3 Biotech (2019) 9:93
https://doi.org/10.1007/s13205-019-1633-8

ORIGINAL ARTICLE

In vitro fermentation of copra meal hydrolysate by human fecal microbiota

Phatcharin Prayoonthien[1] · Robert A. Rastall[2] · Sofia Kolida[2] · Sunee Nitisinprasert[1,3] · Suttipun Keawsompong[1,3]

Received: 20 June 2018 / Accepted: 11 February 2019 / Published online: 19 February 2019
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Abstract

Copra meal hydrolysate (CMH) is obtained by hydrolyzing defatted copra meal with β-mannanase from *Bacillus circulans* NT 6.7. In this study, we investigated the resistance of CMH to upper gastrointestinal tract digestion and the fecal fermentation profiles of CMH. Fecal slurries from four healthy human donors were used as inocula, and fructooligosaccharides (FOS) were used as a positive prebiotic control. Fecal batch cultures were performed at 37 °C under anaerobic conditions. Samples were collected at 0, 10, 24 and 34 h for bacterial enumeration via fluorescent in situ hybridization and organic acid (OA) analysis. In vitro gastric stomach and human pancreatic α-amylase simulations demonstrated that CMH was highly resistant to hydrolysis. Acetate was the main fermentation product of all the substrates. The proportions of acetate production of the total OAs from FOS, CMH and yeast mannooligosaccharides (MOS) after 34 h of fermentation did not significantly differ (69.76, 65.24 and 53.93%, respectively). At 24 h of fermentation, CMH promoted the growth of *Lactobacillus* and *Bifidobacterium* groups ($P<0.01$) and did not significantly differ from the results obtained using FOS. The results of in vitro fecal fermentation of CMH indicate that CMH can promote the growth of beneficial bacteria.

Keywords Coconut · Copra meal hydrolysate · In vitro fermentation · Human fecal microbiota

Introduction

The human large intestine contains a very complex microbial ecosystem that is estimated to contain at least 500 different bacterial species (Gibson 2004). Most bacteria in the colon are strict anaerobes, and the combined density of these species is approximately 10^{11}–10^{12} bacteria/g (Salminen et al. 1998; Dethlefsen et al. 2006; Ley et al. 2006).

The international Scientific Association of Probiotics and Prebiotics (ISAPP) defined a dietary prebiotic as a "selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Gibson et al. 2010). The most established prebiotics for human consumption include inulin as well as its derived fructooligosaccharides (FOSs), galactooligosaccharides (GOSs) and lactulose (Sanz et al. 2006>; Gibson et al. 2010; Cockburn and Koropatkin 2016).

Many studies have recently focused on plant materials that could potentially be developed into prebiotics for human consumption, such as dietary fiber from almond skins (Mandalari et al. 2008, 2010), oligosaccharides from dragon fruit (Wichienchot et al. 2010), mannooligosaccharides from coffee (Asano et al. 2004, 2006; Walton et al. 2010) and mannan from copra meal (Titapoka et al. 2008; Ghosh et al. 2015; Pangsri et al. 2015a, b). Aside from plant cell walls, mannooligosaccharides (MOS) can also be extracted from yeast cell walls. The yeast mannan or yeast MOS from yeast cell wall of *Saccharomyces cerevisiae* are α-linked branched oligosaccharides, which consist of a series of oligosaccharides containing α-(1–2)- and α-(1–3)-linked side chains attached to an α-(1–6)-linked backbone (Jones and Ballou 1969; Spring et al. 2015).

MOSs from yeast cell walls are widely used as an animal feed additive in poultry production. Yeast cell wall

✉ Suttipun Keawsompong
fagisuk@ku.ac.th

[1] Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Ladyaow, Chatuchak, Bangkok 10900, Thailand

[2] Department of Food and Nutritional Sciences, University of Reading, Whiteknights, Reading, UK

[3] Center for Advanced Studies in Agriculture and Food, Institute for Advanced Studies, Kasetsart University, Ladyaow, Chatuchak, Bangkok 10900, Thailand

powder has the ability to improve the immune function and intestinal oxidative status of broiler chickens (Li et al. 2016). Yeast MOS has also been shown to improve the growth performance and increase the intestinal immunoglobulin secretion of broiler chickens (Iji et al. 2001; Hooge and Connolly 2011). M'Sadeq et al. (2015) investigated yeast cell wall extract as an alternative to zinc bacitracin or salinomycin using a necrotic enteritis challenge model. Broilers fed yeast cell wall extract exhibited increased villus height and decreased crypt depth, indicating that yeast cell wall extract could reduce the impact of necrotic enteritis in broilers (M'Sadeq et al. 2015).

Copra meal, or coconut residual cake, is the dried meat that remains after coconut milk extraction and it is the main by-product of coconut oil extraction. Saittagaroon et al. (1983) reported that the percentage approximate composition of copra meal on a dry weight basis was as follows: 43–45% carbohydrate, 19–20% crude protein, 10–11% crude fat and 12% crude fiber. The non-starch polysaccharide of copra meal exists as 26% mannan, 61% galactomannan and 13% cellulose (Balasubramaniam 1976). The galactomannan in copra meal is composed of repeating β-(1,4)-mannose units and a few α-(1,6)-galactose units attached to the β-(1,4)-mannose backbone (Hossain et al. 1996; Ghosh et al. 2015).

Copra meal cannot be utilized by monogastric animals and humans, because the high level of mannan makes it resistant to the digestive enzymes in the gastrointestinal tract. However, β-1,4-mannanase can digest the complex mannan polysaccharide to form MOSs. Phothichitto et al. (2006) isolated 19 bacteria and 4 fungi capable of mannanase production from 23 soil samples. *Bacillus circulans* NT 6.7 showed high mannanase activity (0.306 U/mL), with an optimal pH of 7.0–9.0 and an optimal temperature of 50 °C.

The copra meal hydrolysate (CMH) produced by *B. circulans* NT 6.7 was described by Pangsri et al. (2015a). Defatted copra meal was hydrolyzed by 20 U/mL mannanase at 50 °C for 50 min. The hydrolysate was assessed in pure cultures of twelve strains of intestinal bacteria and five strains of pathogenic bacteria. The results showed that this material promoted the growth of the *Lactobacillus* group as effectively as commercial MOSs. CMH did not support the growth of pathogens, such as *Shigella dysenteriae* DMST 1511, *Staphylococcus aureus* TISTR 029 and *Salmonella enterica serovar Enteritidis* DMST 17368.

This study investigated the in vitro fecal fermentation properties of CMH. Human gastric and small intestine conditions were simulated to estimate this substrate's resistance in the upper digestive tract. In vitro fermentation was carried out to monitor the effect of CMH on the human fecal microbiota.

Materials and methods

Preparation of copra meal

Copra meal residue was collected after coconut milk extraction from the Bangkhen Market (Bangkok, Thailand). The residue was dried at 60 °C for 2–4 h and then blended and milled to obtain the particle size of 0.5 mm. The residual oil was removed from the copra meal by petroleum ether extraction using a Soxhlet apparatus (Gerhardt Soxtherm Multistat/SX PC, Königswinter, Germany) (Horwitz et al. 1970).

Microorganism and mannanase production

The mannanase was produced from *B. circulans* NT 6.7 (modified from Phothichitto et al. 2006). Briefly, a 5 L fermenter (Winpact FS-05; Major Science, California, USA) was filled with 3.5 L of production medium (PM) containing 1% copra meal, 3% polypeptone, 1.5% KH_2PO_4, 0.06% $MgSO_4 \cdot 7H_2O$ and 2.5% corn steep liquor at pH 7.0 and was sterilized at 121 °C for 15 min. The culture was grown at an agitation speed of 600 rpm with aeration at 0.75 vvm (vol. of air/vol. of medium/min) at 45 °C (Feng et al. 2003). Samples were collected from the fermenter at 0, 3, 6, 9, 12, 15, 18, 21 and 24 h for the determination of bacterial growth by standard plate count in Luria broth (LB) agar. The mannanase activity was measured as described by Titapoka et al. (2008). One unit of enzyme activity was defined as the amount of enzyme producing 1 μmole of mannose per minute under the experimental conditions (Titapoka et al. 2008).

Preparation of CMH

CMH was prepared using 1.0% defatted copra meal in 20 U/mL crude mannanase from *B. circulans* NT 6.7 following the method described by Pangsri et al. (2015a). The CMH was lyophilized for further study.

Carbohydrates and oligosaccharide analysis

The amount of reducing sugar in the CMH was determined using the dinitrosalicylic acid (DNS) method (Miller 1959). The total carbohydrate content was measured using the phenol–sulfuric acid method (Dubois et al. 1956). CMH was analyzed for different sugar types using high-performance liquid chromatography (HPLC) (Waters 1525 Binary HPLC, Milford Massachusetts, USA). The sample was centrifuged at 13,000×*g* for 10 min and passed through a 0.45 μm syringe filter. The filtered sample was then injected into an Aminex HPX-87P column (300×7.8 mm, particle size 9 μM Bio-Rad, California, USA). The mobile phase was deionized

water, the flow rate was 0.6 mL/min, a refractive index (RI) detector was used (Waters 2414, Milford Massachusetts, USA), and the column oven temperature was 55 °C. The sugar concentrations were calculated using standard curves for cellobiose, glucose, xylose, galactose, arabinose and mannose.

The oligosaccharide content was determined using an Aminex HPX-42C column (300 × 7.8 mm, particle size: 25 µm, Bio-Rad, California, USA). The sample was analyzed at 75 °C using deionized water as the mobile phase at a flow rate of 0.4 mL/min with an RI detector (waters 2414, Milford Massachusetts, USA). Mannose, mannobiose, mannotriose, mannotetraose, mannopentaose and mannohexaose were used to construct calibration curves.

Degree of polymerization (DP) analysis

CMH and yeast MOS were dissolved in deionized water at a concentration of 10 g/L, centrifuged at 13,000× *g* for 5 min and passed through a 0.22 µm syringe filter. The samples were analyzed by HPLC (Agilent 1100 Series, Waldbronn, Germany) using a BioSep-SEC-S2000 column (300 × 7.8 mm, Phenomenex, California, USA). The column was heated to 30 °C, and the flow rate was adjusted to 0.7 mL/min. The mobile phase was 50 mM $NaNO_3$. The samples were monitored using an RI detector as described by Ho et al. (2014). The DP of the samples was analyzed by comparison with known purified standards. Oligosaccharide standards of mannose, maltose, maltotriose, maltotetraose, maltopentaose and maltohexaose were purchased from Sigma-Aldrich (Poole, United Kingdom). Dextran standards at 1, 5, 6, 12, 50 and 80 kDa were used for external calibration.

The effect of simulated gastric and bile juice hydrolysis

In vitro gastric digestion was performed as described by Hongpattarakere et al. (2012). Five grams of CMH were suspended in hydrochloric acid buffer containing: 8 g/L NaCl, 0.2 g/L KCl, 8.25 g/L $Na_2HPO_4 \cdot 2H_2O$, 14.35 g/L NaH_2PO_4, 0.1 g/L $CaCl_2 \cdot 2H_2O$, and 0.18 g/L $MgCl_2 \cdot 6H_2O$ (Korakli et al. 2002). The pH was adjusted to 2.0 using 5 M HCl, and 3 g/L pepsin (Sigma-Aldrich, Munich, Germany) was added, and the mixture was incubated at 37 °C for 4 h. Samples were taken at 0 and 4 h. Following gastric digestion, the pH was increased to 6.9 by adding 1 M NaOH. Then, 1 U/mL of human pancreatic α-amylase (Sigma-Aldrich, Munich, Germany) was added before incubation at 37 °C for 6 h. Samples were taken at 0 and 6 h. The total carbohydrates and reducing sugars were determined before and after digestion using the phenol–sulfuric acid method (Dubois et al. 1956) and the DNS method (Miller 1959), respectively. Hydrolysis percentages were calculated by dividing the reducing sugar released by the total carbohydrates, as shown by the following formula (Wichienchot et al. 2010):

$$\text{Hydrolysis (\%)} = \frac{(\text{Reducing sugar released} \times 100)}{(\text{Total sugar content} - \text{initial reducing sugar content})}$$

where the reducing sugar released is the difference between the final and initial contents of reducing sugar.

The oligosaccharide contents before and after digestion were determined by HPLC, as described above. One milliliter samples were obtained from the digestion solution and centrifuged at 13,000×*g* for 5 min. The supernatants were filtered using a 0.22 µm filter unit (Millipore, Cork, Ireland).

Substrates for in vitro fermentations

Three types of carbohydrate materials were used as substrates in the batch cultures. CMH from defatted copra meal was digested using mannanase and then lyophilized. Yeast MOS was obtained from Alltech Inc., Thailand (Actigen™, Alltech Inc., Kentucky, USA), and FOS (Orafti® P95; Beneo, Tienen, Belgium) was used as the prebiotic reference.

Fecal inocula

Fresh human fecal samples were obtained from four healthy human volunteers between 30 and 45 years old (three females and one male) who had not taken prebiotics or been subjected to antibiotic treatment for 3 months before the study. The samples were diluted 1:10 (w/w) in anaerobic phosphate-buffered saline (PBS; 0.1 mol/L, pH 7.4) (Oxoid, Basingstoke, United Kingdom), homogenized in a stomacher (Seward Stomacher®, Worthing, United Kingdom) at normal speed for 2 min and then used as an inoculum.

In vitro fermentation

Batch culture fermentation systems were set up in sterile vessels and aseptically filled with 45 mL of sterile basal medium: 2 g/L peptone water (Oxoid, Basingstoke, United Kingdom), 2 g/L yeast extract (Oxoid, Basingstoke, United Kingdom), 0.1 g/L NaCl, 0.04 g/L K_2HPO_4, 0.04 g/L KH_2PO_4, 0.01 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $CaCl_2 \cdot 6H_2O$, 2 g/L $NaHCO_3$, 2 mL Tween-80, (British Drug Houses, Leicestershire, United Kingdom), 0.05 g/L hemin, 10 µL vitamin K1, 0.5 g/L cysteine HCl, and 0.5 g/L bile salts at pH 7.0. Then, the mixture was pre-reduced overnight with oxygen-free nitrogen gas before the substrates were added to the fermentation vessels. The final concentration of carbohydrate substrates added to each vessel was 1% (w/v) in a

50 mL working volume (0.5 g). Each batch culture was inoculated with 5 mL of fresh fecal slurry (1/10, w/w) to reach a final concentration of 10% (w/v). The temperature was kept at 37 °C, and the pH was maintained between 6.7 and 6.9 using a pH controller (Fermac 260; Electrolab, Tewkesbury, United Kingdom). The batch cultures were magnetically stirred, and anaerobic conditions were maintained with oxygen-free nitrogen gas. Five milliliter samples were collected at 0, 10, 24, and 34 h for bacterial enumeration using fluorescent in situ hybridization (FISH) and organic acid (OA) analysis. Fermentations were performed in four replicates, each representing a different donor.

Bacterial enumeration

Human fecal bacterial populations were assessed using FISH with 16S rRNA probes (Table 1). These probes were commercially synthesized and labeled with the fluorescent dye Cy3 (supplied by Eurogentec Ltd., Hampshire, United Kingdom). The 16S rRNA probes were specific for the *Bifidobacterium* group, *Lactobacillus/enterococcus* group, *Atopobium* cluster, *Bacteroides/prevotella* group, *Clostridium histolyticum* group, *Clostridium* cluster IX, *Clostridium coccoides/Eubacterium rectale* group, *Roseburia*, and *Faecalibacterium prausnitzii* and relatives. The probes used were Bif164, Lab158, Ato291, Bac303, Chis150, Prop853, Erec482, Rrec584, and Fpra655, respectively.

The total bacterial counts were achieved using 4′, 6-diamidino-2-phenylindole (DAPI). Samples (375 μL) obtained from the batch culture fermentation were diluted in 1125 μL of filtered 4% (w/v) paraformaldehyde and fixed overnight at 4 °C.

Fixed cells were centrifuged at 13,000×*g* for 5 min and washed twice in 1 mL of filter-sterilized PBS. The pelleted cells were re-suspended in 150 μL of filtered PBS, and then, 150 μL of ethanol (99%) was added. The mixture was vortexed and stored at −20 °C for at least 1 h before further analysis. Samples were diluted in PBS, and 20 μL of the solution was added to each well of a six-well polytetrafluoroethylene/poly-L-lysine-coated slide. Ten millimeter diameter well slides were purchased from Tekdon Inc. (Myakka City, Florida, USA). The slides were dried for 15 min in a drying chamber (50 °C) to permeabilize the cells and then dehydrated using a series of alcohol solutions at 50, 80 and 96% (v/v) ethanol for 3 min in each solution. The slides were dried in a drying chamber for 2 min to evaporate the ethanol. The 100 mL hybridization buffer solution contained 5 M NaCl, 1 M Tris/HCl (pH 8.0), deionized water, and 10% sodium dodecyl sulfate. Pre-warmed hybridization buffer (45 μL) was mixed with 5 μL of probe (50 ng/μL), and 50 μL of hybridization mixture was added to each well. Hybridization was conducted at appropriate temperatures for the probes (Table 1) for 4 h in a hybridization incubator (Grant-Boekel, Cambridge, United Kingdom). After hybridization, the slides were washed in 50 mL of washing buffer (9 mL of 5 M NaCl, 1 mL of 1 M Tris/HCl [pH 8.0], and 40 mL of deionized water) containing 20 μL of DAPI solution (50 ng/ μL) for 15 min in a water bath at the appropriate temperature for the probe used. The slides were dipped in cold deionized water for a few seconds and dried with compressed air. Five milliliters of "antifade" (Sigma-Aldrich, New Jersey, Belgium) was added to each well, and the cover slide was placed on top (20 mm; thickness no. 1; VWR, Lutterworth, United Kingdom). The slides were examined

Table 1 16S ribosomal RNA oligonucleotide probes used in this study

Probe name	Specificity	Sequence (5′-3′)	Temperature (°C)		References
			Hybridization	Washing	
Bif164	*Bifidobacterium* spp	CAT CCG GCA TTA CCA CCC	50	50	Langendijk et al. (1995)
Lab158	*Lactobacillus/enterococcus* group	GGT ATT AGC AYC TGT TTC CA	50	50	Harmsen et al. (1999)
Bac303	Most *Bacteroidaceae* and *Prevotellaceae*, some *Porphyromonadaceae*	CCA ATG TGG GGG ACC TT	46	48	Manz et al. (1996)
Ato291	*Atopobium* cluster	GGT CGG TCT CTC AAC CC	50	50	Harmsen et al. (2000)
Chis150	Most of the *Clostridium histolyticum* group (*Clostridium* clusters I and II)	TTA TGC GGT ATT AAT CTY CCT TT	50	50	Franks et al. (1998)
Erec482	Most of the *Clostridium coccoides/Eubacterium rectale* group (*Clostridium* clusters XIVa and XIVb)	GCT TCT TAG TCA RGT ACC G	50	50	Franks et al. (1998)
Rrec584	*Roseburia* genus	TCA GAC TTG CCG YAC CGC	50	50	Walker et al. (2005)
Fpra655	*Faecalibacterium prausnitzii* and relatives	CGC CTA CCT CTG CAC TAC	58	58	Hold et al. (2003)
Prop853	*Clostridium* cluster IX	ATT GCG TTA ACT CCG GC	50	50	Walker et al. (2005)

using fluorescence microscopy (Eclipse 400; Nikon, Surrey, United Kingdom). At least fifteen random fields were counted per well.

Organic acid analysis

The samples collected from the batch culture fermentation were centrifuged at 13,000×*g* for 5 min. The supernatants were filtered with a 0.22 µm filter unit (Millipore, Cork, Ireland), and 20 µL was injected into an HPLC system (Agilent 1100 Series, Waldbronn, Germany) equipped with an RI detector and automatic injector. The column used was an ion-exclusion Aminex HPX-87H column (300 × 7.80 mm; Bio-Rad, California, USA) maintained at 50 °C. The mobile phase was 5 mM H_2SO_4 in HPLC-grade water, and the flow rate was 0.6 mL/min. Quantification of the samples was performed using calibration curves of lactate, formate, acetate, propionate and butyrate at concentrations of 12.5, 25, 50, 75, and 100 mM.

Statistical analysis

Statistical analysis was performed using SPSS for Windows (version 21.0; SPSS, Inc.). The differences between bacterial numbers and OA production at 0, 10, 24 and 34 h of fermentation for each batch culture were assessed for significance using the paired *t* test. Univariate analysis of variance (ANOVA) and post hoc Tukey's tests were used to determine the significant differences according to the substrate used for the bacterial group population and OA production. The differences were considered significant at $P < 0.05$.

Results

Mannanase production

Samples from the 5 L fermenter were taken at 0, 3, 6, 9, 12, 15, 18, 21 and 24 h for the determination of cell growth by plate count on LB agar and mannanase activity assay. *B. circulans* NT 6.7 showed the highest cell growth and mannanase activity 9.72×10^7 CFU/mL and 21.54 U/mL, respectively, at 15 h (Fig. 1).

Preparation of CMH

The defatted copra meal was hydrolyzed by concentrating crude mannanase at 20 U/mL and lyophilized. The soluble fraction of CMH (50 mg/mL) was analyzed for reducing sugar, total sugar, type of sugar and oligosaccharide content. The amounts of reducing sugar and total carbohydrate were 0.85 ± 0.03 mg/mL and 4.52 ± 0.25 mg/mL, respectively. The monosaccharide content of CMH was

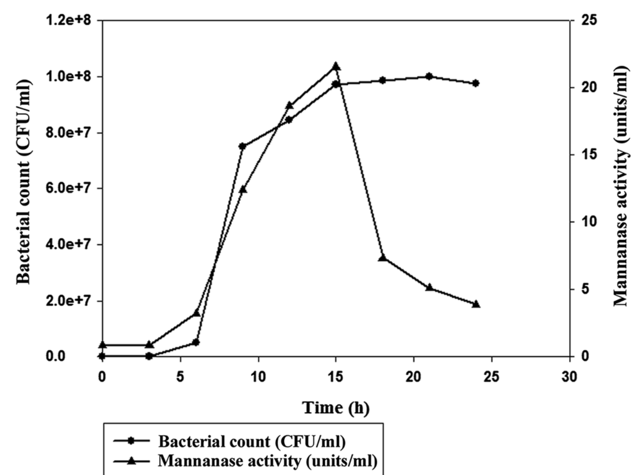

Fig. 1 The mannanase activity of *B. circulans* NT 6.7 grown in PM

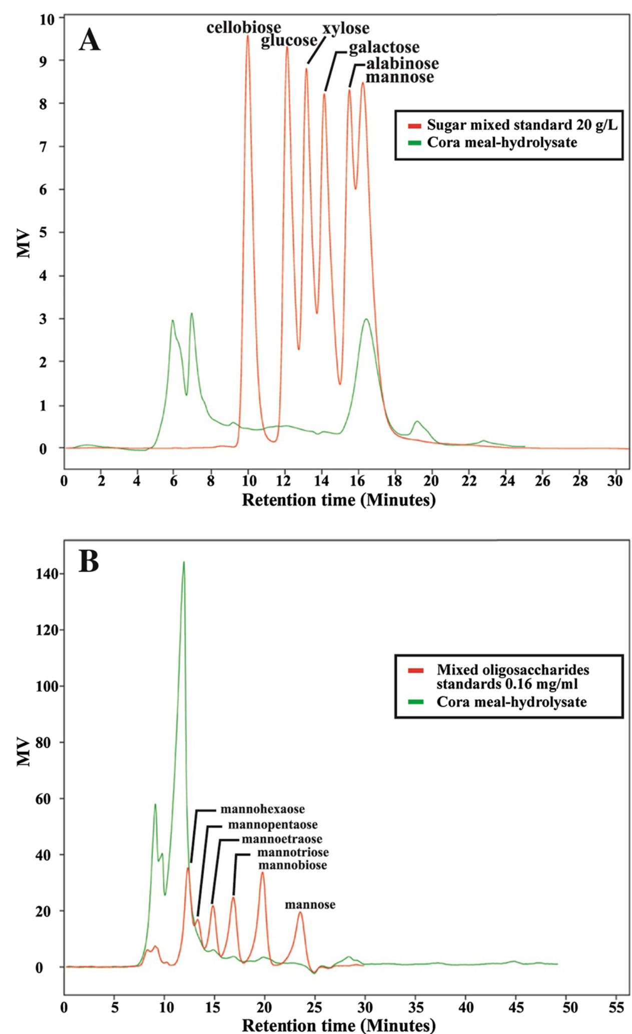

Fig. 2 Sugar composition of the soluble fraction of 50 mg/mL CMH. **a** Types of sugar in CMH analyzed by an Aminex HPX-87P column. **b** Oligosaccharides analyzed by an Aminex HPX-42C column

largely mannose (0.69 ± 0.14 mg/mL) (Fig. 2a), whereas the oligosaccharide content of CMH primarily consisted of mannopentaose and mannohexaose (26.47 ± 1.8 mg/mL) (Fig. 2b). FOSs have an average DP of 4 (Moniz et al. 2016), and yeast MOSs have an average DP of 2–5.

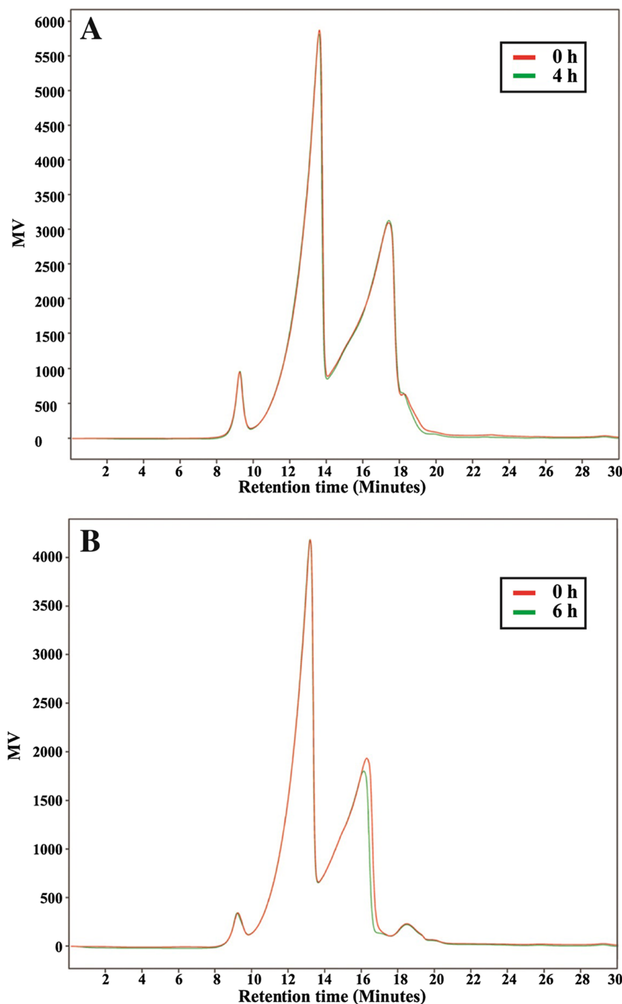

Fig. 3 CMH digestibility profiles obtained by HPLC under simulated gastric juice hydrolysis at 0 and 4 h (**a**) and under human α-amylase hydrolysis at 0 and 6 h (**b**)

CMH after simulation of the upper digestion system

Figure 3 shows the CMH HPLC profiles before and after treatment under gastric digestion conditions. After 4 h of incubation, the mannopentaose and mannohexaose (i.e., the major oligosaccharides comprising CMH) exhibited resistance to the simulated human gastric juice (Fig. 3a). The mannopentaose and mannohexaose profiles did not change after 6 h of incubation with human pancreatic α-amylase, indicating that little was hydrolyzed by α-amylase (Fig. 3B). The percentages of CMH hydrolysis under the stomach and small intestine conditions were 2.11% and 4.10%, respectively (Table 2). According to these results, CMH was resistant to both in vitro digestion conditions.

Effect on fecal microbiota

Batch culture fermentations were used to study the effects of CMH, yeast MOS and FOS supplementation on the human fecal microbiota. Changes in the bacterial population (log10 cells/mL) in batch cultures after 0, 10, 24, and 34 h of incubation with different substrates are shown in Table 3. Total bacterial cell counts significantly increased after 10 h of fermentation for all substrates and decreased at 34 h ($P < 0.01$). There was a significant increase of *Bifidobacterium* populations in response to all substrates at 10 h compared with 0 h ($P < 0.01$). After 24 h, FOS mediated the greatest effect on *Bifidobacterium*, followed by CMH and yeast MOS, however, the magnitude of the impact of different substrates on *Bifidobacterium* populations was not statistically significant.

Concentrations of *Lactobacillus*/*Enterococcus* group started to increase after 10 h of fermentation, similar to FOS and yeast MOS, which showed significant difference ($P < 0.01$) when compared with 0 h.

Significant increases in the *Bacteroidaceae*/*Prevotellaceae* group were noted between 10 and 34 h of fermentation with CMH and yeast MOS ($P < 0.01$). CMH fermentation resulted in a significant decrease ($P < 0.01$) in the *Atopobium* cluster between 24 and 34 h of fermentation. The *C. histolyticum* group and *Faecalibacterium prausnitzii* showed no significant differences between substrate groups, and both did not significantly decrease after 10 h of fermentation ($P > 0.05$). A decrease in the *Eubacterium rectale*/*Clostridium coccoides* group occurred after 34 h for all the

Table 2 CMH before and after in vitro gastric and small intestine digestion

In vitro simulated gastro-intestinal conditions	Incubation time (h)	Total CHO (mg/mL)	Reducing sugar (mg/mL)	Hydrolysis (%)
Stomach	0	4.35 ± 0.06	0.37 ± 0.05	0.00
	4	3.45 ± 0.01	0.43 ± 0.03	2.11
Small intestine	0	2.42 ± 0.03	1.44 ± 0.05	0.00
	6	2.77 ± 0.02	1.50 ± 0.01	4.10

Table 3 Changes in bacterial population (log10 cells/mL) in batch cultures after 0, 10, 24 and 34 h of incubation with various substrates

Probe	Time (h)	Bacterial population (log10 cells/mL)[d]		
		CMH	Yeast MOS	FOS
Bif164	0	8.15 ± 0.09	8.15 ± 0.09	8.15 ± 0.09
	10	8.66 ± 0.07[ab**]	8.56 ± 0.09[b**]	8.80 ± 0.01[a**]
	24	8.76 ± 0.07**	8.59 ± 0.25*	8.85 ± 0.03**
	34	8.38 ± 0.14*	8.16 ± 0.33	8.54 ± 0.07*
Lab158	0	8.42 ± 0.09	8.42 ± 0.09	8.42 ± 0.09
	10	8.98 ± 0.11[b**]	9.21 ± 0.11[a**]	9.18 ± 0.08[ab*]
	24	9.31 ± 0.07**	9.28 ± 0.08**	9.38 ± 0.03**
	34	8.83 ± 0.11**	8.94 ± 0.11**	8.93 ± 0.25
Ato291	0	8.29 ± 0.15	8.29 ± 0.15	8.29 ± 0.15
	10	8.62 ± 0.06*	8.59 ± 0.15**	8.70 ± 0.09
	24	8.79 ± 0.04**	8.77 ± 0.12*	8.75 ± 0.08*
	34	8.69 ± 0.12**	8.69 ± 0.14*	8.69 ± 0.06*
Bac303	0	8.34 ± 0.08	8.34 ± 0.08	8.34 ± 0.08
	10	8.85 ± 0.12**	9.00 ± 0.19**	9.04 ± 0.18*
	24	9.09 ± 0.05[b**]	9.11 ± 0.02[b**]	9.18 ± 0.02[a**]
	34	8.99 ± 0.04[c**]	9.07 ± 0.02[b**]	9.13 ± 0.01[a**]
Chis150	0	8.14 ± 0.07	8.14 ± 0.07	8.14 ± 0.07
	10	8.13 ± 0.33	8.03 ± 0.41	7.97 ± 0.44
	24	8.07 ± 0.32	7.89 ± 0.27	7.70 ± 0.33
	34	7.80 ± 0.37	7.54 ± 0.21**	7.37 ± 0.30
Erec482	0	8.51 ± 0.05	8.51 ± 0.05	8.51 ± 0.05
	10	8.75 ± 0.10*	8.62 ± 0.31	8.56 ± 0.44
	24	8.97 ± 0.07**	8.91 ± 0.09**	8.70 ± 0.25
	34	8.72 ± 0.17	8.79 ± 0.19	8.41 ± 0.57
Rrec584	0	8.09 ± 0.16	8.09 ± 0.16	8.09 ± 0.16
	10	8.16 ± 0.10	7.88 ± 0.53	7.87 ± 0.37
	24	8.21 ± 0.21	8.01 ± 0.12	7.95 ± 0.35
	34	7.93 ± 0.07	7.88 ± 0.10*	7.73 ± 0.37
Fpra655	0	8.21 ± 0.06	8.21 ± 0.06	8.21 ± 0.06
	10	8.17 ± 0.16	7.97 ± 0.26	8.10 ± 0.12
	24	8.01 ± 0.19	7.84 ± 0.20*	8.03 ± 0.28
	34	7.96 ± 0.22	7.77 ± 0.20**	7.97 ± 0.29
Prop853	0	7.74 ± 0.19	7.74 ± 0.19	7.74 ± 0.19
	10	8.05 ± 0.10	7.91 ± 0.25	7.65 ± 0.51
	24	7.98 ± 0.30	7.93 ± 0.37	7.59 ± 0.55
	34	7.59 ± 0.19	7.49 ± 0.1	7.35 ± 0.28
DAPI	0	9.27 ± 0.06	9.27 ± 0.06	9.27 ± 0.06
	10	9.67 ± 0.02[c**]	9.72 ± 0.01[b**]	9.78 ± 0.02[a**]
	24	9.77 ± 0.01[b**]	9.79 ± 0.01[b**]	9.85 ± 0.02[a**]
	34	9.59 ± 0.02[b**]	9.67 ± 0.03[a**]	9.70 ± 0.03[a**]

*Significantly different from the population at 0 h, ($P < 0.05$); **significantly different from the bacterial population at 0 h, ($P < 0.01$)

[a,b,c]Significantly different ($P < 0.05$) between treatments at the same time point

[d]Mean bacterial count ± standard deviation ($n = 4$)

substrates, although no significant differences were determined between the substrate groups ($P > 0.05$).

FOS fermentation was not associated with a significant decrease in the *Clostridium cluster* IX population after 10 h of fermentation, while CMH and yeast MOS caused a decrease (after 24 and 34 h, respectively) with no significant differences ($P > 0.05$).

Organic acid analysis

The OA production results of human fecal fermentations with CMH, yeast MOS, and FOS at 0, 10, 24 and 34 h are shown in Table 4. Total OAs significantly increased for all the substrates after 10 h of fermentation. Acetate was the main fermentation product for all substrates tested, followed by propionate, butyrate, formate and lactate. The proportions of acetate in the total OA production for FOS, CMH and yeast MOS at 34 h were 69.76, 65.24 and 53.93%, respectively.

The highest acetate level was achieved with FOS at all time points, whereas the acetate production from CMH fermentation was similar to that of commercial yeast MOS. However, at 34 h, FOS fermentation resulted in a fivefold increase in acetate concentration relative to the initial fermentation concentration, whereas eightfold and tenfold changes were observed for CMH and yeast MOS. Propionate was produced upon the fermentation of all the substrates. A significant increase in propionate was observed with CMH and yeast MOS ($P < 0.01$) compared with 0 h. The use of yeast MOS yielded the highest propionate concentration at 34 h ($P < 0.01$). There was no significant increase of *Clostridium* cluster IX when compared with its baseline, but an increase was seen in *Clostridium* cluster IX between 10 and 24 h fermentation and a slight decrease after 34 h ($P > 0.05$). Although, the increase in propionate from *Clostridium* cluster IX was not seen. Moreover, propionate was also produced by *Bacteroides*, and *Bacteroides* significantly increased ($P < 0.01$) with all substrates when compared with 0 h which correlated with the significant increase in propionate between 24 and 34 h. Lactate and formate were absent after 24 h of fermentation for all the substrates tested.

Discussion

This paper is an initial report of the in vitro fermentation of CMH by the human fecal microbiota as an initial evaluation into its prebiotic potential. Clear criteria must be satisfied for CMH to be classified as a candidate prebiotic. The first criterion is resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption. The oligosaccharide composition of CMH was only slightly hydrolyzed after being exposed to both the

Table 4 Organic acid (OA) concentrations produced during the fermentation of each carbohydrate source at 0, 10, 24 and 34 h

OA	Time (h)	OA concentration (mM)[d]		
		CMH	Yeast MOS	FOS
Acetate	0	4.33 ± 0.47[b]	3.39 ± 0.77[b]	8.72 ± 0.78[a]
	10	21.78 ± 3.12[b**]	22.37 ± 6.60[b**]	54.52 ± 9.56[a*]
	24	31.03 ± 4.09[b**]	31.60 ± 3.68[b**]	53.36 ± 18.83[a]
	34	36.03 ± 3.07[**]	35.55 ± 4.40[**]	51.26 ± 23.81
Propionate	0	0.41 ± 0.53	0.41 ± 0.49	0.00 ± 0.00
	10	7.66 ± 0.52[**]	9.88 ± 5.93[*]	5.29 ± 4.49
	24	9.24 ± 0.49[b**]	21.71 ± 3.19[a**]	12.41 ± 7.19[ab]
	34	11.19 ± 2.02[b**]	22.46 ± 2.31[a**]	11.83 ± 7.43[b]
Butyrate	0	0.13 ± 0.15[a]	0.16 ± 0.18[a]	0.00 ± 0.00[a]
	10	1.85 ± 0.48[*]	4.01 ± 1.56[*]	3.51 ± 3.43
	24	4.28 ± 1.27[**]	6.12 ± 3.72	10.06 ± 2.86[*]
	34	5.14 ± 2.07[*]	7.39 ± 2.73[*]	10.29 ± 3.79[*]
Lactate	0	0.90 ± 0.16	0.93 ± 0.53	1.57 ± 0.31
	10	0.59 ± 0.21[b]	0.20 ± 0.23[b]	15.85 ± 7.68[a]
	24	0.00 ± 0.00[**]	0.00 ± 0.00[*]	0.00 ± 0.00[*]
	34	0.00 ± 0.00[**]	0.00 ± 0.00[*]	0.00 ± 0.00[*]
Formate	0	4.73 ± 0.63[a]	3.99 ± 0.81[a]	0.00 ± 0.00[b]
	10	1.13 ± 1.31[b**]	4.02 ± 1.43[b]	14.80 ± 5.32[a*]
	24	0.00 ± 0.00[**]	0.00 ± 0.00[*]	0.00 ± 0.00
	34	0.00 ± 0.00[**]	0.00 ± 0.00[*]	0.00 ± 0.00
Total	0	10.50 ± 0.62	8.68 ± 1.54	18.14 ± 11.53
	10	32.98 ± 2.19[b**]	39.08 ± 15.71[b*]	93.97 ± 8.99[a*]
	24	47.53 ± 6.25[**]	57.87 ± 14.58[**]	75.89 ± 23.18
	34	55.22 ± 2.68[**]	65.92 ± 2.55[**]	73.48 ± 30.13

*Significantly different from the OA concentration at 0 h, ($P < 0.05$); **significantly different from the OA concentration at 0 h, ($P < 0.01$)

[a,b,c]Significantly different ($P < 0.05$) between treatments at the same time point

[d]Mean OA concentration ± standard deviation ($n = 4$)

in vitro gastric stomach simulation and human pancreatic α-amylase in the small intestine simulation. Therefore, CMH is expected to reach the colon and become a substrate for colonic bacterial fermentation.

The second criterion is selective fermentation by the intestinal microflora. CMH affected *Bifidobacterium* spp. and the *Lactobacillus*/*Enterococcus* group similarly to medium containing FOS as a positive control. Typically, *Bifidobacterium* and *Lactobacillus* utilize prebiotic substances with low molecular weights more rapidly than those with higher molecular weight (Gibson 2004). Similarly, Olano-Martin et al. (2002) used batch culture fermentations for the preliminary screening of the in vitro utilization of dextran and three sizes of novel oligodextran (I, II and III) by human feces. The results showed that low molecular weight of oligodextran resulted in higher *Bifidobacterium* and lactic acid bacteria populations during fermentation than dextran and oligodextrans II and III. Our observations suggest that the bifidogenic effect on CMH is similar to that of FOS, which has a DP of 4–5.

The major end products of colonic bacterial fermentation are OAs (mainly acetate, propionate and butyrate). Although each bacterial genus produces different types of fermentation products, the fermentation products of some species are substrates or intermediate compounds in the fermentation pathways of other species (Salminen et al. 1998; Chaia and Oliver 2003; Hashizume et al. 2003). Fermentation intermediates, such as lactate and formate tend to accumulate in fecal fermentation of rapidly fermenting substrates such as FOS. In this study, increasing numbers of *Bifidobacterium* spp. and *Lactobacillus*/*Enterococcus* groups were identified upon the fermentation of all substrates. Although both bifidobacteria and lactobacilli produce lactate, significant levels were only observed during the fecal fermentation of FOS at 10 h, indicating rapid fermentation of this substrate. These results were consistent with those of Sarbini et al. (2011), who observed the correlation of *Bifidobacterium* spp. and lactic acid bacteria with lactate levels after 10 h of fermentation which then decreased. These findings may be attributable to lactate being utilized by other bacteria,

which then produced acetate, butyrate and propionate. *Bacteroidaceae/Prevotellaceae* group and *Clostridium* cluster IX contain known propionate producers (Salminen et al. 1998; Gómez et al. 2014) which is in agreement with the increase in propionate observed in this study. Our results showed significant increase in *Bacteroidaceae/Prevotellaceae* populations and a trend for increase in *Clostridium* cluster IX populations. Therefore, we suggest that propionate may have been produced by the *Bacteroidaceae/Prevotellaceae* and *Clostridium* cluster IX.

Batch culture fermentation of all substrates tested, demonstrated that *Clostridium histolyticum* decreased after 10 h, while during this time acetate concentration was continuously increasing with all substrates' fermentation. A number of *Bifidobacterium* spp., *Atopobium* cluster, *Bacteroidaceae/Prevotellaceae, Clostridium coccoides* group increased after 10 h which correlated with the concentration of acetate. Higher acetate concentration may be related to the decrease of *Clostridium histolyticum*. Therefore, acetate may be suppressing the populations of *Clostridium histolyticum* (Wang and Gibson 1993).

Bifidobacteria can break down and utilize inulin-type fructans and complex carbohydrates via the fructose 6-phosphate phosphoketolase pathway or "bifid shunt" of which acetate and lactate are the final metabolites (De Vuyst et al. 2014). In this study, the highest microbial populations of *Bifidobacterium* were found for FOS, and the fermentation of FOS produced the most acetate and lactate. These results may also be attributable to the lower DP of FOS compared to those of CMH and yeast MOS.

CMH stimulated *Bifidobacterium* populations equally well to FOS, consistent with the fact that CMH contains significant amounts of mannose in the form of 1,4-β-D-mannan or β-mannan (Hossain et al. 1996; Ghosh et al. 2015). *Bifidobacterium* spp., the target microorganisms for the Bif 164 probe (Langendijk et al. 1995) is specific for, among others, *B. adolescentis, B. angulatum, B. bifidum, B. breve, B. infantis*, and *B. longum*. *Bifidobacterium adolescentis* (living in the human gut) can produce endo-1,4-β-mannanase or β-mannanase (Kulcinskaja et al. 2013). The yeast MOS or α-mannan of the yeast cell wall from *Saccharomyces cerevisiae* is a series of highly branched oligosaccharides. Side chains are linked by α-(1–2)- and α-(1–3)- linkages to an α-(1–6)-linked backbone (Jones and Ballou 1969; Spring et al. 2015). Yeast MOS resulted in the lowest *Bifidobacterium* populations. This was likely because the *Bifidobacterium* strain could not utilize yeast MOS as well as the other substrates. The present study showed that β-mannan in CMH can be utilized more efficiently than α-mannan in yeast MOS, possibly because *Bifidobacterium* produces β-mannanase which can hydrolyze β-mannan (Kulcinskaja et al. 2013).

This study reports for the first time in vitro fecal fermentation of CMH. CMH had a generally desirable effect on the microbiota which should be confirmed in fecal fermentation models simulating the human colon and human intervention studies.

Acknowledgements We are indebted to The Royal Golden Jubilee (RGJ) Ph.D. Programme, The Thailand Research Fund (TRF), Betagro Science Centre Co., Ltd. and the National Research Council of Thailand (NRCT) for financial support.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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