Effect of *Lactobacillus casei* fermented *Senna tora* L. seeds and its active compound via muscarinic M3 signaling on the improvement of intestinal function in rats

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Abstract We previously reported the potential of *Senna tora* L. seeds fermented by *Lactobacillus casei* (FSL) as a laxative agent in a loperamide-induced constipation rat model. Here, we examine the mechanism of action of FSL and its bioactive compound, revealed herein, on loperamide-induced constipation Sprague Dawley rat model. We identified the compound aurantio-obtusin (AO) using HPLC quantitative analysis. Rats were randomly assigned to six experimental groups (eight rats each)-normal and constipated groups (loperamide, FSL [100, 300, 500 mg/kg], and AO [1 mg/kg]). The FSL and AO-treated group showed an increase in the frequency, amount, and water content of feces in the constipated rat. Moreover, FSL and AO increased the intestinal transit speed in the constipated rat. Histological analysis revealed that FSL and AO recovered the intestinal mucus, the number of goblet cells, as well as thickness of the mucosa layer and muscle. Furthermore, the protein levels of the muscarinic acetylcholine receptor M3, which is involved in intestine contraction, were recovered in the FSL and AO-treated group. Its downstream signaling pathway (p-protein kinase C) was recovered by FSL and AO treatment. In conclusion, fermentation of *S. tora*

L. seeds increases AO, which improves intestinal function, indicating that FSL is effective for treating constipation.

Keywords Constipation · Gastrointestinal transit · *Lactobacillus casei* · Loperamide · Muscarinic receptor · *Senna tora* L. seeds

Introduction

In modern society, the prevalence of constipation is increasing because of the consumption of meat and processed foods and unbalanced dietary habits. Constipation is a common problem that causes inconvenience and decreases the quality of life of those affected [1]. In people who are constipated, the stools remain in the intestines for an extended period of time and become hardened [2]. Approximately 90% of patients with constipation suffer from chronic idiopathic constipation, for which there exists no identifiable cause or explanation [3,4]. Various treatments are used for this condition, such as consumption of fiber or yogurt and administration of chemical drugs [5,6]. In most cases, chemical drugs stimulate bowel movements or act as osmotic agents. However, these drugs have undesirable side effects including diarrhea, severe cramps or pain, and bloody stools [7]. Thus, natural substances may be more desirable for treating constipation.

Senna tora L. is a herbaceous plant belonging to the legume family and is widely distributed in West Asia, East Asia, Northern Australia, and Africa [8]. In oriental medicine, the seeds are known to have several physiological benefits such as improving liver function and vision. They are also used to treat hypertension, hepatitis, cirrhosis ascites, and habitual constipation in India and East Asia [9, 10]. In previous studies, several anthraquinones and naphthopyrones were isolated from *S. tora* L. seeds [11]. Anthraquinones, a group of structurally diverse secondary metabolites,

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are the major constituents of *S. tora* L. seeds. The anthraquinone auranthio-obtusin (AO) has been reported to have a variety of physiological effects [12, 13]. Fermentation is a metabolic process characterized by the anaerobic conversion of carbohydrates, such as starch or a sugar, to alcohol or organic acids [14]. Fermentation can improve the digestibility, bioavailability, and pharmacological activity of molecules [15]. *Lactobacillus casei*, belonging to the family Lactobacillaceae, is found in the human urinary tract and mouth. Many studies have reported that fermented foods containing strains of *L. casei* can improve constipation-related symptoms [16,17].

Previous studies have reported that the expression levels of muscarinic receptor-2 or -3 affects constipation and the therapeutic effects of drugs in rat model [18]. Muscarinic acetylcholine receptor (mAChR) is a membrane receptor molecule that binds to acetylcholine, the most common neurotransmitter, to mediate cellular responses [19]. It is distributed on the surfaces of neurons, smooth muscle, cardiac cells, and other cells including those in colon glands and tissues [20]. There are five subtypes of mAChR (M1-M5) based on their pharmacological activity, especially, mAChR M2 and M3 have been reported to play a major role in regulating intestinal motility [21].

We previously reported that *S. tora* L. seeds fermented by *L. casei* (FSL) has a laxative effect in a loperamide-induced constipation in rat [22]. However, the mechanism action of the laxative effects of FSL has not been elucidated yet. In the present study, we found the compound(AO) that increased after fermentation in *S. tora* L. seeds, and examined that FSL and AO exhibited ameliorating effects via muscarinic M3 signaling on loperamide-induced constipation in Sprague-Dawley (SD) rats.

Materials and Methods

Preparation of FSL

The seeds of *S. tora* L. were purchased from growers in Haenam-Gun, Jeollanam-Do, Korea in November 2017 and identified as *S. tora* L. (Leguminosae) by the National Institute for Korean Medicine Development (NIKOM). After washing and drying at 60 °C for 24 h, the seeds were roasted at 150 °C for 5 min. Next, the seeds were soaked in water for 3 h and autoclaved at 121 °C for 15 min, followed by *L. casei* fermentation at 37 °C for 24 h. The cultured MRS broth (2 L) of *L. casei* isolated from kimchi was added and mixed with the autoclaved seeds (18 kg). After fermentation, the seeds were autoclaved at 121 °C for 15 min and dried at 60 °C for 36 h. The fermented *S. tora* L. seeds (FSL) were pulverized and passed through 300 mesh before administration.

Isolation of AO

FSL was air-dried and milled. Approximately 5 kg of the powdered seeds was boiled in 50% ethanol for 180 min. After cooling the mixture was filtered through filter paper and

concentrated to obtain crude extract (358 g). The extract (50.0 g) was dissolved in MeOH (100 mL) and charged on a column (60×15 cm, C18, 50 µm, YMC, Kyoto, Japan) with a flow rate of 0.5 mL/min. After LC-MS analysis (ESI-TOF, Shimadzu, Kyoto, Japan), fractions with similar retention time and molecular weight were combined. To obtain 19 major fractions were further purified by HPLC (1260 Infinity, Agilent, Palo Alto, CA, USA). Fraction 16 (2 g) was purified using prep-HPLC (Water : ACN=40:60, v/v) to yield pure compound (AO, 30 mg).

Quantitative HPLC analysis of AO

Approximately 200 mg of samples (before and after fermenting seeds form *S. tora* L. powder) were extracted with methanol (10 mL) and filtered through a 0.45 µm PTFE syringe filter. And the filtrate was used as the sample solution. The isolated AO (1.05 mg) was dissolved in MeOH (10 mL) and used as a standard solution. Quantitative HPLC analysis were carried out on a Luna 5u C18 (2) 100A (250×4.60 mm) column, using isocratic elution with water-ACN (70:30, 45 min, v/v) at a flow rate of 1.0 mL/min. For sample preparation, the pulverized plant material (400 mg) was extracted via sonication with 45 mL of methanol for 180 min. The extract was filtered through filter paper and adjusted to a final volume of 50 mL in a volumetric flask. A standard solution containing 2 mg/mL of AO in methanol was also prepared, and 1 mL of this solution was diluted to 10 mL with the same solvent. A linear relationship between peak area and concentration (1.5-25 µg/mL) was observed with a correlation coefficient $r=0.9999$.

Care of animals and experimental design

Healthy male SD rats (4- weeks- old, SPF grade, total 40 rats), with an average weight of 160 g, were purchased from Orient Bio, Inc. (SungNam, Korea) and allowed to adapt for 1 week at the animal laboratory of NIKOM (conventional system, Jangheung-gun, Jeollanam-do, Korea). During the experiment, the rats were housed two per cage and maintained under laboratory conditions with a strict light cycle (12-h light/12-h dark cycle, 23±2 °C and 50±10% relative humidity). We randomly assigned animals to five experimental groups, each with eight rats, as follows: normal (NOR), loperamide (LOP), FSL (100, 300, 500 mg/kg), and AO (1 mg/kg) groups.

Induction of constipation and sample treatment

Constipation was induced in the animals via the subcutaneous injection of loperamide (5 mg/kg body weight, Sigma-Aldrich, Saint Louise MO, USA) twice per day for 1 week, whereas normal rats were injected with distilled water only. Starting on the fifth day, FSL treatment by daily gavage was administered at doses of 100, 300, and 500 mg/kg for 3 days. All experimental procedures were approved by the Institutional Animal Care and Use Committee of National Development Institute of Korea Medicine (NIKOM-IACUC; Approval Number; NIKOM-2017-019, Date: December 4, 2017) and the National Development

Institute of Korea Medicine Animal Laboratory certified by the Korea Food and Drug Administration (Accredited Unit Number-000389). When the experiment ends, all rats were sacrificed by carbon dioxide inhalation in a closed cage, according to the ethical guidelines.

Measurement of total number, weight, and water contents of feces

Each day, feces were collected from the metabolic cages housing each group of rats at 10:00 A.M. for 3 days. All fecal samples were immediately weighed. The number of fecal pellets in the metabolic cages was counted each day. The water content of the feces was calculated as the difference between the weights of wet and dry stools by drying the samples in an oven at 70 °C for 24 h.

Measurement of gastrointestinal motility ratio

We used the method described by Chalazonitis et al [23]. On day 7, 1 mL carmine red (60 mg/mL in carboxymethyl cellulose; Sigma-Aldrich, MO, USA), which is commonly used to estimate intestinal transit time, was orally administered to the rats. After 1 h, the rats were sacrificed, and the intestine was quickly removed. The gastrointestinal motility ratio was measured as the proportion to the length of the intestine and distance traveled by the carmine.

Quantitative determination of mAChRs M2 and M3

Hundred mg of colon tissue was isolated and washed 3 times with PBS. Then, the samples homogenized in 1 mL of PBS and stored at –20 °C. After two freezing and thawing the samples were performed to break the cell membranes, the samples were centrifuged for 5 min at 5,000× g and 4 °C to get rid of cell debris. The supernatant was assayed immediately. The mAChR M2 and M3 concentrations in the supernatant were analyzed using an enzyme-linked immunosorbent assay (ELISA) kit (Rat Muscarinic Acetylcholine Receptor M2, M3 ELISA Kit, CUSABIO, Houston, TX, USA) according to the manufacturer's instructions.

Histological analysis of transverse colon

Transverse colons were fixed with 10% formalin for 48 h, embedded in paraffin, and sectioned to 5 µm. The sections were subsequently deparaffinized with xylene, rehydrated in graded alcohol, and stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA). After 5 min of incubation, the tissue sections were stained with an Alcian Blue solution (Sigma-Aldrich, St. Louis, MO, USA). The mucosa layer and muscle thickness were measured with a microscope (Nikon coolscope II, Tokyo, Japan), and the distribution of goblet cells (mucous secretory cells) was measured and scored in proportion to the scoring criteria.

Immunoblotting

The intestine tissue (100 mg) was isolated, homogenized, and lysed with RIPA buffer (Enzo Life Sciences, Farmingdale, NY,

USA) containing protease and phosphatase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA). The tissue lysate was quantified using the Bradford method. Proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis running gel, and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). Blots were blocked for 2 h at room temperature using 5% bovine serum albumin in tris-buffered saline containing 0.1% tween 20. The membranes were incubated with protein kinase C (PKC, 1:500 dilution), phosphor-PKC (p-PKC, 1:500 dilution), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:100 dilution) antibodies. After the membrane was washed 3 times every 20 min, specific proteins were detected with ECL detection reagent (Merck Millipore, Burlington, MA, USA), and images were taken using an Odyssey FC imaging system (Li-Cor, Lincoln, NE, USA).

Statistical analysis

All data are presented as the mean ± SD based on SPSS version 25 software (SPSS, Inc., Chicago, IL, USA). Statistical significance was considered at $p < 0.05$. Differences in the mean values of the groups were determined by analysis of variance followed by Duncan's multiple-range post-hoc test.

Results

Identification of AO

White pure AO powder was obtained. Molecular weight of AO was determined as m/z 331.0716 $[M+H]^+$ and 329.0673 $[M-H]^-$ by MS spectra. The molecular formula was suggested as $C_{17}H_{14}O_7$ based on the high resolution mass data recorded by the ESI-IT-TOF mass spectrometer. The 1H NMR spectrum of this compound also displayed two singlet aromatic protons at δ_H 7.76 (1H, s) and 7.16 (1H, s) indicating that benzene ring, two methoxy protons, and one methyl proton signals were found at δ_H 3.83 (s, 3H), 3.80 (s, 3H), and 2.28 (s, 3H), respectively. The ^{13}C NMR spectrum showed 17 resonance signals, and proved the presence of the carbonyl group at δ_C 187.6 and 180.8, two methoxy groups at δ_C 61.6 and 60.4, and one methyl group at δ_C 16.9 (Fig. 1, Table 1). Therefore, this compound was identified as AO (Additional file 1: Fig. S1).

Method validation of AO

The selectivity of AO detection was validated by determining the retention times and recording the complete UV spectra (Additional file 1: Fig. S2). To evaluate linearity, five different concentration of AO standard solution were prepared (1.56 to 25 µg/mL). Calibration point was analyzed in three replicates, respectively. Calibration curves were drawn using unweighted linear regression analysis and linearity was expressed by the r^2 -value. All of the regression coefficients were always stable as >0.99 . The regression coefficients were calculated by each analyte and listed in Table 2.

Fig. 1 Structure of aurantio-obtusin (AO) isolated from fermented *S. tora* L. seeds (FSL)

Table 1 ^1H and ^{13}C -NMR data of AO in $\text{DMSO}-d_6$

Position	δ_{H}	δ_{C}
1	-	147.6
2	-	155.9
3	-	132.4
4	7.76 (s, 1H)	126.3
5	7.16 (s, 1H)	108.1
6	-	157.1
7	-	139.8
8	13.25 (s, 1H, OH)	157.4
9	-	187.6
10	-	180.8
11	-	128.9
12	-	111.5
13	-	124.1
14	-	125.3
OCH_3	3.83 (s, 3H)	61.68
OCH_3	3.80 (s, 3H)	60.47
CH_3	2.28 (s, 3H)	16.95

The sigma method was used for the limit of detection (LOD) [24]. The formula for the calculation of LOD used was $\text{LOD} = 3.3r/\text{SP}$

and $\text{LOQ} = 10r/\text{SP}$ (r : The standard deviation value, SP : The slope of the calibration curve). The LOD and LOQ were found to be 0.127 and 0.423 $\mu\text{g/mL}$, respectively.

Quantitative analysis of AO before and after fermentation

The preparation and analysis of three different samples are described in the Materials and Methods Section. The FSL contained more than 0.103% AO. The content of this component before and after the fermentation of *S. tora* L. increased from 0.399 to 1.037 $\mu\text{g/mg}$, respectively (Table 3).

Effect of FSL and AO on the number, weight, and water content of feces in constipated rats

To investigate the effects of FSL on feces parameters, we assessed feces defecation, weight, and water content in constipated rats. The number of fecal pellets was decreased to 19.7 in the loperamide alone group (LOP) compared with 36.3 in the normal group (NOR). The ingestion of FSL (100, 300, and 500 mg/kg) and AO (1 mg/kg) significantly increased the number of feces (34.8, 39.4, 42.4, and 41.8 respectively; Fig. 2). Similarly, the reduced feces weight induced by loperamide was recovered in the FSL and AO-treated groups relative to that in NOR group (Fig. 3A). As shown in Fig. 3B, the total water content of the fecal pellets decreased by 32.3% in the LOP. However, that in FSL and AO-treated groups increased by 33.6, 38.4, 39.7, and 40.2% in the 100, 300, 500 mg/kg FSL groups and 1 mg/kg AO groups, respectively.

Effect of FSL and AO on intestinal transit ratio in constipated rats

On the 7th day, at 1 h after the administration of carmine, the experimental animals were sacrificed to assess the migration distance of the dye reagent in the intestines. In the LOP group (51.1%), the intestinal motility of carmine was significantly decreased compared to that in the NOR group (71.8%). The administration of FSL and AO improved the intestinal transit ratio in loperamide-induced constipation model rats (Fig. 4).

Table 2 Linearity result of AO

Level	Conc. ($\mu\text{g/mL}$)	Area			Mean	SD	%RSD
		1 time	2 time	3 time			
1	25	1419.8	1404.1	1365.1	1396.3	28.2	2.0
2	12.5	718.7	705.1	692.0	705.3	13.4	1.9
3	6.25	365.3	356.7	352.5	358.2	6.5	1.8
4	3.125	189.7	181.0	180.8	183.8	5.1	2.8
5	1.5625	97.4	92.3	92.7	94.2	2.8	3.0

Table 3 Contents of AO in Sample

Sample	1	2	3	AVERAGE	STDEV	RSD
Before ($\mu\text{g/mg}$)	0.394	0.391	0.412	0.399	0.011	2.846
After ($\mu\text{g/mg}$)	1.055	1.030	1.027	1.037	0.013	1.219

Fig. 2 Effects of fermented *S. tora* L. seeds (FSL) and aurantio-obtusin (AO) on number of feces in loperamide-induced constipated SD rats. (A) Mean number of feces. (B) Macroscopic observation of number of feces were presented (n=1). Data represent the mean $\pm$ SD. Different letters are significantly different at $p < 0.05$ by Duncan's multiple range test. NOR; Normal group, LOP; Loperamide treated group, FSL-100; Loperamide and FSL 100 mg/kg treated group, FSL-300; Loperamide and FSL 300 mg/kg treated group, FSL-500; Loperamide and FSL 500 mg/kg treated group, AO-1; loperamide and AO 1 mg/kg treated group (n=8)

Fig. 3 Effects of fermented *S. tora* L. seeds (FSL) and aurantio-obtusin (AO) on (A) weight and (B) water contents of feces in loperamide-induced constipated SD rats. Water content of feces was determined by drying fecal for 24 h. Data represent the mean $\pm$ SD. Different letters are significantly different at $p < 0.05$ by Duncan's multiple range test. NOR; Normal group, LOP; Loperamide treated group, FSL-100; Loperamide and FSL 100 mg/kg treated group, FSL-300; Loperamide and FSL 300 mg/kg treated group, FSL-500; Loperamide and FSL 500 mg/kg treated group, AO-1; loperamide and AO 1 mg/kg treated group (n=8)

Effect of FSL and AO on histology of transverse colon in constipated rats

Cross-sections of the rat transverse colon were stained with hematoxylin and eosin (H&E) and Alcian blue and observed under an optical microscope. As shown in Fig. 5. In the LOP group, mucus stained by Alcian blue was significantly reduced compared to that in the NOR group. Likewise, significant decreases in the length of mucosa layer, muscle thickness, and distribution of goblet cells (mucous secretory cells) were observed in the LOP group compared to those in the NOR group. These decreases were restored by FSL and AO treatment in loperamide-induced constipated rats; particularly, the 500 mg/kg FSL and 1 mg/kg AO groups showed similar levels to those in the NOR group (Table 4).

Effect of FSL and AO on expression of muscarinic receptor (mAChR) M2 and M3 in the constipated transverse colon

Since mAChR M2 and M3 are acetylcholine receptors related to contraction in the gastrointestinal system, we determined the effects of FSL and AO on the protein expression of mAChR M2 and M3 in the intestine by ELISA. mAChR M2 and M3 protein levels were considerably decreased in loperamide-treated (LOP) group compared to those in the NOR group. FSL- and AO-treated groups showed similar protein expression levels of mAChR M2 as the LOP group. However, mAChR M3 protein was remarkably increased in the FSL- and AO-treated groups. Therefore, FSL and AO treatment increased mAChR M3 but not M2 protein in the intestine (Fig. 6).

Fig. 4 Effects of fermented *S. tora* L. seeds (FSL) and aurantio-obtusin (AO) on intestinal transit ratio in loperamide-induced constipated SD rats. Graph of intestinal transit ratio were presented. Data represent the mean $\pm$ SD. Different letters are significantly different at $p < 0.05$ by Duncan's multiple range test. NOR; Normal group, LOP; Loperamide treated group, FSL-100; Loperamide and FSL 100 mg/kg treated group, FSL-300; Loperamide and FSL 300 mg/kg treated group, FSL-500; Loperamide and FSL 500 mg/kg treated group, AO-1; loperamide and AO 1 mg/kg treated group (n=8)

Effects of FSL and AO on muscarinic receptor M3 downstream signaling pathway

To determine the effect of FSL and AO of the mAChR M3 downstream signaling pathway on the constipation model, the relative levels of PKC and p-PKC were determined by western blot. The levels of p-PKC were decreased in the LOP group

compared to those in the NOR group. However, these levels were considerably recovered to similar levels as those in the normal group when FSL and AO were administered (Fig. 7). These findings demonstrate that FSL and AO can ameliorate constipation, which might be related to the regulation of mAChR M3 expression and its downstream signaling in constipated SD rats.

Discussion

Many studies have examined herbal medicines for the treatment of constipation because of their effectiveness and safety [25,26]. Our previous results suggested that fermented *S. tora* L. seeds improve constipation compared to that with non-fermented seeds and that the most effective fermentation time was 24 h in a rat model of constipation [23]. However, it remained unknown why fermented *S. tora* L. seeds are a more effective laxative than non-fermented *S. tora* L. seeds. Therefore, we first investigated alterations in the amounts of anthraquinones in fermented *S. tora* L. seeds. According to previous studies, more than 37 anthraquinones have been identified in Senna species [27]. We identified 12 main compounds through LC/MS and ESI-IT-TOF-MS in *S. tora* L. seeds, namely (1) isorubrofusarin gentiobioside, (2) casiaside, (3) aurantio-obtusin 6-*O*- β -D-glucoside, (4) rubrofusarin 6-*O*- β -D-gentiobioside, (5) obtusifolin 2-*O*- β -D-glucoside, (6) AO, (7) chrysophanol, (8) obtusifolin, (9) emodin, (10) obtusin, (11)

Fig. 5 Effects of fermented *S. tora* L. seeds (FSL) and aurantio-obtusin (AO) on mucous secretion capacity in loperamide-induced constipated SD rats. Cross-sections of rat transverse colon, and the sections were stained H&E and Alcian blue. All groups were observed at magnifications using a light microscope (100X). Data represent the mean $\pm$ SD. NOR; Normal group, LOP; Loperamide treated group, FSL-100; Loperamide and FSL 100 mg/kg treated group, FSL-300; Loperamide and FSL 300 mg/kg treated group, FSL-500; Loperamide and FSL 500 mg/kg treated group, AO-1; Loperamide and AO 1 mg/kg treated group

Table 4 Histopathological alterations in constipated SD rat

Content	Group	NOR	LOP	FSL-100	FSL-300	FSL-500
Mucosa layer thickness (μm)		497.6 $\pm$ 54.5 ^a	155.3 $\pm$ 21.1 ^d	209.8 $\pm$ 21 ^c	285.3 $\pm$ 34.4 ^b	369.9 $\pm$ 39.6 ^b
Muscle thickness (μm)		94.3 $\pm$ 13.4 ^a	39.4 $\pm$ 8.1 ^c	37.6 $\pm$ 6.4 ^c	60.5 $\pm$ 13.7 ^{bc}	74.8 $\pm$ 12.6 ^{ab}
Number of goblet cells (ea)		95.0 $\pm$ 13.0 ^a	58.0 $\pm$ 8.0 ^c	62.0 $\pm$ 8.0 ^{bc}	75.0 $\pm$ 11.0 ^{ab}	86.0 $\pm$ 12.0 ^{ab}

Data represent the mean $\pm$ S.D. Different letters are significantly different at $p < 0.05$ by Duncan's multiple range test. NOR; Normal group, LOP; Loperamide treated group, FSL-100; Loperamide and FSL 100 mg/kg treated group, FSL-300; Loperamide and FSL 300 mg/kg treated group, FSL-500; Loperamide and FSL 500 mg/kg treated group, AO-1; Loperamide and AO 1 mg/kg treated group (n=8)

Fig. 6 Effects of fermented *S. tora* L. seeds (FSL) and auranitio-obtusin (AO) on mAChR (Muscarinic receptor) (A) M2 and (B) M3 levels in loperamide-induced transverse colon tissue. Homogenated 100 mg intestine tissue supernatant was collected, and the levels of mAChR M2 and M3 were analyzed using an enzyme linked immunosorbent assay (ELISA). Data represent the mean $\pm$ SD. Different letters are significantly different at $p < 0.05$ by Duncan's multiple range test. NOR; Normal group, LOP; Loperamide treated group, FSL-100; Loperamide and FSL 100 mg/kg treated group, FSL-300; Loperamide and FSL 300 mg/kg treated group, FSL-500; Loperamide and FSL 500 mg/kg treated group, AO-1; Loperamide and AO 1 mg/kg treated group (n=8)

Fig. 7 Effects of fermented *S. tora* L. seeds (FSL) and auranitio-obtusin (AO) on mAChR M2 and M3 signaling pathway in loperamide-induced constipated SD rats. Equal amounts of protein (20 mg) were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with antibodies against PKC, p-PKC, and GAPDH. Data represent the mean $\pm$ SD. NOR; Normal group, LOP; Loperamide treated group, FSL-100; Loperamide and FSL 100 mg/kg treated group, FSL-300; Loperamide and FSL 300 mg/kg treated group, FSL-500; Loperamide and FSL 500 mg/kg treated group, AO-1; Loperamide and AO 1 mg/kg treated group

rubrofusarin, and (12) isorubrofusarin (Additional file 1: Fig. S3). Using HPLC quantitative analysis, it was observed, that AO levels

significantly increased by 2.6-fold, whereas (2) auranitio-obtusin 6-O- β -D-glucoside levels decreased after fermentation. AO typically accounts for 0.04% by weight of *S. tora* L. [28]. Our results showed that the AO increased from 0.039% to 0.104% in *S. tora* L. seeds during fermentation. Second, we observed that constipation was improved following the administration of FSL and AO. Therefore, we elucidated the molecular mechanism underlying these effects using a loperamide-induced constipated rat model. Stool parameters such as number, weight, and water contents are important factors in constipation [29]. Our results showed that FSL and AO recovered constipation-related factors such as the reduction in number, weight, and water content of stools, induced by loperamide.

The ability of feces to move through the intestine is another important parameter related to constipation, including the movement facilitated by mucus secreted by goblet cells [30]. It was reported that a decrease in mucus reduces mobility through the colon and thus decreases intestinal mobility [31]. Our results showed that FSL and AO alleviated the reduction in the number of goblet cells (mucous secretory cells) and the thickness of intestinal tissues. Additionally, FSL and AO improved intestinal motility and mucus secretion, resulting in improved movement of feces in the intestine.

In gastrointestinal motility, mAChR M2 and M3 are thought to

play a crucial role in intestinal contraction [32], which are one of the main features of bowel movements including peristalsis [33]. mAChR M3 has a primary role in cholinergic contraction, whereas the specific role of mAChR M2 is unclear [34,35]. To determine the biological functions and molecular mechanisms of FSL in a constipation animal model, we examined changes in the protein expression of mAChR M2 and M3 by ELISA. Treatment with loperamide decreased mAChR M2 and M3 protein expression. Following FSL treatment, mAChR M2 levels were unchanged, whereas levels of mAChR M3 were significantly increased in the intestine. Thus, FSL might improve intestinal contraction by increasing M3 protein levels. Additionally, p-PKC, a downstream signaling molecule of mAChR M3, exhibited similar expression levels as M3 protein. Following treatment with FSL and AO, p-PKC levels were similar to those in the normal group. Based on these results, it can be concluded that FSL and AO effectively improved constipation by increasing the motility of the colon muscles by influencing muscarinic receptor signaling in the smooth muscle.

Our findings demonstrate that FSL increases the content of AO after fermentation. In addition, FSL and AO administration ameliorated various features of constipation, including stool parameters (number, weight, and water content), gastrointestinal transit ratios, and the distribution of goblet cells. Our results suggest that the effects of FSL and AO are mediated by the expression and signaling of muscarinic acetylcholine receptor M3. Thus, FSL, being rich in AO, might be useful for treating chronic constipation. Further studies are required to explore how FSL and AO influence the expression of M3 receptor to determine the efficacy of FSL in humans.

Authors' contributions JHJ conducted the majority of the experiments and writing the manuscript. KHL, HJL and HP performed validation analysis of samples. JHN and BDY participated in the animal experiment. HWC, BKA and SRK managed and coordinated responsibility for the research activity planning and execution. JHY contributed to the fermentation of samples. HKJ and RDP supervised the study and checked the final manuscript. All authors have read and agreed to the final version of the manuscript.

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References

- Jun DW, Park HY, Lee OY, Lee HL, Yoon BC, Choi HS, Ham JS, Lee MH, Lee DH, Kee CS (2006) A population-based study on bowel habits in a Korean community: prevalence of functional constipation and self-reported constipation. *Dig Dis Sci* 51: 1471–1477
- Chatoor D, Emmanuel A (2009) Constipation and evacuation disorders. *Best Pract Res Clin Gastroenterol* 23(4): 517–530
- Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC (2006) Functional bowel disorders. *Gastroenterology* 130(5): 1480–1491
- Lembo A, Camilleri M (2003) Chronic constipation. *N Engl J Med* 349: 1360–1368
- Voderholzer WA, Schatke W, Muhldorfer BE, Klauser AG, Birkner B, Muller-Lissner SA (1997) Clinical response to dietary fiber treatment of chronic constipation. *Am J Gastroenterol* 92: 95–98
- Li XR, Liu CJ, Tang XD, Zhang HM, Luo YY, Zhang L, Yang E (2020) Gut microbiota alterations from three-strain yogurt formulation treatments in slow-transit constipation. *Can J Infect Dis Med Microbiol* 2020: 9
- Siegers CP, von Hertzberg-Lottin E, Otte M, Schneider B (1993) Anthranoid laxative abuse—a risk for colorectal cancer? *Gut* 34: 1099–1101
- Pawar HA, Lalitha KG (2014) Isolation, purification and characterization of galactomannans as an excipient from *Senna tora* seeds. *Int J Biol Macromol* 65: 167–175
- Hong L, Guo Z, Huang K, Wei S, Li Bu, Meng S, Long C (2015) Ethnobotanical study on medicinal plants used by Maonan people in China. *J Ethnobiol Ethnomed* 11
- Pawar HA, Lalitha KG (2015) Extraction, characterization, and molecular weight determination of *Senna tora* (L.) seed polysaccharide. *Int J Biomater* 928679
- Dong X, Fu J, Yin X, Yang C, Zhang X, Wang W, Du X, Wang Q, Ni J (2017) Cassiae semen: A review of its phytochemistry and pharmacology (Review). *Mol Med Rep* 16: 2331–2346
- Hou J, Gu Y, Zhao S, Huo M, Wang S, Zhang Y, Qiao Y, Li X (2018) Anti-inflammatory effects of aurantio-obtusin from seed of *Cassia obtusifolia* L. through modulation of the NF- κ B pathway. *Molecules* 23: 1–15
- Mbatchou VC, Tchouassi DP, Dickson RA, Annan K, Mensah AY, Amponsah IK, Jacob JW, Cheseto X, Habtemariam S, Torto B (2017) Mosquito larvicidal activity of *Cassia tora* seed extract and its key anthraquinones aurantio-obtusin and obtusin. *Parasites and Vectors* 10: 1–8
- Hutkins RW (2008) Microbiology and technology of fermented foods, 1st edn. Blackwell publishing, Oxford, pp 15–66
- Çabuk B, Nosworthy MG, Stone AK, Korber DR, Tanaka T, House JD, Nickerson MT (2018) Effect of fermentation on the protein digestibility and levels of non-nutritive compounds of pea protein concentrate. *Food Technol Biotechnol* 56: 257–264
- Koebnick C, Wagner I, Leitzmann P, Stern U, Zunft HJ (2003) Probiotic beverage containing *Lactobacillus casei* Shirota improves gastrointestinal symptoms in patients with chronic constipation. *Can J Gastroenterol* 17: 655–659
- Mazlyn MM, Nagarajah LH, Fatimah A, Norimah AK, Goh KL (2013) Effects of a probiotic fermented milk on functional constipation: a randomized, double-blind, placebo-controlled study. *J Gastroenterol Hepatol* 28: 1141–1147
- Kim JE, Lee YJ, Kwak MH, Ko J, Hong JT, Hwang DY (2013) Aqueous extracts of *Liriope platyphylla* induced significant laxative effects on loperamide-induced constipation of SD rats. *BMC Complement Altern Med* 13: 333
- Campbell N, Reece J (2002) Biology, 6th edn. Pearson Education, San Francisco, California, p 1037
- Gomez A, Martos F, Bellido I, Marquez E, Garcia AJ, Pavia J, Sanchez CF (1992) Muscarinic receptor subtypes in human and rat colon smooth muscle. *Biochem Pharmacol* 9: 2413–2419
- Kim JE, Go J, Sung JE, Lee HA, Yun WB, Hong JT, Hwang DY (2017) Uridine stimulate laxative effect in the loperamide-induced constipation of SD rats through regulation of the mAChRs signaling pathway and mucin secretion. *BMC Gastroenterol* 17: 21
- Nho JH, Jung HK, Lee MJ, Jang JH, Sim MO, Jung JK, Lee KH, An BK, Cho JH, Jang MC, Yong JH, Cho HY (2016) Effect of roasted water extract of fermented *Cassia tora* L. by *Lactobacillus casei* on the

- loperamide-induced constipation model in rats. Korean J Medicinal Crop Sci 24: 471–478
23. Li Z, Chalazonitis A, Huang YY, Mann JJ, Margolis KG, Yang QM, Kim DO, Côté F, Mallet J, Gershon MD (2011) Essential roles of enteric neuronal serotonin in gastrointestinal motility and the development/survival of enteric dopaminergic neurons. J Neurosci 31(24): 8998–9009
 24. Awotwe-Otoo D, Agarabi C, Faustino PJ, Habib MJ, Lee S, Khan MA, Shah RB (2012) Application of quality by design elements for the development and optimization of an analytical method for protamine sulfate. J Pharm Biomed Anal 62: 61–67
 25. Cirillo C, Capasso R (2015) Constipation and botanical medicines: An overview. Phytother Res 29: 1488–1493
 26. Ali MZ, Mehmood MH, Saleem M, Gilani AH (2020) The use of *Euphorbia hirta* L. (Euphorbiaceae) in diarrhea and constipation involves calcium antagonism and cholinergic mechanisms. BMC Complement Med Ther 20: 14
 27. Dave H, Ledwani L (2012) A review on anthraquinones isolated from Cassia species and their applications. Indian J Nat Prod Resour 3: 291–319
 28. Seo CS, Kim JH, Shin HK, Hwang SY, Kim BS (2014) Quantitative analysis of anthraquinones in cassiae semen by processing method. Korean J Pharmacogn 45: 200–208
 29. Sharma A, Rao S (2017) Constipation: pathophysiology and current therapeutic approaches. Handb Exp Pharmacol 239: 59–74
 30. Cornick S, Tawiah A, Chadee K (2015) Roles and regulation of the mucus barrier in the gut. Tissue Barriers 3: 1–2
 31. Cepinskas G, Specian RD, Kvietys PR (1993) Adaptive cytoprotection in the small intestine: role of mucus. Am J Physiol 264: 921–927
 32. Akiho H, Ihara E, Motomura Y, Nakamura K (2011) Cytokine-induced alterations of gastrointestinal motility in gastrointestinal disorders. World J Gastrointest Pathophysiol 2: 72–81
 33. Goyal RK, Hirano I (1996) The enteric nervous system. N Engl J Med 334: 1106–1115
 34. Figueroa KW, Griffin MT, Ehlerl FJ (2009) Selectivity of agonists for the active state of M1 to M4 muscarinic receptor subtypes. J Pharmacol Exp Ther 328: 331–342
 35. Eglen RM, Choppin A, Watson N (2001) Therapeutic opportunities from muscarinic receptor research. Trends Pharmacol Sci 22: 409–414