Depression is a prevalent, stress-related mental disorder that can lead to serious psychiatric diseases with morbidity and high mortality. Although some functional fermented dairy drinks have promising anxiolytic and antidepressant effects, the mechanism is still not clear. To determine the antidepressant-like effect and the potential molecule mechanism of kefir peptides (KP), various behavioral tests, including the elevated plus maze test, open field test, forced swimming test, and tail suspension test, were used. Administration of 150 mg/kg KP in mice reduced the duration of immobility in the forced swimming test and tail suspension test, elevated the time spent in the open arm and center zone in the elevated plus maze test, and tail suspension test, were used. Administration of 150 mg/kg KP in mice reduced the duration of immobility in the forced swimming test and tail suspension test, elevated the time spent in the open arm and center zone in the elevated plus maze test, and increased the total distance traveled, average speed, and time spent in the center zone in the open field test compared with the mock group. These results indicated that KP dramatically ameliorated the depression-like behaviors. Kefir peptides were further isolated and identified using high-performance liquid chromatography and liquid chromatography–tandem mass spectrometry, from which 3 peptides were identified and designated KFP-1, KFP-3, and KFP-5. Among these peptides, administration of KFP-3 (15 AA residues) remarkably decreased immobility time in the forced swimming test and increased mobility time in the tail suspension test. Therefore, KFP-3 may be the major active peptide with antidepressant activity in KP. Overexpression of brain-derived neurotrophic factor, phosphorylated tropomyosin receptor kinase B, and phosphorylated ERK1/2 protein levels could be detected in the hippocampus under KP administration. Therefore, we suggest that KP improves depressive-like behaviors by activating the brain-derived neurotrophic factor–phosphorylated tropomyosin receptor kinase B signaling pathway. Kefir peptides may serve as a new type of antidepressant dairy product and may provide potent antidepressant effects for clinical use.

**Key words:** kefir peptide, depressive-like behavior, brain-derived neurotrophy factor, tropomyosin receptor kinase B, extracellular signal-regulated kinase 1/2

**INTRODUCTION**

Depression, a mental health and behavioral disorder that affects both mental and physical health with high morbidity and mortality, represents a major public health problem that substantially contributes to disease burden globally (Ferrari et al., 2013; Abou-Saleh et al., 2017; Jia et al., 2017). Because of the complexity and heterogeneity of the progression of depression, current clinical therapy shows limited effect on most patients with depression (Murphy and Byrne, 2012). In addition, currently prescribed conventional antidepressant drugs have some adverse effects, such as prolonged onset of therapeutic effectiveness, which may increase risk of suicidal ideation and self-injury in patients with depression (Paschos et al., 2009; Pozzi et al., 2016). Thus, development of a more effective therapeutic approach for depression is urgently needed.

Kefir, a fermented dairy product made from kefir grains, is produced by the symbiotic fermentation of milk (Bourrie et al., 2016). Because of the broad
health benefits of kefir bioactive peptides, such as angiotensin-converting enzyme inhibition activity and antithrombotic, mineral binding, opioid, immunomodulating, antimicrobial, and antioxidative functions (Eisele et al., 2013; Ebner et al., 2015; Chen et al., 2020; Tu et al., 2020), numerous studies focused on the possible pharmacological effects and application of kefir. Kefir has exhibited some potential health benefits in the treatment of obesity, hyperlipidemia, digestive disease, allergies, and asthma; antimicrobial and tumor suppressor activities; an ability to increase the speed of wound healing; and beneficial effects in the prevention of hypertension and ischemic heart disease (Farnworth and Mainville, 2003; Bourrie et al., 2016; Chen et al., 2016).

The hippocampus is a key area of the brain for recording emotional information and converting it into memory. In addition, the hippocampus plays a role in regulating the major mediator of systemic stress responses (i.e., the amygdala and hypothalamic-pituitary-adrenal axis). Previous studies have shown that a decrease in hippocampal cell viability may occur in the progression of depression and that those changes result in hippocampal volumes that are approximately 5% smaller in patients with major depressive disorder than in healthy controls (Videbech and Ravnkilde, 2004). In a postmortem histological study, Stockmeier et al. (2004) demonstrated the occurrence of hippocampal atrophy in patients with depression compared with healthy controls.

Brain-derived neurotrophy factor (BDNF) plays a crucial role in the regulation of neuron survival and differentiation during hippocampal development, which may correlate with the function of depressive regulation. Based on meta-analysis studies, lower BDNF levels in the serum of patients with depression have been confirmed, and antidepressant drug treatment restores serum BDNF to basal levels (Yu and Chen, 2011; Levy et al., 2018). According to preclinical and clinical studies, a critical action of antidepressant drugs is to induce overexpression of BDNF and to release and then activate its receptor, tropomyosin-related kinase B (TrkB), for downstream signaling (Saarelainen et al., 2003; Thompson et al., 2011).

This downstream signaling, including extracellular signal-regulated kinase/cAMP response element binding protein (ERK/CREB), has been widely studied (Lin et al., 2014; Jin et al., 2019). Yi et al. (2014) showed that administration of oleanolic acid improved depressive-like behaviors, mainly through activation of the BDNF/TrkB-ERK/CREB signaling cascade in a rat model (Yi et al., 2014). Some traditional Chinese medicine extracts, such as Chaihu Shugan San and Schisandra chinensis extract, could improve depressive-like mood status through activation of the hippocampal BDNF/TrkB-ERK/CREB signaling pathway in a chronic unpredictable mild-stress mouse model (Yan et al., 2017; Chen et al., 2018).

Whether kefir peptides (KP) exert antidepressant-like actions through regulation of the BDNF/TrkB-ERK/CREB signaling pathway in mice has yet to be elucidated. In this study, various behavioral tests, including the elevated plus maze test (EPM), open field test (OFT), forced swimming test (FST), and tail suspension test (TST), were used to evaluate the antidepressant-like efficacy of KP and its active peptide in a mouse model. Serum serotonin levels and the protein expression levels of BDNF, total and phosphorylated TrkB, and total and phosphorylated ERK 1/2 in the hippocampus were examined to explore the possible mechanism of KP.

MATERIALS AND METHODS

KP Preparation

Kefir was purchased from Phermep Co. (Kefep). Kefir peptides were made as previously described (Chen et al., 2015; Tu et al., 2015; Tung et al., 2018). Briefly, kefir grain was fermented in sterilized milk and filtered through a 3-kDa molecular weight cutoff filter, and then the products were lyophilized and used as KP powder. The composition of peptides in the KP powder was calculated as a triglycine equivalent in grams per 100 g, which was 23.1 g/100 g in the sample.

Isolation and Identification of KP

To separate the KP mixture, the semipreparative HPLC on a pump (Jasco; model PU-980) equipped with a UV detector and a TSK-GEL G2000SWXL column (Sigma-Aldrich: 300 × 7.8-mm i.d., 5-μm particle size) was used as previously described (Tung et al., 2018). The mobile phase consisted of 100 mM KH2PO4, 1 M NaCl, and 1 mM EDTA (pH = 6.5) at a flow rate of 0.5 mL/min, and detection was performed at 215 nm. The molecular weights of the peptides were determined by liquid chromatography–tandem MS, and tandem mass spectra were used to identify which isolated peptides correlated with which milk protein subset in the SwissProt database.

Animals

Male CD-1 mice (aged 5 wk; 25–30 g of BW) were purchased from the National Laboratory Animal Cen-
ter (Taipei, Taiwan), provided a standard laboratory diet (Altromin no. 1320) and distilled water ad libitum, and maintained on a 12-h light–dark cycle at 22 to 24°C and 60 to 70% relative humidity. Five mice were housed in a plastic cage (7.0 × 11.5 × 5.0 cm) with a filter lid for 6 wk to adapt to the environment before initiation of the study. According to the criteria of Rouen depressed mice, CD-1 mice with high immobility score (>115 s) on the TST were selected based on their individual responsiveness and depression- and anxiety-like behaviors (Cryan and Mombereau, 2004; El Yacoubi et al., 2013). The selected CD-1 mice with depressive-like behavior were then divided into 2 experiment groups. In experiment 1, 24 mice were randomly assigned to 4 groups (n = 6) and were administered the following via oral gavage: distilled water (referred to as the mock treatment), milk powder (MP; 150 mg/kg, dissolved in water), KP (150 mg/kg, dissolved in water), and trazodone hydrochloride (TH; 10 mg/kg, dissolved in water). The dosage used in this study followed that in our previous study (Chen et al., 2019). In experiment 2, 30 mice were randomly assigned to 5 groups (n = 6): mock (distilled water), KP (150 mg/kg, dissolved in water), and 3 peptides designated KFP-1 (10 mg/kg, dissolved in water), KFP-3 (10 mg/kg, dissolved in water), and KFP-5 (10 mg/kg, dissolved in water). All treatments were administered by oral gavage for 8 d. This animal study was repeated twice and approved by the Institutional Animal Care and Utilization Committee of National Chung Hsing University (IACUC approval no. 100-104). The animals and study design are shown in Figure 1. At the end of the experiment, each mouse was anesthetized by carbon dioxide asphyxiation before blood collection, and the hippocampal tissue was immediately isolated and stored at −80°C for reverse transcription (RT)-PCR and western blot analysis.

Detection of the Content of Serum Serotonin

The levels of serum serotonin were determined using a serotonin ELISA kit (Abcam) according to the manufacturer’s instructions. Briefly, the serum samples were diluted in PBS (1:10). Labeled alkaline phosphatase conjugate and serotonin antibody were added to samples, and then the para-nitrophenylphosphate substrate was added to produce a yellow solution. The absorbance values were detected at 405 nm.

EPMT

A schematic drawing of the EPMT for mice is shown in Figure 1. The maze consisted of 2 opposing open arms (32 × 6 cm) intersected (center platform) by 2 opposing closed arms (32 × 6 cm) with 15-cm-high walls. The maze apparatus was 50 cm above the floor. This test was performed 6 h after administration of the treatment on d 7. Mice were individually placed in the center of the maze facing an open arm. During the 5-min observation period, the time spent in each arm was measured, and automated quantitative analysis was performed using a DSP CCD camera (model KMS-63F4; Awon) connected to a computer with Noldus software (Ethovision version 4.0, Noldus Information Technology) for data acquisition (Chen et al., 2012). Animal clues and smells, including fecal boli and urine, were removed from the apparatus using 95% ethanol spray and wipes before starting the next test.

OFT

A schematic drawing of the OFT for mice is shown in Figure 1. The experiment was performed according to the method described previously (Yang et al., 2019). Six hours after administration of the treatment on d 8, mice were individually placed on the open field in a brown paper box (45 × 45 cm; 40 cm high). The total distance traveled, speed, time spend in the center, and time spent immobile were recorded over 5 min using a DSP CCD camera connected to a computer with Noldus software for data acquisition. Animal clues and smells, including fecal boli and urine, were removed from the apparatus using 95% ethanol spray and wipes before starting the next test.

FST

A schematic drawing of the FST for mice is shown in Figure 1. The experiment was performed according to the method described previously (Yankelevitch-Yahav et al., 2015). Thirty minutes after administration of the treatment on d 7, mice were placed in a transparent glass cylinder (25-cm diameter; 30 cm high) filled with water (25 ± 1°C) to a depth of 10 cm for 6 min. Immobility time (characterized by floating in the water without any active movements) and mobility time (characterized by performing active swimming or circular movements) were measured and recorded during the last 4 min of the test using a DSP CCD camera connected to a computer with Noldus software for data acquisition.

TST

A schematic drawing of the TST for mice is shown in Figure 1. The experiment was performed according to the method described previously (Yang et al., 2019).
Thirty minutes after administration of the treatment on d 8, mice were individually suspended 50 cm above the surface of a paper box using adhesive tape placed approximately 1 cm from the tip of the tail for 6 min. Immobility time (when they hung passively and completely motionless) was measured during the last 4 min of the test.

**RT-PCR Analysis of mRNA Expression**

Total RNA from hippocampal tissue was extracted using TRizol reagent (Invitrogen) as specified by the manufacturer. The RNA was reverse transcribed to cDNA using an MMLV Reverse Transcription kit (Lan et al., 2015, 2019). Aliquots of the cDNA were used for

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**Figure 1.** Schedule of the animal study of kefir peptide treatment on depressive-like behavior. The mock group was orally fed distilled water, the positive mock group was orally fed trazodone hydrochloride (TH, 10 mg/kg), and the other groups were orally fed milk powder (MP, 150 mg/kg), kefir peptides (KP, 150 mg/kg), or isolated KFP-1, KFP-3, or KFP-5 (10 mg/kg) for 8 d. The elevated plus maze test (EPMT) and forced swimming test (FST) were performed on d 7, and the open field test (OFT) and tail suspension test (TST) were performed on d 8, after administration of the treatment. L = length; W = width; H = height.
PCR amplification of BDNF and CREB. The amplified RT-PCR products were subjected to electrophoresis in a 1.5% agarose gel for 22 min. The relative mRNA expression levels were quantified using ImageJ software (National Institutes of Health) and normalized to that of the reference ACTB gene (Tung et al., 2018).

**Western Blot Analysis**

The indicated protein expression in hippocampal tissue was measured by western blot analysis as described previously (Chen et al., 2019). Briefly, hippocampal tissues were homogenized in 300 µL of radioimmunoprecipitation assay buffer. Thirty micrograms of total protein was loaded onto each lane, and the proteins were separated by SDS-PAGE in 10% polyacrylamide and electrotransferred onto a polyvinylidene fluoride membrane. The membranes were blocked with 5% BSA in Tris-buffered saline with 0.1% Tween 20 at room temperature for 1 h and then incubated with the appropriate primary antibody (BDNF, phosphorylated ERK1/2, ERK1/2, phosphorylated TrkB, TrkB, and β-actin; Abcam) for 2 h. After washing, each protein was identified with an enhanced chemiluminescence western blot detection system (Thermo Fisher Scientific Inc.) using an appropriate horseradish peroxidase-conjugated secondary antibody for 1 h. Detection and quantification of protein levels in the western blot were analyzed using an LAS-4000 chemiluminescence detection device (Fujifilm; Huang et al., 2019).

**Statistical Analysis**

All results are expressed as means ± standard deviation (n = 6). For multiple comparisons, 1-way ANOVA was used, followed by Tukey’s post hoc test. Results with P < 0.05 were considered significant.

**RESULTS**

**Effect of KP on the EPMT**

As shown in Figure 2, mice fed the MP treatment spent more time in the center zone than mice in the mock group, and mice in the TH (10 mg/kg) and KP (150 mg/kg) groups showed a remarkable increase in time spent in the center zone compared with mice in the MP and mock groups (P < 0.05; Figure 2F). In addition, mice in the KP group spent significantly more time in the open arms and less time in the closed arms compared with mice in the mock, MP, and TH groups (P < 0.05; Figure 2G and H).

**Effect of KP on the OFT**

According to the tacking line recorded by a camera during the 5-min observation period in the OFT (Figure 3A–D), the total travel distance in the TH and KP groups was significantly increased compared with that in the mock and MP groups (Figure 3E; P < 0.05). In addition, mice in the TH and KP groups had increased average speed and time spent in the center zone compared with mice in the mock and MP groups (Figure 3F and G; P < 0.05) during a 5-min observation period. Although immobility time in the OFT was not significantly different among groups, there was a slight trend for a reduction in immobility time in the TH and KP mice (Figure 3H).

**Effects of KP on the FST and TST**

The FST was used to evaluate the antidepressant effects of KP, as shown in Figure 4A. The immobility and mobility times of the mock group were 1.5 ± 0.3 and 2.5 ± 0.3 min, respectively. In contrast, the TH and KP groups showed significantly lower immobility times (0.7 ± 0.2 and 0.9 ± 0.2 min, respectively; P < 0.05) and significantly higher mobility times (3.3 ± 0.3 and 3.1 ± 0.3 min, respectively; P < 0.05) compared with the mock group.

In the TST behavior test, the immobility and mobility times of the mice in the mock group were 2.2 ± 0.3 and 1.8 ± 0.3 min, respectively. As anticipated, the TH and KP groups showed a significant decrease in immobility times (0.8 ± 0.2 and 1.1 ± 0.2 min, respectively; P < 0.05) and a significant increase in mobility times (3.2 ± 0.3 and 2.9 ± 0.3 min, respectively; P < 0.05) compared with the mock group (Figure 4B).

**Bioassay-Guided Fractionation of KP**

The KP showed antidepressant activity, indicating that the biologically active antidepressant peptides were enriched in the total KP mixture. Thus, the constituent characteristics of KP were further investigated in this study. To examine the difference between the MP and KP, they were first separated on a TSK-GEL G2000SWXL column under isocratic conditions of 100 mM KH₂PO₄, 1 M NaCl, and 1 mM EDTA (pH = 6.5). The low-molecular-weight protein contents in the KP mixture were higher than those in MP, possibly because kefir grain components digested the milk proteins into peptides. Therefore, the biologically active peptides released by microbial enzymes were among the active antidepressant peptides. The KP were further isolated and identified using HPLC and liquid chromatography–
Figure 2. Effects of kefir peptide treatment on the tracks (A–D), total arm entries (E), and time spent in the center (F), open arms (G), and closed arms (H) in the elevated plus maze test (EMPT). The red tracking lines (A–D) were recorded by a DSP CCD camera (Awon) during the 5-min observation period in the EMPT. Values (E–H) are presented as means ± SD (n = 6). The mock group received an oral administration of distilled water alone as a blank control; the MP group received an oral administration of 150 mg/kg milk powder as a negative control; the KP group received an oral administration of 150 mg/kg kefir peptides as a test group; and the TH group received an oral administration of 10 mg/kg trazodone hydrochloride as a positive drug-treated group. *P < 0.05 or **P < 0.01 compared with the mock group, which received an oral administration of distilled water.
Figure 3. Effects of kefir peptide treatment on the tracks (A–D), total travel distance (E), speed (F), time spent in the center (G), and time spent immobile (H) in the open field test (OFT). The red tracking lines (A–D) were the total travel distances recorded by a DSP CCD camera (Avoni) during the 5-min observation period in the OFT. Values (E–H) are presented as the means ± SD (n = 6). *P < 0.05 or **P < 0.01 compared with the mock group, which received an oral administration of distilled water.
tandem MS, and 3 kefir-fermented peptides (KFP-1, KFP-3, and KFP-5) were isolated (Figure 5A). The molecular weights of KFP-1, KFP-3, and KFP-5 were determined by tandem MS to be 1,749.94, 1,668.96, and 1,561.95 m/z, respectively, and tandem mass spectra were correlated with a milk protein subset of the Swiss-Prot database. The sequence of KFP-1 was observed in κ-CN, and the sequences of KFP-3 and KFP-5 were observed in β-CN.

Effects of Isolated Active KP on the FST and TST

In the FST test, immobility and mobility times of the mice in the mock group were 1.6 ± 0.3 and 2.4 ± 0.3 min, respectively. Immobility times of the KP, KFP-1, KFP-3, and KFP-5 mice were 0.8 ± 0.2, 1.4 ± 0.3, 0.7 ± 0.2, and 1.3 ± 0.3 min, respectively, and mobility times were 3.2 ± 0.3, 2.6 ± 0.3, 3.3 ± 0.2, and 2.7 ± 0.3 min, respectively. Results showed that KP and KFP-3 caused a significant decrease in immobility time (P < 0.05) and a significant increase in mobility time (P < 0.05) compared with the mock group (Figure 5B).

In addition, the TST showed that immobility and mobility times of the mice in the mock group were 2.2 ± 0.3 and 1.8 ± 0.3 min, respectively. Immobility times in the KP, KFP-1, KFP-3, and KFP-5 mice were 1.1 ± 0.2, 1.5 ± 0.3, 1.0 ± 0.2, and 1.8 ± 0.3 min, respectively, and mobility times were 2.9 ± 0.3, 2.5 ± 0.3, 3.0 ± 0.2, and 2.2 ± 0.3 min, respectively. Results showed that KP and KFP-3 caused a significant decrease in immobility time (P < 0.05) and a significant increase in mobility time (P < 0.05) compared with the mock group (Figure 5C). As anticipated, mice in the KFP-3 group showed remarkably decreased immobility time (P < 0.05) and increased mobility time (P < 0.05); the same trend was shown in the KP group. Therefore, our data showed that KFP-3 has antidepressant activity and may be the major active peptide in the KP mixture.

Effects of KP on Serum Serotonin and BNDF/TrkB mRNA and Protein Levels

Antidepressant agents can increase serotonin levels to activate BDNF/TrkB signaling, which subsequently improves depression-like behaviors (Svenningsson et al., 2013). Serotonin level significantly increased in the MP, KP, and TH groups compared with the mock group (Figure 6A; P < 0.05). The mRNA expression levels of BDNF and CREB in the hippocampi of mice after treatment with KP and TH were analyzed by quantitative RT-PCR (Figure 6B). BDNF mRNA expression was significantly upregulated in the TH and KP groups compared with the mock group (P < 0.05; Figure 6C), whereas CREB mRNA expression did not significantly change among the groups (Figure 6D). The BDNF protein expression levels in the hippocampi of mice were also assessed by western blot analysis (Figure 6E). The results showed that BDNF protein levels were significantly increased by more than 2-fold in both the

Figure 4. Effects of kefir peptide treatment on the forced swimming test (A) and the tail suspension test (B). The immobility and mobility times in the forced swimming test and tail suspension test were recorded in the different groups. Values are presented as the means ± SD (n = 6). *P < 0.05 compared with the mock group, which received an oral administration of distilled water.
In addition, the protein levels and phosphorylated states of the downstream targets of BDNF (TrkB and ERK1/2) were detected by western blot analysis (Figure 7). After treatment with TH or KP, phosphorylated ERK1/2 (Figure 7C) and phosphorylated TrkB (Figure 7F) were overexpressed, whereas the total protein expression of TrkB (Figure 7B) and ERK1/2 (Figure 7E) was not significantly altered.

**DISCUSSION**

Incidences of depression and other mood disorders are increasing rapidly and are estimated to affect approximately 300 million people worldwide according to the World Health Organization (Can et al., 2017). Therefore, discovering novel antidepressant pharmacological options to overcome the existing problems with current treatment, including prolonged onset of therapeutic effectiveness, high incidences of nonresponding
Figure 6. Effects of kefir peptide administration on serum serotonin levels and on mRNA (BDNF and CREB) and protein (BDNF) expression levels. (A) Serum serotonin concentration and (B) the mRNA expression of BDNF and CREB in the hippocampi of mice after treatment with kefir peptides and trazodone hydrochloride were analyzed by quantitative reverse-transcription PCR. The quantitative data for the BDNF (C) and CREB (D) mRNA expression levels normalized to β-actin mRNA transcript levels as an internal control. (E) The BDNF protein expression in the hippocampi of mice after treatment with kefir peptides and trazodone hydrochloride were assessed by western blot analysis. (F) The quantitative data of BDNF protein expression levels normalized to β-actin housekeeping protein levels as an internal control. BDNF = brain-derived neurotroph factor. Values are presented as the means ± SD (n = 6). *P < 0.05 compared with the mock group, which received an oral administration of distilled water.
Figure 7. Effects of kefir peptide administration on the protein expression and phosphorylation levels of tropomyosin-related kinase B (TrkB) and extracellular signal-regulated kinase 1/2 (ERK1/2). (A) The protein expression levels of TrkB in the hippocampi of mice after treatment with kefir peptides and trazodone hydrochloride were assessed by western blot analysis. The quantitative data of total TrkB (TrkB/β-actin; B) and phosphorylated TrkB (pTrkB/TrkB; C) protein expression levels were analyzed by quantitative densitometry. (D) The protein expression levels of ERK1/2 in the hippocampi of mice after treatment with kefir peptides and trazodone hydrochloride were assessed by western blot analysis. The quantitative data of total ERK1/2 (ERK1/2/β-actin; E) and phosphorylated ERK1/2 (pERK/ERK; F) protein expression levels were analyzed by quantitative densitometry. Values are presented as the means ± SD (n = 6). *P < 0.05 compared with the mock group, which received an oral administration of distilled water.
patients, and unwanted adverse effects (Paschos et al., 2009), and elucidating their potential mechanisms is crucial for the treatment of depression.

Kefir originated in the Caucasus Mountains and is made from kefir grains that comprise a mixture of lactic acid bacteria and yeasts combined with AA, partially digested proteins, peptide macroelements, microelements, and complex sugars. Fermented dairy products provide a myriad of health benefits, such as antihypertensive, anti-inflammatory, antioxidant, immunomodulatory, anti-diabetic, anti-proliferative antimicrobial, hypcholesterolemic, gut microbiota modulation, and antinociceptive properties (Hernández-Ledesma et al., 2014; Chen et al., 2020; Farag et al., 2020). Noori et al. (2014) indicated that kefir has the potential to treat depression, anxiety, and cognition impairment in a nicotine cessation-induced animal model. As anticipated, in the present study, mice in the KP treatment showed remarkable improvement on behavior tests; this indicates that antidepressant peptides are enriched in the KP mixture and that kefir has potential as an antidepressant fermented dairy product. We further showed that KFP-3 (YQEPVLGPVRGPFPI), but not KFP-1 or KFP-5, may be a major active antidepressant peptide that could be used to treat depression-like behavior (Figure 5).

The peptide sequence YQEPVLGPVRGPFPI may have originated from different species, and sources of cheeses and milks have been reported to have various beneficial bioactivities. The peptide purified from fresh bovine colostrum played a role in limiting microbial pathogen contamination, possibly through modulating macrophage properties (Birkemo et al., 2009; Zhang et al., 2017). In addition, the bitter peptide related to the flavor attributes in Gouda or aged Cheddar cheese was found to have antihypertensive and antimicrobial effects (Karametsi et al., 2014). The peptide from ovine and caprine cheese-like systems, such as Fresco cheese (Torres-Llanez et al., 2011) or Gouda cheese (Saito et al., 2000), exerted angiotensin I-converting enzyme inhibitory and free radical scavenging activities that decreased the blood pressure of a spontaneously hypertensive animal model in an in vitro examination (Durak and Turan, 2020). With this work, we are the first to demonstrate a new function of this peptide: improving depression-like behavior in a mouse model.

The AA profiles of KP showed that the levels of valine, isoleucine, methionine, lysine, threonine, phenylalanine, and tryptophan are higher compared with those in unfermented milk, and some of them play a crucial role in the nervous system (Farag et al., 2020). Thus, after oral ingestion, the KFP-3 peptide may advance the hydrolytic actions in the gastrointestinal tract by pepsin and pancreatic peptidases, including trypsin, chymotrypsin, and carboxypeptidases, to yield peptides and free AA. The ExPASy Peptide Cutter tool (http://web.expasy.org/peptide_cutter/) was used to predict the cleavage sites of proteases; numerous cleavages sites for trypsin, chymotrypsin, pepsin, and elastase were found in the sequence of KFP-3.

The KFP-3 peptide could be cleaved with chymotrypsin and pepsin to yield tyrosine. Current antidepressant drug strategies increase serotonergic and catecholaminergic neurotransmission. Plasma levels of tyrosine, a precursor for catecholamines, were lower in patients with endogenous depression compared with healthy controls in a study by Parker and Brotchie (2011), and oral administration of tyrosine as a therapeutically beneficial supplement showed a marked antidepressant effect in a double-blind crossover study by Gelenberg et al. (1990). The peptide KFP-3 is approximately 26.7% proline and is referred to as a proline-rich polypeptide complex. Proline-rich polypeptides have regulatory effects on immunological response and antioxidant activity, and have beneficial effects on neurological diseases, including Alzheimer disease and depressive-like symptoms (Sochocka et al., 2019; Zablocka et al., 2020). In addition, administration of proline-rich polypeptides significantly increase levels of BDNF in the brain and serum, which leads to enhanced levels of dopamine and serotonin in the brain, considered an effective antidepressant therapy (Dobrzyński and Bednarek, 2016). Furthermore, KFP-3 can be cleaved with trypsin to yield GPFPI, which has been suggested to be a cathepsin B inhibitory peptide (Lee and Lee, 2000). Cathepsin B is a neuronal lysosomal protease in the brain implicated in a variety of cell damage and apoptosis pathways. Studies in rats treated with an antidepressant drug (paroxetine) have shown that expression of cathepsin B dramatically decreased downregulation of the proapoptotic protein and upregulation of neuron survival, which may be important for therapeutic efficacy (Karanges et al., 2013; Zuo et al., 2018). As an antidepressant agent, KFP-3 peptide could more effectively relieve depression-related symptoms because of its antiapoptotic effects and neurotrophic actions.

Bear et al. (2020), in a preclinical model study, showed that altered gut microbiota composition influences brain function and behavior in individuals with depression, which differ from that in healthy controls. Administration of Lactobacillus kefiranofaciens ZW3 from Tibetan kefir could improve depression-like behaviors in a mouse model through modulation of gut microbiota composition (Sun et al., 2019). Van de Wouw et al. (2020) revealed that administration of kefirs Fr1 and UK4 can change the composition of the host microbiota toward modulating immunological and behavioral effects, including repetitive and reward-
seeking behaviors. De Melo et al. (2020) reported that kefir exhibited antidepressant effects through modulation of intestinal microbiota composition and the gastrointestinal immune system in zebrafish; however, the mechanisms are still unclear. The gut microbiota has been explored at the genus level; a high abundance of *Anaerostipes* (Cheung et al., 2019) and *Romboutsia* (Sun et al., 2019) and a low abundance of *Oscillibacter* (Jiang et al., 2015) and *Alistipes* (Zheng et al., 2016) have been found in people or animal models with depression. In this study, we found that the bacterial taxa in *Anaerostipes* and *Romboutsia* were attenuated and *Oscillibacter* and *Alistipes* were increased following KP treatment in depressive mice (Supplemental Figure S1, https://figshare.com/s/7e2ee6a28108ea650ce9).

In an experimental depression-like animal model (Sun et al., 2020) or patients with depression (Emon et al., 2020), serum serotonin levels were decreased. Serotonin supplementation may attenuate depressive symptoms (Matraszek-Gawron et al., 2019) through upregulating *BDNF* mRNA and subsequently activating BDNF/TrkB signaling (Martinowich and Lu, 2008). The level of BDNF is regarded as an appropriate biomarker for investigating the brain pathology of neurological disorders and neuropsychiatric diseases. Stress strongly reduces the level of BDNF in the hippocampus and accelerates depression-like symptoms; thus, protecting against depression-induced BDNF downregulation is thought to have an antidepressant effect (Gumuslu et al., 2014; Fu et al., 2016). Mature BDNF binds to the TrkB receptor to induce tyrosine phosphorylation, which may in turn activate the ERK/MAPK pathway, the phospholipase C-gamma pathway, the phosphatidylinositol 3-kinase pathway, and other signaling pathways that trigger downstream CREB activation to promote the survival and antiapoptotic effects of nerve cells and increase neurogenesis action by overexpression of BDNF and survival-related genes (Yan et al., 2016; Palasz et al., 2020). Several natural products can directly reverse the downregulation of BDNF by upregulating the BDNF activator phosphorylated ERK1/2 in the hippocampus and frontal cortex in human and

![Figure 8](https://figshare.com/s/7e2ee6a28108ea650ce9)

**Figure 8.** Schematic mechanism of kefir peptides (KP) on improving depressive-like behaviors. The potential antidepressant action of kefir peptides through activating brain-derived neurotrophin factor (BDNF) signaling by increasing phosphorylation of tropomyosin-related kinase B (TrkB) and extracellular signal-regulated kinase 1/2 (ERK1/2) in the mouse hippocampus. In addition, kefir peptides increased serotonin level and activated downstream signaling to boost *BDNF* mRNA and protein levels.
animal depression models (Wang et al., 2013; Ge et al., 2014). Mounting evidence has demonstrated that drugs appear to activate BDNF-dependent TrkB-ERK-CREB signaling pathways and may ameliorate depression-like behaviors (Chen et al., 2018; Jin et al., 2019).

Administration of KP may boost the mRNA and protein levels of BDNF to improve depression-like behaviors, possibly by activating BDNF signaling via increased phosphorylation of TrkB and ERK1/2 in the mouse hippocampus. In addition, KP increases serotonin levels and activates its downstream signaling to increase BDNF expression (Figure 8). To the best of our knowledge, this study is the first to demonstrate that KP, especially KFP-3 (YQEPVLGPVRGPFPI), has antidepressant-like effects in mice. Moreover, the results indicated that KP may be a potential functional fermented food for treating or alleviating depression-like symptoms through the BDNF/TrkB pathway. Therefore, KP may represent a new type of antidepressant product and could provide potent antidepressant effects for clinical use.

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