# In vivo Functional Effects of Weissella confusa VP30 Exopolysaccharides on Loperamide-Induced Constipation in Rats

**Se-Ho Park**
BIFIDO Co., Ltd
**Mi-Ra Lee**
Hongcheon Institute of Medicinal Herb
**Yang Su Young**
BIFIDO Co., Ltd
**Lee Ju Yeon**
BIFIDO Co., Ltd
**Lee Hyun Ha**
BIFIDO Co., Ltd
**Yeong-Je Seong**
BIFIDO Co., Ltd
**Bohye Kim**
Hongcheon Institute of Medicinal Herb
**Hee-Jun Kim**
Hongcheon Institute of Medicinal Herb
**Hui Jin**
Seoul National University
**Tony V. Johnston**
Middle Tennessee State University
**Seockmo Ku**
Middle Tennessee State University
**Myeong Soo Park** ( bifidopark@bifido.com )
BIFIDO Co., Ltd

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# Abstract

In our previous work, we characterized the in vitro biofunctionality and evaluated the biosafety of exopolysaccharide (EPS)-producing Weissella confusa VP30 (VP30), newly isolated from healthy children's feces. Since the purified EPS (pEPS) from fermented milk with VP30 (VP30-EPS) showed significant water holding capacity, it was theorized that its consumption would relieve constipation. In this work, the in vivo functionalities of VP30-EPS and pEPS were evaluated for their effect on constipation using an experimental constipated rat model. Rats were randomly divided into four groups: (i) negative control (PBS administered normal group), (ii) loperamide treated positive control (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 treated positive control group. No significant changes in dietary intake or serum hepatocellular necrosis maker levels were observed in any experimental group. These results suggest that the addition of VP30-EPS or pEPS potentially improves the functional laxative effects of commercial products. No other published research relating in vivo functional effects of EPS from Weissella spp. on constipation could be identified. This study suggests the possibility that VP30-EPS and pEPS can be applied to fermented and/or functional foods to relieve constipation.


# 1. 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 (Mathur et al. 2020; Avivi et al. 2020; Quinto et al. 2014). LAB can be (or are) added to fermented/processed fo ods 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; Szutowska 2020; Rakhmanova et al. 2018). 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, Tetragonococcus, 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 this total 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 or 2022.

**Table 1** Functionality of EPS produced by bacteria

| No | Species | Model (*in vitro*/ *in vivo*/clinical study) | Dosing level (Stimulation reagent, experimental day) | Efficacy | Ref |
|---|---|---|---|---|---|
| 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)<br><br>2. Antioxidant activity: scavenging rate (> 65%)<br><br>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; *S. typhimurium*, 33.5, 37.0, 36.5 µg/ml)<br><br>2. Antioxidant activity: 53%, 58%, 64% of scavenging | Vosough et al. 2021 |

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| | | | | activity (25 mg/ml) | |
| 6 | EPS produced by *L. mesenteroides* SN-8 | *In vitro* | | 1. Antioxidant activity: 57.42% of scavenging activity (10 mg/ml)<br><br>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) | Karadavi et al. 2021 |
| 8 | EPS produced by *L. paracasei* IJH-SONE68 | *In vivo* (C57BL/6J mice, IBD model) | 1 mg/ml<br><br>(Dextran sulfate sodium, 2 weeks) | 1. Stool consistency score: about 1.4 (about 1.8 of positive control)<br><br>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%), *S. enterica* (55.18%), *S. aureus* (54.22%), *E. coli* (42.39%), respectively (2 mg/ml)<br><br>2. Antioxidant activity: 71.65% of scavenging activity (3 mg/ml) | Bai et al. 2021 |
| 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 | Amer et al. 2021 |

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| | | | | activity (80 μg/ml)<br><br>2. Antiviral effect: 99.99% of inhibition (1.5 mg/ml)<br><br>3. Anti-cancer activity ($IC_{50}$): 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 | |
| 12 | EPS produced by *L. kimchi* SR8 | *In vitro / In vivo* (Kunming mice, aging model) | 200 mg/kg<br><br>(D-galactose, 4 weeks) | 1. Antioxidant activity: 71.39% of scavenging activity (1 mg/ml)<br><br>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<br><br>(Diethylnitrosamine, 8 weeks) | 1. Anti-inflammatory activity (TLR2): about 2.8-fold gene expression (about 6.7-fold of positive control) | Khedr et al. 2022 |
| 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, Lh43, 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 |

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 lactic acid bacteria 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 $3.8 \times 10^6$ Da, composed of 96.5% α (1→ 6) glycosidic bonds and 3.5% α (1→ 3) branches. 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 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.

# 2. Materials And Methods

*2.1. Reagents*

Loperamide (L4762), Tween 20 (P9416), activated charcoal (C9157), Gum Arabic (G9752), sodium acetate (S2889), disodium phosphate (1.06586), ammonium citrate (09833), Tween 80 (P5188), magnesium sulfate (M2643), manganese sulfate (M7899), sucrose (S0389), and L-cysteine (C7352) were purchased from the Sigma-Aldrich Co. Ltd., (Manassas, VA, USA). Dextrose (215530), beef extract (212610), yeast extract (212750), and skim milk (232100) were purchased from Becton, Dickinson and Company (Franklin Lakes, New Jersey, USA) and meat peptone (MB-M0306) was purchased from the Kisan Bio Ltd., (Seoul, Korea).

*2.2. Cultivation of VP30*

Overnight cultured VP30 was inoculated 3% (v/v) in modified MRS media with 2% dextrose, 1% meat peptone, 1% beef extract, 0.5% yeast extract, 0.5% sodium acetate, 0.2% disodium phosphate, 0.1% Tween 80, 0.01% magnesium sulfate, 0.005% manganese sulfate, 0.05% L-cysteine (pH 6.7). After 12 h of incubation under anaerobic at 37°C, the optical density was measured using an ELISA reader at 550 nm.

*2.3. Preparation of VP30-EPS*

Activated VP30 cells were inoculated at 5% (v/v) into reconstituted (10%) sterilized skimmed-milk (BD, Difco®, Becton Dickinson, USA) containing 10% (w/v) sucrose. To produce VP30-EPS, the sample was anaerobically incubated for 20 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 70°C for 100 minutes. The sterilized VP30-EPS was stored at 4°C until used.

*2.4. Exopolysaccharide 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 (18,000 Í g) 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 minutes (18,000 Í g) to recover crude EPS. The obtained crude EPS was washed with 70% ethanol and centrifuged for 20 minutes (18,000 Í g). 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.

*2.5. 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 $H_2SO_4$ solution was added to the pEPS-phenol mixture and held at 90°C for 5 minutes. After lowering the sample temperature to 27°C, absorbance was measured at 490 nm.

*2.6. Animal and induction of constipation.*

Male Sprague-Dawley 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) once daily at the same time, with the exception of the normal group, which were not treated. VP30-EPS (1 g/kg) and pEPS (0.6 g/kg) were orally administered to the test groups twice a day for 3 days, after which all animals were sacrificed (Table 2). 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.

**Table 2** Animal design of VP30-EPS in loperamide-treated SD rats

| Group | Abbreviation | Treatment |
|---|---|---|
| Normal | NC | PBS |
| Loperamide | LP | 2 mg/kg |
| Loperamide + VP30-EPS | LP+VP30-EPS | 1 g/kg |
| Loperamide + purified EPS | LP+pEPS | 0.6 g/kg |

*2.7. 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.

*2.8. Intestinal transit ratio.*

Intestinal transit ratio was measured using a modified method from Jo et al. (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 minutes 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.

*2.9. 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; ThermoFisher Scientific, Waltham, MA, USA).

*2.10. 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 $p<0.05$.

# 3. Results And Discussion

*3.1 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 3.

**Table 3** 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.

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. (Shimotoyodomo et al. 2000) reported that loperamide administration induced abdominal distension and decreased food intake in experimental

animals. 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.

*3.2. 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 three 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 AQP3, 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 ± 0.22g/rat, $p<0.05$) (Table 4).

**Table 4** 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 | $3.19 \pm 0.44^{a}$ | $2.49 \pm 0.40^{a}$ | $22.05 \pm 3.15^{b}$ |
| LP | $2.36 \pm 0.22^{b}$ | $2.12 \pm 0.20^{a}$ | $9.95 \pm 3.97^{c}$ |
| LP+VP30-EPS | $3.31 \pm 0.42^{a}$ | $2.39 \pm 0.37^{a}$ | $27.97 \pm 4.47^{a}$ |
| LP+pEPS | $3.19 \pm 0.34^{a}$ | $2.17 \pm 0.31^{a}$ | $32.00 \pm 5.86^{a}$ |

$^{a-d}$ Mean values with different letters are significantly different ($p < 0.05$) according to Duncan's multiple range test.

However, when LP was administered to rats along with VP30-EPS or pEPS, the wet fecal weights were not different from that of the NC group (3.31 ± 0.42, 3.19 ± 0.34 and 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, 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 was 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 (Fig. 1 A). The pattern of third day data (the last day of experiment) for all

groups was consistent with the pattern of average feces numbers (Fig. 1 A and B). These results suggest that pEPS has a positive effect on stool frequency.

Zannini et al. reported that EPS produced by lactic acid bacteria attaches to intestinal mucosa to form an EPS biofilm, helping the adhesion and growth of lactic acid bacteria (Zannini 2015). In addition, it is reported that EPS produced by lactic acid bacteria 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.

*3.3. 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 were measured. There was no significant difference in the volume of feces in the intestine or intestinal length in all groups (Table 5, Fig. 2).

**Table 5** Effects of VP30-EPS or pEPS on transit ratio and intestine length in loperamide-treated SD rats

| | Transit ratio (%) | Small intestine length (cm) | Large intestine length (cm) |
|---|---|---|---|
| NC | 73.80±4.71$^{a}$ | 54.55±6.05$^{b}$ | 10.75±0.53$^{a}$ |
| LP | 62.10±5.13$^{b}$ | 59.69±4.41$^{b}$ | 10.80±1.70$^{a}$ |
| LP+VP30-EPS | 70.18±4.19$^{a}$ | 55.84±2.31$^{ab}$ | 11.60±0.70$^{a}$ |
| LP+pEPS | 68.52±2.62$^{a}$ | 59.00±5.18$^{a}$ | 12.34±0.35$^{a}$ |

$^{a, b}$ Mean values with different letters are significantly different ($p < 0.05$) according to Duncan's multiple range test.

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 level of the control group. Various research groups have reported a positive correlation between milk fermented by lactic acid bacteria and fecal transit ratios. Ge et al. reported that *L. acidophilus* and *B. bifidum* release neuro-messengers that promote intestinal motility (Ge et al. 2017). Milk fermented with *B. lactis* and *B. 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 s*pp. 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 lactic acid bacteria, or lactic acid bacterial EPS or microbial fermented foods can enhance *in vivo* host intestinal peristalsis (Table 6).

**Table 6** Constipation improvement efficacy reporting case of probiotics or produced EPS

| No | Species | Model (*in vitro*/ *in vivo*/ clinical study) | Dosing level (Stimulation reagent, experimental day) | Efficacy | Ref |
|---|---|---|---|---|---|
| 1 | *B. bifidum* G9-1 | *In vivo* (Sprague-Dawley rat) | 1.0 x $10^{10}$ CFU (Loperamide, 4 days) | 1. Fecal pellet number: 40 pellets/day (30 pellets/day of control) 2. Fecal water content: 60 % (50% of control) | Makzaki et al. 2021 |
| 2 | Mixture of *B. adolescentis* CCFM626, CCFM667, CCFM669 | *In vivo* (BALB/c mice) | 1.0 x $10^{10}$ CFU (Loperamide, 17 days) | 1. Fecal weight: 1 g (0.5 g of control) 2. Fecal moisture: 60% (about 40% of control) 3. Intestinal transit ratio: 100% (30% of control) | Wang et al. 2017 |
| 3 | *L. plantarum* NCU116 | *In vivo* (Kunming mice) | 1.0 x $10^{9}$ CFU (Loperamide, 15 days) | 1. Fecal pellet number: 15.22 pellets (8.78 pellets of control) 2. Fecal pellet weight: 0.41 g (0.21 g of control) 3. Fecal moisture: 49.86% (34.65% of control) 4. Intestinal transit ratio: 73.83% (50.40% of control) | Li et al. 2015 |
| 4 | *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) 2. Fecal pellet number: 40 pellets (22 pellets of control) 3. Fecal moisture: 45% (20% of control) 4. Intestinal transit ratio: 80.5% (25.2% of control) | Yi et al. 2019 |
| 5 | Mixture of *L. rhamnosus* CCFM1068, FFJND15-L2, FHeNJZ7-1, FTJDJ11-1, FZJHZ11-7 | *In vivo* (BALB/c mice) | 5 x $10^{9}$ CFU (Loperamide, 29 days) | 1. Fecal moisture: 60% (40% of control) 2. Intestinal transit ratio: 60% (40% of control) | Wang et al. 2020 |
| 6 | Heat-killed *L. plantarum* nF1 | *In vivo* (Sprague-Dawley rat) | 1.6 x $10^{11}$ CFU (Loperamide, 5 weeks) | 1. Fecal moisture: 90% (75% of control) 2. Intestinal transit ratio: 95% (55% of control) | Park et al. 2021 |
| 7 | *L. paracasei* NTU101 | *In vivo* (Sprague-Dawley rat) | 2.3 x $10^{10}$ CFU | 1. Fecal pellet number: 67.43 pellets (44.83 pellets of control) | Chen et al. 2020 |

| | | | | | |
|---|---|---|---|---|---|
| | | | (Loperamide, 20 days) | 2. Fecal weight: 14.05 g (10.63 g of control)<br>3. Fecal moisture: 47.54% (36.50% of control)<br>4. Intestinal transit ratio: 62.57% (47.43% of control) | |
| 8 | *L. plantarum* LRCC5193 | *In vivo* (Sprague-Dawley rat) | 2.5 x $10^{10}$ CFU<br>(Loperamide, 2 weeks) | 1. Fecal weight: 0.4 g (0.25 g of control)<br>2. Fecal moisture: 40% (25% of control)<br>3. Intestinal transit ratio: 60% (35% of control) | Eor et al. 2019 |
| 9 | Mixture of *B. subtilis* CBD-2, KMKW4 | *In vivo* (BALB/c mice) | 1.0 x $10^{7}$ CFU<br>(Loperamide, 2 weeks) | 1. Fecal pellet number: 15 pellets (10 pellets of control)<br>2. Fecal weight: 0.15 g (0.1 g of control)<br>3. Fecal moisture: 45% (35% of control)<br>4. Intestinal transit ratio: 50% (35% of control) | Kim et al. 2014 |
| 10 | *B. subtilis* CBD-2-fermented SD-P2A2 | *In vivo* (BALB/c mice) | 1.0 x $10^{4}$ CFU<br>(Loperamide, 2 weeks) | 1. Fecal pellet number: 15 pellets<br>(10 pellets of control)<br>2. Fecal weight: 0.15 g (0.10 g of control)<br>3. Fecal moisture: 55% (45% of control)<br>4. Intestinal transit ratio: 50% (40% of control) | Kim et al. 2016 |
| 11 | *L. kefiranofaciens* | *In vivo* (Sprague-Dawley rat) | 300 mg/kg of EPS<br>(low-fiber diet, 57 days) | 1. Fecal weight: 3 g (1g of control)<br>2. Fecal moisture: 55% (40% of control) | Maeda et al. 2004 |
| | | Clinical study | Functional constipation patient<br>(4 weeks) | 1. Stool frequency (before/after of administration): 2/5<br>2. Stool consistency (before/after of administration): 12/6 (hard); 6.5/12.5 (normal); 1/1 (Loose) | Turan et al. 2014 |
| 12 | *L. kefiranofaciens* DN1 | *In vivo* (BALB/c mice) | 2.0 x $10^{8}$ CFU | 1. Fecal weight: 0.194 g (0.150 g of control) | Jeong et al. 2017 |

| | | |
|---|---|---|
| | (2 weeks) | 2. Fecal moisture: 45.66% (28.99% of control) |

Specifically, Maeda et al. (2004) artificially induced constipation in Sprague-Dawley (SD) rats by treatment with a low-fiber diet and orally administered EPS isolated from *L. 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. 2004). 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 Í $10^8$ 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). The 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.

*3.4. 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 (Table 7).

**Table 7** Effects of VP30-EPS or pEPS on ALT, AST in loperamide-treated SD rats

| Group | ALT (U/L) | AST (U/L) |
|---|---|---|
| NC | 31.00±9.37[a] | 108.13±17.67[b] |
| LP | 30.58±7.51[a] | 130.40±13.83[a] |
| LP+VP30-EPS | 25.90±6.15[a] | 102.14±9.30[b] |
| LP+VP30 EPS | 27.50±5.28[a] | 94.14±11.41[b] |

[a–c] Mean values with different letters are significantly different ($p < 0.05$) according to Duncan's multiple range test.

Serum AST was statistically increased by LP treatment, but both VP30-EPS and pEPS maintained serum AST levels within the normal range. 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).

# 4. 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.

# Declarations

### Ethics approval and consent to participate

Not applicable.


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### Competing interests

The author have no relevant financial or non-financial interests to disclose.


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### Authors' contributions

SHP, MRL, BK, HJK and MSP were involved in the conception, design and writing of the manuscript. Experiments were designed by YSY, LJY, LHH and performed by SHP, MRL, BK and HJ. SHP, MRL, YJS and TVJ performed the data analysis and interpretation of the results. The later modified as per corresponding author's comments on previous versions of the manuscript. SK and MSP were revised the manuscript (co-corresponding author). All authors read and approved the final manuscript.

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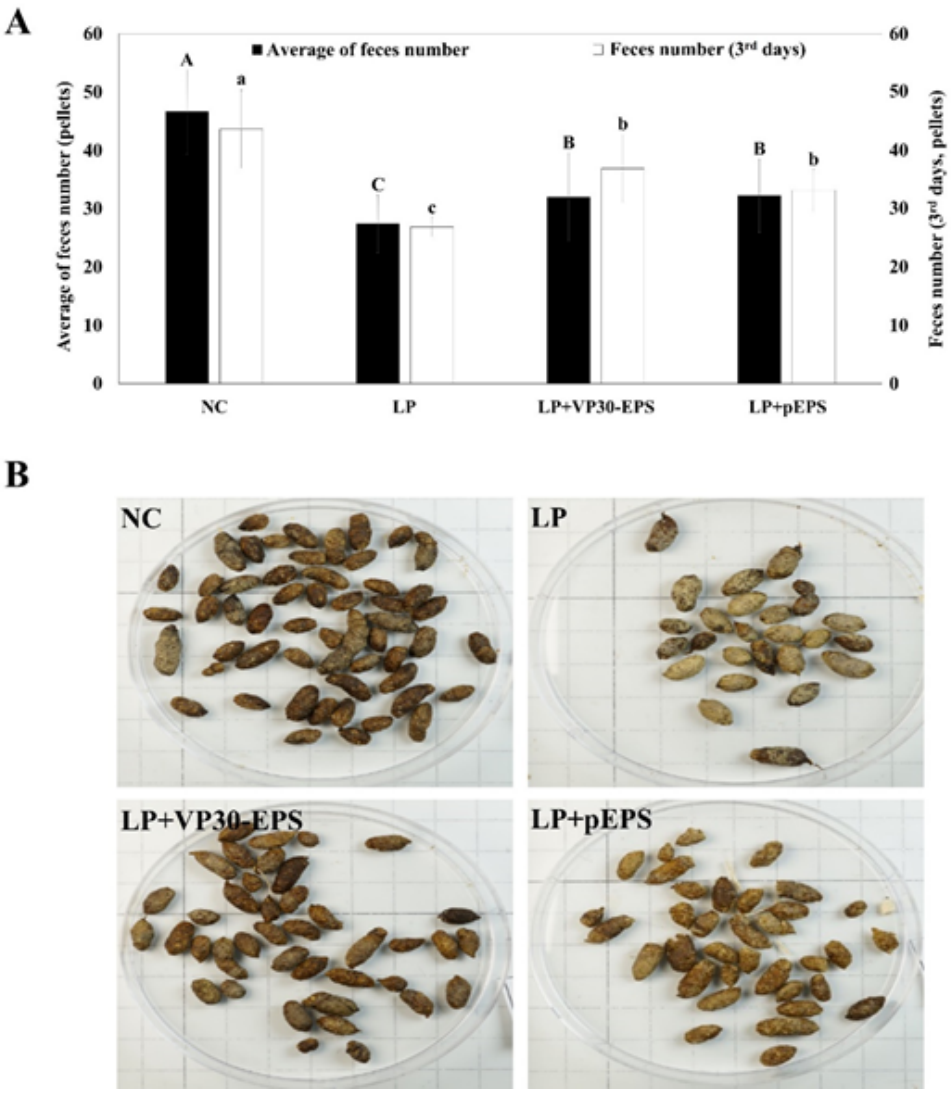
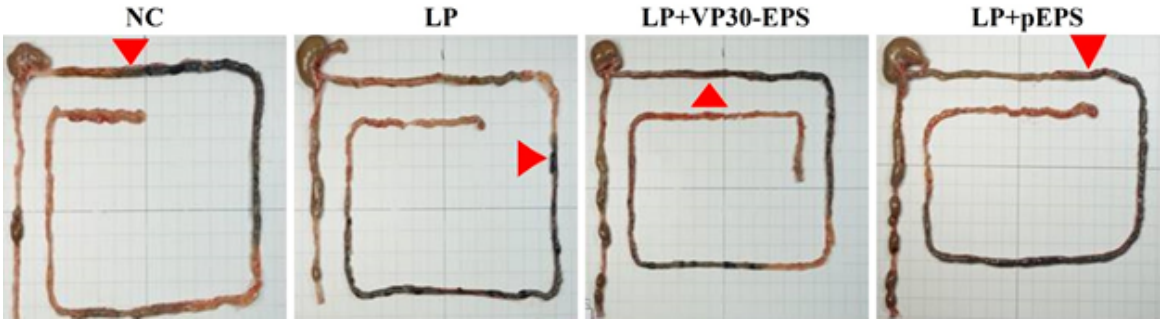
# Figures




## Figure 1

Effects of VP30-EPS or pEPS on feces number in loperamide-induced rats. (A) Average of feces number and feces number depending on VP30-EPS or pEPS administration for 3 days, and (B) observation of feces depending on VP30-EPS or pEPS administration for 3 days. NC: normal group, LP: loperamide treated group (2 mg/kg), LP+VP30-EPS: loperamide (2 mg/kg) and fermented milk from *W. confusa* VP30 (1 g/kg) treated group, LP+pEPS: loperamide (2 mg/kg) and pEPS (0.6 g/kg). Values with different superscript letters are significantly different ($p<0.05$) by Duncan's multiple range test using SPSS software. Mean values with different letters over bars are significantly different ($p < 0.05$) according to Duncan's multiple range test.



## Figure 2

Effects of VP30-EPS or pEPS on transit ratio in loperamide-induced rats. These images of excised intestinal tracts show charcoal meal transit through the intestine. Red arrows indicate charcoal meal transit distance.