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Evaluation of mannooligosaccharide production from konjac by using recombinant mannanase of *Bacillus* sp. SWU60 as a prebiotic *in vitro* and *in vivo*

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ABSTRACT

Prebiotics consist of non-digestible foods that have many benefits to promote health. In the present study, mannooligosaccharide (MOS) produced from degradation of konjac by recombinant mannanase from *Bacillus* sp. SWU60 (rManS2) was investigated for its prebiotic properties both *in vitro* and *in vivo*. The prebiotic properties were evaluated by *in vitro* fermentation of the MOS (M2-M5) with *Lactobacillus plantarum* N25, and compared with the fermentation of the FOS. In the *in vivo* study, testing was performed on mice (BALB/cAJcl). The short-chain fatty acids (SCFAs) were evaluated both *in vitro* and *in vivo*. The results of the high -performance anion-exchange chromatography (HPAEC) indicated that the digested products were MOSs, including mannobiose, mannotriose, mannotetraose, and mannopentose. The antioxidant activity of MOS determined by a DPPH assay was 643.06 ± 0.05 µg/mL. These products promoted the growth of a probiotic strain, which was significantly different from the results of the control groups. In terms of the prebiotic effects *in vivo*, the results showed that all mice treated with MOS were normal, compared with the control group, and there were no deaths. The SCFAs, including acetate and lactate, were detected both *in vitro* and *in vivo*. In conclusion, the *in vitro* studies revealed that the MOS from konjac by rManS2 had a potential as an antioxidant, prebiotic property and had no toxic effect on mice.

Keywords: mannooligosaccharide; short-chain fatty acids; prebiotic; probiotic; mice

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1. INTRODUCTION

In the current global situation, in addition to the usual illnesses, there are still problems with emerging diseases that have no drug treatment or prevention methods. The strengthening of bodily health is necessary in terms of fighting various diseases (Warburton and Bredin, 2017). If you have a healthy body, the various working systems, including the immune system perform well. There are many ways to build good health, such as exercise, eating healthy food, and/or adding beneficial nutrients (Harvard Health, 2014). The best well-known and interesting nutrients

today are prebiotics. Prebiotics are the dietary fiber that is not digested in the human gut system and found in many foods, vegetables and some whole grains, which are responsible for promoting the growth of healthy microbes, also known as probiotics, such as *Lactobacillus* spp. and *Bifidobacterium* spp. (Carlson et al., 2018; Peterson et al., 2018; Rizzoli, 2019). When the intestines are balanced, there are more types of probiotics than the pathogens, causing the digestive system to function normally. The prebiotic substances that are commercially available include fructooligosaccharides (FOS), galactooligosaccharides, insulin, etc., which are expensive and limited to a few types (Thomas et al., 2010; Sabater et al., 2016; Lafontaine et al., 2020). So, the production of substances with other prebiotic properties that come from nature, at low cost, safe and interesting.

Konjac is an important economic plant and popular in Thailand because it has many benefits, for example, it is high in fiber and has no calories. Mannooligosaccharide (MOS) from konjac are oligosaccharide consisting of a linear chain of glucose and mannose. The MOS was produced from agro-waste by digestion with endo-β-(1,4)-mannanase. It has significant stability against human enzymes, and stimulates good bacteria in the gastrointestinal tract, which is an essential parameter for oligosaccharides to serve as a prebiotic (Liu et al., 2020). The MOS derived from agro-wastes of palm kernel cake and copra meal (CM) showed good properties as prebiotics (Jana and Kango, 2020). Furthermore, the MOS can promote proliferation of normal bacterial flora such as *L. acidophilus* and *B. infantis* that promoted host health (Singh et al., 2017) and inhibited pathogenic microorganisms that have been reported to stimulate the immune system against pathogens, such as *Vibrio harveyi* in Pacific white shrimp (Torrecillas et al., 2014; Yamabhai et al., 2016). Therefore, this study aimed to examine the production of MOS from konjac, using the rManS2 enzyme from thermotolerant *Bacillus* sp. SWU60.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

The pET32a-manS2 in *Escherichia coli* BL21 (DE3) was prepared from the genes of the *Bacillus* sp. SWU60 (Seesom et al., 2017). The HisTrap HP affinity chromatography column (5 mL) was obtained from GE Healthcare UK Ltd. (Buckinghamshire, UK). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). FOS was purchased from Sigma-Aldrich (Bangkok, Thailand). The konjac powder was prepared from konjac in a laboratory at Srinakharinwirot University (Bangkok, Thailand). Azurine-cross-linked (AZCL)-galactomannan was purchased from Megazyme Ltd. (Bangkok, Thailand).

2.2 Microorganisms

The probiotic strain, *Lactobacillus plantarum* N25, was purchased from the Thailand Institute Scientific and Technological Research (Bangkok, Thailand).

2.3 Glucomannan preparation from konjac

The konjac was cleaned with water and peeled. After that, it was sliced and rinsed with distilled water. Then, it was dried in an incubator at 60°C for 2-3 days or until absolutely dry. Then, the konjac powder was ground into a powder using a blender and stored in a dry cabinet.

2.4 Preparation of rManS2 enzyme

The preparation of rManS2 enzyme was followed by Seesom et al. (2017). The pET32a-manS2 constructed in *E. coli* BL21 (DE3) was inoculated into 10 mL of Luria-Bertani (LB) medium containing an antibiotic (ampicillin, 50 µg/mL), incubated at 37°C with shaking at 220 rpm for 16 h. Then, the pre-culture was added to 1 L of LB medium containing ampicillin (50 µg/mL), and incubated in same conditions for 1.30 h or OD_{600} between 0.6-0.8. After that, gene expression was induced using 100 µL IPTG (0.1 mM as final concentration) and continued incubation for 3 h. The cell pellet was harvested by centrifugation at 5000 rpm for 20 min, rinsed twice with 20 mM potassium phosphate, with a pH 6.0. Then, the enzyme was extracted from the cells by sonification. The mannanase activity was examined by using 0.02% AZCL-galactomannan, specific synthetic substrate, that dissolved in 20 mM KPB pH 6.0, then incubated at 60°C for 30 min. After this reaction, the solution with mannanase activity changed to a blue color.

2.5 MOS production

The konjac powder was dissolved in 20 mM potassium phosphate buffer pH 6.0, the concentration 1.0% and autoclave at 121°C for 15 min as a substrate solution. Enzyme digestion was performed with methods modified from Seesom et al. (2017), using 1 L of 1% konjac substrate solution, then adding rManS2 (7U) and incubating at 45°C for 24 h. The reaction was stopped by boiling for 10 min and filtrated through a 0.45 µm membrane filter. Then, the MOS in hydrolysate was purified by using an activated charcoal column. The adsorbed MOS on charcoal was eluted by using 1-60% ethanol and analyzed by using high performance anion exchange chromatography (HPAEC).

2.6 Antioxidant activity of MOS by DPPH assay

A serial dilution of MOS was performed from 0 to 1000 µg of MOS (M2-M5) in 1 mL of double-distilled water. One milliliter of each MOS solution was added into 1 mL of 0.1 mM DPPH solution (ratio 1:1), then incubated the solution at room temperature for 30 min in darkness. After that, the absorbance of the solution was measured at a wavelength of 517 nm using double-distilled water with 0.1 mM DPPH as a blank. In addition, ascorbic acid was used to perform a serial dilution from 0 to 1000 µg in 1 mL double-distilled water and used as a standard antioxidant (Bortolomeazzi et al., 2010; Cheng et al., 2006). The experiment was performed in triplicate.

The measurement of the DPPH radical scavenging assay for the analytical sample at each concentration was performed by calculation IC_{50} using ascorbic acid as a positive control. The experiment was repeated in triplicate. The IC_{50} calculated equation was as follows:

$$A\% = [1-(A_0-A_t)/(A_{0'}-A_{t'})] \times 100$$

where A_0 and $A_{0'}$ are the absorbance at time zero of the sample and the control, respectively, whereas A_t and $A_{t'}$ are the absorbance of the sample and the control at 30 min, respectively.

2.7 Prebiotic effect on probiotics *in vitro*

One milliliter of overnight cultures of *L. plantarum* N25 was harvested by centrifugation at 5000 rpm for 20 min, and then washed twice with phosphate buffer saline (PBS), pH 7.0. The cell pellet was added into 5 mL of basal medium supplemented with 10 mg/mL MOS as a carbon source and cultivated anaerobically at 37°C for 48 h. The growth of microorganisms was assessed by determining the OD_{600} values at 0, 4, 8, 12, 16, and 24 h, compared to the growth of the probiotic from the basal medium with 10 mg/mL FOS as a carbon source. The growth of probiotics in a basal medium without being supplemented was used as control.

2.8 Detection of short chain fatty acids (SCFAs) *in vitro* of MOS tested

The culture of *L. plantarum* N25 at 0, 24 and 48 h were collected for analysis of SCFAs including acetic, propionic, butyric and lactic acids. The culture was centrifuged at 13000 rpm for 5 min, the supernatant was filtrated through 0.22-µm paper filter. The analysis of SCFAs was achieved by high performance liquid chromatography, using a 300x8.0 mm Shodex SUGAR SH1011 column (Tokyo, Japan), and used 5 mM H_2SO_4 as eluent solvent (1 mL/min flow rate.)

2.9 Properties of MOS as prebiotic *in vivo*

For the prebiotic effect study *in vivo*, 20 male BALB/cAJcl mice aged five weeks were detained to investigate abnormality and adaptation in the laboratory (Animal Housing, Faculty of Medicine, Srinakharinwirot University), with feeding formula 082G and drinking water at all times (ab lib). Ethical approval for this study was obtained from the Srinakharinwirot University Research Ethics Committee (Reference Number 2/2561; Approved 7 March 2018). The mice were divided into three experimental groups and one control group or five mice per group (n = 5) as follows:

Group 1 (Control): PBS pH 7.0
Group 2 (Prebiotic): MOS in PBS pH 7.0
Group 3 (Probiotic): *L. plantarum* N25 in PBS pH 7.0
Group 4 (Synbiotic): *L. plantarum* N25 and MOS in PBS pH 7.0

Each group was treated by oral gavage administration, once a day for 14 days. The MOS solution was prepared for feeding dose at 1000 mg/kg per day, and *L. plantarum* N25 was prepared at 10^8-10^9 cells or the OD_{600} ranged from 0.8 to 1.0 and dissolved in phosphate pH 7.2. The volume that the mice could accept was 0.5 mL. All experiments were based on the National Research Council Guidelines of Laboratory Animals (National Research Council, 2011). All mice were observed to have symptoms, including body weight, and the mice were euthanized on the 15th day using isoflurane. Their feces were collected from the caecum for analysis of SCFA production and the number of probiotics *in vivo*.

2.10 Statistical analysis

The results were performed as a mean ± S.D. for at least three experiments. The data were analyzed using SPSS for Windows (version 26.0). The differences were considered significant at $p<0.001$.

3. RESULTS

3.1 MOS preparation from konjac degrading by rManS2 enzyme

The enzymatic products of konjac with rManS2 at 0 h and 24 h wereanalyzed by HPAEC. The MOS products from konjac at 24 h of degradation were mannose (M1), mannobiose (M2), mannotriose (M3), mannotetraose (M4), mannopentose (M5) and gluco-mannotetraose (Figure 1). The 32.01% level of mannobiose was the highest amount, followed by 25.63% of gluco-mannotetraose (Table 1).

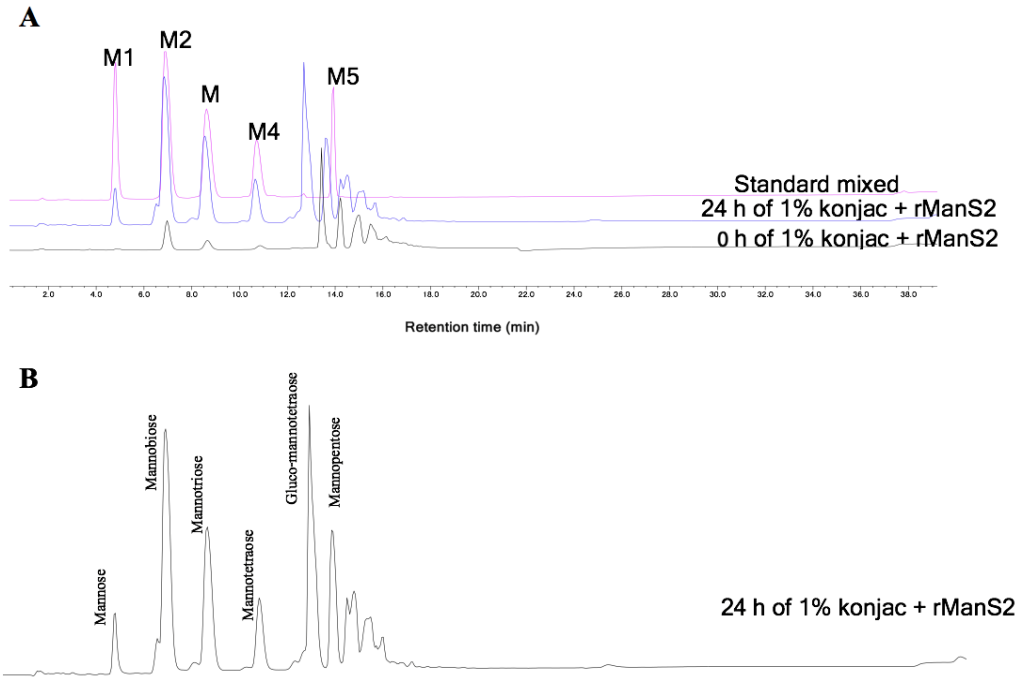

Figure 1. HPAEC chromatograms of analysis; (A) 1% konjac with rManS2 at 0, 24 h and (B) 1% konjac with rManS2 at 24 h
Note: M1 = mannose, M2 = mannobiose, M3 = mannotriose, M4 = mannotetraose, and M5 = mannopentose

Table 1. Percent yield of MOS from degradation of konjac by the rManS2 enzyme

Products	Yield of product (%)
Mannose	4.32
Mannobiose	32.01
Mannotriose	17.70
Mannotetraose	7.50
Gluco-mannotetraose	25.63
Mannopentose	12.84
Total MOS from konjac	100

3.2 Antioxidant activity of MOS by DPPH assay

The DPPH radical scavenging assay showed that the IC_{50} of the MOS products from konjac was 643.06 ± 0.05 µg/mL. The IC_{50} was calculated from the equation, y = 0.0774x + 0.1851, R_2 = 0.9907, while the IC_{50} of ascorbic acid was 494.72 ± 0.15 µg/mL (Figure 2).

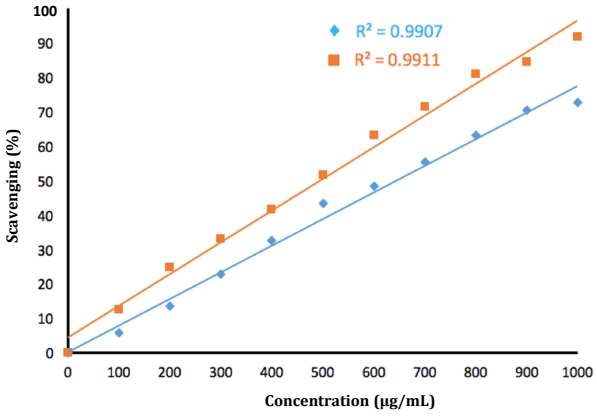

Figure 2. Percent scavenging by DPPH assay; (◆) MOS product and (■) ascorbic acid

3.3 Prebiotic effect on probiotics

The amount of *L. plantarum* N25 was increased in the culture medium with MOS from konjac and FOS added when compared with the control group. The results showed that the MOS product from konjac stimulated the growth of *L. plantarum* N25 *in vitro* when compared to the control, but it slightly promoted the growth of probiotics when compared with FOS (Figure 3).

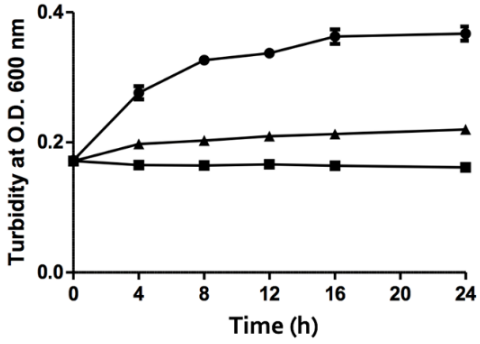

Figure 3. Growth curve of *L. plantarum* N25 at 0 to 24 h after incubation with various carbon sources in basal medium; (●) FOS as carbon source, (▲) MOS as carbon source, and (■) no carbon source as control

3.4 Detection of SCFAs *in vitro*

The results of SCFAs production *in vitro* showed that when *L. plantarum* N25 used MOS as a carbon source, the fermented products were lactic acid and acetic acid. The amount of acetic acid from fermentation with MOS increased when compared with control (without MOS) (Table 2). It also found that *L. plantarum* N25 that used MOS as carbon source could not produce propionic and butyric acids (Figure 4).

Table 2. SCFA products and *in vitro* concentration after incubation of MOS from konjac with *L. plantarum* N25

	Time (h)	Concentration (mM)			
		Lactic acid	Acetic acid	Propionic acid	Butyric acid
Control	0	5.43	1.24	0	0
	24	5.07	2.28	0	0
	48	4.98	2.96	0	0
MOS	0	5.96	1.56	0	0
	24	5.15	4.01	0	0
	48	3.37	7.53	0	0

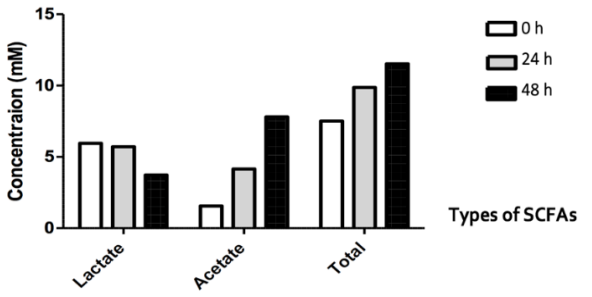

Figure 4. Concentration of lactate, acetate, and total SCFAs at 0, 24, and 48 h after incubating MOS from konjac with *L. plantarum* N25 *in vitro*

3.5 Properties of prebiotics *in vivo* study

To study the properties of prebiotics *in vivo*, the quantity of *L. plantarum* N25 and concentration of SCFAs in each experimental group included prebiotics (only MOS), probiotic (only *L. plantarum* N25) and synbiotic (MOS and *L. plantarum* N25) were compared to test whether or not the MOS from konjac promoted probiotic growth. The results indicated that the body weight of all mice groups was unaffected by MOS supplements in the food compared to the control group. The study demonstrated that the increased amount of *L. plantarum* N25 in synbiotic group was higher than the probiotic group, which treated with the same amount of *L. plantarum* N25 as shown in Figure 5. The results of SCFAs analysis from the supernatant

obtained from feces extraction from each of the mice group show that four SCFAs, including lactate, acetate, propionate and butyrate, were found (Figure 6).

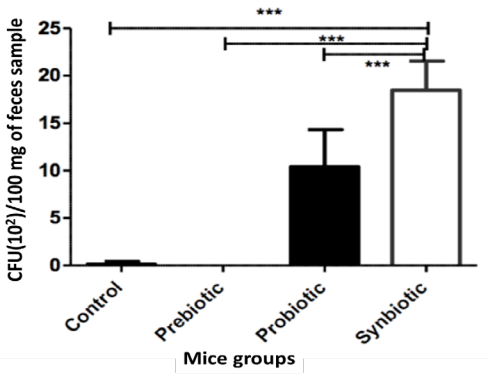

Figure 5. The number of *L. plantarum* N25 per 100 mg of feces sample from each mice group including the control, prebiotic, probiotic, and symbiotic groups

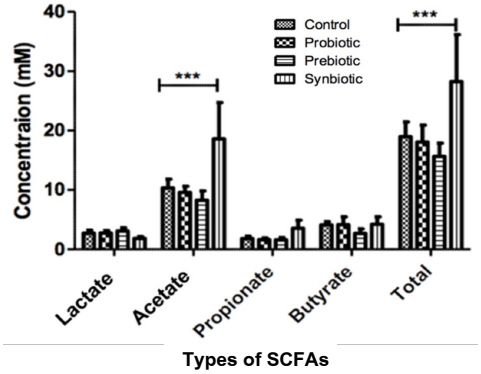

Figure 6. Concentration of short chain fatty acids (SCFAs) production in each mice group. The statistics used for the analysis were mean ± SD, compared between groups using two-way ANOVA

4. DISCUSSION

The recombinant enzyme rManS2 of *Bacillus* sp. SWU60 was constructed in bacteria by Seesom et al. (2017). Its molecular weight is 38 kDa with optimum temperature and a pH at 60°C and 6.0, respectively. In this study, konjac mannan was used as substrate for rManS2 digestion at 45°C for 24 h. The main products were MOS. In general, the structure of konjac mannan consisting of residues of glucose and mannose molecules linked by β-1,4-glycosidic bonds. The rManS2 is an endo-mannanase enzyme that randomly digests in the backbone structure of the mannan polysaccharides and generates products with different structure sizes (Yang et al., 2017). In this study, the DPPH method was used for the measurement of free-radical scavenging property of a MOS product from konjac. The results of the DPPH assay are shown in terms of the IC_{50}. Normally, the lowest value of IC_{50} correlated with the highest antioxidant activity. Ascorbic acid was used as a standard substance for comparison (Olorugbami et al., 2015). Although the value of antioxidants of MOS was less than the standard, but the MOS had an antioxidant value of up to 70% compared with ascorbic acid. The antioxidant activity of oligosaccharides derived from a hydroxyl group (R-OH) of monosaccharide sugar in the structure which served as its bioactive property (Jana and Kango, 2020). Furthermore, another study found that the MOS from konjac showed DPPH radical scavenging activity based on amount of oligosaccharide molecules and a different structural base of sugar against the MOS from other natural polymannans (Amna et al., 2018). These results demonstrated that the MOS product from konjac can take advantage of the preparation of functional foods. After investigating the prebiotic efficiency of MOS from konjac on *L. plantarum* N25 by focusing on the growth promotion. This is similar to the result of a previous research, which found that *L. plantarum* grown in the medium with a FOS was higher than that grown the medium with MOS, but showed lower surface adhesion properties and aggregation rates (Cao et al., 2019). Although, MOS can stimulate growth of *L. plantarum* N25 less than FOS, but it has been reported that MOS is one of the important prebiotic substances that promotes the growth of probiotics (Rolim, 2015). The ability of prebiotics to stimulate the growth of probiotics are different, because of the different prebiotic structures and/or specificity of each probiotic species. In addition, a few research studies reported the prebiotic properties of MOS; for example, MOS from CM (Thongsook and Chaijamrus, 2018). Therefore, the results of this study informed that MOS from konjac could be produced as a natural source of prebiotics. It can be seen that the MOS had some prebiotic properties. In addition, the production of SCFAs *in vitro* was investigated by using supernatant of cultured media to detect SCFAs products including lactic, acetic, propionic, and butyric acid. These results were different from some studies in which the MOS from coffee grounds fermented showed slight levels of propionate and butyrate (Pérez-Burillo et al., 2019), the MOS from locust bean galactomannan fermented showed acetate and propionate (Suryawanshi and Kango, 2021), possibly due to differences in the composition of the MOS from different sources. Furthermore, the amount of lactic acid decreased while the amount of acetic acid increased. It can be explained by the fact that lactic acid was used as the primary product for synthesis of acetic acid, a secondary product in the fermentation process of the lactic acid bacteria (LAB) (Castro-Alba et al., 2019). This may be due to the limitation of each strain in which different types of SCFAs are produced *in vitro* and the type of prebiotics that have different effects on probiotics. For example, *B. bifidum* and *L. gasseri* could express propionic acid in significant amount, but not produce butyric acid, while *Bifidobacterium sp.* could produce butyrate from insulin, and acetate and lactate from FOS (Rossi et al., 2005; Dong et al., 2012). For the *in vivo* study, the body weight of mice from prebiotic, probiotic, and synbiotic groups was unaffected by the groups of MOS-treated when compared to the control group (p<0.001) (data not shown). Furthermore, there were no symptoms and deaths reported during the experimental period. It is indicated that MOS from konjac was dose-tested and considered as a safe prebiotic for mice. The increasing of probiotics *in vivo* was performed by plate count method. The synbiotic group showed the highest number of *L. plantarum* N25 (p<0.001). These results were consistent with previous study, which found that mice treated MOS from coffee had

higher number of probiotics compared with control group (Asano et al., 2004). Therefore, it can be seen that MOS products from konjac has prebiotic property to promote growth of probiotics *in vivo*. The SCFAs production *in vivo* was detected from the feces of mice. The concentration of acetate and the total SCFAs of synbiotic group were significantly higher than the control group (p<0.001) whereas, propionate and butyrate was not detected by an *in vitro* study. This may be due to the limitation of growth conditions of probiotics, such as carbohydrate sources and different environments, compared to *in vivo* (Kahouli et al., 2015). In the animal model, the synbiotic group (*L. plantarum* N25 and MOS) produced higher amounts of SCFAs, such as acetic acid and propionic acid, and total SCFAs which differed from the control group, while the concentration of lactate and butyrate were similar to all mice groups. In addition, the amount of SCFAs such as acetic, lactic, propionic, and butyric acids were different from *in vitro* because butyric acid and propionic acid were found *in vivo*, which may be due to in the gastrointestinal tract of the mammalian composition of the several gastrointestinal microflorae. Normally, SCFAs are obtained from bacteria fermentation. These results were similar with previous studies, which found that the concentration of SCFAs in mice treated with MOS were significantly higher than the control group (Yazbeck et al., 2019; Asano et al., 2004). In this study, the MOS was fermented by anaerobic pathway of probiotic bacteria. In the case of MOS hydrolysis, the bacteria produced SCFAs through the anaerobic glycolysis, also known as lactic acid system (Wolfe, 2015). Generally, the bacteria produced acetic, propionic and butyric acids were released at a ratio of 60:20:20. These products are a source of energy for metabolism in the body. In addition, it was found that the SCFAs have also been shown to provide other benefits for health such as protecting the intestines from attaching and growing of pathogens, changing the gut environment to an acidic one that is unsuitable for the growth of pathogens (Besten et al., 2013). Butyric acid is a particularly important source of energy for the colonocytes, which has a positive effect on preventing gastrointestinal disorders, reducing the risk of inflammation bowel disease, or preventing the occurrence of certain cancers, including colon cancer (Baxter et al., 2019). Therefore, it can be seen that SCFAs are useful and necessary for health to maintain a good balance of the body (Thursby and Juge, 2017).

5. CONCLUSION

MOS from glucomannan of konjac had the ability to be antioxidant *in vitro*. It also had prebiotic property which promote the growth of the probiotic strains used in this study. Furthermore, the MOS could be produced SCFAs *in vitro* and *in vivo* whereas butyric acid was detected only *in vivo*. Therefore, MOS can be used as prebiotics, which can be applied in various industries, such as pet food, livestock, infant formulas, pharmaceuticals and other medical fields.

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