIP decreased β -Amyloid accumulation and prevented brain atrophy in the 5xFAD mouse model of Alzheimer's disease [44]. In our study, however, there were significantly lower levels of VIP in the ABX and ABXL groups compared to the LPS group. Most of the previous studies of probiotics on VIP evaluated serum levels, which could be the reason for the contrasting results observed in this study. Furthermore, these studies did not evaluate cognitive function. In contrast, *L. fermentum* Suo attenuated HCl/ ethanol-induced gastric injury in mice and increased VIP level through its antioxidant effects [45]. *Lactobacillus fermentum* Zhao significantly increased the serum levels of MTL, Gas, ET, AChE, SP, and VIP [46]. However, in another study, *Lactobacillus* isolated from yak yogurt increased levels of somatostatin and VIP in mice serum [47].

Ghrelin is a novel growth hormone recently isolated from human and rat stomachs [48]. Ghrelin undergoes esterification, binds to GHSR-1a, and is transported via receptor-mediated transport across BBB. It is synthesized predominantly in the stomach and secreted into circulation [49]. Two-thirds of circulating ghrelin is produced by X/A-like cells of the oxyntic mucosa of the stomach and the remainder in the intestine [50]. Ghrelin is involved in glucose and lipid metabolisms and higher brain functions such as learning and memory; it influences mitochondrial respiration and exerts neuroprotective effects. It participates in neurodegenerative disorders, representing a link between metabolism and neurodegeneration [51]. The ghrelin level in the hippocampus of rats fed ABXL was significantly higher than ABX. In line with the present study, [52] indicated significantly increased levels of serum ghrelin in 18- and 24-week-old treated AD mice compared to their respective controls.

GLP-1 is a 30-amino acid long peptide hormone derived from the tissue-specific post-translational processing of the proglucagon peptide. It is produced and secreted by intestinal enteroendocrine L-cells and specific neurons within the nucleus of the solitary tract in the brainstem upon food consumption [53]. GLP-1 improved memory function and increased hippocampal neuronal numbers in a senescence-accelerated mouse model of AD [54]. The results from the present study found no differences between the various groups. The role of probiotics on GLP-1 concerning cognitive function is unclear. There have been no previous studies.

In a study on metabolic syndrome, the levels of acetate and GLP-1 were increased by *Bifidobacterium animalis* ssp. *lactis* GCL2505 (BlaG) treatment in both the gut and plasma of a five-week-old male mouse model [55]. In vancomycin-treated SPF mice, the diversity of the gut microbiota was significantly reduced, and the abundance of lactobacilli was markedly increased. Significant increases in body weight, cecum weight, plasma GLP-1 level, and colonic GLP-1/GPR43 expression were also noted relative to the controls [56]. In a study that used antibiotics with markedly different antibacterial spectra to modulate the gut microbiome in a diet-induced obesity mouse model, ceftazidime showed significant, dose-dependent improvement in key metabolic variables, including glucagon-like peptide-1 (GLP-1) [57]. Treatment of C57BL/6J mice with the antibiotics (vancomycin [V] and bacitracin [B]) in drinking water before diet-induced obesity (DIO) significantly decreased both Firmicutes and Bacteroidetes in the gut as revealed by pyrosequencing of the microbial 16S rRNA gene. Concomitantly, systemic glucose intolerance,

hyperinsulinemia, and insulin resistance in DIO were ameliorated via augmentation of GLP-1 secretion [58].

CONCLUSION

Altogether, the results showed that the antibiotics rats showed a similar pattern of cognitive impairment to that of the LPS rats. LAB12 improved cognitive function but did not reverse memory impairment in LPS-ABX rats suggesting that the germ-free induced memory impairment may be chronic (combination of LPS and ABX). The LAB12, however, impacted especially the serotonin but not GABA and the gut hormone ghrelin but not the neuropeptides. It can be concluded that LAB12 mediated crosstalk between the ENS and the CNS through modulation of neurotransmitters, neuropeptides, gut hormones, and mitochondrial function.

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AUTHOR'S CONTRIBUTION

Muhammad Syukri Noor Azman performed the experiments, analyzed the data, and wrote the original draft. Siong Meng Lim was involved in project administration and supervision. Fei Tieng Lim analysed the data. Kalavathy Ramasamy was involved in conceptualization, project administration, and supervision. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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