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Characterization of prebiotics and their synergistic activities with *Lactobacillus* probiotics for β-glucuronidase reduction

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ABSTRACT: The role of synbiotics for enriching health and well-being in addition to suppressing disease is gaining interest. Synergistic activities of four candidate prebiotics as exopolysaccharides (EPSs) derived from *Lactobacillus fermentum* TISTR 2514 (EPS-TISTR 2514), *Pediococcus acidilactici* TISTR 2612 (EPS-TISTR 2612), manno-oligosaccharides and rice syrup-oligosaccharides were characterized and evaluated for decreasing the risk of colorectal cancer (CRC). Results revealed that one or more candidate prebiotics stimulated the growth of *Lactobacillus casei* DSM 20011, *Lactobacillus plantarum* DSM 2648, and *Lactobacillus rhamnosus* DSM 20021 by at least two orders of magnitude higher than positive control (using FOS as carbon source) within 24 h *in vitro*. Simulated gastrointestinal (pH 1) and α-amylase (pH 7) resistance were tested. Results showed more than 75% remaining after incubation at 37 °C after 6 h for all treatments except rice syrup. *L. plantarum* DSM 2648 + manno-oligosaccharides (Tr.1), *L. plantarum* DSM 2648 + EPS-TISTR 2612 (Tr.2), *L. rhamnosus* DSM 20021 + rice syrup-oligosaccharides (Tr.3), *L. casei* DSM 20011 + EPS-TISTR 2514 (Tr.4), and *L. rhamnosus* DSM 20021 + EPS-TISTR 2514 (Tr.5) significantly decreased bacterial β-glucuronidase activity in rat feces demonstrating the potential of synergistic activity to reduce CRC risk. Synergistic activity of *L. plantarum* DSM 2648 + EPS-TISTR 2612 (Tr.2) showed the highest percentage reduction at 57.94 and 50.72% in male and female rats, respectively. Effectiveness of synbiotics in reducing the risk of CRC can be applied to future functional food development.

KEYWORDS: β-glucuronidase, prebiotics, probiotics, synbiotics

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the fourth leading cause of cancer-related death worldwide. CRC is anticipated to increase by 60% to more than 2.2 million new cases and 1.1 million cancer deaths by 2030[1]. Around one third of deaths from cancer are due to behavior and dietary risks. Incidence of CRC is significantly related to age, high body mass index, low fruit and vegetable intake, lack of physical activities, tobacco and alcohol use[2] and high consumption of red and processed meat[3]. Generally, diet and antibiotics directly influence gut microbiota diversity relating to colonic disease and colon cancer[4]. Two groups of intestinal microbiota which exhibit a pivotal role in human health are beneficial and detrimental bacteria. Some strains of *Lactobacillus* and *Bifidobacterium* have been reported as probiotics with ability to stimulate the immune system, inhibit pathogen colonization, improve bowel motility, produce short-chain fatty acids (butyrate, acetate and propionate), and vitamin K and B groups via carbohydrate fermentation[5]. Detrimental bacteria such as *Escherichia coli*, *Bacteroides fragilis*, *Fusobacterium nucleatum*, and *Clostridium perfringens* are associated with proteolytic fermentation and fecal bacteria production. Some protein products of proteolytic fermentation including phenolic compounds, amines, ammonia, N-nitroso compounds, hydrogen sulfide and indoles are toxic to the host[6]. Goldin and Gorbach[7] recorded rat fecal bacterial enzyme activities involving β-glucuronidase, azoreductase and nitroreductase at

significantly higher levels when rats were fed with meat. These enzymes had the capacity to convert procarcinogens to carcinogens through hydrolysis of glycosidic bonds. Furthermore, previous studies displayed higher activity of fecal β-glucuronidase in patients with colon cancer than in the healthy control[8]. Conversely, specific activities of fecal enzymes were reduced after *L. acidophilus* was consumed by meat-fed rats and human volunteers[9]. Several reports suggested that the synergic activity of pro- and prebiotics showed more potential effects in the host. The combination of *Bifidobacterium longum* and inulin was more efficient at decreasing β-glucuronidase activity on colonic aberrant crypt foci (ACF) than *B. longum* or inulin alone[10]. Roller[11] found that proliferative response of lymphocytes (in Peyer's patches) from AOM-treated (azoxymethane-treated) rats was suppressed in the combination of maltodextrin and probiotics-supplemented rats. Similarly, Bomba[12] found that prebiotics (maltodextrin KMS X-70 and FOS) potentiated the probiotic effect in the small and large intestines, respectively. Non-digestible oligosaccharides have recently received increasing interest as a result of their prebiotic status. This encourages host health by stimulating beneficial bacteria and inhibiting pathogen growth[13] leading to reduced risk of diseases. Prebiotics are varied, depending on their origin as bacteria, algae, fungi and higher plants. Natural origins of resistant starch or synthetic origins such as xylo-oligosaccharides, pyrodextrin, and isomalto-oligosaccharides are now being increasingly considered as novel prebiotics and entering world markets[14]. Here, exopolysaccharides (EPSs) derived from *L. fermentum* TISTR 2514 (EPS-TISTR 2514) and *P. acidilactici* TISTR 2612 (EPS-TISTR 2612), rice syrup-oligosaccharides derived from *Aspergillus oryzae* TISTR 3222 fermentation process, and manno-oligosaccharides from defatted copra meal hydrolysis and probiotics were synergized. Then, the effects of combination (synbiotics) on reducing β-glucuronidase activity *in vivo* were investigated, leading to the development of functional food ingredients that reduced the risk of colorectal cancer.

MATERIALS AND METHODS

Candidate prebiotic preparation

Exopolysaccharide producing lactic acid bacteria

Single colonies of *L. fermentum* TISTR 2514 and *P. acidilactici* TISTR 2612 were used as starters in MRS broth (HiMedia, India). Fresh overnight cultures were incubated at 37 °C for 48 h in anaerobic jar. Samples were centrifuged at 7510 × *g* for 10 min. The supernatants were then collected and solutions were concentrated using a rotary evaporator to one third of the starting volumes. EPS was precipitated with two volumes of cold 95% ethanol. The mixtures were stored at 0 °C for 48 h and then centrifuged at 7510 × *g* for 10 min. The supernatants were discarded and EPS resuspended with 10–50 ml of distilled water. Compositions of oligosaccharides were analyzed by the HPLC technique. EPS solutions (EPS-TISTR 2514 and EPS-TISTR 2612) were freeze-dried and kept at −20 °C until required for experimentation.

Manno-oligosaccharides

Two percent defatted copra meal (w/v) was digested with 50 units/ml mannanase enzyme (Mannanase BGM Amano, Japan) in citric buffer pH 4.5 at 70 °C for 6 h. The reaction was stopped by boiling at 100 °C for 15 min. After cooling to room temperature, the solution mixture was centrifuged at 7510 × *g* for 15 min and the supernatant was collected for concentration by a rotary evaporator. Compositions of oligosaccharide were analyzed by the HPLC technique. Oligosaccharide solutions were freeze-dried and kept at −20 °C until required for experimentation.

Rice syrup-oligosaccharides

Rice syrup was prepared from *A. oryzae* TISTR 3222 fermentation process using sticky rice as a substrate as described by Saman[15]. Spore suspensions were prepared after a 5-day sporulation period and diluted with 0.85% NaCl to required density (108 spores/ml) using hemocytometer estimation. The sterilized solid substrate was inoculated with 1 ml of the prepared inoculum, and contents were mixed thoroughly and incubated at 30 °C for 7 days. The fermented mass was added with 0.03 g $CaCl_2$, adjusted to pH 6 and incubated at 50 °C and 60 °C for 30 min and 60 min, respectively. Finally, the fermentation was adjusted to pH 5.6 and incubated at 70 °C for 60 min. The reaction was stopped by boiling for 30 min, and the fermented solution was filtrated using filter paper (Whatman No. 1). Compositions of rice syrup were analyzed by HPLC, then freeze-dried and kept at −20 °C until required for experimentation.

Growth-stimulation effect on probiotics

Three strains of probiotics consisting of *L. casei* DSM 20011, *L. plantarum* DSM 2648 and *L. rhamnosus* DSM 20021 were purchased from DSMZ Bacteria Collection (Germany). All candidate prebiotics were tested for their growth stimulation effect on probiotics as described by Chaiongkarn[16]. Briefly, 1% of inocula (with OD_{600} of 0.5) was inoculated in basal medium broth using different carbon sources supplemented with 1% w/v of glucose (negative control), FOS (Bornnet Corporation Co., Ltd.) (positive control), candidate prebiotics (EPS-TISTR 2514, EPS-TISTR 2612, manno-oligosaccharides and rice syrup-oligosaccharides), and then incubated at 37 °C for 48 h in anaerobic jar. Growth of probiotics was enumerated as CFU/ml, counting colonies which appeared on MRS agar+0.5% $CaCO_3$ using drop plate technique and incubated in anaerobic jar at 37 °C for 48 h (samples were taken every 12 h).

Simulated gastrointestinal (SGI) and α-amylase tolerance test

Hydrolysis of EPSs, manno-oligosaccharides and rice syrup-oligosaccharides to SGI pH 1 and α-amylase pH 7 were described by Wichienchot[17]. Here, the commercial prebiotic (FOS, Bornnet Corporation Co., Ltd.) was used as a positive control. Percentage of hydrolysis was analyzed after incubation for 1, 2, 3, 4 and 6 h. Oligosaccharides remaining after digestion were measured by phenol-sulfuric acid and the DNS method[18,19]. Hydrolysis degrees of samples were calculated according to the following formula, where reducing sugar released is the difference between reducing sugar content at a specified time and initial reducing sugar content.

$$\text{Hydrolysis degree}\,(\%) = \frac{\text{Reducing sugar released} \times 100}{\text{Total sugar} - \text{Initial reducing sugar}}$$

β-glucuronidase assay *in vitro*

One percent of *E. coli* TISTR 887 and probiotics (*L. plantarum* DSM 2648, *L. casei* DSM 20011 and *L. rhamnosus* DSM 20021) at $OD_{600} = 0.5$ was used as a starter inoculum in basal culture medium consisting of peptone water 2.00 g, yeast extract 2.00 g, NaCl 0.10 g, K_2HPO_4 0.04 g, $MgSO_4 \cdot 7H_2O$ 0.01 g, $CaCl \cdot 2H_2O$ 0.01 g, $NaHCO_3$ 2.00 g, L-cysteine HCl 0.50 g, bile salts 0.50 g, and hemin 0.05 g followed with liquid addition of resazurin (0.05 g/l) 4.00 ml, vitamin K 10 μg, and Tween-80 2.00 ml. The mixture was adjusted to 1000 ml and sterilized at 121 °C for 15 min. For β-glucuronidase enzymatic assay, *E. coli* was cultured in basal medium broth using different carbon sources supplemented with 2% (w/v) glucose, FOS and the four candidate prebiotics. Combination of candidate prebiotics and probiotics was compared at 37 °C for 48 h in anaerobic jar. The cell suspension was centrifuged at $7510 \times g$ for 10 min at 4 °C, then discarded supernatant and cell pellet was resuspended in 30 ml of pH 7 phosphate buffer. Cell suspension was sonicated (4 cycles, 30 s, 0 °C) and centrifuged at $7510 \times g$ for 10 min at 4 °C. The supernatant was collected, transferred to a clean tube and kept at −80 °C until required for β-glucuronidase enzymatic assay. Fecal β-glucuronidase activity was assayed in triplicate for release of phenolphthalein from phenolphthalein mono-p-glucuronide (Sigma Chemical Co., St. Louis, MO) and measured at 540 nm by a spectrophotometer (Milton Roy Co, Rochester, NY).

Bacteriological methods

A single probiotic colony (*L. casei* DSM 20011, *L. plantarum* DSM 2648 and *L. rhamnosus* DSM 20021) was cultured in 15 ml of MRS broth (Hi-Media, India) in anaerobic jar at 37 °C for 24 h. Then, the probiotic was scaled up to 200 ml in MRS broth by 1% starter cell solution (measuring 0.5 at OD_{600}) and grown in the same condition for 48 h. Before cell harvesting, 500 μl of cell solutions were collected for serial dilution in 4.5 ml of normal saline and growth of probiotics was enumerated as CFU/ml, counting colonies using the drop plate technique. The supernatant was removed after centrifugation at $7510 \times g$ for 10 min, and the cell pellet was resuspended with sterile distilled water and centrifuged to wash the remaining media at least twice. Finally, the cell pellet was resuspended with 30–50 ml of UHT milk and 2% (w/v) of candidate prebiotics were added.

Animals and treatments

Wistar rats, 7-week-old males and females, with starting weights of 276.8–281.2 g and 211.6–209.0 g, respectively were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakhon Pathom. The rats were kept in cages with sterilized wood shavings as bedding at 24 ± 2 °C in 12 h light/dark cycles and fed with standard diet and reverse osmosis (RO) water ad libitum. All rats were acclimatized for 7 days prior to the experiments. The study was approved by the TISTR Animal Ethical Committee (No. 59001). Rats were randomly divided into six groups of each gender, consisting of five experimental groups and

one control group ($n = 5$ in each group). All animals were fed the normal diet (Mouse and rat diet No. 082, NLAC, Thailand) and given UHT milk (control group) with diet containing 2% (w/v) of candidate prebiotics (feeding rate of 5 g of candidate prebiotics per kg rat body weight), containing log 9–log 10 CFU/ml of probiotics per day in all experiments for 8 weeks. After dosing, all animals were observed once daily and body weight was recorded weekly and at the end of the test. Mean body weight gain of the animals in the test groups was calculated in comparison to rats of the control group using ANOVA ($p \leqslant 0.05$). At the end of the experiment, all survivors were humanely killed by CO_2 asphyxiation and a necropsy was performed to examine gross pathological lesions.

Feces preparation for β-glucuronidase assay

Methods for β-glucuronidase assay were described by Goldin and Gorbach[7]. Fresh feces of each rat in the treatment group were taken from the large intestinal part of the cecum after death and placed in sterile aluminum foil. The feces were then homogenized using a spatula and fecal samples (1.5 g) were dissolved in 30 ml of phosphate buffer at pH 7 and sonicated for 2 min (4 cycles, 30 s, 0 °C). A suspended solution of feces was centrifuged at $7510 \times g$ for 10 min at 4 °C. The supernatant was collected in a clean tube and kept at −80 °C until required for assessment of β-glucuronidase. The β-glucuronidase was assayed in triplicate as described above. Results were displayed as units/g solid ± SD.

Statistical analysis

All analyses were performed in triplicate using SPSS (version 18) with results displayed as mean ± SD. Difference between the means was analyzed by Tukey's method and level of significance was defined at $p < 0.001$–0.05.

RESULTS

Exopolysaccharides, manno-oligosaccharides and rice syrup-oligosaccharides: characterization and their effect on growth stimulation of probiotics

Compositions of EPSs, manno-oligosaccharides and rice syrup-oligosaccharides were characterized using the HPLC technique. Results showed that the monosaccharide composition of EPS-TISTR 2514 was 0.47 g/l galactose, 0.43 g/l rhamnose, 0.38 g/l glucose and 0.37 g/l mannose, while EPS-TISTR 2612 consisted of 0.87 g/l glucose and 0.36 g/l mannose. Composition of manno-oligosaccharides consisted of 6 g/l mannobiose, 8 g/l mannotriose, 16 g/l mannotetraose and 25 g/l mannopentaose, while the composition of rice syrup-oligosaccharides was 135 g/l glucose, 81 g/l maltose, 12 g/l maltotriose and isomalto-oligosaccharide (IMO) including 44 g/l isomaltose, 10 g/l panose and 7 g/l isomaltotriose.

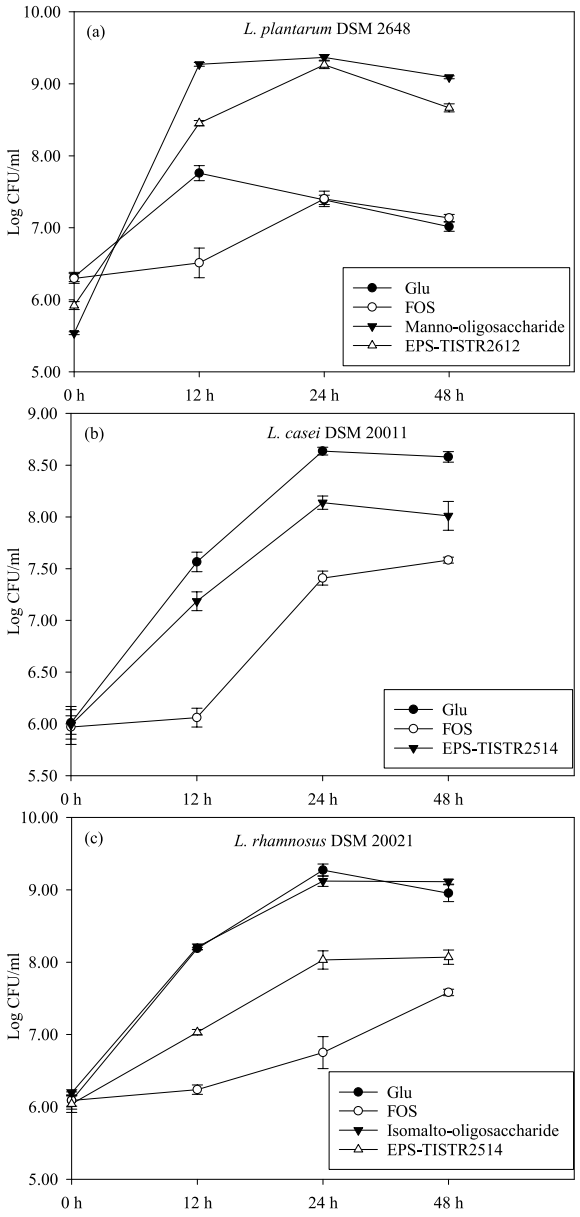

Fig. 1 Capability of candidate prebiotics for higher growth stimulation on (a) *L. plantarum* DSM 2648, (b) *L. casei* DSM 20011 and (c) *L. rhamnosus* DSM 20021 as compared to the one fed FOS.

The effect of candidate prebiotics on probiotics growth in basal medium *in vitro* showed that all oligosaccharides were capable of promoting growth of probiotic organisms greater than FOS within 24 h (Fig. 1). Results indicated that manno-oligosaccharides and EPS-TISTR 2612 had the ability to stimulate growth of *L. plantarum* DSM 2648 from log 5.52 ± 0.02 to log 9.35 ± 0.04 and from log 5.52 ± 0.05 to log 9.26 ± 0.06 CFU/ml, respectively. EPS-TISTR 2514 enhanced the growth of *L. casei* DSM 20011 and *L. rhamnosus* DSM 20021 from log 6.03 ± 0.05 to log 8.17 ± 0.02 and from log 6.02 ± 0.08 to log 8.08 ± 0.06 CFU/ml, respectively[16]. *L. rhamnosus* DSM 20021 showed the highest growth when cultured in basal medium broth supplemented with rice syrup-oligosaccharides from log 6.22 0.04 to log 9.10 0.07 CFU/ml within 24 h. The rice syrup consisted of numerous digestible sugars including glucose and maltose that were easily utilized by probiotics. Furthermore, isomalto-oligosaccharides (isomaltose, panose and isomaltotriose) were found to improve intestinal microbiota and prevent dental caries[20,21]. Kohmoto[22] found that isomalto-oligosaccharides were utilized by *Bifidobacterium* but not by *E. coli*, *Clostridium* and other bacteria. The capability of candidate prebiotics to stimulate growth of probiotics was used as a criterion for optimizing combination between candidate prebiotics and probiotics. Five synergistic couples were selected, consisting of *L. plantarum* DSM 2648 + manno-oligosaccharides, *L. plantarum* DSM 2648 + EPS-TISTR 2612, *L. rhamnosus* DSM 20021 + rice syrup-oligosaccharides, *L. casei* DSM 20011 + EPS-TISTR 2514 and *L. rhamnosus* DSM 20021 + EPS-TISTR 2514.

SGI and α-amylase tolerance

Resistance of all oligosaccharides to SGI pH 1 and α-amylase was tested and results are summarized in Table 1. FOS had the highest tolerance to α-amylase with the highest degree of hydrolysis when incubated in SGI pH 1 at 37 °C for 6 h. Non-digestible oligosaccharide properties showed that at least 65% of EPS-TISTR 2612, EPS-TISTR 2514 and manno-oligosaccharides were tolerant to SGI pH 1 and α-amylase pH 7. Rice syrup-oligosaccharides demonstrated acidity resistance but did not show tolerance to α-amylase. The composition of rice syrup was abundant with fermented sugar (glucose, maltose and maltotriose) but the highest hydrolysis would be possible when some production of rice syrup was not successful due to a range of alpha glucosidic linkage of remained-isomaltodextrin. Therefore, they were simply digested by amylase. However, more than 90% of rice syrup-oligosaccharides were resistant to SGI pH 1 for at least 6 h.

Table 1 Percentage hydrolysis of α-amylase and SGI compared with FOS (commercial prebiotics).

Candidate prebiotic	Hydrolysis (%) after incubation (6 h)		
	α-amylase	SGI	Total
EPS-TISTR 2612	3.54 ± 0.08	20.44 ± 0.32	23.98
EPS-TISTR 2514	17.29 ± 0.62	17.40 ± 0.17	34.69
Manno-oligosaccharides	14.52 ± 3.80	15.76 ± 2.40	28.28
Rice syrup-oligosaccharides	116.58* ± 5.36	4.29 ± 0.23	120.87
FOS	0.68 ± 0.08	126.16* ± 1.10	126.84

* indicates $p < 0.01$; EPS-TISTR 2612 and EPS-TISTR 2514 refer to EPS-producing *P. acidilactici* TISTR 2612 and *L. fermentum TISTR* 2514, respectively.

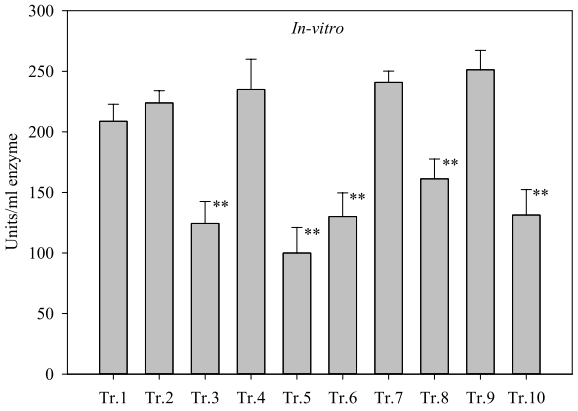

Fig. 2 β-Glucuronidase activity (units/ml) of *E. coli* when cultured in basal medium with different carbon sources *in vitro* were compared with control (*E. coli* + glucose). ** refers to $p < 0.01$. (Tr.1 = *E. coli* + glucose, Tr.2 = *E. coli* + EPS-TISTR 2612, Tr.3 = *E. coli* + EPS-TISTR 2612 + *L. plantarum* DSM 2648, Tr.4 = *E. coli* + EPS-TISTR 2514, Tr.5 = *E. coli* + EPS-TISTR 2514 + *L. rhamnosus* DSM 20021, Tr.6 = *E. coli* + EPS-TISTR 2514 + *L. casei* DSM 20011, Tr.7 = *E. coli* + manno-oligosaccharides, Tr.8 = *E. coli* + manno-oligosaccharides + *L. plantarum* DSM 2648, Tr.9 = *E. coli* + rice syrup-oligosaccharides, Tr.10 = *E. coli*+ rice syrup-oligosaccharides + *L. rhamnosus* DSM 20021).

Effect of probiotics and candidate prebiotics on reduction of β-glucuronidase activity *in vitro*

From the candidate prebiotic properties, we next evaluated the effect of candidate prebiotics with and without probiotics (*L. plantarum* DSM 2648,

L. casei DSM 20011 and *L. rhamnosus* DSM 20021) for the reduction of β-glucuronidase activity in *E. coli* (*in vitro*). *E. coli* was cultured in basal medium broth using different carbon sources supplemented with 2% (w/v) of four candidate prebiotics compared with glucose. Results showed that the activity of β-glucuronidase did not decrease when using EPS-TISTR 2612 (Tr.2), EPS-TISTR 2514 (Tr.4), manno-oligosaccharides (Tr.7) and rice syrup-oligosaccharides (Tr.9) as a carbon source (Fig. 2), showing, 223.96 ± 10.03, 235.00 ± 24.97, 240.83 ± 9.36 and 251.25 ± 16.02 units/ml enzyme, respectively. Similarly, in treatments of *E. coli* cultured with *L. plantarum* DSM 2648, *L. casei* DSM 20011 or *L. plantarum* DSM 20021 supplemented with glucose in the same conditions for 48 h, activity of β-glucuronidase did not significantly decrease from the control ($p = 0.525$, data not shown). These results indicated that supplementation of candidate prebiotics or probiotics alone did not influence the activity of β-glucuronidase in *E. coli*. However, when *E. coli* was cultured with a combination of candidate prebiotics and probiotics, the results showed that the activity of β-glucuronidase significantly decreased from culture with glucose and prebiotics alone ($p < 0.01$) in all treatments as 124.38 ± 18.21, 100.00 ± 21.07, 130 ± 19.69, 161.25 ± 16.35 and 131.25 ± 21.13 units/ml enzyme for EPS-TISTR 2612 + *L. plantarum* DSM 2648 (Tr.3), EPS-TISTR 2514 + *L. rhamnosus* DSM 20021(Tr.5), EPS-TISTR 2514 + *L. casei* DSM 20011 (Tr.6), manno-oligosaccharides + *L. plantarum* DSM 2648 (Tr.8), and rice syrup-oligosaccharides + *L. rhamnosus* DSM 20021 (Tr.10), respectively (Fig. 2). Competition evaluation indicated that probiotics utilized EPS-TISTR 2612, EPS-TISTR 2514, manno-oligosaccharides and rice syrup-oligosaccharides better than *E. coli*. This suggested that lactic acid bacteria directly influenced a lowering of pH in basal medium. Studies in human have demonstrated that the capacity of probiotics to decrease bacterial enzyme activity is strain specific. For example, *L. casei* Shirota significantly decreased enzymatic activity[23] while *L. acidophilus* A1, *L. rhamnosus* DR20 and *L. plantarum* 299V were unable to decrease β-glucuronidase activity[24,25]. Enzymatic assessments suggested that the synergistic activities of candidate prebiotics, *L. plantarum* DSM 2648, *L. rhamnosus* DSM 20021 and *L. casei* DSM 20011 showed a significant decrease of enzymatic activity *in vitro* but the effect of synergistic activity on decreasing β-glucuronidase activity *in vivo* was required.

Table 2 Percentage of decreasing β-glucuronidase activity *in vivo* compared with control.

Treatment	Male rat	Female rat
Tr.1	15.31	15.25
Tr.2	57.94	50.72
Tr.3	34.63	19.29
Tr.4	15.50	23.12
Tr.5	31.83	15.62

Effect of synbiotics on reduction of β-glucuronidase activity *in vivo*

To study the effect of synbiotics on decreasing β-glucuronidase activity *in vivo*, we orally administered synbiotics to the rats for 8 weeks. Combination of five candidate prebiotic and probiotic experimental diets were administered as *L. plantarum* DSM 2648 + manno-oligosaccharides (Tr.1), *L. plantarum* DSM 2648 + EPS-TISTR 2612 (Tr.2), *L. rhamnosus* DSM 20021 + rice syrup-oligosaccharides (Tr.3), *L. casei* DSM 20011 + EPS-TISTR 2514 (Tr.4), and *L. rhamnosus* DSM 20021 + EPS-TISTR 2514 (Tr.5) compared to the control (fed UHT milk at the same volume). Range of viable probiotics in the experimental diets was found between log 9–10 CFU/ml of diet, and 5 g of candidate prebiotics per kg of rat body weight per day was used in all periods of experiments. After feeding the five diets for 8 weeks, there were no significant differences in final body weight between dietary groups in the same gender (404.00–415.40 g; $p = 0.816$ in male rats and 246.6–251.40 g; $p = 0.976$ in female rats). Highest reduction (more than 50%) was found in Tr.2 which was significantly less than the others ($p = 0.000$) for both male and female rats (Fig. 3). In the *in vitro* experiments, percentages of enzyme activity decrease from the control for Tr.3, Tr.5, Tr.6 and Tr.10 were similar in all combination of synbiotics (except in Tr.8) but *in vivo* results were dramatically different because *in vivo* gut microbiota is more diverse and complex than *in vitro*. Besides *E. coli*, some bacterial strains of *B. fragilis*, *F. nucleatum*, and *C. perfringens* have been found in association with fecal bacterial enzyme production[12].

Results also indicated that male and female rats fed with *L. plantarum* DSM 2648 and EPS-TISTR 2612 showed a greater reduction of β-glucuronidase activity than rats supplemented with *L. plantarum* DSM 2648 and manno-oligosaccharides. Rats fed *L. rhamnosus* DSM 20021 showed the greatest reduction of enzyme activity when fed with rice syrup-

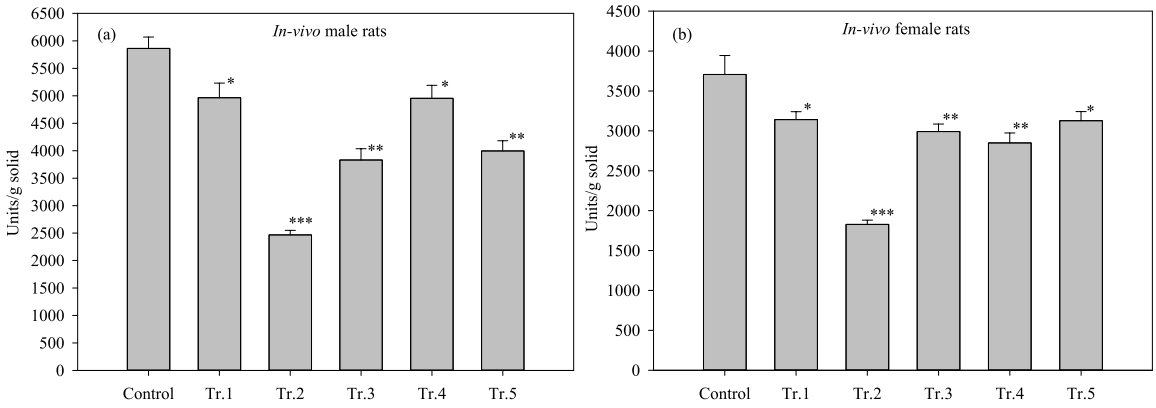

Fig. 3 Comparison of β-glucuronidase activity (units/g solid) between the control (fed UTH milk at the same volume) and *in vivo* treatments in (a) male rats and (b) female rats (fed five candidate prebiotic and probiotic diets): Tr.1 = *L. plantarum* DSM 2648 + manno-oligosaccharides, Tr.2 = *L. plantarum* DSM 2648 + EPS-TISTR 2612, Tr.3 = *L. rhamnosus* DSM 20021 + rice syrup-oligosaccharides, Tr.4 = *L. casei* DSM 20011 + EPS-TISTR 2514 and Tr.5 = *L. rhamnosus* DSM 20021 + EPS-TISTR 2514 were indicated. * and ** refer to $p < 0.01$ and $p < 0.001$ from control, respectively. *** refers to $p < 0.0001$ from the other combinations.

oligosaccharides. A small difference from *L. rhamnosus* DSM 20021 and EPS-TISTR 2514 was observed but the trend of decreasing percentage in male and female rats was similar (Table 2). Results indicated that the efficacy of synergistic activity was specific to strains and prebiotics supplementation. Thus, bacterial enzyme assessment in this study displayed that the combination of probiotics and prebiotics showed significantly decreasing β-glucuronidase activity from the control for both *in vitro* and *in vivo*. Although *L. plantarum* DSM 2648 has been reported for its tolerance to gastrointestinal conditions, enhancing intestinal barrier function increased the transepithelial electrical resistance (TEER) 135% more than *L. rhamnosus* HN001 and showed greatest inhibitory effect on the adherence of entero-pathogenic *E. coli* (EPEC) strain[26]. However, the effect of *L. plantarum* DSM 2648 on β-glucuronidase reduction depended on prebiotic supplementation. Lower enzyme activity was probably due to EPS-TISTR 2612 which selectively stimulated growth and activity of *L. plantarum* DSM 2648 on modulation of gut microbiota.

DISCUSSION

Decreased β-glucuronidase activity may confer benefits by limiting the microbial production of aglycons involved in the pathogenesis of colorectal cancer[27]. β-glucuronidase can hydrolyze many glucuronides in the lumen of the gut. Toxic and carcinogen compounds from this reaction are detoxified by glucuronide formation in the liver and then enter the bowel via bile. In this way, toxic aglycons can be regenerated in situ in the bowel by bacterial β-glucuronidase. Rat carcinogenicity study results suggested that combined administration of probiotics and prebiotics significantly reduced colonic ACF in AOM-induced rats[10,28]. Roller[11] found that oligofructose-enriched inulin with or without *B. lactis* stimulated IL-10, and prevented AOM-induced suppression of NK cell-like cytotoxicity in Peyer's patches. Depletion of NK cells *in vivo* is associated with enhanced tumor formation in mouse model[29]. Furthermore, studies showed that patients fed with oligofructose-enriched inulin + *L. rhamnosus* GG and *B. lactis* Bb 12 exhibited significant changes in fecal microflora (*Bifidobacterium* and *Lactobacillus* increased while *C. perfringens* decreased), and decreased colonic cell proliferation[30]. Several research findings supported that colonic microbiota have critical roles, beneficial or detrimental to health, controlling epithelial cell proliferation and differentiation through host nutrition via metabolism[31]. However, here, we only emphasized the effect of the combination of probiotics and prebiotics on lowering colorectal cancer risk via demonstration of bacterial enzyme reduction. Effects of the combination between *L. plantarum* DSM 2648 and EPS-TISTR 2612 on AOM-treated rats and colorectal cancer patients are interesting.

By studying prebiotic properties of EPS-TISR 2514, EPS-TISTR 2612, manno-oligosaccharides and rice syrup-oligosaccharides to enhance probiotics and reduce the risk of colorectal cancer

in vitro, we found that manno-oligosaccharides and EPS-TISTR 2612 stimulated the growth of *L. plantarum* DSM 2648 from log 5 to log 9 while rice syrup-oligosaccharides and EPS-TISR 2514 enhanced *L. rhamnosus* DSM 20021 from log 6 to log 9 and from log 6 to log 8, respectively. *L. casei* DSM 20011 showed the highest growth when supplemented with EPS-TISR2514 within 24 h. For non-digestible oligosaccharide properties, EPS-TISTR 2612 had the greatest tolerance to α-amylase and artificial human gastric juice, with more than 75% able to reach the colon. *In vivo* studies showed that activity of bacteria significantly decreased in all experiments, especially rats fed with *L. plantarum* DSM 2648 + EPS-TISTR 2612 which showed the greatest reduction (more than 50% from control). Similar to previous study[32], the combinations of *L. plantarum* + inulin enriched with oligofructose (PRO+PRE) and *L. plantarum* + *Lini oleum virginale* (PRO+O) reduced β-glucuronidase in 1,2-dimethylhydrazine treated (DMH-treated) rats, 35% and 43% reductions from untreated rats as control and 55% and 61% reductions from DMH-treated rats, respectively. The beneficial effect of synergism against β-glucuronidase was dependent on an appropriate mixture of probiotics and prebiotics, as well as strain specificity. Bacterial enzyme assessment confirmed that *L. plantarum* DSM 2648 and *L. rhamnosus* DSM 20021 were more effective for bacterial β-glucuronidase reduction in male and female rats when supplemented with EPS-TISTR 2612 and rice syrup-oligosaccharides, respectively.

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