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Coadministration of Lactulose with Probiotics Ameliorates Loperamide-Induced Constipation in Mice

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ABSTRACT: We evaluated the efficacy of mixtures of lactulose with probiotic strains to ameliorate constipation and to identify suitable probiotic strains. Constipation was induced in Institute of Cancer Research mice (6-week-old, male) by the administering loperamide (5 mg/kg, twice a day) orally for 5 days, whereas the control group was not treated. To evaluate the laxative effects of the lactulose-probiotic and lactulose-magnesium hydroxide mixtures, fecal parameters, the gastrointestinal (GI) transit ratio, and fecal short-chain fatty acid (SCFA) content were analyzed. The administration of lactulose and *Bacillus licheniformis* or *Saccharomyces boulardii* significantly improved stool number and water content, which were reduced by loperamide. The GI transit ratio was significantly increased compared with that of the control group. The combined administration of lactulose and probiotics (*B. licheniformis* or *S. boulardii*) increased total SCFA content, including that of acetate, more effectively compared with lactulose alone. Similarly, coadministration of lactulose and magnesium hydroxide improved the loperamide-induced changes in fecal parameters and GI transit as well as increased total SCFA content. Overall, the combination of lactulose and probiotics relieves the symptoms of constipation by increasing SCFA content and is more effective compared with lactulose alone.

Keywords: *Bacillus licheniformis*, constipation, lactulose, magnesium hydroxide, *Saccharomyces boulardii*

INTRODUCTION

Constipation is a common gastrointestinal (GI) disease that occurs worldwide. In Korea, it has a prevalence of 16.5% (Cho et al., 2023). Constipation results in bowel movements that occur less than three times a week. It is associated with difficulty in passing stools because of it being hard and it causes abdominal pain and bloating (Milosavljevic et al., 2022). Various dietary treatments, such as exercise or the consumption of rich dietary fiber, are used to ameliorate constipation. If constipation cannot be improved with dietary interventions, laxatives are administered in parallel. Lactulose, sorbitol, and polyethylene glycol (PEG) are hyperosmotic laxatives. Lactulose, which is a disaccharide composed of galactose and fructose, is a representative laxative (Karakan et al., 2021). It is not absorbed into the small intestine and increases osmotic action to soften stools and facilitate defecation (Zhao et al., 2021). Furthermore, unabsorbed lactulose is metabolized by intestinal bacteria and fermented into acetate and lactate, which lowers intestinal pH and enhances peristalsis (Zhang et al., 2021). Saline laxatives, such as magnesium hydroxide, are osmotic laxatives that promote water absorption in the intestinal tract to facilitate bowel movements (Shin et al., 2015). Lactulose syrup can be safely administered over a long period of time at doses of 10∼15 mL/d in adults; however, a limitation of lactulose is that it requires a high dose, but its efficacy is similar to or lower compared with that of other agents. Therefore, it is necessary to identify new compounds that can be used in combination with lactulose to increase its effectiveness.

Recently, probiotics have become increasingly used to improve intestinal health (Roobab et al., 2020; Li et al., 2023). They promote intestinal motility by regulating the concentration of neurotransmitters and short-chain fatty acids (SCFAs), and improving gut health by regulating the gut microbiome (Wang et al., 2017). In particular, SCFAs produced by intestinal bacteria are considered metabolites that are involved in improving intestinal health. They are major energy sources for intestinal epithelial cells and

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influence the intestinal environment by regulating intestinal pH (Martin-Gallausiaux et al., 2021). Certain probiotic strains are also used to treat intestinal disorders (Dimidi et al., 2020). In the present study, specific probiotic strains (*Lactobacillus*, *Bacillus*, and *Saccharomyces* genera) used to treat constipation were evaluated in combination with lactulose. *Lactobacillus helveticus* produces bioactive peptides with anti-inflammatory, antioxidant, and antithrombotic properties and reduces fecal pH (Chelladhurai et al., 2023). Wang et al. (2020) demonstrated that *Lactobacillus rhamnosus* alleviates constipation by restoring intestinal neurotransmitter levels and promoting GI motility. *Bacillus licheniformis* inhibits the growth of pathogenic microorganisms, regulates the immune system, and improves various diseases, such as GI diseases, diabetes, and obesity. Additionally, *Saccharomyces boulardiid* is an acid- and heat-resistant probiotic that exerts health benefits, such as producing antibacterial compounds, suppressing oxidative stress, and improving intestinal microbial dysbiosis (Ansari et al., 2023).

In this study, we determined the effects of administration of mixtures of lactulose and probiotic strains or magnesium hydroxide using a mouse model of loperamide-induced constipation. The effect of the combination of lactulose and probiotics on the intestinal environment was assessed by analyzing fecal parameters, GI transit rate, and SCFA content of cecal feces in a loperide-induced mouse model.

MATERIALS AND METHODS

Sample preparation

Lactulose syrup and the four probiotic strains were provided by the JW Pharmaceutical Corporation. The probiotic strains included probiotic 1 (*Bacillus subtilis* and *Enterococcus faecium*), probiotic 2 (*L. rhamnosus* R0011 and *L. helveticus* R0052), probiotic 3 (*S. boulardii*), and probiotic 4 (*B. licheniformis*). Magnesium hydroxide [$Mg(OH)_2$] was purchased from Sigma-Aldrich.

Animals

Institute of Cancer Research (ICR) mice (6-week-old, male) were purchased from Orient Bio and maintained at room temperature (23±2°C) with 55±5% relative humidity and a 12 h light/12 h dark cycle. The mice were provided food and water *ad libitum*. All animal experiments were approved by the Animal Research Working Committee of Korea University (approval number: KUIACUC-2022-0059; approval date: 07-11-2022).

Experimental groups

After a 1-week adaptation period, the mice were randomly distributed into nine experimental groups with six animals per group (two mice/cage) as follows: NOR (normal group, 0.9% saline), CON (loperamide-control group, 0.9% saline), LAC (3 mL/kg of lactulose), PB1 (3 mL/kg of lactulose+6×10^8 CFU/kg/d probiotics 1), PB2 (3 mL/kg of lactulose+8×10^8 CFU/kg/d probiotics 2), PB3 (3 mL/kg of lactulose+2×10^9 CFU/kg/d of probiotics 3), PB4 (3 mL/kg of lactulose+3×10^8 CFU/kg/d of probiotics 4), Mg-L [100 mg/kg of $Mg(OH)_2$+3 mL/kg of lactulose], Mg-H [200 mg/kg of $Mg(OH)_2$+3 mL/kg of lactulose]. Based on a previous method (Hayeeawaema et al., 2020), all experimental groups, except the NOR group, were administered loperamide (5 mg/kg) orally twice a day for 5 days to induce constipation. After the induction of constipation, the sample was orally administered once daily for 2 weeks.

Measurement of body and organ weight

The body weight of the mice was measured once per week. At the end of the experiment, the animals were sacrificed by CO_2 inhalation anesthesia and the weight of the liver, heart, kidney, spleen, and total intestine was measured. Total intestinal weight was measured by collecting intestines containing feces from the duodenum to the rectum (Park et al., 2007).

Fecal parameters

To evaluate the induction of constipation, the number, weight, and water content of the feces were measured 5 days following loperamide administration. During sample administration period, feces were collected once a week at a fixed time, and the number, weight, and water content were measured (Kim et al., 2020). The wet weight of the feces was measured immediately after collection. To measure water content, the feces were dried at 70°C for 24 h and the dry weight was measured. Fecal water content was calculated using the following formula:

$$\text{Fecal water content (\%)} = [(\text{wet weight of feces} - \text{dry weight of feces})]/(\text{wet weight of feces})] \times 100$$

Measurement of the intestinal transit time using eosin solution

Intestinal transit time was measured using a 5% eosin solution (Sigma-Aldrich) (He et al., 2022). On the 10th day of sample administration, the time to the first red fecal defecation following the oral administration of an eosin solution was measured. Feces were also collected 6 h after oral administration of the eosin solution and the number of red stools was confirmed over 6 h.

GI transit ratio

Changes in the GI transit ratio following sample administration were measured using activated carbon (Kim et

al., 2020). After fasting for 18 h, the mice were administered an activated carbon solution (5% activated carbon in 0.5% carboxymethyl cellulose solution) orally. After 30 min, the mice were sacrificed and the GI transit rate was calculated using the following formula:

GI transit rate (%)
=(distance traveled by activated charcoal)/(total length of the GI tract)×100

SCFA analysis

The SCFA content of feces in the cecum was measured by gas chromatography (GC). Following sacrifice, the cecum was extracted. Next, 100 mg of feces in the cecum was collected, extracted using 0.8 mL of 80% methanol, and filtered through a 0.45 μm filter (Millipore). Acetic acid, propionic acid, butyric acid, and valeric acid were used as standards, and 2-ethylbutyric acid was used as an internal standard. All of the compounds were purchased from Sigma-Aldrich. A GC instrument (GC 7890, Agilent) equipped with a flame ionization detector and a DB-FFAP 123-3253 Column (50 m×0.32 mm×0.50 μm; Agilent) was used for SCFA determination based on as previously described (Jang et al., 2020).

Statistical analysis

The results of the animal experiments are presented as the mean±standard error of the mean. Statistical significance was determined using IBM SPSS Statistics ver. 23.0 (IBM Corp.) and an analysis of variance. The difference between experimental groups was tested for significance at $P<0.05$ using Tukey's test.

RESULTS

Effect of lactulose mixture on body and organ weight

The effects of the lactulose combination on body and organ weights were determined using a loperamide-induced constipation model. For all loperamide-treated groups, body weight tended to decrease slightly compared with that in the NOR group; however, no significant differences between the experimental groups were observed (Table 1). No significant differences were observed in the weights of the liver, heart, kidney, spleen, and intestine in any of the experimental groups and no toxicity was evident in the liver or kidney, even after 2 weeks of sample administration (Table 1). Therefore, treatment with lactulose alone or in combination for 2 weeks did not result in changes in body or organ weight.

Effect of lactulose mixture on fecal parameters

After the 2nd week of sample administration, feces were collected and fecal parameters (number of stools, weight, and water content) were measured (Fig. 1). The number of stools ($P<0.001$; Fig. 1A) and water content ($P<0.001$; Fig. 1C) were significantly decreased in the CON group, which was only administered loperamide, compared with that in the NOR group. Moreover, the weight of feces in the CON group tended to decrease compared with that in the NOR group, although not significantly (Fig. 1B). Thus, loperamide administration was effective at inducing constipation in mice.

The number of stools in the LAC group administered lactulose alone was 67.3±2.8 feces/d, which was significantly higher compared with that in the CON group (44.3 ±2.1 feces/d) ($P<0.05$; Fig. 1A). Fecal water content was also significantly higher in the LAC group compared with

Table 1. Effects of lactulose-probiotics and lactulose-magnesium hydroxide mixtures on body and organ weight in loperamide-induced mice

Group	Body weight (g)	Organ weight (g/100 g of body)				
		Liver	Heart	Kidney	Spleen	Intestinal
NOR	35.07±1.27^{ns}	5.45±0.24^{ns}	0.65±0.04^{ns}	2.10±0.13^{ns}	0.27±0.02^{ns}	7.96±0.29^{ns}
CON	33.45±0.58	5.69±0.23	0.66±0.03	2.11±0.09	0.32±0.01	8.17±0.29
LAC	33.67±0.42	6.23±0.41	0.65±0.02	2.14±0.03	0.36±0.02	8.08±0.52
PB1	34.40±0.64	5.23±0.16	0.56±0.02	1.76±0.11	0.30±0.02	7.72±0.25
PB2	34.12±0.44	5.13±0.13	0.57±0.02	1.87±0.12	0.29±0.02	7.90±0.27
PB3	32.48±0.79	4.92±0.30	0.55±0.04	1.84±0.06	0.29±0.02	7.54±0.17
PB4	33.09±0.65	5.33±0.22	0.58±0.03	1.83±0.07	0.34±0.03	7.36±0.07
Mg-L	33.90±0.45	5.07±0.15	0.60±0.03	1.93±0.07	0.31±0.02	7.45±0.08
Mg-H	33.63±0.51	4.69±0.21	0.61±0.04	1.86±0.09	0.35±0.02	6.79±0.08

Values are presented mean±SEM (n=6). ns, not significant.
NOR: normal group (0.9% saline), CON: loperamide-control group (0.9% saline), LAC: lactulose (3 mL/kg), PB1: lactulose 3 mL/kg+probiotic 1 (*Bacillus subtilis* and *Enterococcus faecium*, 6×10^8 CFU/kg/d), PB2: lactulose 3 mL/kg+probiotic 2 (*Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052, 8×10^8 CFU/kg/d), PB3: lactulose 3 mL/kg+probiotic 3 (*Saccharomyces boulardii*, 2×10^9 CFU/kg/d), PB4: lactulose 3 mL/kg+probiotic 4 (*Bacillus licheniformis*, 3×10^8 CFU/kg/d), Mg-L: lactulose 3 mL/kg+$Mg(OH)_2$ (100 mg/kg), Mg-H: lactulose 3 mL/kg+$Mg(OH)_2$ (200 mg/kg).
In all experimental groups except the NOR group, constipation was induced by administering loperamide (5 mg/kg) orally twice a day.

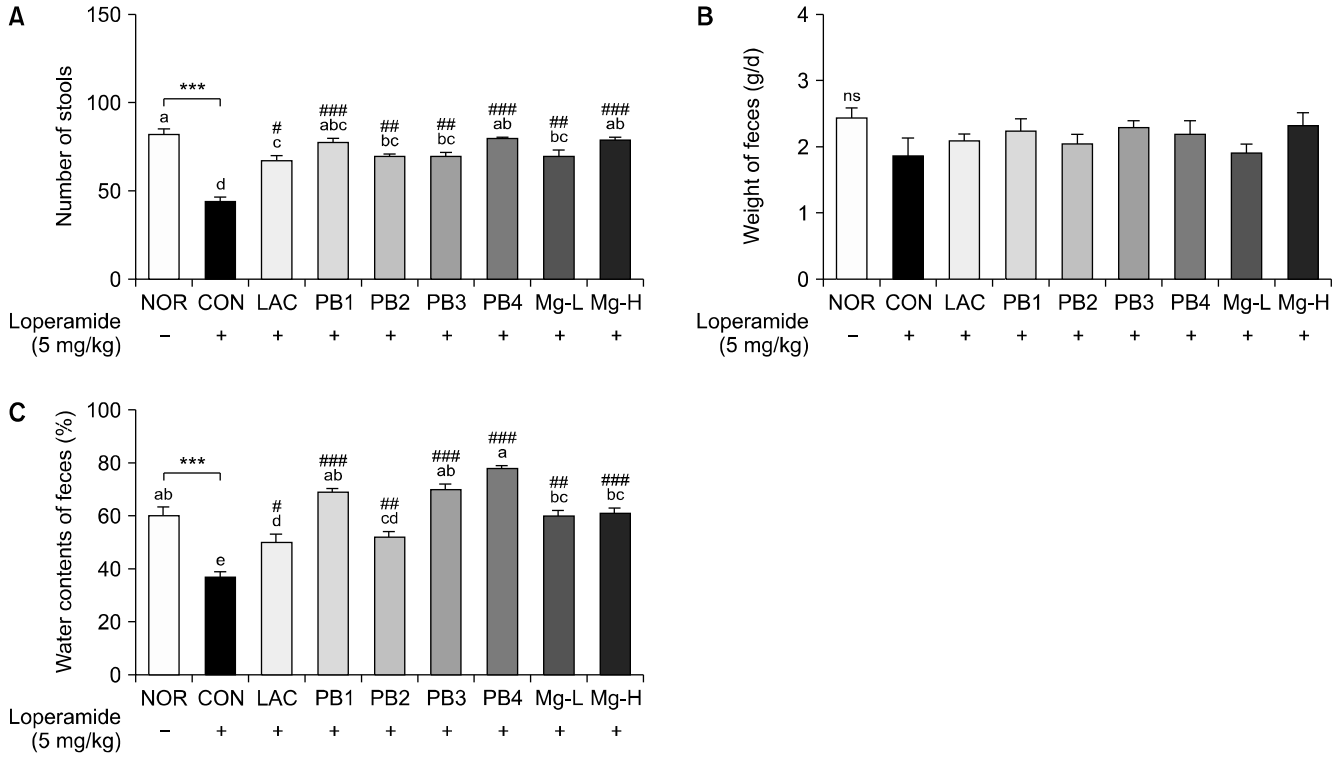

Fig. 1. Effect of lactulose-probiotics and lactulose-magnesium hydroxide mixtures on (A) number of stools, and (B) weight and (C) water content of feces in mice with loperamide-induced constipation at 2 weeks. Data are presented as mean±SEM (n=6). NOR: normal group (0.9% saline), CON: loperamide-control group (0.9% saline), LAC: lactulose (3 mL/kg), PB1: lactulose 3 mL/kg+probiotic 1 (*Bacillus subtilis* and *Enterococcus faecium*, 6×10^8 CFU/kg/d), PB2: lactulose 3 mL/kg+probiotic 2 (*Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052, 8×10^8 CFU/kg/d), PB3: lactulose 3 mL/kg+probiotic 3 (*Saccharomyces boulardii*, 2×10^9 CFU/kg/d), PB4: lactulose 3 mL/kg+probiotic 4 (*Bacillus licheniformis*, 3×10^8 CFU/kg/d), Mg-L: lactulose 3 mL/kg+$Mg(OH)_2$ (100 mg/kg), Mg-H: lactulose 3 mL/kg+$Mg(OH)_2$ (200 mg/kg). In all experimental groups, except the NOR group, constipation was induced by the oral administration of loperamide (5 mg/kg) twice a day. Different symbols indicate significance at $^{***}P<0.001$ vs. NOR group, and $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ vs. CON group, according to Tukey's test. Different letters (a-d) represent significant differences ($P<0.05$) among experimental groups as assessed by Tukey's multiple range test. ns, not significant.

that in the CON group ($P<0.05$; Fig. 1C). The groups treated with lactulose and the probiotic combination (PB1, PB2, PB3, and PB4) exhibited a significant increase in the number of stools compared with the CON group ($P<0.01$ and $P<0.001$, respectively; Fig. 1A). The PB1 (77.7±2.2 feces/d) and PB4 (80.0±0.6 feces/d) groups were associated with a high frequency of defecation. The groups treated with lactulose and probiotic mixtures exhibited significantly improved fecal water content compared with the CON group (37.0±1.8%), whereas the PB4 group had the highest water content (78.0±1.2%). In the lactulose and $Mg(OH)_2$ treatment group, the number of stools and water content of the feces was increased in a dose-dependent manner compared with those in the CON group ($P<0.01$ and $P<0.001$, respectively). The number of stools and water content was improved when probiotics or $Mg(OH)_2$ were administered in combination with lactulose ($P<0.05$).

Effect of lactulose mixture on the intestinal transit time as assessed using eosin solution

From the time of the first red stool to defecation following the administration of a 5% eosin solution, the intestinal transit time was significantly increased in the CON group (197.3±12.6 min) compared with that in the NOR group (131.6±9.2 min) ($P<0.05$; Fig. 2A). In the lactulose combination groups, the red stool defecation time tended to decrease compared with that in the CON and lactulose alone (LAC) group. In particular, the PB3 group (141.0±14.9 min) exhbited a significant decrease compared with that of the CON group ($P<0.05$; Fig. 2A).

The number of red stools excreted 6 h after eosin treatment tended to decrease in the CON group compared with that in the NOR group, but there was no significant difference (Fig. 2B). The LAC and probiotic mixed groups (PB1, PB2, and PB4) showed a tendency of an increased number of red stools compared with the CON group. The number of red stool samples in the PB3 group was significantly higher compared with that in the CON group ($P<0.05$). Even in the high-dose $Mg(OH)_2$ mixed administration group (Mg-H), the number of red feces was significantly higher compared with that in the CON group ($P<0.001$; Fig. 2B).

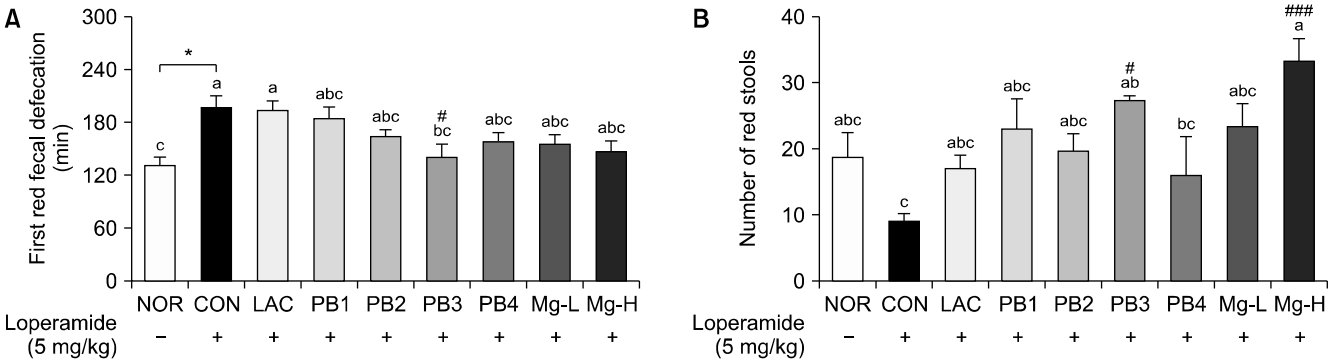

Fig. 2. Effect of lactulose-probiotics and lactulose-magnesium hydroxide mixtures on (A) time to the first red fecal defecation and (B) number of red stools in mice with loperamide-induced constipation. Data are presented as mean±SEM (n=6). NOR: normal group (0.9% saline), CON: loperamide-control group (0.9% saline), LAC: lactulose (3 mL/kg), PB1: lactulose 3 mL/kg+probiotic 1 (*Bacillus subtilis* and *Enterococcus faecium*, 6×10^8 CFU/kg/d), PB2: lactulose 3 mL/kg+probiotic 2 (*Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052, 8×10^8 CFU/kg/d), PB3: lactulose 3 mL/kg+probiotic 3 (*Saccharomyces boulardii*, 2×10^9 CFU/kg/d), PB4: lactulose 3 mL/kg+probiotic 4 (*Bacillus licheniformis*, 3×10^8 CFU/kg/d), Mg-L: lactulose 3 mL/kg+$Mg(OH)_2$ (100 mg/kg), Mg-H: lactulose 3 mL/kg+$Mg(OH)_2$ (200 mg/kg). In all experimental groups except the NOR group, constipation was induced by the oral administration of loperamide (5 mg/kg) twice a day. Different symbols indicate significance at $^*P<0.05$ vs. NOR group, and $^{\#}P<0.05$, and $^{\#\#\#}P<0.001$ vs. CON group according to Tukey's test. Different letters (a-c) indicate significant differences ($P<0.05$) among experimental groups as assessed using the Tukey's multiple range test.

Effect of lactulose mixtures on the GI transit ratio

To determine the effect of lactulose mixtures on intestinal motility, the GI transit rate was measured using activated charcoal (Fig. 3). Loperamide treatment of the CON group (34.6±1.5%) significantly decreased the GI transit rate compared with that in the NOR group (57.4±4.9%) ($P<0.05$; Fig. 3). The LAC group (51.6±6.2%) exhibited an increased GI transit rate compared with the CON group; however, the difference was insignificant. The groups treated with the lactulose and probiotic combinations (PB2: 61.8±4.9%; PB3: 69.3±2.0%; PB4: 72.7±5.2%) exhibited a significant improvement in GI transit ratio compared with the CON group ($P<0.01$ and $P<0.001$, respectively). Lactulose plus $Mg(OH)_2$ significantly increased the GI transit ratio in a dose-dependent manner (Mg-L: $P<0.05$; Mg-H: $P<0.001$). Mixtures with probiotics or $Mg(OH)_2$ also significantly improved the GI transit rate, which was reduced by loperamide compared with lactulose treatment alone.

Effect of lactulose mixture on SCFA production

GC was used to determine effect of the lactulose mixture on the production of SCFAs (Fig. 4). The CON group exhibited significantly lower butyric acid ($P<0.001$) and total SCFA ($P<0.05$) content compared with the NOR group (Fig. 4B and 4E). Acetic acid and valeric acid content tended to decrease in the CON group compared with that in the NOR group, but the difference was not significant (Fig. 4A and 4D). The acetic acid content of the PB3 ($P<0.05$) and PB4 ($P<0.01$) groups was significantly increased compared with that in the CON group and was higher compared with that in the LAC group (Fig. 4A). The Mg-H group showed a significantly higher acetic acid content compared with the CON group, which was the highest among all of the experimental groups ($P<0.001$). The butyric acid content increased significantly in the PB4 group compared with that in the CON group ($P<0.001$; Fig. 4B) and valeric acid content increased significantly in the PB3 group ($P<0.05$; Fig. 4D); however, there was no significant difference in propionic acid con-

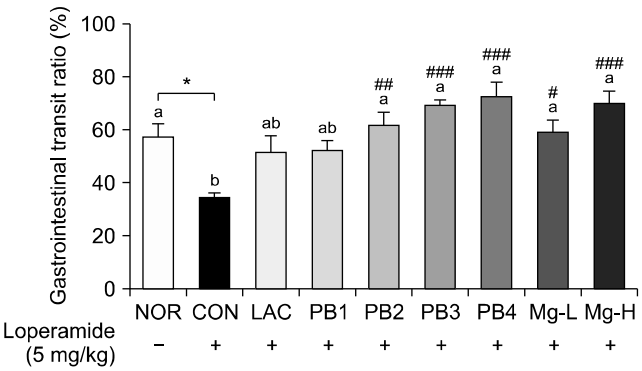

Fig. 3. Effect of lactulose-probiotics and lactulose-magnesium hydroxide mixtures on the gastrointestinal transit ratio in mice with loperamide-induced constipation. Data are presented as mean±SEM (n=6). NOR: normal group (0.9% saline), CON: loperamide-control group (0.9% saline), LAC: lactulose (3 mL/kg), PB1: lactulose 3 mL/kg+probiotic 1 (*Bacillus subtilis* and *Enterococcus faecium*, 6×10^8 CFU/kg/d), PB2: lactulose 3 mL/kg+probiotic 2 (*Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052, 8×10^8 CFU/kg/d), PB3: lactulose 3 mL/kg+probiotic 3 (*Saccharomyces boulardii*, 2×10^9 CFU/kg/d), PB4: lactulose 3 mL/kg+probiotic 4 (*Bacillus licheniformis*, 3×10^8 CFU/kg/d), Mg-L: lactulose 3 mL/kg+$Mg(OH)_2$ (100 mg/kg), Mg-H: lactulose 3 mL/kg+$Mg(OH)_2$ (200 mg/kg). In all experimental groups except the NOR group, constipation was induced by the oral administration of loperamide (5 mg/kg) twice a day. Different symbols indicate significance at $^*P<0.05$ vs. NOR group, and $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ vs. CON group according to Tukey's test. Different letters (a,b) indicate significant differences ($P<0.05$) among experimental groups as assessed using Tukey's multiple range test.

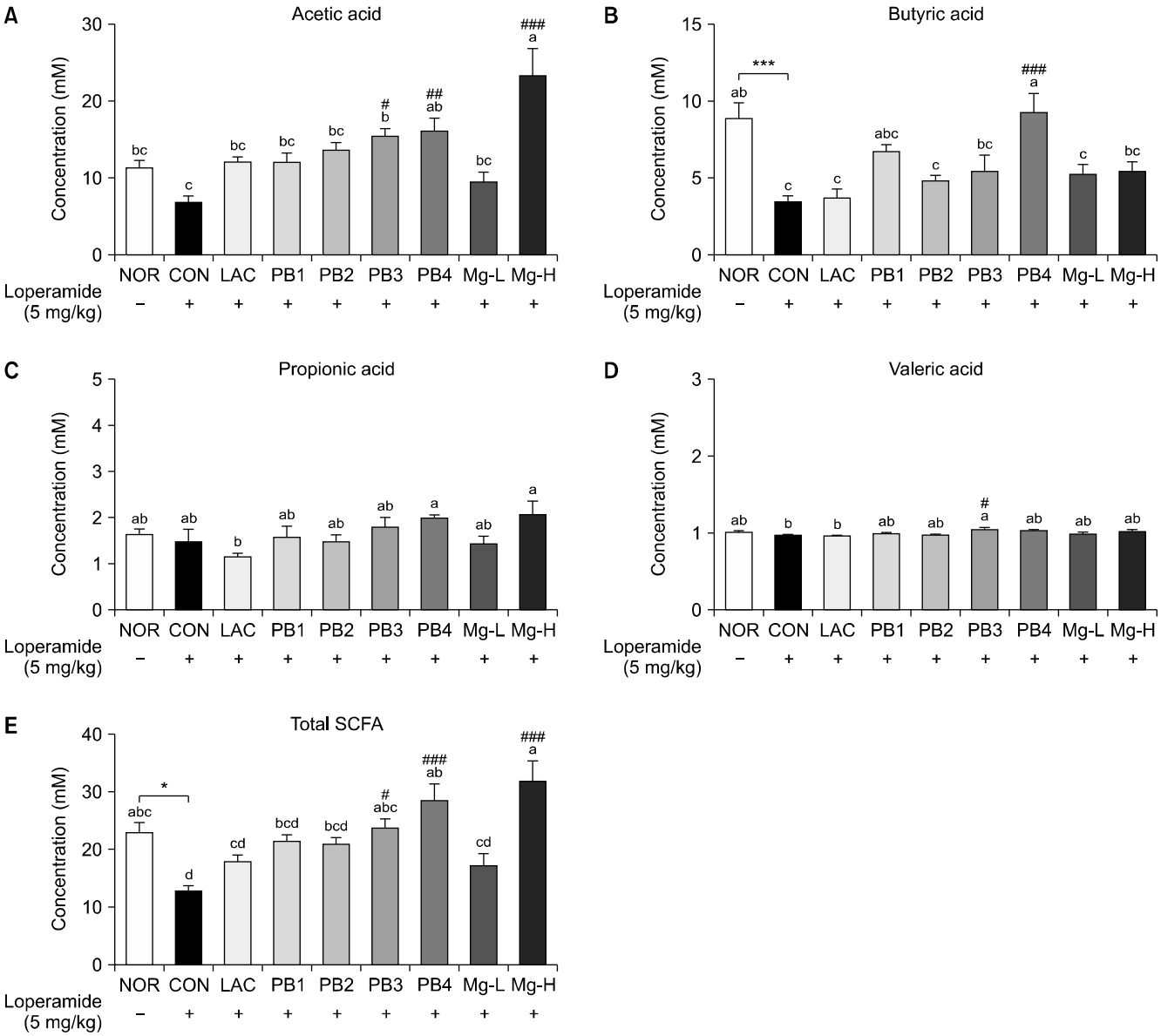

Fig. 4. Effect of lactulose-probiotics and lactulose-magnesium hydroxide mixtures on (A) acetic acid, (B) butyric acid, (C) propionic acid, (D) valeric acid, and (E) total short-chain fatty acid (SCFA) production in mice with loperamide-induced constipation. Data are presented as mean±SEM (n=6). NOR: normal group (0.9% saline), CON: loperamide-control group (0.9% saline), LAC: lactulose (3 mL/kg), PB1: lactulose 3 mL/kg+probiotic 1 (*Bacillus subtilis* and *Enterococcus faecium*, 6×10^8 CFU/kg/d), PB2: lactulose 3 mL/kg+probiotic 2 (*Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052, 8×10^8 CFU/kg/d), PB3: lactulose 3 mL/kg+probiotic 3 (*Saccharomyces boulardii*, 2×10^9 CFU/kg/d), PB4: lactulose 3 mL/kg+probiotic 4 (*Bacillus licheniformis*, 3×10^8 CFU/kg/d), Mg-L: lactulose 3 mL/kg+$Mg(OH)_2$ (100 mg/kg), Mg-H: lactulose 3 mL/kg+$Mg(OH)_2$ (200 mg/kg). In all experimental groups except the NOR group, constipation was induced by the oral administration of loperamide (5 mg/kg) twice a day. Different symbols represent significance at $^*P<0.05$, and $^{***}P<0.001$ vs. NOR group, and $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ vs. CON group according to Tukey's test. Different letters (a-d) indicate significant differences ($P<0.05$) among experimental groups as assessed using Tukey's multiple range test.

tent among the groups (Fig. 4C). Total SCFA content in the PB3 ($P<0.05$), PB4 ($P<0.001$), and Mg-H ($P<0.001$) groups was significantly higher compared with that in the CON group (Fig. 4E). In particular, the PB4 and Mg-H groups exhibited significantly higher SCFA content compared with the LAC group ($P<0.05$).

DISCUSSION

A decrease in the moisture content of feces is a characteristic feature of constipation, which disrupts the passage of feces through the intestines (Forootan et al., 2018). Loperamide is an antidiarrheal agent that is commonly used to induce constipation in animal models (Kim et al., 2017; Zhang et al., 2021). This drug modulates the transport of water and electrolytes into the GI tract, thus inhibiting water secretion and decreasing fecal water con-

tent (Regnard et al., 2011). In the present study, the water content of the feces was reduced by loperamide and ameliorated by administering lactulose alone or a lactulose mixture (Fig. 1). Moreover, loperamide is a μ-opioid receptor agonist, which acts on the intestinal muscle plexus to reduce peristalsis and increase intestinal transit time (Holzer, 2009).

Intestinal metabolites produced by the fermentation of intestinal bacteria are closely related to constipation. Patients with constipation exhibit a decrease in SCFA levels, including acetate, propionate, and butyrate, compared with normal individuals (Shi et al., 2016). In particular, butyrate regulates intestinal peristalsis and maintains the integrity of the intestinal mucosa (Morrison and Preston, 2016). In the present study, the reduction of butyrate and total SCFA content by loperamide was improved by the combination of lactulose and *B. licheniformis* (Fig. 4). Similarly, the administration of *B. licheniformis* alleviated chronic stress-induced gut microbiome dysbiosis, increased the abundance of butyrate-producing strains, and ameliorated inflammation through branched SCFA production (Feng et al., 2022). Additionally, a mixture of lactulose and galactose-oligosaccharide ameliorated loperamide-induced constipation by increasing the levels of the beneficial bacteria bifidobacteria and by enhancing the production of SCFAs in the intestine (Kwon et al., 2018). SCFAs also promote the production of 5-hydroxytryptamine (5-HT), which regulates intestinal motility in the colon (Reigstad et al., 2015). The administration of *L. rhamnosus* increases 5-HT production and to accelerate the intestinal transit time by modulating the secretion of GI regulatory hormones (Wang et al., 2020).

Lactulose is a hyperosmotic laxative that is not absorbed into the blood and does not alterblood sugar levels. Therefore, it is used to treat constipation in patients with diabetes, the elderly, and children (Prasad and Abraham, 2017). Lactulose is safe even when consumed over a long period; thus, it is often used as an initial treatment for constipation (Panesar and Kumari, 2011). Although the main mechanism underlying lactulose action is unclear, it increases the water content of materials in the large intestine by increasing the osmotic pressure (Lee-Robichaud et al., 2010). In the present study, lactulose treatment alone improved the water content and the number of stools in a loperamide-induced constipation model (Fig. 1). Lactulose promotes excretion resulting from an increase in the volume of stool and is fermented into acetic acid and lactic acid in the large intestine, which lowers the intestinal pH over several hours. Hydrogen and carbon dioxide that is subsequently generated promotes intestinal peristalsis (Ruszkowski and Witkowski, 2019). Magnesium hydroxide, which is also used as an osmotic laxative, promotes defecation by maintaining osmotic pressure without being absorbed into the large intestine (Maheshwari and Sood, 2022); however, the excessive use of magnesium causes hypermagnesemia. Therefore, it is not used in patients with renal failure and caution is must be used when taking it (Liu, 2011). Combining magnesium hydroxide with another laxative may improve these side effects by reducing the dose of both laxatives. Shin et al. (2010) found that the combined administration of magnesium hydroxide and PEG increased bowel clearance compared with PEG treatment alone during colonoscopy in patients with constipation. Similarly, the combination of magnesium hydroxide and lactulose significantly improved stool parameters and increased total SCFA production, including acetic acid, compared with lactulose alone (Fig. 1 and 4).

Various probiotic strains, such as *Bacillus*, *Lactobacillus*, and *Saccharomyces* spp., have been used in the search for strains that can be administered in combination with lactulose. *B. licheniformis* is a safe probiotic that inhibits the growth of pathogenic bacteria, is resistant to heat and acids, and exhibits antioxidant effects (Zeng et al., 2022). In the present study, the combination of *B. licheniformis* and lactulose ameliorated constipation symptoms by increasing the GI transit rate and improving fecal parameters. *Lactobacillus* sp. is a widely used probiotic strain and the consumption of a probiotic drink containing *Lactobacillus casei* can relieve constipation symptoms in patients with slow-transit constipation by reducing colonic transit time (Krammer et al., 2011). Li et al. (2015) demonstrated that the administration of *Lactobacillus plantarum* NCU116 improved fecal parameters and SCFA content in a loperamide-induced mouse model. Similarly, the combination of *L. rhamnosus* R0011 and lactulose significantly improved stool parameters and the GI transit ratio, which was reduced by loperamide.

S. boulardii is a live yeast and a probiotic strain that is widely used for the prevention and treatment of GI disorders (McFarland, 2017). *S. boulardi* exhibits antibacterial and antitoxic effects, and improves the barrier function of the GI tract by ameliorating the inflammatory response of the intestinal mucosa (Kelesidis and Pothoulakis, 2012; Pais et al., 2020). *S. boulardii* produces large amounts of acetic acid, a major SCFA in intestinal epithelial cells, which regulates intestinal pH and improves inflammatory and immune responses (Offei et al., 2019). The combination of *S. boulardii* and lactulose increased acetic acid production (Fig. 4). Compared with the group treated with lactulose alone, the water content, number of stools, and fecal excretion rate were significantly higher in the *S. boulardii*-treated group (Fig. 1 and 2).

We demonstrated that the combination of lactulose with magnesium hydroxide and probiotic strains alleviates constipation by improving the intestinal environment and increasing SCFA content. In particular, the combination of lactulose with magnesium hydroxide or probiotics (*S.*

boulradii and *B. licheniformis*) relieves constipation more effectively compared with lactulose alone. However, few studies have identified probiotic strains that can be used with lactulose and to confirm the efficacy of a mixture of lactulose and magnesium hydroxide for improving constipation. Therefore, additional studies on the underlying mechanism of improving constipation by combinations of lactulose, probiotic strains, and magnesium hydroxide are warranted.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: HJS, YA. Analysis and interpretation: CWP, JL, YA. Data collection: CWP, JL, YHH, YSK. Writing the article: CWP, JL, YA. Critical revision of the article: YHH, YSK, HJS, YA. Final approval of the article: all authors. Statistical analysis: CWP, JL. Obtained funding: HJS. Overall responsibility: YA.

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