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In vivo evaluation of holocellulose and cellulose isolated from kumaizasa (*Sasa senanensis*) powder on bowel movements in rats

Hideo Hara[1,2], Ryusuke Mifuru[2], Yoshiro Ishikura[2], Ryo Yokotani[3], Naobumi Ishida[4], Takaaki Hara[2] and Shuji Ozawa[5]

[1]Graduate School of Life Science, Hokkaido University, Sapporo, Hokkaido, Japan; [2]UNIAL Co., Ltd., Hokkaido Bioresources Innovation Center, Itabashi-ku, Tokyo, Japan; [3]Safety Research Institute for Chemical Compounds Co., Ltd., Sapporo, Hokkaido, Japan; [4]Clinical Support Corporation, Ltd., Sapporo, Hokkaido, Japan; [5]Rakuno Gakuen University, Department of Sustainable Agriculture, Laboratory of Natural Products Chemistry, Ebetsu, Hokkaido, Japan

Corresponding authors: Naobumi Ishida, PhD, Clinical Support Corporation, Ltd., 4-1 South 1 West 8, Chuo-ku, Sapporo, Hokkaido 060-0061 Japan and Yoshiro Ishikura, Ph.D. UNIAL Co., Ltd., Hokkaido Bioresources Innovation Center,63-10 Nakajuku, Itabashi-ku, Tokyo 173-0005, Japan

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ABSTRACT

Background: Kumaizasa (*Sasa senanensis* Rehder) is a representative natural plant growing in Hokkaido, Japan, and has a history of being used in herbal medicine and as a health food option. Nishihira et. al. (2019) confirmed in a clinical trial that the kumaizasa dry powder had the effect of improving bowel movements of healthy volunteers. In this study, we evaluated the effect of the components of kumaizasa powder, like holocellulose (hemicellulose + cellulose) and cellulose, involved in the bowel movement of rats and tried to elucidate the role of each component.

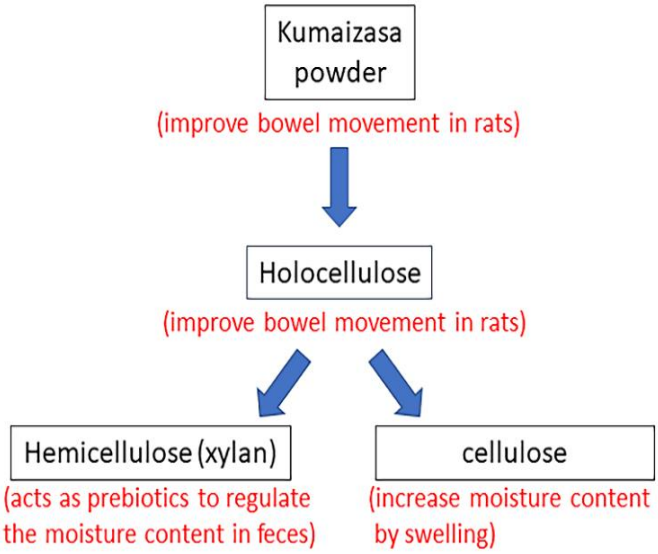
Methods: Male rats (Slc:SD, weight 79~93 g) were administered kumaizasa powder (3000 mg/kg/day) orally, holocellulose (1500 mg/kg/day), cellulose (900mg /kg/day) or water using a stomach tube twice a day for 14 days.

Results: Among the intervention groups (kumaizasa powder, holocellulose, cellulose group) and control group (water), no significant differences were observed with changes in body weight and food consumption. All the feces were normal,

with one exception of watery feces on day 2 in the cellulose group. Multigroup comparison by the Tukey-Kramer method showed that the dry weight of feces collected at day 14 in the kumaizasa powder group significantly increased as compared with that of the control group ($p<0.01$). This result confirmed that kumaizasa powder had the effect of increasing fecal amount. A paired t-test between each kumaizasa group and control group indicated that the cellulose increased the fecal dry weights and moisture content in feces, while the kumaizasa powder and the holocellulose increased only fecal dry weights.

Conclusion: It is shown that the holocellulose and cellulose of kumaizasa powder have the effect of increasing the bowel movement of rats by oral administration. It is also suggested that the cellulose increases the moisture content in the feces by swelling, while hemicellulose in the holocellulose acts as a prebiotic to regulate the moisture content in feces.

Kumaizasa



Keywords: Kumaizasa, *Sasa senanensis* Rehder, hemicellulose, cellulose, holocellulose, bowel movements

INTRODUCTION:

Kuma bamboo grass is a plant of the genus *Sasa* in the family Poaceae and consists of four main species in Japan: the large-sized *Sasa kurilensis*, the medium-sized *Sasa senanensis* (kumaizasa), the small-sized *Sasa japonica*, and the *Sasa borealis* [1-2]. Kuma bamboo grass is a traditional material that is described in "Compendium of Materia Medica" and has been used as a crude drug in Chinese medicine [3-4].

Among Kuma bamboo grass, kumaizasa is a symbolic wild plant in Hokkaido, Japan and has been used in herbal and functional food situations. Kumaizasa extract is reported to have ameliorating activities against various diseases like bacterial infections, cancers, and inflamm-atory diseases in animal models [5-7]. Kumaizasa powder is also reported to improve the

intestinal environment and alleviate constipation, or to at least activate NK cell in human trials [8]. Recently, Nishihira et al. confirmed the effect of kumaizasa powder on bowel movements in healthy Japanese adults by a randomized, double-blinded, placebo-controlled parallel-group trial [9].

Relating to the safety of the kumaizasa powder, it was shown that the powder was not toxic in a 28-day repeated dose toxicity test and a micronucleus test at a dose of 2,000 mg/kg in male rats [unpublished data]. In clinical studies of kumaizasa powder, laboratory test abnormal-ities and adverse events such as abdominal pain or diarrhea were not observed during the continuous intake of 4.2 g/day for 2 to 4 weeks [8-9]. It has not been reported that the commercially available kumaizasa products caused health hazards. As health foods, the kumaizasa products have been consumed since 1957, and ingested about forty tons per year; that is more than 2,500 tons in the past 65 years in Japan [9]. It is obvious that the kumaizasa powder is safe and has sufficient history of being eaten as a food product.

In this study, we evaluated the effect of holocellulose (composed of cellulose and hemicellulose) and cellulose, which are the main components of the kumaizasa powder, on the bowel movement in rats. We also tried to elucidate the different roles of each component in the bowel movement, because hemicellulose in holocellulose seems to be unique to kumaizasa.

METHODS

Sample preparation: The kumaizasa powder (SanSTAGE®) was provided by UNIAL Co., Ltd (Tokyo, Japan). All the steps were processed in the quality-controlled manu-facturing plant. The powder conformed the standard for labeling the food as safe. As shown in Figure 1, High Performance Liquid Chromatogram of kumaizasa powder in SanSTAGE® shows a qualitative indicator peak at around Rt 18 min, which is specific to kumaizasa and does not appear in the chromatograms of powder of leaves of barley, kale, or mulberry (data not shown). Therefore, the samples of holocellulose and cellulose used in this study were confirmed to be derived from kumaizasa. Using this kumaizasa powder, the holocellulose was produced after various steps. At first, the kumaizasa powder was refluxed in hexane-ethanol (2: 1 [V / V]) for 6 hours, and then the remaining residue was dried to obtain defatted kumaizasa powder. The defatted kumaizasa powder was suspended with ion-exchanged water, sodium chlorite and 90% acetic acid. The powder was heated at 90 - 95° C for 1 hour and then cooled until 85° C or lower. These steps were repeated a total of five times. Thereafter, the sample was adjusted to a pH of 7.0 using a 24% sodium hydroxide solution. The filtrated residue was washed with ion-exchanged water and then acetone, and the dried powder was obtained as the holocellulose. Kumaizasa cellulose was obtained from the holocellulose by the following process: the holocellulose was heated at 20°C in 17.5% aqueous sodium hydroxide solution for 1 hour, and the filtrated residue was washed by ion-exchange water and then by acetone. The dried powder was obtained as the cellulose.

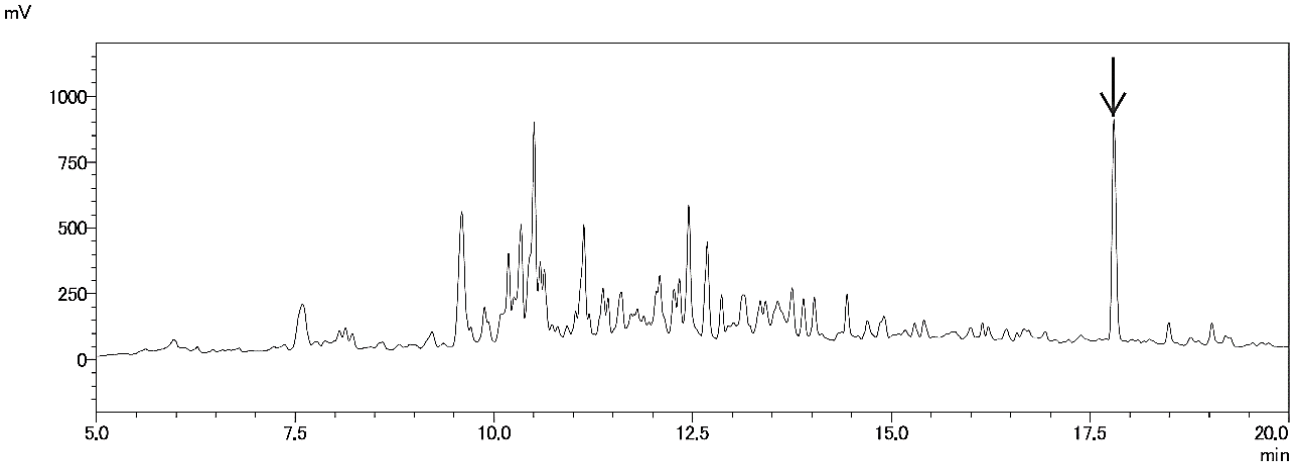


Figure 1. High Performance Liquid Chromatogram of the kumaizasa powder. Column: ACQUITY UPLC BEH C18 (ϕ2.1mm × 100mm, 1.7µm), Gradient going from 0.5 - 100% acetonitrile with 0.1% formic acid in 50 min, 40°C, 0.4 ml/min, Equipment: Nexera (LC-30AD, SPD-20A, SHIMADZU CORPORATION)

In vivo experiment: Male rats (Slc:SD, SPF grade, weight 79 - 93 g, 4-weeks-old) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Rats were quarantined and acclimatized for 6 days, having solid feed CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water ad libitum following their arrival. On day 6 following arrival, body weight, general conditions, and feces conditions of rats were observed. Then, the rats without any abnormal characteristics in feces and in general condition were divided into 4 groups (8 rats/group) by the method of stratified random sampling so that the weight of each group was as uniform as possible. Rats were raised in metal bracket-type cages (260 W x 380 D x 180 H) with wire mesh floors. During the quarantine and acclima-tization period, three rats were kept in one cage, and after grouping, one rat was kept in an individual cage.

As the experimental intervention, the kumaizasa powder, the holocellulose, or the cellulose was orally fed to each group. The dosages of the holocellulose and the cellulose were adjusted to be roughly the same amounts in the kumaizasa powder, respectively. The kumaizasa powder, the holocellulose and the cellulose were suspended in Japan pharmacopeia purified water (NikP, Nichi-Iko Pharmaceutical Co., Ltd., Toyama, Japan) adjusting to 100 mg/mL, 50 mg/mL, and 30 mg/mL, respectively. These samples were administered by oral gavage into the stomach of rats using a stomach tube twice a day (15 mL/kg in the morning and 15 mL/kg in the afternoon). Therefore, the respective daily dosages were 3000 mg/kg for the kumaizasa powder group, 1500 mg/kg for the holocellulose group, and 900 mg/kg for the cellulose group (Table 1). For the control group, NikP water was given. Table 2 summarized the amount of cellulose, hemicellulose and moisture contained in each intervention sample.

Each group was given its intervention sample twice a day for 14 days with solid feed CRF-1 and drinking water (tap water) ad libitum. The temperature and the humidity in the animal room were set to 22±3°C and 50±20%, respectively. The rats were fed under the conditions of light and dark, a 12-hour cycle (light period 8:00 - 20:00).

Table 1. Study group setting and daily dosage

Group	Number of rats	Concentration (mg/mL)	Dosage (mL/kg)	Dosage (mg/kg)
Kumaizasa powder	8	100	30	3000
Holocellulose	8	50	30	1500
Cellulose	8	30	30	900
Control*	8	-	30	-

*Japan pharmacopoeia purified water NikP

Table 2. Summary of intervention food

	Kumaizasa powder	Holocellulose	Cellulose
Total	3000 mg	1500 mg	900 mg
Cellulose (mg)	894.0	724.5	764.1
Hemicellulose (mg)	654.0	403.5	48.6
Water (mg)	123.0	82.5	52.2

Fecal properties and amount: The fecal properties of individual rats were classified into four groups: "normal stool", "loose stool", "diarrhea stool", and "watery stool" and recorded every day from day 1 to day 14 of administration. Then individual rat was transferred to a metabolic cage and kept over a 24-hour period on the previous day of day 1 (day 0), day 7, and day 14 of administration. All the feces were collected, and the fecal wet weights were measured. In addition, the feces were dried at 55 °C for 48 hours and the dry weights were measured. The difference between the wet weight and the dry weight was calculated as the moisture content in the feces.

Other observations: From the start to day 15, visual appearance, vital sign and other data of rats were carefully observed. Body weight of individual rat was measured on day 0, 1, 3, 7, 10, 14 and 15. Further, the supplied feed amount was measured on day 1, 3, 7 and 10. The remaining amount of feed was also measured on days 3, 7, 10 and 14, respectively. The amount of food intake per day was calculated by dividing the difference between the supplied amount and the remaining amount by the number of days between measurements.

Ethics statement and management of animals: All animal experimental procedures were approved on Oct 23, 2015 (approval number: NP015-36-02) by the Institutional Animal Care and Use Committee of Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo, Japan). All the experiments were performed in compliance with Act on Welfare and Management of Animal [10] and other relevant standards.

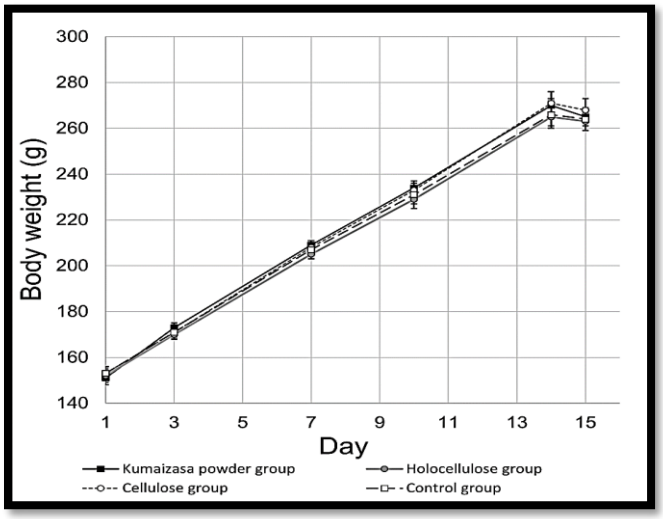
The animal experiment was conducted during Oct. 28 to Nov. 18, 2015. The rats not selected at grouping and the rats finished the experiment were euthanized by exsanguination of abdominal aortic amputation under isoflurane anesthesia.

Statistical analysis: As for fecal wet weight, fecal dry weight and fecal moisture content, a Bartlett's test was performed among the groups for each measurement point. Multiple comparisons were performed for the entire groups by the Tukey-Kramer method in the case of equal variance, and by the Steel-Dwass' test in the case of unequal variance. In addition, the measured values of each group were arranged in ascending order at each measurement point, and the values of the same rank were regarded as "corresponding data", and the control group and other groups were compared by the method of a paired t-test. The quartile deviation method was used to determine outliers.

RESULTS

Transition of weight and food intake in rats: During the experimental period, any cases of death or other abnormalities were not observed in the intervention groups nor control group. Fig. 2 shows the transition of the weight and feeding amount of the 4 groups. In each group, the rats grew normally, and their body weight increased along with food intake. In the intergroup comparison, no significant differences in body weight and feeding amount were found among all the groups.

A



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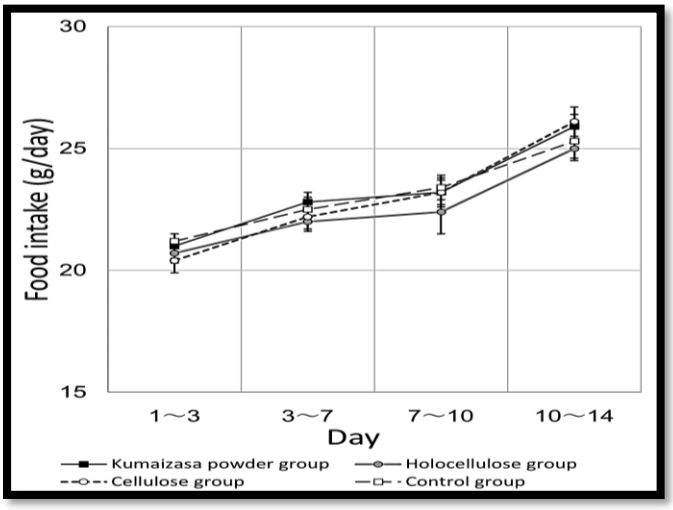

Figure 2. Transitions of body weight (A) and food intake (B) of rats

FECAL OBSERVATION

Fecal properties: In the cellulose group, a transient watery stool was found in one rat on day 2 of administration, however all other rats were observed with normal stools.

Fecal amount: In items of the fecal amount, there was one rat whose stool amount was extremely small in each intervention group. Using quartile deviation method, measurements determined to be outliers that deviated in the smaller direction were excluded from the analysis. Measurements that deviated greatly were used for analysis because the possibility of the effect of the test substance used in the intervention cannot be ruled out. Thus these 3 rats were removed from the analysis as outliers. In addition, to prevent bias among the groups, one rat that showed the lowest value in the total fecal wet weight in the control group was also excluded from the analysis. The results of changes in fecal wet weights, fecal dry weights and moisture content in feces were shown in Table 3.

Regarding multigroup comparison, the dry weight of feces collected at day 14 and total sum of those at day 0, 7 and 14 in the kumaizasa powder group significantly increased as compared with those of control group (day 14; $p<0.01$, sum of day 0, 7 and 14; $p<0.05$, by Tukey-Kramer method). These results suggest that kumaizasa powder has the effect of increasing fecal amount. Other measurements of fecal amount were not significantly different among the intervention and control groups.

Then, the ratios between the fecal amount values of intervention group and control group using each average value were calculated. We also performed a paired t-test using the corresponding data where the measured values of the same order when arranged in ascending order for each intervention group vs. control group were regarded as paired. The results were shown in Table 4. The ratios between fecal dry weights in cellulose group and control group exceeded 1 and the differences of fecal dry weights were statistically significant at day 7 ($p<0.05$), day 14 ($p<0.001$) and the total ($p<0.05$) showing that cellulose in kumaizasa powder has the effect of increasing fecal amount. Relating to moisture content in feces, the ratio between cellulose group and control group also exceeded 1 and the differences were significant at day 7 ($p<0.05$), day 14 ($p<0.05$) and the total ($p<0.001$) showing that cellulose has the effect of increasing moisture content in feces.

Regarding the effect of holocellulose, the paired t-test suggested that holocellulose had effect of increasing fecal dry weights on day 14 but no effect of increasing moisture content in feces despite that the holocellulose sample contains almost same amount of cellulose in cellulose sample as shown in Table 2. The results of kumaizasa powder on fecal dry weights coincided well with the results of the cellulose. As to the moisture content in feces, the results of kumaizasa powder coincided well with those of holocellulose.

Table 3. Transition of wet weight, dry weight, and moisture content in feces of rats

Fecal sample	Treatment group[1)]	Sampling days after treatment[2)]			
		0	7	14	Total
Fecal wet weight (g)	Kumaizasa powder	4.72±0.18	6.21±0.28	8.04±0.47	18.97±0.78
	Holocellulose	4.70±0.15	5.96±0.30	8.33±0.54	18.99±0.85
	Cellulose	4.61±0.29	6.29±0.30	8.33±0.30	19.24±0.40
	Control	4.80±0,15	5.85±0.21	7.67±0.30	18.32±0.38
Fecal dry Weight (g)	Kumaizasa powder	3.05±0.11	4.00±0.16	5.26±0.10**	12.31±0.33**
	Holocellulose	3.02±0.07	3.65±0.14	4.76±0.23	11.44±0.35
	Cellulose	2.88±0.15	3.77±0.12	4.70±0.12	11.35±0.17
	Control	3.02±0.08	3.60±0.11	4.42±0.12	11.04±0.22
Fecal moisture content (g)	Kumaizasa powder	1.67±0.09	2.21±0.13	2.78±0.45	6.66±0.58
	Holocellulose	1.68±0.11	2.31±0.18	3.57±0.32	7.56±0.53
	Cellulose	1.74±0.14	2.51±0.19	3.63±0.22	7.88±0.25
	Control	1.79±0.08	2.25±0.14	3.24±0.19	7.28±0.20

1) Data of 7 rats/group without an outlier were used.

2) Number in table 3 was shown as an average ± standard deviation.

*: <0.05, **: <0.01 (Tukey—Kramer method compared with control)

Table 4. Mean ratio of fecal wet weight, fecal dry weight and fecal moisture content compared with the control value as 1

Fecal sample	Treatment group[1)]	Days collected fecal sample			
		0	7	14	Total
Fecal wet weight[2)]	Kumaizasa powder	0.98	1.06 **	1.05	1.04
	Holocellulose	0.98	1.02	1.09 *	1.04
	Cellulose	0.96	1.08 *	1.09 *	1.05 **
Fecal dry weight[2)]	Kumaizasa powder	1.01	1.11 ***	1.19 ***	1.12 ***
	Holocellulose	1.00	1.01	1.08 *	1.04
	Cellulose	0.95	1.05 *	1.06 ***	1.03 *
Fecal moisture content[2)]	Kumaizasa powder	0.93 *	0.98	0.86	0.91
	Holocellulose	0.94	1.03	1.10	1.04
	Cellulose	0.97	1.12 *	1.12 *	1.08 ***

1) Data of 7 rats/group without outlier were used.

2) the ratio to control group

*: <0.05, **: <0.01, ***: <0.001 (Comparison with the control group by the corresponding t--test in the same rank)

DISCUSSION

Dietary plant fibers such as cellulose, hemicellulose, and pectin are resistant to digestion by human digestive enzymes and reach to human colon [11]. Dietary fiber exhibits a variety of physiological activities, including the ability to retain water in the stool and to increase specific bacteria such as genus of *Bifidobacterium* [12-13] in the human gut.

Recently, Nishihira et al. reported that the kumaizasa powder (SanSTAGE® UNIAL Co., Ltd. (Tokyo, Japan)) showed the significant increase of the stool frequency and improved the stool odor after 2-week ingestion (4.2 g/day) compared with those of the placebo (starch), in the clinical trial of a placebo-controlled, randomized, double-blinded, parallel-group comparison using 80 healthy Japanese adults (age 20 to 65) [9]. In another study, stool volume and stool frequency were also significantly improved by continuous ingestion (4.2 g/day) of kumai-zasa powder for 4 weeks in female subjects with chronic constipation [8].

In this study, the effects of the kumaizasa powder and its components, i.e., the holocellulose (hemicellulose + cellulose) and cellulose on the bowel movement in rats were evaluated to elucidate each role of holocellulose and cellulose. Multigroup comparison by the Tukey-Kramer method showed that the dry weight of feces collected at day 14 in the kumaizasa powder group significantly increased as compared with that of control group. This result confirmed that kumaizasa powder had the effect of increasing fecal amount in rats. A paired t-test between each kumaizasa intervention group and control group indicated that cellulose increased the fecal dry weights as well as moisture content in feces, while the kumaizasa powder and holocellulose increased fecal dry weights but not moisture content in the feces despite both samples containing similar amounts of cellulose. This difference seems to be attributed to hemicellulose that is rich in kumaizasa powder and the holocellulose samples but not in the cellulose sample.

The kumaizasa holocellulose is comprised of cellulose and hemicellulose and is produced by removing liposoluble components and lignin from the kumaizasa powder. The main component of cellulose is known as alpha-cellulose. Aoyama et al. reported that xylan was solubilized from kumaizasa by steaming treatment [14]. Xylan is supposedly the main component of hemicellulose in kumaizasa powder. From the results of this study, it is clear that alpha-cellulose increases the moisture content of feces due to water retention and/or swelling action. The transient watery stool observed in one rat in the cellulose group may be due to the excessive swelling action of cellulose. Xylan in holocellulose is known as a novel prebiotic derived from 5 carbon sugar xylose and is connected via beta bonds [15]. Its mechanism of action on the bowel movement is now elucidated as follows: xylan is degraded by enzymes in Bacteroides and Prevotella in the gut microbiota to yield a soluble xylooligosaccharide that Bifidobacteria can feed [16]. It is confirmed by an *in vitro* study using human feces that xylan and xylooligo-saccharides grew Bifidobacteria to produce short-chain fatty acids, such as acetic acid, that improve the bowel movement [17-18]. It is also confirmed in human trials that the consumption of xylooligosaccharides improved the bowel movement by increasing Bifidobacteria and stool frequency [19]. Moreover, Clostridium subcluster XIVa identified by terminal restriction fragment length poly-morphism (T-RELP) of 16S rRNA increased in pig flora that was fed with the kumaizasa powder [20]. Clostridium sub-cluster XIVa is known as a butyrate-producer and its reduced number is related to the decline in intestinal tract function commonly seen in elderly individuals [21-22].

Based on the present and other studies, it is indicated that the effect of kumaizasa powder on bowel movement is derived from a function of the cellulose, as well as a function of the hemicellulose whose main component is supposedly xylan, a novel prebiotic. The holocellulose meets the condition as part of the active ingredients of the kumaizasa powder.

The holocellulose derived from kumaizasa powder (SanSTAGE®) is a functional substance of Functional Food with Claims in Japan. It is a safe natural health food material that can improve bowel movement with favorable gut microbiota. Further studies are warranted to elucidate its detailed mechanism of action.

CONCLUSION

This study confirmed that kumaizasa powder had the effect of increasing fecal amount in rats. It is suggested that the cellulose increases the moisture content in the feces by swelling, while hemicellulose in the holocellulose acts as a prebiotic to regulate the moisture content in feces.

Competing interests: H.H. and T.H. are employers, and R.M. and Y.I. are employees of the UNIAL Co., Ltd., which provided financial support for this study but did not contribute in any other way to the conduct of the study.

Authors' contributions: Conceptualization, H.H., T.H. and S.O.; Methodology, H.H., R.M., Y.I. and R.Y.; Software, R.Y.; Validation, H.H., R.M. and Y.I.; Formal Analysis, R.Y.; Investigation, H.H., R.M., Y.I. and R.Y.; Resources, H.H. and T.H.; Data Curation, H.H., R.M., Y.I., R.Y. and N.I.; Writing-Original Draft Preparation, H.H., R.M., Y.I., R.Y. and N.I.; Writing-Review & Editing, H.H., R.M., Y.I., N.I. and S.O.; Visualization, H.H., R.M., Y.I. and N.I.; Supervision, H.H. and T.H.; Project Administration, H.H., R.M., and Y.I.; Funding Acquisition, H.H. and T.H.

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