Preventive effect of peptides derived from fermented milk on chronic stress-induced brain damage and intestinal dysfunction in mice

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ABSTRACT

This study investigated the preventive effects of peptides derived from milk fermented with the probiotic strain Lactobacillus gasseri 505 (505) against stress-related brain damage and anxiety-like behavior. The peptides MKPWIQPPTKVPYVRYL (Pep14) and VYQHQKAMKPWIQPPTKVPYVRYL (Pep21), which exhibit high antioxidant and anti-inflammatory activities, were administered to stressed mice. The results showed that the stress mechanism in the gut-brain axis was regulated by pretreatment with both peptides, leading to inhibition of neurodevelopment and neuroinflammation through the hypothalamic-pituitary-adrenal (HPA) axis, based on the expression of related mRNA and proteins. The expression of colonic inflammation-related mRNA and proteins was also reduced. Moreover, anxiety-like behavior was significantly reduced in mice treated with Pep14 and Pep21, derived from milk fermented with 505, may prevent stress-induced brain damage and anxiety-like behavior via regulation of the HPA axis.

Key words: chronic stress, Lactobacillus gasseri 505, milk peptide, fermented milk, gut-brain axis

INTRODUCTION

Stressors cause physiological and behavioral responses as the essential stress response to restore homeostasis (de Kloet et al., 2005). Stress could activate several neuropeptide-secreting systems, which leads to widespread release of noradrenaline and adrenaline from synapses and the adrenal medulla, respectively (de Kloet et al., 2005). Neuropeptides modulate the activity of neurotransmitters and together coordinate behavioral and metabolic responses to stress (de Kloet et al., 2005). Moreover, changes in corticosterone and serotonergic systems via the hypothalamic-pituitary-adrenal (HPA) axis play a central role in the stress response of the brain. Accumulating data indicate that chronic stress leads to dysregulation of HPA axis and corticotropin-releasing factor (CRF) signaling, which are associated with impaired the central nervous system functions, including learning and memory, and with anxiety-like behavior (Smith and Vale, 2006; Pentkowski et al., 2009; Pentkowski et al., 2022). Physiological adaptation to and anxiety-like behavioral properties in the stress response, including increased cardiovascular and metabolic processes, and respiratory system, are initiated along with the suppression of general vegetative functions such as digestion, immunity, growth, and reproduction (Smith and Vale, 2006). Stress-induced behavioral and physiological disorders are related to systemic physiology via neuroendocrine, autonomic, immune, and metabolic mediators in the brain. Therefore, the brain is a target for stressful experiences (McEwen, 2017).

Stress is also a crucial risk factor for the gastrointestinal (GI) disorder such as irritable bowel syndrome (IBS), which reflects pathological alteration of gut-brain axis homeostasis. Several experimental studies have shown that IBS is induced by a stressed brain as well as a combination of irritable bowel (Qin et al., 2014). Stress alters the quantity of neurotransmitters including 5-hydroxytryptamin (5-HT) and inflammatory cytokines, which are considered to mediate gut functions, mucosal immune activation, intestinal motility, secretion, and permeability in IBS (Feng et al., 2012). In addition, stress may affect the gut microbiota composition, which helps maintain bidirectional network of signaling pathways between the gut and the brain (Konturek et al., 2011). Under stress conditions, abnormal gut microbiota interacts with the nervous and immune systems, which may cause GI tract disorders (Cryan and Dinan, 2012; Ringel and Maharshak, 2013). Moreover, several studies have been reported a strong correlation between IBS severity and comorbid psychological disorders, particularly anxiety and depression.
(O’Malley et al., 2011; Singh et al., 2012). Thus, the gut microbiota is considered as an important factor for the regulation of the gut-brain axis during the treatment to the stress-related disorders such as IBS. In our previous study, fermented milk (FM) with a specific probiotic strain, *Lactobacillus gasseri* 505 (505), improved antioxidative and anti-inflammatory activities and preventive effects on stress-induced damage such as testicular dysfunction with decreased corticosterone levels in mice (Joung et al., 2022). Therefore, we investigated the effects of the peptides derived from the FM as well as 505 and FM on stress-induced brain damage and intestinal inflammation.

**MATERIALS AND METHODS**

**Identification and Preparation of Peptides Produced from Fermentation of Milk**

Milk fermented with the probiotic strain *Lactobacillus gasseri* 505 (505) was manufactured as described in our previous study (Oh et al., 2016a). Briefly, milk was pasteurized at 85°C for 15 min and cooled down to 41°C. Then, the pasteurized milk was inoculated with 505 to approximately 10⁷ cfu/mL and incubated at 41°C for 48 h. Peptides produced from milk fermentation were identified using MALDI-TOF/MS/MS, and the AA sequence of the total of 21 peptides was presented in Supplemental Table S1 (https://doi.org/10.6084/m9.figshare.24320818.v1, Joung et al., 2023; Oh et al., 2016b). The peptides used in this study were chemically synthesized according to the AA sequence (Peptron Inc., Daejeon, South Korea).

**Functional Properties of Peptides**

**Determination of Antioxidative Activity.** The antioxidative activity of peptides generated during milk fermentation was determined by estimating the reducing power and the radical scavenging activity using the ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC), 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid: ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays as described by Oh et al. (2018). In brief, for FRAP assay, FRAP reagent, the mixture of sodium acetate buffer, tripyridil-s-triazin, and FeCl₃·6H₂O, was mixed with sample and incubated at 37°C for 30 min. Then, the absorbance was read at 562 nm to measure the ferric reducing power of sample. Oxygen radical absorbance capacity assay estimated the antioxidant capacity of a sample by quantifying its ability to scavenge free radicals. The ABTS solution (a mixture of ABTS and potassium persulfate) or DPPH solution in ethanol were mixed with sample and the radical scavenging activities were measured, as the absorbance of mixture was read at 734 nm for ABTS assay and 517 nm for DPPH assay. Each assay was performed in triplicates.

**Determination of Cytotoxicity and Anti-inflammatory Activity in HT-29 Cells.** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay in colon cancer (HT-29) cells was used for determination of cytotoxicity of the peptides generated during FM production (Rubinstein et al., 1990). To determine the anti-inflammatory activity of the peptides, HT-29 cells were treated with 50 μ or 100 μg/mL of peptides and 100 ng/mL of LPS. All assays were performed in 6 replicates. Nitric oxide (NO) production was measured using the Griess reaction as described by Oh et al. (2018).

**Animal Experiments**

Male C57BL/6J mice at 5 wk of age were purchased from DBL (Seoul, Korea). After an acclimation period of 1 wk, mice were administered a normal diet, 505, FM, and selected peptides: MKPWIQPDKTVIPYVRYL (Pep14) and VYQHQKAKPKPIQKTVIPYVRYL (Pep21) (Figure 1A). All groups, excluding the control group (CON), were exposed to unpredictable chronic mild stress (UCMS) after sample treatment. The mice were randomly housed 6 per cage and allocated to the following groups (n = 12): nonstressed and normal diet (CON), UCMS and normal diet (UCMS), UCMS and 505-treated group (10⁷ cfu/d; PRO), UCMS and FM-treated group (1,500 mg/kg per d; FM), UCMS and Pep14-treated group (20 mg/kg per d; Pep14), and UCMS and Pep21-treated group (20 mg/kg per d; Pep21). The cages are 22 cm wide × 28 cm long × 13 cm tall polycarbonate covered with a filter top and stainless steel lid. All diet and water were provided to the mice with ad libitum access. Behavioral properties such as UCMS-induced anxiety and depressive-like behaviors were determined according to those in previous studies (Mineur et al., 2006; Jung et al., 2014). The stressors, which are cold water (4°C, 15 min), empty cage (overnight), food deprivation (8 h or overnight), foreign cage (overnight), illumination (3,000 lx), restraint (within a box, 13 cm × 10 cm × 10 cm, 4 h), sleep cycle change (reverse regular 12-h light-dark cycle), tilted cage (45°, 8 h or overnight), water deprivation (8 h), wet cage (overnight) were randomly exposed to the mice each day for 6 wk (Supplemental Table S2, https://doi.org/10.6084/m9.figshare.24320818.v1, Joung et al., 2023). The mice were maintained under standard conditions; temperature 23°C ± 2°C, humidity 50% ± 10%, 12-h light and dark cycle. Experiments were performed according to the guidelines of the Institutional Animal
Physiological Tests

**Serum Analysis.** The concentration of corticosterone in serum was measured as previously described (Oh et al., 2020). Briefly, serum samples were collected immediately after the mice were euthanized by cardiac puncture. The serum corticosterone was estimated using the corticosterone ELISA kit (Abnova, Taipei, Taiwan) in triplicates.

**Quantitative Real-Time PCR.** Total RNA was extracted from the brain and the colon tissues and mRNA expression was evaluated using quantitative real-time PCR (qRT-PCR) as previously described (Oh et al., 2020). Relative target gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences of target genes used in this study are presented in Supplemental Table S3 (https://doi.org/10.6084/m9.figshare.24320818.v1, Joung et al., 2023). All experiments were performed in triplicates.

**Western Blot Analysis.** Protein expression related to brain function and gut health was analyzed by western blotting using the method described in Oh et al. (2020). In brief, protein extracted from dissected brain and colon tissues was loaded to sodium dodecyl sulfate-PAGE. The gel was transferred to a polyvinylidene difluoride membrane, and incubated with the primary and secondary antibodies. The antibodies used in this study are listed in Supplemental Table S4 (https://doi.org/10.6084/m9.figshare.24320818.v1, Joung et al., 2023). The protein expression was normalized to those of β-actin.

**Histopathological Analysis**

For histological analysis, the colon tissues were sectioned and stained with hematoxylin (Sigma-Aldrich)
and eosin (Thermo Fisher), then images were captured using a Leica DM3000 microscope (Leica Microsystems Corp., Wetzlar, Germany) using method of Oh et al. (2020).

### Behavioral Tests

**Elevated Plus Maze Test.** The elevated plus maze test (EPM) was performed to measure anxiety-like behavior and was slightly modified from a previous report (Komada et al., 2008). The apparatus (opaque white acrylic, w × d × h, 65 cm × 65 cm × 40 cm, elevated from the floor) had a center platform (5 cm × 5 cm), 2 open arms, and 2 closed arms with walls (height 15 cm). The mice were placed on the central platform and allowed to freely explore the maze for 5 min. The test was recorded and analyzed using Any-maze 6.0 software (Stoelting, Wood Dale, IL).

**Light and Dark Test.** The light and dark box test (LDB) was performed to measure anxiety, as described previously, with slight modifications (Schramm et al., 2001). The apparatus (white and black acrylic, w × d × h, 45 × 26 × 26 cm) consisted of 2 compartments with one-third dark and two-thirds light zones (500×). The mice were placed in the light zone and allowed to explore freely for 5 min. The test was recorded and analyzed using Any-maze software.

**Forced Swimming Test.** The forced swimming test (FST) was performed to evaluate despair-like behaviors as described in a previous study (Porsolt et al., 1977). A clear glass beaker (Ø × h, 19 cm × 28 cm) filled with water (16 cm, 24°C ± 1°C) was used for this test. Mice were placed in a beaker filled with water for 6 min. The immobility time was measured over the last 4 min. After the test, the mice were placed in a drying cage with a heat lamp until their coats dried. The test was recorded and measured using Any-maze software.

### Statistical Analysis

All data are presented as mean ± standard error of the mean. The statistical significance of between-group differences was assessed using an independent sample t-test. SPSS (version 22.0; IBM, Chicago, IL) was used for the statistical analyses.

### RESULTS

**Antioxidant and Anti-inflammatory Activities of Peptides Derived from Fermentation of Milk with 505**

A total of 21 peptides produced through the fermentation of milk with 505 were evaluated for their antioxidant and anti-inflammatory activities (Tables 1 and 2). All the antioxidant activity tests, such as FRAP, ORAC, ABTS, and DPPH assays, revealed that peptide13 (Pep13), Pep14, and Pep21 exhibited high antioxidant power. Pep13, Pep14 and Pep21 exhibited FRAP values of 256.0, 180.3, and 193.8 μM Fe$_{2}$SO$_{4}$.
respectively; ORAC values of 81.6, 86.9, and 85.3 μM TE respectively; 33.0%, 34.1%, and 35.8% for ABTS scavenging activity respectively; and 26.7%, 29.9%, and 59.9% for DPPH scavenging activity respectively. However, Pep13 exhibited toxicity, with less than 5% cell viability in HT-29 cells at concentrations of 0.5 and 1 mg/mL. Nitric oxide production in LPS-treated HT-29 cells was evaluated to determine the anti-inflammatory activities of the peptides. The selected peptides, Pep14 and Pep21, inhibited LPS-induced NO production at concentration of 0.1 mg/mL. Therefore, Pep14 and Pep21 were used in further animal studies.

**Behavioral Properties**

Elevated plus maze test and light and dark test were conducted to investigate the effects of FM on stress-induced anxiety-like behavior. The UCMS group exhibited decreased total distance ($P < 0.05$) and distance in the open arms ($P < 0.001$) in the EPM test compared with the CON group (Figure 2A). However, 505- and FM-treated mice exhibited increased total distance ($P < 0.05$) and open distance ($P < 0.05$) compared with the UCMS group. Pep21 increased total distance and distance in the open arm. Unpredictable chronic mild stress-treated mice spent less time in the center zone ($P < 0.05$) and open arms ($P < 0.001$) than CON mice. In contrast, FM-treated mice spent more time in the center zone ($P < 0.05$) and open arms ($P < 0.05$). The other groups did not exhibit any significant differences compared with the UCMS group. In light and dark test, the UCMS group exhibited a diminished distance in the light zone ($P < 0.001$) and time in the light zone ($P < 0.001$) compared with the CON group. In contrast, the PRO-, FM-, Pep14, and Pep21 groups exhibited increased distance ($P < 0.001$, $P < 0.05$, $P < 0.005$, respectively) and time in the light zone ($P < 0.001$, $P < 0.005$, $P < 0.001$, respectively) compared with the UCMS group. The FST was performed to investigate the effects of FM on UCMS-induced despair-like behavior. Immobility time in the UCMS group was significantly higher than that in the CON group (Figure 2C). Pep14 treatment induced immobility similar to that observed in the UCMS group. In contrast, the immobility times of 505-, FM, and Pep21 treated mice were significantly lower than those of the UCMS mice.

**Neuroprotective Effects of 505, FM, and Peptides Produced in FM**

To investigate the preventive effects of probiotic strains 505, FM, and peptides against UCMS-induced

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Table 2. Cytotoxicity and inhibitory effects of peptides produced from fermentation of milk on nitric oxide production in RAW264.7 cells induced with LPS

<table>
<thead>
<tr>
<th>Item</th>
<th>50 μg/mL</th>
<th>100 μg/mL</th>
<th>50 μg/mL</th>
<th>100 μg/mL</th>
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<tr>
<td>LPS</td>
<td>83.61 ± 6.84</td>
<td></td>
<td>74.78 ± 2.32</td>
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</tr>
<tr>
<td>Peptide 1</td>
<td>71.42 ± 3.80*</td>
<td>74.38 ± 5.98*</td>
<td>70.64 ± 0.00*</td>
<td>62.83 ± 2.65</td>
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<tr>
<td>Peptide 2</td>
<td>81.73 ± 2.22</td>
<td>84.02 ± 1.40</td>
<td>65.48 ± 1.55*</td>
<td>57.59 ± 1.44</td>
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<tr>
<td>Peptide 3</td>
<td>66.66 ± 1.26*</td>
<td>75.51 ± 3.21*</td>
<td>64.08 ± 0.22*</td>
<td>55.95 ± 0.66</td>
</tr>
<tr>
<td>Peptide 4</td>
<td>65.49 ± 1.62*</td>
<td>80.62 ± 7.71</td>
<td>65.41 ± 0.77*</td>
<td>57.13 ± 0.11</td>
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<tr>
<td>Peptide 5</td>
<td>71.46 ± 0.45*</td>
<td>72.11 ± 0.31*</td>
<td>71.97 ± 0.11</td>
<td>61.42 ± 1.77</td>
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<tr>
<td>Peptide 6</td>
<td>71.74 ± 4.69*</td>
<td>83.03 ± 0.45</td>
<td>67.98 ± 1.33*</td>
<td>59.39 ± 0.88</td>
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<tr>
<td>Peptide 7</td>
<td>78.29 ± 3.13</td>
<td>82.11 ± 4.55</td>
<td>69.39 ± 1.33</td>
<td>57.13 ± 0.11</td>
</tr>
<tr>
<td>Peptide 8</td>
<td>78.79 ± 1.38*</td>
<td>79.96 ± 0.06*</td>
<td>68.45 ± 1.33*</td>
<td>61.73 ± 0.22</td>
</tr>
<tr>
<td>Peptide 9</td>
<td>77.77 ± 1.82*</td>
<td>80.12 ± 0.44*</td>
<td>66.66 ± 0.11*</td>
<td>58.22 ± 1.22</td>
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<tr>
<td>Peptide 10</td>
<td>62.15 ± 1.02*</td>
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<td>59.16 ± 1.88*</td>
<td>35.88 ± 0.55</td>
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<tr>
<td>Peptide 11</td>
<td>5.00 ± 0.30*</td>
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<td>69.63 ± 4.53</td>
<td>61.97 ± 2.54</td>
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<td>Peptide 12</td>
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<td>66.27 ± 5.08*</td>
<td>44.55 ± 0.44</td>
</tr>
<tr>
<td>Peptide 13</td>
<td>5.00 ± 0.50*</td>
<td>60.80 ± 0.39*</td>
<td>67.95 ± 1.10*</td>
<td>53.26 ± 2.54</td>
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<tr>
<td>Peptide 14</td>
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<td>58.84 ± 1.22</td>
</tr>
<tr>
<td>Peptide 15</td>
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<td>66.81 ± 1.44*</td>
<td>57.20 ± 4.86</td>
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<tr>
<td>Peptide 16</td>
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<td>90.08 ± 1.52</td>
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<td>57.20 ± 1.99</td>
</tr>
<tr>
<td>Peptide 17</td>
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<td>71.50 ± 2.02*</td>
<td>64.94 ± 0.33*</td>
<td>57.59 ± 1.22</td>
</tr>
<tr>
<td>Peptide 18</td>
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<td>87.92 ± 4.01</td>
<td>64.47 ± 5.45*</td>
<td>57.05 ± 3.09</td>
</tr>
<tr>
<td>Peptide 19</td>
<td>76.35 ± 4.99</td>
<td>75.77 ± 9.91</td>
<td>69.55 ± 3.09</td>
<td>61.27 ± 1.77</td>
</tr>
<tr>
<td>Peptide 20</td>
<td>65.72 ± 6.15*</td>
<td>79.86 ± 0.14*</td>
<td>69.31 ± 1.44</td>
<td>60.25 ± 2.32</td>
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<tr>
<td>Peptide 21</td>
<td>71.92 ± 1.88*</td>
<td>82.34 ± 3.48</td>
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</tbody>
</table>

1Data are expressed as the mean ± SEM (n = 6).
2RAW 264.7 cells were treated with peptide for 24 h before exposure to LPS (100 ng/mL) for 18 h.
*P < 0.05, in comparison to LPS (independent sample t-test).
brain damage and anxiety-like behaviors, 505, FM, Pep14, and Pep21 were administered to UCMS-exposed mice. Stress and sample treatment did not significantly affect the body weight of mice during the experimental period (Figure 1B). However, UCMS exposure significantly increased serum corticosterone levels ($P < 0.05$), but the UCMS-induced increase in serum corticosterone level was significantly inhibited by PRO ($P < 0.001$), FM ($P < 0.005$), and Pep21 ($P < 0.005$) treatment (Figure 3A). Moreover, treatment with 505, FM, or both peptides significantly suppressed the chronic stress-induced overexpression of corticotropin-releasing hormone (CRH; $P < 0.05$, $P < 0.001$, $P < 0.005$, and $P < 0.05$, respectively; Figure 3B). The expressions of CRHr1 and CRHr2 were significantly altered, and the expression of NMDA2R was slightly increased after UCMS exposure ($P = 0.09$). The expression levels of CRHr1, CRHr2, and NMDA2R in the sample-treated groups remained unchanged despite stress treatment. The neurodegenerative marker brain-derived neurotrophic factor (BDNF), the ratio of Bax to Bcl-2, and caspase-3 were significantly affected by UCMS exposure (Figure 3B). In particular, the levels of the neuroprotective molecule BDNF were higher in the PRO and Pep21 groups than in the nonstressed group despite exposure to stress. In addition, treatment with FM and both peptides suppressed the UCMS-induced changes in bcl-2, Bax, and caspase-3 expression to a greater degree. Furthermore, the effects of the samples on UCMS-induced inflammation and barrier dysfunction in the brain revealed significant suppression of inflammatory markers, such as NF-κB, iNOS, and COX2, but no statistical changes in the expression of barrier-related proteins (Figure 4). Significant increases in the expression of iNOS ($P < 0.005$) and COX2 ($P < 0.001$) induced by UCMS were drastically downregulated in FM-treated mice ($P < 0.05$ and $P < 0.001$, respectively). Administration of 505, Pep14, and Pep21 significantly inhibited UCMS-induced increase in COX2 expression.

505, FM, and Peptides Alleviated Intestinal Inflammation in UCMS-Induced Mice

The effect of the samples on intestinal health, colon length, and expression of inflammation- and barrier function-related proteins in colon tissue were evaluated, and histopathological analysis of the colon was performed. Exposure to UCMS significantly reduced colon length ($P < 0.001$). However, all samples significantly suppressed the UCMS-induced decrease in colon length ($P < 0.001$; Figure 5A). Histopathological analysis revealed damage to epithelial cells after stress treatment; however, pretreatment with samples prevented UCMS-induced damage to the colon epithelium (Figure 5B). In addition, the increase in NF-κB, iNOS,
and COX2 expression due to stress was attenuated in all the sample-treated groups (Figure 5C). TPH-1, an isoenzyme of tryptophan hydroxylase, was significantly downregulated by stress exposure \((P < 0.005)\); however, treatment with FM, Pep14, and Pep21 significantly attenuated UCMS-induced changes in THP-1 levels \((P < 0.05, P < 0.05, P < 0.005, \text{respectively})\). Furthermore, the levels of the intestinal barrier-related proteins Zo-1, Occludin, and Claudin-5, were observed to have a tendency to decrease in the UCMS group.

Figure 3. (A) Neuroendocrine-related protein expression and serum corticosterone level and (B) neurodevelopment-related protein expression in the brain of mice treated with 505, FM, and milk peptides under UCMS. CON = nonstressed and normal diet; UCMS = unpredictable chronic mild stress and normal diet; PRO = UCMS and 505-treatment; FM = UCMS and fermented milk treatment; Pep14 = UCMS and Pep14 treatment; Pep21 = UCMS and Pep21 treatment. Data are expressed as the mean ± SEM \((n = 12)\). # symbolizes significant difference between CON and UCMS (#\(P < 0.05\), ##\(P < 0.005\), independent sample t-test). * symbolizes significant difference with UCMS (*\(P < 0.05\), **\(P < 0.005\), ***\(P < 0.001\), independent sample t-test).
However, these proteins were overexpressed in the sample-treated groups (Figure 5C). Especially, Pep21 induced the highest expression of all intestinal barrier-related proteins.

**DISCUSSION**

Stress is a normal response to everyday pressures or demands, and highly associated with many neuro-psychological disorders such as depression and bipolar disorder (Lee et al., 2020). Chronic stress induces structural remodeling and functional loss in the brain, including cognitive impairment, depression, and anxiety (Lupien et al., 2009; McEwen et al., 2016). In this study, chronic stress caused brain dysfunction, including HPA axis activation, neuronal inflammation, and anxiety-like behaviors. However, fermented milk-derived peptides, Pep14 and Pep21, as well as 505 and FM, ameliorated the stress-induced brain damage and anxiety-like behaviors. Several studies have determined the beneficial effects of milk-derived bioactive peptides, such as antioxidative, antihypertensive, lipid-lowering, and immunomodulatory effects (Meisel, 1997; Dziuba et al., 1999; Marcone et al., 2017). Peptides are gener-
ated by enzymatic hydrolysis, microbial fermentation, and by in vivo digestive enzymes including trypsin and enzymes produced by gut microbes (Marcone et al., 2017). In our previous study, the most peptides derived from fermentation of milk with 505 were originated from β-casein, followed by αS1- and αS2-casein, and had been previously determined their antimicrobial, anti-hypertensive, antioxidant, and angiotensin-converting enzyme inhibitory activities (Oh et al., 2016b). However, Pep14 and Pep21 were originated from αS2-casein, especially, Pep14 is a novel peptide, which had not been reported. Both peptides, which exhibited antioxidant
and anti-inflammatory activities, as well as their intact form of FM and the fermenting strain of 505 were investigated the preventive effects on stress-related disorders. Corticotropin-releasing factor, including CRH, is known to play a central role in stress response which is associated with the regulation of the HPA axis (Smith and Vale, 2006). In particular, CRF triggers a cascading events that culminate in the release of glucocorticoids from the adrenal cortex (Smith and Vale, 2006). Glucocorticoids also play a crucial role in regulating activation of HPA axis; neuronal and endocrine systems, metabolic response, cardiovascular process, immune system, and behavioral process (Charmandari et al., 2005). CRH primarily binds to CRHr1, thus stimulating adenylate cyclase and activating cyclic adenosine monophosphate (cAMP) pathway events that culminate in the release of adrenocorticotropic hormone (ACTH) from pituitary corticotropes (Dautzenberg and Hauger, 2002). In contrast, activation of CRHr2 ameliorates stress responses by facilitating negative feedback of the HPA axis (Bell et al., 2021). Elevated corticosterone due to increased ACTH release activates glucocorticoid receptors and N-methyl-D-aspartate receptor (NMDAR), which results in suppression of neuronal cell proliferation and neurogenesis (Yuste and Katz, 1991). Particularly, overactivation of NMDAR causes an excessive influx of Ca2+ leading to excitotoxicity, a process induced by excessive activation of glutamate receptors and consequent neuronal dysfunction (Yuste and Katz, 1991). In this study, the administration of samples (i.e., probiotic strain 505, milk fermented with 505, and peptides derived from FM) significantly prevented stress-induced increases in CRH and serum corticosterone levels. Similar to this study, several studies have demonstrated that the administration of probiotics decreased CRF signaling by modulating the barrier function (Murakami et al., 2017; Lach et al., 2018). Moreover, the expression of BDNF, Bax, Bel-2, and caspase-3, which are involved in neurogenesis, was significantly regulated by sample administration. Brain-derived neurotrophic factor is one of the most abundant and studied neurotrophins in the brain, which affects neuronal survival and differentiation and is associated with synapse formation and the regulation of synaptic plasticity (Morse et al., 1993; Martinowich and Lu, 2008). Pretreatment of the samples may directly or indirectly influence neuroprotection against stress through regulation of HPA axis. Additionally, UCMS caused neuroinflammation, as reflected by the significant increase in inflammatory mediators including NF-κB, iNOS, and COX2 in the brain, whereas sample treatment suppressed the UCMS-induced increases in pro-inflammatory factors. CRH release in response to stress regulates neuronal and endocrine systems, which are highly associated with immune processes. Accumulating data indicate that elevated stress hormones initiate an inflammatory response involving the release of inflammatory markers such as pro-inflammatory cytokines. The activation of HPA axis by UCMS in this study, could contribute to the increase of inflammatory markers and further neurodegeneration. Interestingly, CRH elicits peripheral organ effects through autocrine, paracrine, and endocrine mechanisms (Bell et al., 2021). We also found that exposure to chronic stress decreased colon length and damaged colon epithelium. Moreover, inflammatory markers such as NF-κB, iNOS, and COX2 were significantly increased in the colon and the brain of UCMS-treated mice. However, treatment with peptide-containing samples significantly reduced UCMS-induced changes. Intestinal barrier dysfunction, such as increased intestinal permeability, may occur due to different types of stress, which is correlated with the release and transfer of acetylcholine, glucocorticoids, and CRH driven by the activation of the parasympathetic nervous system (Lambert, 2009). Intestinal barrier dysfunction affects inflammatory reactions and immune activation by regulating neuroendocrine–immune pathways, leading to abnormal GI function (Akiho et al., 2010; Schmulson and Chey, 2012). We also found that the administration of samples prevented the UCMS-induced decrease in the expression of intestinal barrier function-related genes such as ZO-1, occludin, and claudin-5. According to these results, treatment with Pep21, as well as 505 and FM, reduced the stress-induced deterioration of intestinal health, such as inflammation and barrier dysfunction. This treatment could potentially lead to the recovery of neuronal dysfunction by regulating gut-brain interactions. In addition, it could be possible that the treatment of samples including 505, FM, and milk-derived peptides improved the intestinal microbiota, which in turn led to gut health, and influenced brain health. Furthermore, according to previous studies of the gut-brain-microbiome axis, exposure to stress and specific diet such as probiotics can alter the intestinal microbiota composition, resulting in changes in behavioral properties (Clarke et al., 2013). Decreased activity in the light zone of the LDB test and the open arms of the EPM test reflects anxiety-like behavior. Unsurprisingly, the UCMS group exhibited anxiety-like behaviors in the LDB and EPM tests. PRO-, FM-, Pep14, and Pep21 ameliorated anxiety-like behavior compared with the UCMS group. All pretreatments, except for Pep14, ameliorated despair-like behaviors in the forced swim test. Further studies using a metagenomic approach are necessary to better understand the
mechanisms underlying intestinal microbiota-induced stress modulation.

In this study, we determined that UCMS negatively affects brain and intestinal functions and induces anxiety- and depressive-like behaviors. However, treatment with PRO, FM, Pep14, and Pep21 prevented stress-induced dysfunction of the brain and intestine, as well as anxiety and depressive behavior. These findings suggest that peptides, the end products of milk fermentation, PRO, and FM, exhibited health-promoting effects against chronic stress. Therefore, these samples, including milk-derived peptides, can be used as high-value food ingredients and therapeutic alternatives for treating stress-related damage.

ACKNOWLEDGMENTS

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry (Jeollanam-do, South Korea) through the National Research Foundation of Korea funded by the Ministry of Education (NRF368 2022M3A915018286). The supporting information is as follows: the AA sequences of 21 peptides (Supplemental Table S1), unpredictable chronic mild stress procedure for mice (Supplemental Table S2), and the primers and antibodies used in this study (Supplemental Tables S3 and S4); UCMS- and sample-induced changes in the expression of brain function-related mRNA in mice (Supplemental Figure S1, https://doi.org/10.6084/m9.figshare.24320818.v1, Joung et al., 2023). The authors have not stated any conflicts of interest.

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