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Original Research Article

Probiotics from Thai Fermented Foods Reduced Anxiety and Enhanced Neuroplasticity in a Wistar Rat Model

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ABSTRACT

Probiotics have a significant impact on mental health through the gut-brain axis mechanism. From Thai fermented foods, various probiotic bacterial strains have been isolated. The present study was aimed at investigating the effects of probiotics from Thai fermented foods on anxiety and neuroplasticity in a Wistar rat model. Six bacterial strains were isolated from Thai fermented foods and used to prepare a probiotic cocktail. Male rats were divided into three groups. Group 1 (CON) rats were given sterile, distilled water for 21 days. Group 2 (ANT) rats received four antibiotics over 21 days. Each rat in Group 3 (PRO) was administered antibiotics every day for seven days, followed by 14 days of probiotics. Compulsiveness, anxiety-like behavior, depression, and neuroplasticity were evaluated. The rat brain tissue specimens were collected at the end of the experiment and analyzed histologically. The expressions of the genes linked with brain inflammation, neuroplasticity, and HPA axis regulation were also examined. The results showed that 14 days of probiotic mixture treatment in the PRO group significantly improved anxiety levels ($p<0.05$), compared to the rats in the ANT group, but did not affect depression. Probiotics showed positive effects on neuroplasticity by lowering *Bax* but increasing *Igf-1* and *Gr* mRNA gene expressions in PRO compared to ANT. They also demonstrated neuroprotectivity in the dentate gyrus. The findings of this study suggest that probiotics from Thai fermented foods decrease anxiety and improve neuroplasticity, thereby having the potential to become a functional food, enriched with psychobiotics to promote human mental behavior.

Keywords: Anxiety, Depression, Gene expression, Neuroplasticity, Probiotic.

Introduction

Many of the lactic acid bacteria (LAB) that are used as probiotics are isolated from a variety of traditional Asian naturally-fermented foods, which are well-known for having high probiotic content and belong to genera like *Lactobacillus*, *Streptococcus*, and *Leuconostoc*.[1] Probiotics that have mental health benefits for psychopathology when consumed in the right doses are referred to as psychobiotics. The term "psychobiotics" was first coined to describe this novel probiotic subclass in 2013. In patients and animal models, psychobiotics have been found to create neurotransmitters and psychotropic effects.[2] Rat models are frequently utilized in antibiotic therapy to evaluate the psychobiotic effects of probiotics. Antibiotics usually disrupt the intestinal microbiota, which can have a variety of effects on rats, including depression, anxiety-like behavior, and cognitive alterations in the gut. The gut-brain axis network alters mental behaviors in rats by modulating the neural, endocrine, and immune systems related to brain activity.[3,4]

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Recently, a probiotic cocktail containing isolated bacteria such as *Lactococcus lactis* subsp. *lactis* TBRC 375, *Pedicoccus pentosaceus* WS11, *Bifidobacterium adolescentis* TBRC 7154, *Lactobacillus brevis* TRBC 3003, *Lactobacillus plantarum* SK321, and *Lactobacillus fermentum* SK324 from Thai fermented foods significantly reduced anxiety and improved locomotor performance and short-term memory in rats.[5] These probiotics also greatly improved enzyme and non-enzyme cerebral antioxidant defenses and showed neuroprotective benefits in rats' cerebral cortexes.[5] However, the influence of these probiotics from Thai fermented foods on rat cerebral neuroplasticity has not been studied.

Therefore, the aim of this research was to investigate the effects of probiotics from Thai fermented foods on anxiety and neuroplasticity in Wistar rats.

Materials and Methods

Sources of probiotic bacteria and preparation of the probiotic cocktail

Lactobacillus lactis subsp. *lactis* TBRC 375, *P. pentosaceus* WS11, and *B. adolescentis* TBRC 7154 were isolated from pickled cabbage, water kefir, and adult intestine, respectively. Also, *L. brevis* TRBC 3003, *L. plantarum* SK321, and *L. fermentum* SK324 were isolated from pickled cabbage, Pak-Sian Dong, and Pak-Sian Dong, respectively. All these were obtained from Thai fermented foods and used as bacterial consortia.[5]

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Source of animals and experimental groupings
Male Wistar rats (eight-week-old) were purchased from the Northeast Laboratory Animal Center, Khon Kaen University, Thailand. All rats were randomly allocated to one of three experimental groups (n = 7 in each group). Normal rats were administered sterile, distilled water (1 mL per rat daily via oral gavage) as a sham medication for 21 days in Group 1 (CON). Rats in Group 2 (ANT) received four antibiotics (ampicillin, neomycin sulfate, vancomycin, and metronidazole, at doses of 1.75, 1.75, 0.875, and 1.75 mg/day, respectively) for 21 days (1 mL per rat daily via oral gavage). In Group 3 (PRO), each rat received 1 mL of antibiotics every day for seven days, followed by 14 days of probiotics (1 mL per rat per day). The rats were housed according to a previous study.[5]

Ethical clearance
Ethical approval for this study was obtained from the Animal Ethics Committee at Khon Kaen University in Thailand with the approval number IACUC-KKU-60/62.

Nestlet shredding test to evaluate compulsiveness and anxiety-like behaviour
Rat anxiety and compulsive behavior were measured in this study using the nestlet shredding method.[6] Each rat was given a two-layered nestlet (6 g, 4 cm thick) placed in the middle of the cage bed and allowed to move freely for 30 min without being disturbed while being observed using a Sony Action Camera (FDR-X3000R). Four parameters of anxiety/compulsiveness were determined, which included digging time (s), freezing time (s), and grooming time (s), while the amount of nestlet shredding by each rat in 30 min was calculated using the nestlets' initial weight before the test. Three independent experiments were conducted.

Depression assessment by sucrose preference test
The rats were given free access to a 1% sucrose solution from one of two water bottles in the rat cage for 48 h, after which they were not allowed to drink any liquid for 14 hours.[7] After that, the rats were confined and given an hour's worth of water and a 1% sucrose solution. Both bottles were randomly positioned on the right or left side to reduce spatial distortion. The bottles were weighed after 1 hour to determine the percentage preference for sucrose using the formular:

$$\%\ \text{sucrose preference} = \frac{\text{sucrose solution intake}}{\text{total water intake}} \times 100$$

Collection of rat brain tissue specimens
An anesthetic induction chamber and isoflurane euthanasia were used on rats on day 22 of the experiment. An expert technician dissected the rat brains in formalin after decapitation to check for antioxidant activity. The brains were then placed on dry ice in -80°C freezer and kept there until required for histological analysis.

Histological analysis of rat brain tissue specimens
Using a cryostat, the brain samples were divided into sections with a 5 µm thickness. Selective fragments were dried in an automated tissue processor for 24 hours before paraffin-embedded brain sections were cut through with a microtome. The paraffin slices were floated in a warm water chamber at 31 °C for 1 min.[8] The tissues of the dentate gyrus were stained with hematoxylin and eosin before being examined under a microscope.

RT-PCR for assessing gene expression
Trizol reagent (Invitrogen, USA) was used to isolate total RNA from the rat prefrontal cortex and hippocampus. Total RNAs were used to synthesize cDNAs using an OneScript® Plus cDNA Synthesis Kit (Thermo Scientific, USA). Then, cDNA products were employed in a PCR process as templates. The reference gene *β-actin* as well as *IL-1, IL-6, Bcl-2, Bcl-xl, Bax,* and *TNF-α,* are the six gene involved in brain inflammation.[9] The six genes involved in brain neuroplasticity, which include *VegfA, Bdnf, Igf-1, Trek2, TrkB,* and *Creb1*, and the six genes involved in HPA axis regulation, including *Crh-bp, Crh-r1, Crh-r2, Mr, Gr,* and *11-hsd1* were all examined with *Gapd* as a reference gene.[10] The PCR master mix containing 100 mM Tris-HCl (pH 9.1), 0.1% Triton TMX-100, 200 mM dNTP, 1.5 mM $MgCl_2$, 0.005 U Taq DNA polymerase, and 0.2 M forward and reverse primers was used in the reaction, which had a total reaction volume of 20 µL (adjusted with nuclease-free water). The PCR thermocycler (Thermo Scientific Hybaid, Px2) was programmed for 10 min denaturation at 94°C for one cycle, followed by 40 cycles of denaturation for 30 s at 94°C, an annealing step of 30 s, an extension step of 1 min at 72°C, and a final extension step of 1 cycle for 7 min at 72°C. The PCR products were analyzed by gel electrophoresis, and the band densities were estimated using ImageJ software (version 1.46r; U.S. National Institutes of Health).

Statistical analysis
One-way analysis of variance (ANOVA) was carried out using GraphPad software, and the analytical outcomes were expressed using average and standard deviation (SD). A Tukey's multiple comparison test was used to examine the variations between the groups. The significant differences were evaluated at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

Results and Discussion

Probiotic consortia reduced compulsiveness and anxiety
This study employed the nestlet shredding test to measure rat anxiety, compulsive behavior, and repetitive activity. This test is simple to implement and provides accurate and spontaneous results of rat actions.[6] Nestlet shredding testing complements marble burying and can be used to evaluate a wide range of medicines for repetitive rat behavior.[11] Stress and anxiety-related stimuli affect grooming behaviors.[12] Fright in a new location is demonstrated by the time spent grooming and freezing.[13] In rats, self-grooming can be caused by motor neuron and neural circuit dysfunction. Stressed rats spend more time grooming themselves. However, rat anxiety should not be inferred from self-grooming alone.[14]
The ANT group shredded more nestlet (44%) than the PRO group (37%), as shown in Figure 1A, while a similar result was obtained in the CON group compared to the ANT group. Both the ANT and CON groups were more obsessive and nervous in the absence of probiotics. Similarly, the times spent digging (170 sec) and grooming (48 sec) were the longest in the ANT group as an indicator of the most anxious state (Figures 1B and D), whereas the time spent freezing was insignificantly different among the three groups (Figure 1C). Probiotic exposure in the PRO group significantly reduced digging time, indicating that probiotics may help reduce impulsiveness in rats. Furthermore, probiotics also appeared to be capable of reducing obsessive and anxious behavior. The CON group showed a certain degree of nestlet shredding with displacement (Figure 2B) from their original locations (Figure 2A). In contrast to the ANT group, which displayed the most nestlet shredding and detachment of the two-layered nestlets, the PRO group showed less nestlet shredding and less detachment of the two-layered nestlet (Figures 2C and D).
Topographical changes in the bedding surface were also evaluated. Before testing, the CON and PRO groups showed that bedding was not interrupted (Figures 2B and 2C) with the level of the bed surface, but the ANT group showed significantly undulating surfaces (Fig. 2D). Rats under stress that were examined with the nestlet shredding test had their obsessive behavior improved by *Lactobacillus reuteri*, while it was found that *Lactobacillus* administration prevented the pathway that initiates IDO1 via the generation of reactive oxygen types (i.e. H_2O_2), thereby directly regulating kynurenine metabolism.[15] *Lactobacillus reuteri* was able to increase the expression of GABA receptors in the central nervous system, which had a beneficial effect on dejection and anxiety-like behavior. The vagus nerve transports peripheral impulses and regulates inflammatory and stress responses.[16]
In this study, it was hypothesized that the six probiotics would influence rat anxiety and compulsivity via the vagus nerve. The vagus nerve transports peripheral signals and modulates inflammatory and stress responses, and *L. reuteri* was able to enhance GABA receptor expression in the central nervous system, which had a positive impact on despair and anxiety-like behavior.[16]

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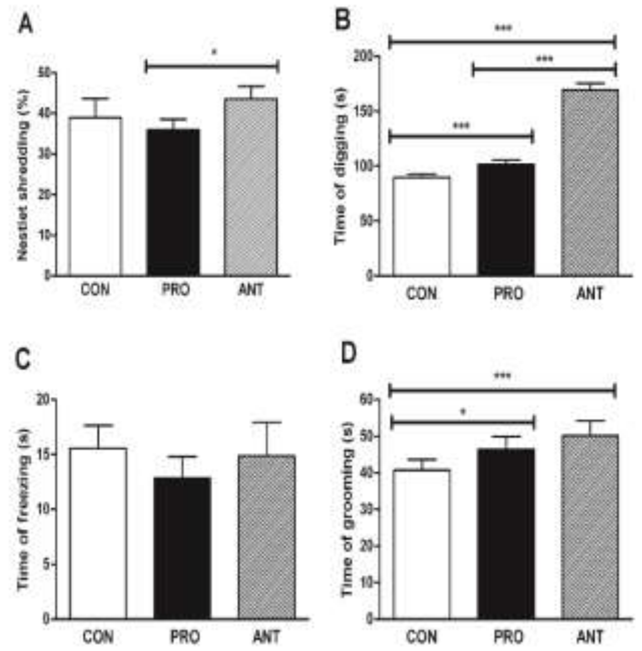

Figure 1: Anxiety assessment using the nestlet shredding test.
A: Nestlet shredding (%); B: Digging time; C: Freezing time; D: Grooming time; CON: Control group; PRO: Probiotic group; ANT: Antibiotics group. The mean ± standard deviation (n = 7) is shown in the results. *** p < 0.001; ** p < 0.01; and * p < 0.05 significance was determined by Tukey's multiple comparison test.

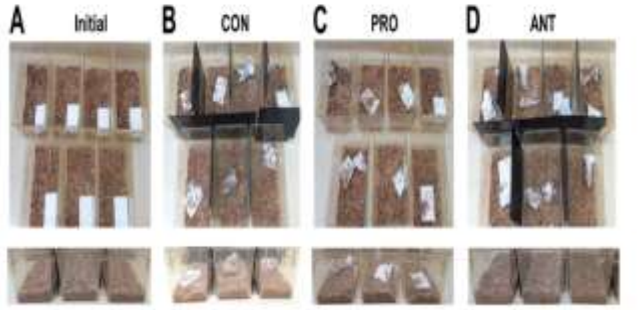

Figure: 2: Overview of the nestlet shredding test.
A: The first time nestlets were placed in the cages without being disturbed. After 30 min, pictures were taken; B (CON): Control group; C (PRO): Probiotic group; D (ANT): Antibiotics group.

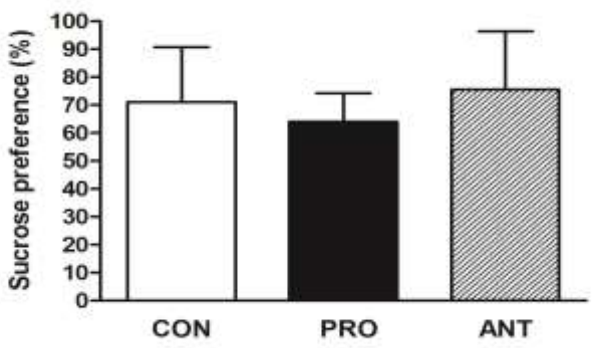

Figure 3: Depression evaluation via the sucrose preference test. Results are presented as mean ± standard deviation (n = 7).

In this study, it was hypothesized that the six probiotics play a role in modulating rat compulsiveness and anxiety via the vagus nerve.

Effects of the probiotic mixtures on depression
Sucrose preference (%) was similar among the three groups (Figure 3), ranging from 65% (PRO) to 75% (ANT), indicating that probiotics did not alleviate depression while reducing anxiety in the nestlet shredding test. According to emerging research, the gut microbiota has been related to stress in recent years. The probiotics were then medically used for the treatment of depression diseases. *Bifidobacterium longum* R0175, *Lactobacillus plantarum* R1012, and *Lactobacillus helveticus* R0052 were found to have antidepressant effects in mice with chronic moderate stress (CMS), as reported by a previous study.[17] CMS-induced anxiety and depressive-like behaviors were reduced by probiotics while *Lactobacillus* abundance was increased, leading to immunological changes in the hippocampus. As a result, *Lactobacillus* species can be linked to the gut microbiota-inflammation-brain axis as the likely mechanism behind probiotics' antidepressant-like activity.

Probiotic mixtures enhanced neuroplasticity
As shown by the RT-PCR results of gene expressions in neuroplasticity (Figure 4A), probiotics appear to reduce brain inflammation and apoptosis. PRO had lower *Bax* expressions than ANT. The results indicated that probiotics reduced the expression of the pro-apoptotic gene *Bax*, which in turn decreased the levels of the protein *Bax* and the release of cytochrome c, slowing the rate of apoptotic cell death.[18] The *Bcl-2*/*Bax* ratio is a key regulator of apoptosis, so a change in any of these proteins could result in cell survival or death. Compared to PRO, the ANT group showed an increase in *Igf-1* mRNA expression (Figure 4B), corresponding to an insulin-like growth factor involved in brain neuroplasticity. The ANT group also revealed an increase in *Gr* mRNA expression (Figure 4C), corresponding to a glucocorticoid receptor involved in HPA axis regulation. GR signaling dysfunction has been linked to the development of psychological diseases such as mood disorders, schizophrenia, and bipolar disorder.[19] However, the expression of *11-hsd1* mRNA, which represents the 11-hydroxysteroid dehydrogenase type 1 enzyme that converts cortisol into cortisol and regulates glucocorticoids in brain regions,[20] was higher in ANT than PRO as presented in Figure 4C. This occurred because antibiotics can cause *11-hsd1* gene expression. The 11-hsd1 enzyme is a target of antidepressant and anti-anxiety medication.[21]

Probiotic mixtures enhanced neuroprotectivity
The hippocampal formation includes the dentate gyrus, an essential part of the brain's cortical area.[22] The dentate gyrus is also linked to anxiety disorders' pathophysiology, in addition to its role in memory and learning.[23] When compared to the ANT group, the results of this study showed that PRP had a neuroprotective impact on the dentate gyrus (Figure 5). The dentate gyrus histology of the CON group was largely preserved. The CON group had a thicker granular cell layer, indicating that the layer was still intact. Antibiotics lowered the thickness of the pyramidal cell layer considerably in the ANT and PRO groups. Similar to the earlier findings, researchers used monosodium glutamate, which drastically reduced the number of *Cornu ammonis* (CA1 and CA3) cells as well as the thickness of the pyramidal cell layer in the control group.[24] Cytoplasmic vacuolation, disorganized neuropils, wider gaps between neurons, and dark nuclei were less prevalent in the PRO group than in the ANT group, implying that probiotics may play a role in neuron cell protection after antibiotic exposure.

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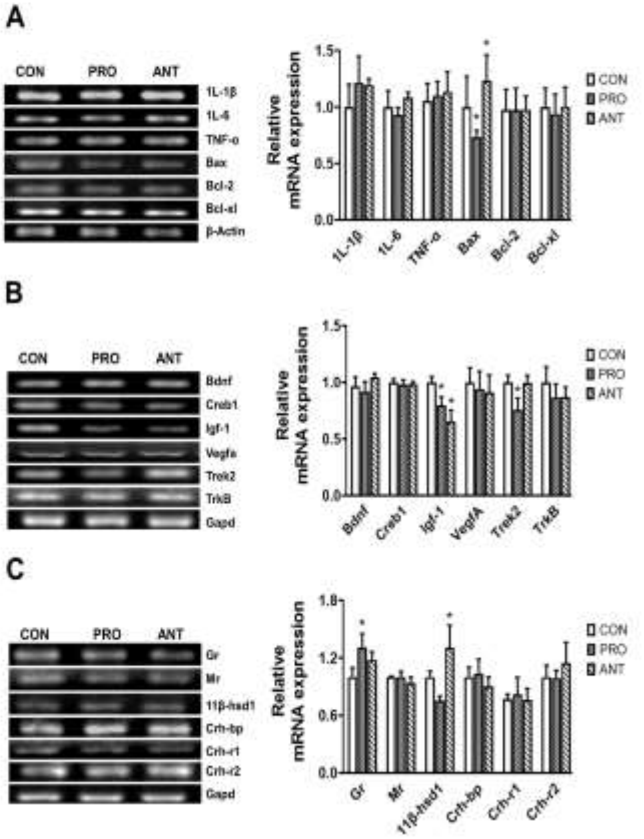

Figure 4: RT-PCR of gene expressions in neuroplasticity. A: Brain inflammation gene; B: Brain neuroplasticity gene; C: HPA axis regulation gene.

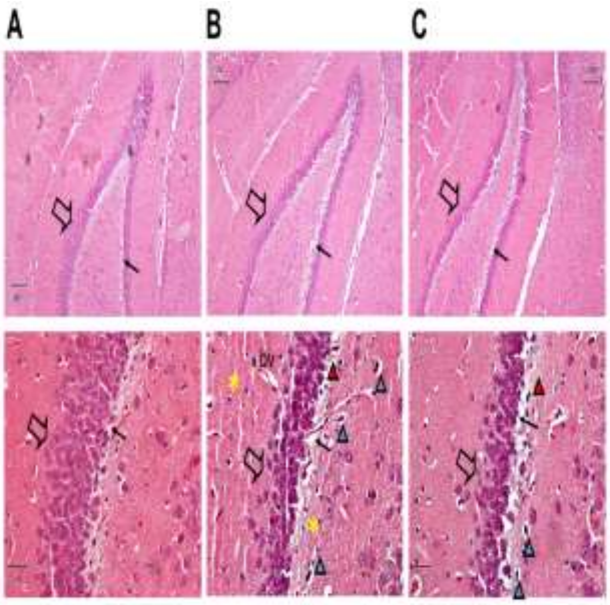

Figure 5: Histological results of dentate gyrus in the rat brain. A (CON): Control group; B (ANT): Antibiotics group; C (PRO): Probiotic group; Micrographs in the upper (hippocampus overview) and lower (enlarged hippocampus) panels at 100x and 400x magnifications, respectively. Thickness of granular cell layer (hollow arrows), vacuolation (arrows), blood vessels (bv), dark nuclei (red triangles), vacuolation (blue triangles), disorganized neuropil (yellow asterisks).

Conclusion

The findings of this study are the first to suggest that a combination of probiotic strains made up of isolated bacteria from fermented Thai foods reduces anxiety, enhances neuroplasticity, and promotes rat neuroprotection. This finding supports the need for further investigation into the use of probiotic mixtures derived from Thai fermented foods for improving and promoting psychological health through the use of these probiotic strains as supplemental foods.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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