In vivo functional effects of *Weissella confusa* VP30 exopolysaccharides on loperamide-induced constipation in rats

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Received: 8 June 2022 / Revised: 15 August 2022 / Accepted: 19 August 2022 / Published online: 6 September 2022 © The Korean Society of Food Science and Technology 2022

**Abstract**

In this work, the in vivo functionalities of milk fermented with *Weissella confusa* VP30 (VP30-EPS) and purified exopolysaccharide (pEPS) from the milk fermented with *Weissella confusa* VP30 were evaluated for their effect on constipation using an experimental constipated rat model. Rats were randomly divided into four groups: (i) control group (PBS administered normal group), (ii) loperamide treated group (constipation group), (iii) constipation with loperamide plus VP30-EPS (1 g/kg), and (iv) constipation with loperamide plus pEPS (0.6 g/kg) groups. Loperamide treatment induced animal constipation and significantly reduced the frequency of defecation, intestinal transit ratio, and water content of feces. However, all four fecal parameters were improved in both the loperamide plus VP30-EPS and pEPS administered groups as compared to the loperamide group. These results suggest that the addition of VP30-EPS potentially improves the functional laxative effects of commercial products. This study suggests the possibility that VP30-EPS can be applied to fermented and/or functional foods to relieve constipation.

**Keywords** Constipation · Exopolysaccharide · Fecal water content · Water holding capacity · *Weissella confusa* VP30
Introduction

Various chronic, non-infectious, degenerative diseases (a.k.a. civilization diseases) such as obesity, hyperlipidemia, diabetes, cancer, cardiovascular diseases, autoimmune diseases, and constipation, are related to the modern Western diet (high fat, high protein, high calorie, high simple sugar, low dietary fiber) and lifestyle of food consumers over the past few decades. Of these, constipation is a very common functional gastrointestinal disorder encountered in clinical practice and is classified as a multifactorial and/or chronic disease. About 30% of the general population suffers from or has experienced constipation and it is known to negatively impact the psychological quality of human life, result in higher health care expenses, and potentially increase the risk of colon cancer. The ability to defecate regularly without difficulty is one of the indicators used to evaluate intestinal health. Constipation can cause a fatal pulmonary embolism, which can be preceded by bloating, vomiting, intestinal obstruction and perforation. The Roman III criterion for functional constipation includes six major clinical symptoms: straining, lumpy and/or hard stools, sensation of incomplete evacuation, sensation of anorectal obstruction or blockage, digital maneuvers and < 3 defecations/week).

Patients suffering two of these symptoms are diagnosed as clinically constipated. It has been reported that the prevalence of constipation varies with environment, sex, and age. A survey of men and women over 65 years of age revealed 26% of women and 16% of men suffered from constipation. When the age group of the study subjects was extended to 84 years or older, the prevalence of constipation increased by 34% for women and 26% for men. It is also known that more than 80% of long-term care or nursing home residents have suffered from constipation (Schuster et al., 2015). Constipation management work by nursing staff in long-term care settings is time-consuming, labor intensive and costly. As the average human lifespan increases, the need for relief or prevention of senile constipation is expected to increase.

A variety of commercially available laxatives (osmotic, stimulant, stool softener, lubricant, saline and prokinetic laxatives) are prescribed as medicines or supplements to relieve constipation. However, overuse of these laxatives can adversely affect, and ultimately damage, the colonic nervous system. Laxative overuse can lead to a vicious cycle of decreased bowel movements followed by enlarged colon, worsened constipation, and laxative abuse (Roerig et al., 2010), and their misuse can lead to side effects including cramping, loose stools, rectal irritation, diarrhea, nausea, urine discoloration, and burping.

The most commonly used treatments for chronic constipation are intended for short-term treatment and are not known to alleviate underlying intestinal problems. Therefore, alternatives to laxatives for constipation relief/prevention are highly desirable. Several studies have reported the positive effects of lactic acid bacteria (LAB) in the management of gut health (Ayivi et al., 2020; Mathur et al., 2020; Quinto et al., 2014). LAB can be (or are) added to fermented/processed foods and/or nutraceuticals to improve gastrointestinal function. Many groups have reported the positive effects of lactic acid bacterial supplements in the management of gut health (De Filippis et al., 2020; Rakhmanova et al., 2018; Szutowksa, 2020). Although many genera of microorganisms are able to biosynthesize lactic acid as a primary metabolite, common LAB are classified as bacteria of the Lactobacillales order including Lactobacillus, Carnobacterium, Lactococcus, Streptococcus, Enterococcus, Vagococcus, Leuconostoc, Oenococcus, Pediococcus, Tetragenococcus, Aerococcus and Weissella.

Modification of eating habits is frequently recommended as a way of preventing constipation. Specifically, it is desirable to reduce the consumption of simple or refined carbohydrates. Consumption of oligo- and polysaccharides with complex chemical structures has also been recommended for the promotion of intestinal peristalsis (Cruz-Rubio et al., 2018). These complex carbohydrates are known to absorb water and become viscous, gelatin-like substances which enhance bulking and promote regularity.

The disadvantage of probiotics is that as living organisms, they are susceptible to processing heat and the acidic environment in our digestive system. The total amount of probiotics that reach the small and large intestine are also limited; some of these totals are ultimately excreted without colonizing the intestine (Kim and Park, 2021). Postbiotics, however, can compensate for these shortcomings. Postbiotic molecules being studied in academia and industry include short-chain fatty acids, antibacterial peptides, neurotransmitters, enzymes, minerals, cell lysates, polysaccharides, cell surface proteins and EPS. Representative functionalities of EPS include antioxidant capabilities, immunomodulatory effects, and anti-tumor and anti-viral effects. Table 1 lists EPS functionalities reported via published research during 2021.

It must be noted that none of the studies summarized in Table 1 were clinical (in vitro and in vivo only), and most of the functionalities of EPS produced by LAB were related to anti-oxidant, anti-bacterial, and anti-tumor effects.

In our previous work, a novel LAB, W. confusa VP30 (VP30), isolated from children’s feces, was found to produce the most exopolysaccharide (EPS) among 156 LAB cell strains tested (Jin et al., 2019). The maximum purified VP30 EPS (pEPS) was 36.47 ± 0.87 g/L in modified MRS media containing 10% (w/v) sucrose. The pEPS showed significant water holding capacity and was found to be a dextran of 3.8 × 10⁶ Da, composed of 96.5% α (1 → 6) glycosidic
| Nos | Species                          | Model (in vitro/in vivo/clinical study) | Dosing level (stimulation reagent, experimental day) | Efficacy                                                                                                                                                                                                 | References                  |
|-----|----------------------------------|----------------------------------------|------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------|
| 1   | EPS produced by *L. plantarum* S123 | In vitro                               |                                                      | 1. Antibacterial activity: Gram-positive (*Staphylococcus aureus*, 7.2 mm), Gram-negative (*Escherichia coli*, 11.5 mm) 2. Antioxidant activity: scavenging rate (> 65%) 3. Water holding capacity: 326.6 ± 0.5% | Saleem et al. (2021)             |
| 2   | EPS produced by *B. aerophilus* rk1 | In vitro                               |                                                      | 1. Antioxidant activity: 56.6% of scavenging activity (4 mg/mL)                                                                                                                                     | Ravi et al. (2021)           |
| 3   | EPS produced by *B. licheniformis* AG-06 | In vitro                              |                                                      | 1. Antioxidant activity: about 40% of scavenging activity (2 mg/mL)                                                                                                                                   | Vinothkanna et al. (2021)   |
| 4   | EPS produced by *W. cibaria* MD2  | In vitro                               |                                                      | 1. Antioxidant activity: about 60% of scavenging activity (1 mg/mL)                                                                                                                                    | Lakra et al. (2021)          |
| 5   | EPS produced by *E. durans* K48, *E. faecium* R114, and *E. faecium* T52 | In vitro                               |                                                      | 1. Antibacterial activity (MIC): Gram-positive (*Staphylococcus aureus*, 5.25, 6.15, 8.35 µg/mL), Gram-negative (*Escherichia coli*, 13.7, 18.6, 10.5 µg/mL; *Salmonella typhimurium*, 33.5, 37.0, 36.5 µg/mL) 2. Antioxidant activity: 53%, 58%, 64% of scavenging activity (25 mg/mL) | Vosough et al. (2021)        |
| 6   | EPS produced by *L. mesenteroides* SN-8 | In vitro                               |                                                      | 1. Antioxidant activity: 57.42% of scavenging activity (10 mg/mL) 2. Anti-tumor effect: about 40% of inhibition ratio in HepG2 cells (1 mg/mL)                                                                 | Wu et al. (2021)             |
| 7   | EPS produced by *A. gonensis* YK25 | In vitro                               |                                                      | 1. Anti-tumor effect: 5.5, 9.61, 15.62, 17.35 mg/mL, respectively (A-549, DU-145, SH-SY5Y, HT-29 cells)                                                                                             | Karadayi et al. (2021)       |
| 8   | EPS produced by *L. paracasei* IJH-SONE68 | In vivo (C57BL/6 J mice, IBD model) | 1 mg/ML (dextran sulfate sodium, 2 weeks) | 1. Stool consistency score: about 1.4 (about 1.8 of positive control) 2. Disease activity score: about 2.2 (about 2.9 of positive control)                                                        | Noda et al. (2021)           |
| 9   | EPS produced by *P. acidilactici* MT41-11 | In vitro                               |                                                      | 1. Anti-biofilm effect: *L. monocytogenes* (55.28%), *Salmonella enterica* (55.18%), *S. aureus* (54.22%), *E. coli* (42.39%), respectively (2 mg/mL) 2. Antioxidant activity: 71.65% of scavenging activity (5 mg/mL) | Bai et al. (2021)            |
| Nos | Species | Model (in vitro/in vivo/clinical study) | Dosing level (stimulation reagent, experimental day) | Efficacy | References |
|-----|---------|---------------------------------------|----------------------------------------------------|----------|------------|
| 10  | EPS produced by *L. mesenteroides* LM187 | In vitro | | 1. Antioxidant activity: 50.2% of scavenging activity (5 mg/mL) | Zhang et al. (2021a) |
| 11  | EPS produced by *W. paramesenteroides* MN2C2 | In vitro | | 1. Antioxidant activity: 45.8% of scavenging activity (80 µg/mL) 2. Antiviral effect: 99.99% of inhibition (1.5 mg/mL) 3. Anti-cancer activity (IC₅₀): Caco-2 (3.50 mg/mL), HepG2 (2.60 mg/mL), MCF-7 (4.80 mg/mL), A-549 (16.50 mg/mL), Wi-38 (26.10 mg/mL), respectively | Amer et al. (2021) |
| 12  | EPS produced by *L. kimchi* SR8 | In vitro/In vivo (Kunming mice, aging model) | 200 mg/kg (α-galactose, 4 weeks) | 1. Antioxidant activity: 71.39% of scavenging activity (1 mg/mL) 2. Anti-aging effect (MDA level): 1.43 nmol/mg (1.99 nmol/mg of control) | Zhang et al. (2021b) |
| 13  | EPS produced by *L. acidophilus* ATCC 4356 | In vivo (Swiss albino rats, hepatocarcinogenesis model) | 100 mg/kg (diethylnitrosamine, 8 weeks) | 1. Anti-inflammatory activity (TLR2): about 2.8-fold gene expression (about 6.7-fold of positive control) | Khedr et al. (2021) |
| 14  | EPS produced by *L. rhamnosus* ZFM231 | In vitro | | 1. Antioxidant activity: 86.2% of scavenging activity (5 mg/mL) | Hu et al. (2021) |
| 15  | EPS produced by *L. helveticus* SIM12, SIS16, Lb3, 1734 | In vitro | | 1. Anti-inflammatory activity (IL-12): about 0.8 ng/mL (about 0.05 ng/mL of control) | Zago et al. (2021) |
constipation and 3.5% α (1 → 3) branches (Jin et al., 2019). Furthermore, VP30 was found to produce more EPS than any LAB reported to date. Therefore, we hypothesized that the high-water holding capacity of pEPS could improve constipation by maintaining fecal moisture and increasing bowel activity. In this research, we sterilized fermented milk with VP30 (VP30-EPS) and 10% (w/v) sucrose and investigated the in vivo biofunctionality of VP30-EPS and pEPS for the alleviation of constipation symptoms.

**Materials and methods**

**Reagents**

Loperamide (L4762), activated charcoal (C9157), gum arabic (G9752) were purchased from the Sigma-Aldrich Co. Ltd., (Manassas, VA, USA).

**Cultivation of VP30**

Overnight cultured VP30 (1 × 10⁹ CFU/mL) was inoculated 3% (3 mL, 3 × 10⁷ CFU/mL, v/v) in modified MRS media with 3% soy peptone, 1% yeast extract, 2% maltose, 0.2% disodium phosphate, 0.5% sodium acetate, 0.005% manganese sulfate, 0.01% magnesium sulfate, 0.05% L-cysteine, 0.01% maleic acid, 0.031% taurine, 0.3% Tween 80, 0.02% vitamin A, 0.02% vitamin E (pH 6.8) (final volume 100 mL). After 16 h of incubation under anaerobic at 37 °C, the optical density was measured using an ELISA reader at 550 nm.

**Preparation of VP30-EPS**

Activated VP30 cells (1 × 10⁹ CFU/mL) were inoculated at 5% (50 mL, final concentration of VP30 cells: 5 × 10⁷ CFU/mL, v/v) into reconstituted (10%) sterilized skimmed-milk (Samik Dairy and Food Co., Ltd., Korea) containing 10% (w/v) sucrose (final volume 1 L). To produce VP30-EPS, the sample was anaerobically incubated for 17 h at 30 °C. To compare whether VP30 EPS alone exhibits constipation relief effects comparable to dietary fiber, we sterilized the prepared VP30-EPS at 75 °C for 120 min. The number of viable cells in the VP30-EPS was qualitatively observed by the number of colonies using the LAB growth selection broth. The VP30-EPS did not show live bacteria in the LAB growth selection broth (data not shown). The sterilized VP30-EPS was stored at 4 °C until used.

**pEPS purification and quantification**

The VP30-EPS sample, which contained EPS produced by VP30, was heat-treated at 100 °C for 15 min, followed by the addition of 85% trichloroacetic acid (17% v/v) and storage at room temperature for 2 h. The acid-treated sample was centrifuged (18,000xg) for 25 min and the supernatant was collected. The supernatant was treated with 95% ethanol (−20 °C) at a ratio of 1:5 (w/v) and cooled at 4 °C for 1 h. The cooled sample was centrifuged again for 20 min (18,000xg) to recover crude EPS. The obtained crude EPS was washed with 70% ethanol and centrifuged for 20 min (18,000xg). After centrifuge, obtained EPS pellet was dissolved in 5 mL of distilled water and then passed through a membrane tube (Cat No. 132697, Spectra/Por®4 dialysis membrane standard RC tubing MWCO: 12–14 kDa, Spectrum Laboratories, Inc., CA, USA) at 4 °C for 2 days.

**pEPS measurement**

pEPS production by VP30 was quantified by the phenol–sulfuric acid method (Jin et al., 2019; Ku et al., 2009; Mendi and Aslim, 2014) and dextran was used as a standard. The pEPS solution was diluted to 800-fold and then 0.5 mL of pEPS was reacted with 0.5 mL of 5% (v/v) phenol solution at room temperature. Then, 2.5 mL of 1 N H₂SO₄ solution was added to the pEPS–phenol mixture and held at 90 °C for 5 min. After lowering the sample temperature to 27 °C, absorbance was measured at 490 nm. The following equation was used to assess the extraction yield percentage.

$$\text{Extraction yield} (%) = \frac{\text{The weight of pEPS}}{\text{The weight of VP30-EPS}} \times 100.$$

The calculated extraction yield of pEPS in VP30-EPS was 13.5 ± 0.6%.

**Animal and induction of constipation**

Male Sprague–Dawley (SD) rats (160–180 g, 6 weeks old) were purchased from Dae Han Bio Link Co. Ltd. (Eumsung-gun, Korea) and housed in individual cages. The experimental animals were kept at a temperature of 23 ± 1 °C and relative humidity of 50–55% for 5 days of adaptation. The animals were then subcutaneously injected with LP (2 mg/kg, n = 7) once daily at the same time with the exception of the normal group (n = 7), which were not treated. VP30-EPS (1 g/kg, n = 7) and pEPS (0.6 g/kg, n = 7) were orally administered to the test groups twice a day for 3 days, after which all animals were sacrificed. All animal experimental protocols in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the Hongcheon Institute of Medicinal Herbs (HIMH A21-05). Animal body weight, food intake, and water intake were measured for 3 days, once per day.
Frequency of feces, weight of fecal pellets, and fecal water content

During the period of loperamide-induced constipation, the number of feces, weight of fecal pellets, and fecal water content were measured once per day. Fecal water content was determined by comparing initial weight to the weight after drying at 60 °C for 24 h.

Intestinal transit ratio

Intestinal transit ratio was measured using a modified method from Jo et al. (2019). To investigate the effect of administration of LP + VP30-EPS or LP + pEPS on intestinal transit, food was removed from all animals for 16 h, followed by oral administration of an activated carbon diet (10% charcoal meal, 1.5 mL). All animals were sacrificed 30 min later, and their gastrointestinal tracts excised. Intestinal length was measured by summing the length of the small intestine and the large intestine, and the intestinal transit ratio was calculated by dividing the intestinal distance traveled by the charcoal meal by total length of the intestinal tract.

Blood chemistry

Serum samples were prepared by centrifuging the collected blood (4,000 rpm for 15 min at 4 °C) and stored at − 80 °C for assay. Alanine transferase (ALT), aspartate transaminase (AST) levels were analyzed using an automated clinical chemistry analyzer (Konelab 20XT; Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

Results were expressed as mean ± standard deviation (SD) and were analyzed using the Statistical Package for Social Sciences version 28.0 (SPSS, Inc., USA). An analysis of variance (ANOVA) test was used to determine differences among samples at a significance level of \( p < 0.05 \).

Results and discussion

Changes of rat body weights

Loperamide is known as an antagonist that activates the µ-opioid receptor. Activation of µ-opioid receptors in myenteric muscle induces blockage of intracellular calcium channels, which affects signaling pathways such as membrane hyperpolarization (Bohn and Raehal, 2006). As a result, intestinal motility is reduced by inhibiting excitatory neurotransmission (Klein et al., 2013).

Loperamide administration causes decreased fecal water and dietary intake due to decreased intestinal peristalsis in rats, which results in constipation and weight loss of the specimen. The residence time of feces in the intestine becomes longer due to decreased intestinal movement, allowing fecal water more time to be absorbed into the body. However, microbial EPS has been shown to have significant water-holding capacity due to its hydrated polymer network mediating function (Zannini, 2015). We, therefore, theorized that loperamide-induced clinical symptoms would be alleviated by EPS containing VP30-EPS or pEPS ingestion. The initial body weight, final body weight, body weight gain, food intake, and water intake for each rat group are shown in Table 2.

Compared to the loperamide-free normal group, all loperamide-administered groups showed significantly decreased food intake, which resulted in lower final body weights and body weight gains compared to the normal group (\( p < 0.05 \)). However, groups treated with VP30-EPS or pEPS showed increased body weight gain and water intake compared to the group treated with only loperamide. Shimotoyodomo et al. reported that loperamide administration induced abdominal distension and decreased food intake in experimental animals (Shimotoyodome et al., 2000). In addition, continuous administration of loperamide decreased large intestine peristalsis, thereby lowering fecal movement rates (Cepinskas et al., 1993). Therefore, the observed decrease in dietary intake due to continuous administration of loperamide is explained.

Table 2  Effects of VP30-EPS or pEPS on body weight, food intake, and water intake in normal and loperamide-treated SD rat groups

| Group       | Initial body weight (g/rat) | Final body weight (g/rat) | Body weight gain (g/rat) | Food intake (g/day) | Water intake (g/day) |
|-------------|-----------------------------|---------------------------|-------------------------|---------------------|----------------------|
| NC          | 180.40± 8.56\( ^{a} \)      | 194.60± 12.34\( ^{a} \)   | 12.20± 4.09\( ^{a} \)   | 13.52± 2.32\( ^{a} \) | 26.80± 8.77\( ^{a} \) |
| LP          | 178.60± 3.91\( ^{a} \)      | 181.72± 4.52\( ^{ab} \)   | 3.12± 3.31\( ^{b} \)    | 11.21± 1.54\( ^{c} \) | 20.98± 9.86\( ^{a} \) |
| LP + VP30-EPS | 172.67± 5.13\( ^{a} \)    | 178.67± 11.02\( ^{b} \)   | 6.00± 6.08\( ^{b} \)    | 12.58± 1.42\( ^{b} \) | 26.30± 12.72\( ^{a} \) |
| LP + pEPS   | 180.50± 7.19\( ^{a} \)      | 184.50± 6.86\( ^{ab} \)   | 4.00± 0.82\( ^{b} \)    | 11.30± 1.46\( ^{c} \) | 21.82± 7.64\( ^{a} \) |

\( ^{a-c} \) Mean values with different letters are significantly different (\( p < 0.05 \)) according to Duncan’s multiple range test.
Effect of VP30-EPS or pEPS on the fecal weights, fecal water contents and number of feces of experimental animals

After administration of VP30-EPS or pEPS to the test groups for 3 days, changes in fecal weight, fecal water content and number of feces were evaluated. Loperamide lowers the moisture content of feces and causes constipation. Recently, the aquaporin family of cell membrane water channel proteins (AQPs) has been identified as playing an important role in the cellular water transport system (Adeoye et al., 2022). AQP3, AQP4, and AQP8 have been identified as the most important. Intestinal water transport is achieved by the activity of AQPs, and it is reported that this protein enhances water absorption by intestinal epithelial cells. In particular, it has been reported that in constipation caused by loperamide, the AQP3 level is reduced due to decreased AQP3-driven mRNA and protein expression (Yi et al., 2019). Therefore, the fecal water content and number of feces in the experimental groups can be used as biomarkers to compare and evaluate the degree of constipation relief for the experimental groups. Administration of loperamide significantly reduced the wet feces weight of rats (2.36 ± 0.22 g/rat, p < 0.05, Table 3).

However, when LP was administered to rats along with VP30-EPS or pEPS, the wet fecal weights were different from that of the NC group (3.31 ± 0.42, 3.19 ± 0.44 g/rat, respectively). These results showed that intestinal transit ratio of the normal, LP + VP30-EPS and LP + pEPS groups were significantly higher than that of the negative control group (LP group). There was no significant difference in dry fecal weight levels of all groups. The level of fecal water content of the normal, LP + VP30-EPS and LP + pEPS groups were 22.05 ± 3.15%, 9.95 ± 3.97%, 27.97 ± 4.47%, and 32.00 ± 5.86%, respectively. In spite of LP treatment, it was observed that fecal water contents were significantly increased when VP30-EPS or pEPS was administered (p < 0.05). Fecal pellet numbers after induced constipation in the LP, LP + VP30-EPS and LP + pEPS groups were significantly different from that of the normal control group (Fig. 1). The three-day averages of fecal pellet number of the LP + VP30-EPS and LP + pEPS groups were significantly higher than that of the LP group (p < 0.05), although the fecal pellet numbers on Day 0 in all groups were similar (data not shown). The pattern of third day data (the last day of experiment) for all groups was consistent with the pattern of average feces numbers (Fig. 1A, B). These results suggest that pEPS have a positive effect on stool frequency.

Zannini et al. reported that EPS produced by LAB attaches to intestinal mucosa to form an EPS biofilm, helping the adhesion and growth of LAB (Zannini, 2015). In addition, it is reported that EPS produced by LAB exhibits strong hydrophilicity and has a high water-holding capacity that enables the survival of microorganisms in a dry intestinal environment (Zannini et al., 2015). We reported in our previous study that the pEPS exhibited significant hydrophilicity with strong water-holding capacity.

Effect of VP30-EPS or pEPS on the intestinal transit ratio

It is also known that the interstitial cells of Cajal (ICC), which regulate intestinal motility, are not differentiated, developed, or maintained by loperamide, and this phenomenon results in decreased intestinal motility, leading to constipation (Hao et al., 2019). Contraction and relaxation of intestinal smooth muscle cells are reduced by loperamide. The known gene markers related to the contraction and relaxation of intestinal smooth muscle cells are mAchR M2 and M3. Two types of markers are decreased by loperamide, and it is known that contraction and relaxation of intestinal smooth muscle cells affect the decrease. If intestinal peristalsis is reduced due to the administration of loperamide, the intestinal movement distance of feces is reduced, which leads to the accumulation of feces in the intestine.

We expected that supplying rats with VP30-EPS and/or pEPS, which have high water-holding capacity, would increase the moisture content of the rat’s feces and reduce the transit time through the large intestine by softening the stool, thereby relieving constipation. For the calculation of intestinal transit ratio, the length of small and large intestines was measured. There was no significant difference in the intestine or intestinal length in all groups (Table 4).

In LP-treated rats, the transit ratio significantly increased when the rats were treated with VP30-EPS or pEPS. VP30-EPS or pEPS consumption increased the transit ratio to the

### Table 3

Fecal parameters following oral administration with VP30-EPS or pEPS in loperamide-treated SD rats

| Group       | Wet fecal weight (g/rat) | Dry fecal weight (g/rat) | Fecal water content (%) |
|-------------|--------------------------|--------------------------|-------------------------|
| NC          | 3.19 ± 0.44<sup>a</sup>  | 2.49 ± 0.40<sup>a</sup>  | 22.05 ± 3.15<sup>b</sup>|
| LP          | 2.36 ± 0.22<sup>b</sup>  | 2.12 ± 0.20<sup>c</sup>  | 9.95 ± 3.97<sup>c</sup> |
| LP + VP30-EPS | 3.31 ± 0.42<sup>a</sup>  | 2.39 ± 0.37<sup>a</sup>  | 27.97 ± 4.47<sup>a</sup>|
| LP + pEPS   | 3.19 ± 0.34<sup>a</sup>  | 2.17 ± 0.31<sup>a</sup>  | 32.00 ± 5.86<sup>a</sup>|

<sup>a-d</sup> Mean values with different letters are significantly different (p < 0.05) according to Duncan’s multiple range test.
Various research groups have reported a positive correlation between milk fermented by LAB and fecal transit ratios. Ge et al. reported that *Lactobacillus acidophilus* and *Bifidobacterium bifidum* release neuro-messengers that promote intestinal motility (Ge et al., 2017). Milk fermented with *Bifidobacterium lactis* and *Bifidobacterium animalis* reduced constipation symptoms and colonic transit time in humans and animals, respectively (Agrawal et al., 2008; Bouvier et al., 2001). Marteau et al. also reported that regular intake of milk fermented with *Bifidobacterium* spp. has a positive effect on large intestine functionality (Marteau et al., 2002). NC and LP denote normal control and loperamide respectively. Various groups have reported that live active or inactivated LAB, or lactic acid bacterial EPS or microbial fermented foods can enhance in vivo host intestinal peristalsis (Table 5).

Specifically, Maeda et al. (2004) artificially induced constipation in SD rats by treatment with a low-fiber diet and orally administered EPS isolated from *Lactobacillus kefiranofaciens* at a dose of 100 or 300 mg/kg for 14 days. They reported that fecal moisture content and weight increased in a dose-dependent manner with bacterial EPS (Maeda et al., 2017).
| Nos | Species                  | Model (in vitro/in vivo/clinical study) | Dosing level (stimulation reagent, experimental day) | Efficacy                                                                 | References            |
|-----|-------------------------|----------------------------------------|-----------------------------------------------------|--------------------------------------------------------------------------|-----------------------|
| 1   | *W. confusa* VP30       | In vivo (Sprague–Dawley rat)           | 1.0 g/kg (loperamide, 3 days)                        | 1. Average fecal number: 32 pellets (27.38 pellets of control)         | This study            |
| 2   | *B. bifidum* G9-1       | In vivo (Sprague–Dawley rat)           | $1.0 \times 10^{10}$ CFU (loperamide, 4 days)      | 1. Fecal pellet number: 40 pellets/day (30 pellets/day of control)      | Makzaki et al. (2021) |
| 3   | Mixture of *B. adolescentis* CCFM626, CCFM667, CCFM669 | In vivo (BALB/c mice)                  | $1.0 \times 10^{10}$ CFU (loperamide, 17 days)     | 1. Fecal weight: 1 g (0.5 g of control)                                  | Wang et al. (2017)    |
| 4   | *L. plantarum* NCU116   | In vivo (Kunming mice)                 | $1.0 \times 10^{9}$ CFU (loperamide, 15 days)      | 1. Fecal pellet number: 15.22 pellets (8.78 pellets of control)         | Li et al. (2015)      |
| 5   | *L. plantarum* CQPC02-fermented soybean milk | In vivo (Kunming mice)                 | 2 mL (loperamide, 17 days)                           | 1. Fecal weight: 0.83 g (0.45 g of control)                              | Yi et al. (2019)      |
| 6   | Mixture of *L. rhamnosus* CCFM1068, FFJND15-L2, FHeNIZ7-1, FTJD111-1, FZHJZ11-7 | In vivo (BALB/c mice)                 | $5 \times 10^{9}$ CFU (loperamide, 29 days)         | 1. Fecal moisture: 60% (40% of control)                                  | Wang et al. (2020)    |
| 7   | Heat-killed *L. plantarum* nF1 | In vivo (Sprague–Dawley rat)           | $1.6 \times 10^{11}$ CFU (loperamide, 5 weeks)     | 1. Fecal moisture: 90% (75% of control)                                  | Park et al. (2021)    |
In a follow-up study they treated 20 patients with symptoms of chronic constipation for 4 weeks with purified EPS isolated from *L. kefiranofaciens* and observed the clinical effects. All 20 patients showed increased excretion frequency and decreased stool retention time (Turan et al., 2014). Jeong’s group identified *L. kefiranofaciens* DN1 with the best EPS production ability among 22 *L. kefiranofaciens* strains (Jeong et al., 2017), and orally administered *L. kefiranofaciens* DN1 (2 $10^8$ CFU) to BALB/c female mice. They reported that the fecal weight and moisture contents of experimental animals were increased by oral administration of *L. kefiranofaciens* DN1 (Jeong et al., 2017).
EPS yield of *L. kefiranofaciens* was 2.5 g/L, a value about 14 times lower than the EPS yield of VP30 (36.47 g/L). Although *L. kefiranofaciens* EPS has been shown to reduce constipation through various studies, the commercialization potential of *L. kefiranofaciens* EPS is limited due to low EPS production.

**Blood analysis**

The microbiological safety of VP30 was verified in our previous study but in vivo toxicity evaluation studies using VP30-EPS or pEPS extract were not conducted. Therefore, we conducted toxicity evaluations using ALT and AST to determine whether the intake of VP30-EPS or pEPS induces hepatotoxicity. Serum ALT was within the normal range in all group (data not shown).

Serum AST was statistically increased by LP treatment, but both VP30-EPS and pEPS maintained serum AST levels within the normal range (data not shown). Based on these results, the intake of VP30-EPS and pEPS is considered harmless to humans. The intake of $10^7$ to $10^{10}$ CFU/day of *Bifidobacterium* or *Lactobacillus* increased stool weight, stool moisture content, number of stools, and bowel movement distance. Most *Bifidobacterium* and *Lactobacillus* are known to produce EPS, but their productivity, chemical structures and molecular sizes differ (Kavitake et al., 2020).

In conclusion, we investigated the constipation-relieving effects of VP30-EPS and pEPS via LP-treated SD rats. Both VP30-EPS and pEPS administration effectively relieved LP-induced constipation in rats. Although LP induced constipation, oral administration of VP30-EPS or pEPS increased stool water content, which promoted colonic peristalsis with intestinal transit ratio reduction. Through animal testing, we verified the hepatotoxicity safety of VP30-EPS and pEPS. These data suggest that VP30-EPS and pEPS can restore normal intestinal conditions in constipated patients by changing the physical properties of their feces. This study is the first showing the effect of reducing constipation through animal experiments by applying EPS produced by a *Weissella* strain to foods. Further clinical experiments with VP30-EPS or pEPS should be accomplished.

**Acknowledgements** Not applicable.

**Author contributions** Methodology and analysis: SHP, MRL., BK, HJK, MSP, SK; Investigation and center management: SHP, MRL; Consultation and sampling: YSY, LJY, LHH, YJS; Data curation and writing: SHP, MRL, BK, HI, TVJ, SK; Funding: MSP. All authors read and approved the final manuscript.

**Funding** This work was supported by the Industrial Strategic Technology Development Program (20014744), Development of K-beauty material for better immune balance and skin health based on probiotics and its industrialization) funded by the Ministry of Trade, Industry and Energy (MOTIE, Korea).

**Declarations**

**Conflict of interest** SHP, YSY, LJY, LHH, YJS, MSP are directly employed by BIFIDO Co., Ltd. as researchers. Other authors declare no conflicts of interest. SK and TVJ participated this work based on a nondisclosure research agreement between Middle Tennessee State University and BIFIDO Co., Ltd.

**Statement of Animal Rights** All institutional and national guidelines for the care and use of laboratory animals were followed.

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