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Prebiotic potential of resistant starch derived from native, phosphorylated and cross-linked sago starches

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Abstract

The objectives of this study were to find out the prebiotic index (PI) and short-chain fatty acid (SCFA) level of the resistant starch (RS) type 4, determined by the fermentation method. The RS from native, phosphorylated and cross-linked sago starches were fermented using a faecal inoculum. The production of SCFA was chromatographically characterized. The grown bacteria were identified using the selective media to enumerate bifidobacteria, lactobacilli, clostridia and *Bacteroides* to calculate the PI. The results showed that the RS enhanced the growth of bifidobacteria and lactobacilli, resulting in a higher PI (1.19) than the value for inulin (1.15) following incubation for 24 hrs. The fermentation of RS from native and phosphorylated sago starches was faster than that of RS from cross-linked sago starch. The increase in the numbers of bifidobacteria and lactobacilli during fermentation of RS gave higher SCFA production with a consequence of lower pH.

1. Introduction

A prebiotic was first defined as a 'non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health' (Gibson and Roberfroid, 1995). Roberfroid (2007) suggests a revision of the definition of prebiotic as a 'selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health.' Prebiotics of proven efficacy can modulate the gut microbiota by stimulating indigenous beneficial flora while inhibiting the growth of pathogenic bacteria, such as clostridia and Enterobacteriaceae by the production of short-chain fatty acids (SCFA) and antimicrobial compounds, as well as by competition for growth substrate and adhesion sites (Lievin *et al*., 2000).

Not all dietary carbohydrates are prebiotic, and clear criteria need to be established for classifying a food ingredient as a prebiotic. These criteria are 1) resistance to gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption; 2) fermentation by intestinal microflora; and 3) selective stimulation of the growth and/or activity of those intestinal bacteria (Gibson *et al*., 2004). This concept implies that prebiotics must be stable in the stomach (Bindels *et al*., 2015), i.e., that acid would not influence them, and they should not be absorbed in the small intestine and thus able to reach the colon (Roberfroid, 2001), where they are selectively fermented by specific bacteria, which exert the beneficial effect on the host (Gibson *et al*., 2010). Prebiotics can be a selective substrate for one of the number of beneficial bacteria colon, which plays a role in the modulation of the immune system and affects the host's health. SCFA production through the provision of fermentable carbohydrates gives a lower pH (Madhukumar and Muralikrishna, 2010; Rathore *et al*., 2012). Lowering pH also leads to the dissociation of alkaline compounds with toxic or carcinogenic potential and so inhibits their absorption (Bird *et al*., 2000).

Several in vivo studies in experimental animals and humans suggest that starch and non-starch polysaccharides have been regarded as unlikely prebiotics (Bird *et al*., 2000). Although several oligosaccharides have been proposed as prebiotics, only inulin-type fructans, transgalacto-oligosaccharides and lactulose achieved the status (Gibson *et al*., 2004; Davani -Davari *et al*., 2019). The most commonly investigated oligosaccharides for prebiotic activity are fructo-

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oligosaccharides and galacto-oligosaccharides but isomalto-oligosaccharides, xylo-oligosaccharides, gluco-oligosaccharides lactosucrose (Carlson *et al*., 2018), and inulin (Oliveira *et al*., 2011) all present prebiotic characteristics, but certain other carbohydrates appear to have the prebiotic potential.

Previous research found that sago and cross-linked sago starches were resistant to an enzyme α-amylase (Haska and Ohta, 1992) and heat at 95°C (Polnaya *et al*., 2012, 2013), thus might be potential as prebiotic. It is likely that in addition to crystallinity, size of granule, and phosphorus content (Polnaya *et al*., 2012) sago starch might reduce the enzymatic digestibility. The starch that avoids hydrolysis by amylolytic enzymes in the small intestine and passes to the large bowel for fermentation is defined as resistant starch (RS) (Sajilata *et al*., 2006). It may be desirable to increase the level of RS in foods by modifying native starches by chemical methods, such as phosphorylation or cross-linking with sodium tripolyphosphate or phosphorous oxychloride, respectively (Sang *et al*., 2010; Polnaya *et al*., 2018). Prebiotic action does not seem to be limited to high amylose starches but also chemically-modified variants (i.e., RS4) (Bird *et al*., 2000). Several's recent researches showed that phosphorylation was used to improve the functional properties of starch associated with RS (Sang *et al*., 2010; Polnaya *et al*., 2018). It is interesting to apply sago starch as a local food that can be developed as a novel prebiotic.

The objectives of this study were to find out the PI and SCFA levels of the RS type 4 determined by the fermentation method and to clarify the effect of phosphate linkage on the prebiotic potential.

2. Materials and methods

2.1 Materials

Substrates used in this study were as follows: RS from native, phosphorylated (DS 0.008), cross-linked (DS 0.018) sago starches and inulin. The RS content of native sago starch was 9.52%, while the phosphate and crosslinked sago starches were 24.41% and 41.38%, respectively, which were samples from the results of previous studies (Polnaya *et al*., 2018). Inulin was used as a standard in this in vitro fermentation experiment bought from Sigma-Aldrich (Germany).

Human faecal samples from male volunteers were pooled to serve as the source of inoculum for the experiment. All donors consumed their normal diet, were approximately 35-37 years old, were free of gastrointestinal disease, and had not received antibiotics at least six months before or during the study. Fresh faecal samples were brought to the laboratory 15 mins after defecation to ensure the viability of microbial populations. All chemicals used for analyses were of analytical grade.

2.2 Preparation and collection of faecal samples

Faecal samples were obtained from a healthy human volunteer who had not been prescribed antibiotics for at least 6 months prior to the study and had no history of any gastrointestinal diseases. Samples were collected on-site and used immediately following collection. Fresh faecal samples were first diluted (1:1) in an anaerobic buffer containing 0.1 M phosphate-buffered saline (PBS) solution, pH 7, and then the samples homogenized in a stomacher for 2 mins. The use of a sample ensures that a representative range of bacterial species is exposed to the test material.

2.3 Preparation and operation of batch fermenters

Basal nutrient mediums were prepared as previously described (Vulevic *et al*., 2004). The composition was: pepton water 2 g/L, *yeast* extract 2 g/L, NaCl 0.1 g/L, K_2HPO_4 0.04 g/L, KH_2PO_4 0.04 g/L, $MgSO_4 \cdot 7H_2O$ 0.01 g/L, $CaCl_2 \cdot 6H_2O$ 0.01 g/L, $NaHCO_3$ 2 g/L, Tween 80 2 mL/L, haemin 0.02 g/L, vitamin K_1 10 µL/L, and L-cysteine·HCl 0.5 g/L, bile salts 0.5 g/L pH 7.0 and gassed overnight with oxygen-free nitrogen. Batch culture fermentation vessels were filled with 90 mL base nutrient medium. Substrates 1% (w/v) were added to vessels, and then sterilized at with 121°C for 20 mins. Prior to the addition of the faecal slurry, culture temperature was set at 37°C.

A 10 mL portion of the faecal slurry was added to a 90 mL basal medium in 100 mL batch fermentation vessels. The vessels were maintained under anaerobic conditions by a gas generation kit. The temperature was controlled at 37°C. At each experiment, a 1 mL sample was taken from each vessel at 0, 24, and 48 hrs.

2.4 Bacterial enumeration

Differences in bacterial populations were assessed through the use of a selective growth medium according to the method of Vardakou *et al*. (2008), with slight modification. The samples from each vessel were immediately serially diluted with pre-reduced half-strength peptone water, pH 7, supplemented with 0.5 g cysteine-HCl/L. Portions of 20 mL from each dilution were plated, onto agar plates. The selective growth mediums used were: Columbia agar containing per litre 5 g glucose, 0.5 g cysteine HCl, and 0.5 mL propionic acid, pH 5.0, for *Bifidobacterium* spp.; Tryptone soya agar, supplemented per litre with 5 mL Brucella, 75 mg kanamycin, 5 mg haemin, 75 mg vancomycin and 50 ml laked horse blood, for *Bacteroides* spp.; Rogosa agar,

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supplemented per litre with 1.32 mL glacial acetic acid, for *Lactobacillus* spp. and reinforced clostridial agar, supplemented per litre with 8 mg novobiocin and 8 mg colistin, for *Clostridium* spp. The mediums were incubated at 37°C and ran for a period of 24 hrs and samples were obtained at 0, 24 and 48 hrs.

2.5 Prebiotic index

The value PI used to quantify the prebiotic effect of crosslinked starch with selective fermentation (Palframan *et al*., 2003) was calculated as follows:

PI = (*Bif* / *Total*) – (*Bac* / *Total*) + (*Lac* / *Total*) – (*Clos* / *Total*)

Where *Bif* is bifidobacteria numbers at sample time/ numbers at inoculation, *Bac* is *Bacteroides* numbers at sample time/numbers at inoculation, *Lac* is lactobacilli numbers at sample time/numbers at inoculation, *Clos* is clostridia numbers at sample time/numbers at inoculation and the *Total* is total bacteria numbers at sample time/ numbers at inoculation. The PI assumes that an increase in the population of bifidobacteria and/or lactobacilli has a positive effect; in contrast, an increase in Bacteroides and clostridia was deemed negative.

2.6 pH

pH samples were measured at 0, 12, 24, and 48 hrs using a pH meter.

2.7 Short-chain fatty acid analysis

The SCFA content was determined according to the method of Hernot *et al*. (2009), and samples were obtained at 0, 24 and 48 hrs. The 2 mL aliquot of fluid removed from the sample tubes for SCFA and lactate analyses were immediately added to 0.5 mL of 25% metaphosphoric acid, precipitated for 30 min, and centrifuged at 5000 rpm for 30 mins. The supernatant was decanted and frozen at -20°C in microfuge tubes. After freezing, the supernatant was thawed and centrifuged in microfuge tubes at 5000 rpm for 20 mins. Concentrations of acetate, propionate, and butyrate were determined in the supernatant using a Shimadzu GC 8A gas chromatograph and a glass column (200 cm × 3 mm id) packed with 10% SP-1200/1% H_3PO_4. The temperature of the injector and column were 220 and 130°C, respectively. The type and temperature of the detector were Flame Ionisation Detector and 230°C, and nitrogen gas carrier 1.3 mL/cm^2. The production of acetic, propionic and butyric acids in each sample was calculated with an external calibration curve using standard acetic, propionic and butyric acids.

2.8 Statistical analysis

Data were statistically analyzed by variance analysis test procedure with two replications, and significant differences were identified by Tukey's test ($P < 0.05$) using SAS 9.0 software (SAS, Inc.).

3. Results and discussion

3.1 Prebiotic Index

The bacteria target group were observed covering *bifidobacteria, lactobacilli*, *Bacteroides*, and *clostridia* (Bird *et al.*, 2000; Gibson *et al.*, 2004), grown on selective media, incubated at 37°C for 48 hrs. *Bifidobacteria* and *lactobacilli* genera play a significant role in fermented prebiotics (RS) because both strains of bacteria and the substrate are specific, that may have health-promoting properties (Tuohy *et al*., 2005) and certain species of *bifidobacteria* and *lactobacilli* may be more desirable than others (Gibson *et al*., 2004). Similarly, *clostridia*, particularly *C. butyricum* able to use oligosaccharides during fermentation in the colon to produce acetate, propionate and butyrate acids (Bird *et al*., 2000). On the other hand, *Bacteroides vulgatus* shows a high production of acetate and formiate (Gullón *et al*., 2011). So, it can be argued that the four of them were a specific group of bacteria that were vital to the process of RS fermentation in the colon.

At 0 and 48 hrs, bacterial populations were measured using a selective growth medium. For all substrates (RS and inulin), 48 hrs of in vitro, fermentation increased bacteria count (Table 1). The increase in several cells of bacteria might be due to the substrate containing carbon compound and consequently, utilising it as a nutrient source in metabolism processes. The metabolism processes had the tendency to increase with the increase of energy for cell proliferation. This was expected because higher energy increases cell bacteria count. Ghoddusi *et al*. (2007) showed that increased bifidobacteria, lactobacilli, *Bacteroides* and clostridia after 8 hrs of in vitro fermentation for all substrates.

The higher number of bifidobacteria and lactobacilli compared to that of *Bacteroides*, and clostridia were caused by the incorporation of RS from native, phosphorylated and cross-linked sago starches, and inulin. This was shown that RS was selectively fermented by specific bacteria and caused an increase of bifidobacteria and lactobacilli groups and then suppressed the growth of *Bacteroides* and clostridia groups.

Grootaert *et al*. (2009) reported that the availability of prebiotic allows fermentation to run optimally,

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thereby increasing resistance to pathogens in the gut. SCFA, as the main fermentation product, contributes directly to the inhibitory activity of gastrointestinal pathogens. The number of bifidobacteria and lactobacilli that grew in the media containing RS from native and phosphorylated sago starch increased within 24 hrs and then decreased until 48 hrs. These might have been due to the rapidly available fermentable substrate, while the decrease was due to the diminishing substrate. The unavailability of the substrate caused inhibition of the growth of bifidobacteria and lactobacillus, while *Bacteroides* and clostridia were still able to grow. The number of bifidobacteria and lactobacilli that grew in the media containing RS from cross-linked sago starch and inulin, increased in 24 hrs and then slightly decreased until 48 hrs. This might have been due to the RS substrates being good carbon sources and showing slow rates of the fermentable substrate with up to 48 hrs of incubation. Hernandez-Hernandez *et al.* (2012) showed that the three strains of *Lactobacillus* sp. (*L. bulgaricus* ATCC7517, *L. casei* ATCC11578, *L. plantarum* ATCC8014, and *L. plantarum* WCFS1) were grown on a galacto-oligosacharide as a carbon source, to grow the maximum 24 hrs incubation time, is stable up to 48 hrs, and then decreased to 120 hrs.

Availability of substrate determines the sustainability of the fermentation process. This effect is in line with what was stated by Grootaert *et al*. (2009) that the proliferation of beneficial intestinal bacteria is influenced by the availability of prebiotic. These results indicate that the RS can modify specific microflora, thus lowering the carbon source can decrease metabolic activity. Protein and amino acids are used as a dominant source of metabolic energy for distal colonic bacteria if carbohydrates are not available. This led to an increasing population of pathogenic bacteria in the gut because proteins and amino acids are the primary source of nutrients for bacterial pathogens. A variety of other compounds are also formed during protein metabolism, and yield branched-chain fatty acids such as isobutyrate, 2-methylbutyrate, and isovalerate) (Macfarlane *et al*., 1992) and toxic materials such as phenol and indole are released from the breakdown of aromatic amino acids (Macfarlane and Gibson, 1994). Therefore, the RS is required to maintain the balance of intestinal microflora, which eventually led to the concept of prebiotic.

The RS from cross-linked sago starch showed an influenced growth of bifidobacteria and lactobacilli higher than that of RS from native and phosphorylated sago starches, but relatively the same with inulin (Table 1). The number of bifidobacteria and lactobacilli was increased while the number of Bacteroides and clostridia decreased, indicating that substrates were better utilized by the gut bacteria (Vardakou *et al*., 2008). RS from cross-linked sago starch and inulin was selectively fermented by specific bacteria and increased population, which exert a beneficial effect on the healthy colon.

The selectivity of RS from native, phosphorylated and cross-linked sago starches, is supposedly because during fermentation, starch hydrolyzed to polymers with a degree of polymerization (DP) lower so easily used by probiotic bacteria as a carbon source for cell growth. Oligosaccharides are a short–chain of carbohydrates of 3 to 10 monomers. Moura *et al*. (2007) suggest that xylooligosaccharide (XOS) with DP 2 has prebiotic properties better than XOS with DP 5–6.

In order to obtain a general quantitative measure of

Table 1. Change in bacterial population after 0, 12, 24, and 48 hrs of *in vitro* fermentation of RS from native (RS NSS), phosphorylated (RS MSP), cross-linked sago starches (RS DSP), and inulin using human faecal microflora.

Organism(s)	Incubation Time (hrs)	Bacterial Population (log CFU/mL)			
		RS NSS	RS MSP	RS DSP	Inulin
Total bacteria	0	7.40	6.86	6.64	5.94
	24	8.17	9.39	8.12	6.72
	48	8.67	10.01	8.21	7.74
Bifidobacteria	0	6.48	6.57	6.51	5.80
	24	6.76	8.43	7.12	6.11
	48	7.86	9.91	8.08	7.57
Bacteroides	0	5.92	5.78	5.58	5.86
	24	6.99	7.49	7.12	6.51
	48	6.63	8.52	5.71	7.51
Lactobacilli	0	5.26	4.52	5.26	4.15
	24	8.10	8.14	7.95	5.74
	48	7.57	8.36	7.19	6.46
Clostridia	0	4.93	4.57	5.51	5.14
	24	6.76	7.51	5.97	5.48
	48	7.86	8.59	7.37	5.84

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the prebiotic effect, a value of PI was calculated for the RS. The PI was determined as reported by Palframan *et al.* (2003), a measure quantitatively using a faecal inoculum (Wichienchot *et al.*, 2006). The PI represents a comparative relationship between the growth of "beneficial" bacteria, such as bifidobacteria and lactobacilli, and that of the "less desirable" ones, such as Bacteroides and clostridia, concerning the change in the total number of bacteria. The PI assumes that an increase in the population of bifidobacteria and/or lactobacilli has a positive effect, in contrast, an increase in Bacteroides and clostridia was deemed contrary (Palframan *et al*., 2003).

For the substrates, the PI values obtained at 24 hrs of incubation (PI 0.33-1.19) were higher than those at 48 hrs (0.13-0.96) (Figure 1). Several other studies showed the same relative PI values of different types of prebiotics such as FOS, trans-galactooligosacharide (TOS), and FOS: TOS (50:50) on the fermentation of 37°C for 24 hrs by colonic bacteria, respectively, were 0.2, 0.8, and 1.3 (Vulevic *et al*., 2004). Bacterial changes determine a positive PI suggesting that the growth of bifidobacteria and lactobacilli is higher than that of Bacteroides and clostridia.

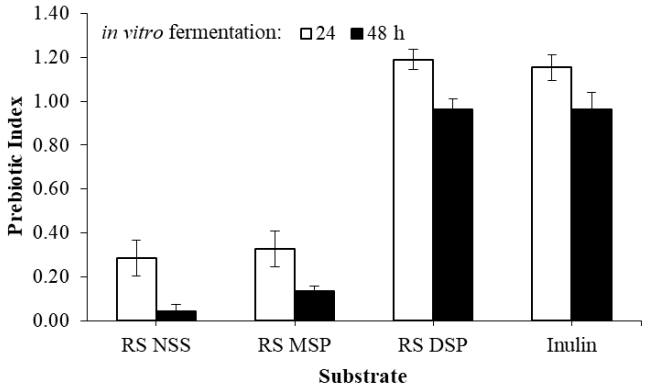

Figure 1. Prebiotic index after 24 and 48 hrs of *in vitro* fermentation of RS from native (RS NSS), phosphorylated (RS MSP), cross-linked sago starches (RS DSP), and inulin using human fecal microflora.

The PI value of RS from native sago starch obtained at 24 h of incubation (PI 0.29) was not different from the PI value of RS from phosphorylated starch (PI 0.33) (Figure 1). Based on the PI value, it turned out that RS from monostarch phosphate gave a similar effect to the RS from native sago starch.

The RS from cross-linked sago starch gave a higher PI value (1.19) than that RS from native and phosphorylated sago starches, but insignificantly different compared to inulin (1.15). These might have been caused by the difference in the structure of phosphate linkage. The bound phosphate can be released by enzymes produced from bacteria that can be used by bacteria to support growth. The enzyme that can release the C6 bond is glucose-6-phosphate dehydrogenase (Thitipraphunkul *et al.*, 2003). These results indicate that the RS from native, phosphorylated, and cross-linked sago starches can be categorized as prebiotic candidates based on the PI value.

3.2 Short-chain fatty acids

SCFA production within RS degradation by extracellular enzymes provided by colonic bacteria into glucose. Throughout fermentation, glucose is undergoing a process of glycolysis that produces pyruvic acid, which furthers during the metabolism. Pyruvic acid is converted to acetate, propionate and butyrate. Colonic bacteria are a group of bacteria that are facultative heterofermentative to produce SCFA by fermentation of RS through the hexose monophosphate pathway or usually called a pentose phosphate pathway (Bernalier-Donadille, 2010).

Effects of SCFA may be subdivided into general and specific. The former related substantially to the fact that the significant SCFA is organic acids with pKa values of approximately five so that their production leads to acidification of the intracolonic environment. It is a common finding that the promotion of SCFA production through the provision of additional fermentable carbohydrates gives a lower pH value compared with diets low that (Leu *et al.*, 2005). The increased SCFA content acts to improve the health of colon (Bird *et al.*, 2000; Gibson *et al.*, 2004).

For the substrates were suitable substrates for intestinal microbiota, enhancing the production of SCFA (acetate, butyrate and propionate) ($P < 0.01$) during a long fermentation (Table 2). The production of SCFA is a consequence of the metabolism of the microbiota present in the fermentation media (Rycroft *et al*., 2001). Production of SCFA indicated that RS from native, phosphorylated, cross-linked sago starches and inulin were suitable substrates for fermentation and did function as prebiotic.

Several studies suggest that RS was the selective substrate for intestinal microbiota, fermented and produced SCFA, preventing the degradation of primary bile acids to carcinogens, stimulating the gut epithelium and immune responses as well as protecting against microbial infections and regulating the pro-inflammatory response (Licht *et al*., 2012). SCFA is a relevant regulator of colonic physiological processes and appears essential for maintaining healthy bowel function.

The total SCFA of RS from native and phosphorylated sago starch obtained at 24 hrs of incubation were 25.79 mM and 32.67 mM, respectively,

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Table 2. Acetate, propionate, butyrate, and total SCFA producing following 0, 12, 24, and 48 hrs of *in vitro* fermentation RS from native (RS NSS), phosphorylated (RS MSP), cross-linked sago starches (RS DSP), and inulin using human faecal microflora.

Fatty Acid(s)	Incubation time (hrs)	Concentration (mM)*			
		RS NSS	RS MSP	RS DSP	Inulin
Total SCFA	0	0.00^{h}	11.69^{g}	3.64^{h}	4.46^{h}
	12	0.00^{h}	17.55^{f}	25.16^{e}	42.61^{c}
	24	25.79^{e}	32.67^{d}	69.01^{a}	53.71^{b}
	48	0.00^{h}	0.06^{h}	53.32^{b}	49.08^{b}
Acetate	0	0.00^{j}	6.91^{i}	0.00^{j}	0.00^{j}
	12	0.00^{j}	9.30^{h}	15.69^{g}	37.26^{d}
	24	16.20^{g}	22.12^{f}	43.47^{b}	45.31^{a}
	48	0.00^{j}	0.00^{j}	29.51^{e}	41.28^{c}
Propionate	0	0.00^{f}	4.23^{de}	3.64^{e}	3.57^{e}
	12	0.00^{f}	7.50^{bc}	9.47^{a}	3.66^{e}
	24	3.59^{e}	9.01^{ab}	10.27^{a}	6.05^{cd}
	48	0.00^{f}	0.00^{f}	9.28^{ab}	5.56^{d}
Butyrate	0	0.00^{f}	0.55^{ef}	0.00^{f}	0.89^{def}
	12	0.00^{f}	0.75^{ef}	0.00^{f}	1.69^{cde}
	24	6.00^{b}	1.54^{cde}	15.27^{a}	2.35^{c}
	48	0.00^{f}	0.06^{f}	14.53^{a}	2.24^{cd}

Values are presented as mean. Values with different superscripts within the same row of the same group of fatty acid are statistically significantly different by Tukey's test ($\alpha = 0.05$).

and then around 100% decrease at 48 hrs of incubation (Figure 1). These phenomena were in line with the data on the growth of bacteria. Increased total SCFA might have been due to increased bifidobacteria and lactobacilli, while the decrease after 24 hrs of incubation was due to the decrease of both bacteria. Mandalari *et al*. (2008) suggested that the composition of bacteria influenced fermentation products.

The total SCFA of RS from cross-linked sago starch (69.01 mM) obtained at 24 hrs on incubation was higher than that of inulin (53.71 mM), and then 22.73% and 8.62% respectively, decreased at 48 hrs incubation. These data show that the total SCFA of both RS from cross-linked sago starch and inulin was higher than RS from native and phosphorylated sago starches until 48 h rsincubation. This might be due to the RS from cross-linked sago starch, and inulin showed slow rates of fermentable with up to 48 hrs of incubation. Availability of substrate, determine the sustainability of the fermentation process to produce SCFA.

The RS from native and phosphorylated sago starches has the same pattern of bacteria growth and the production of SCFA might be due to having the same molecular structure. RS from native sago starch contained a phosphate group with monostarch phosphate formation. RS from cross-linked sago starch has a different structure than the RS from native and phosphorylated sago starches. A cross-linked structure causes a phosphate group of RS to bind more glucose, which is two-fold.

The results revealed that the concentration of acetate, propionate and butyrate differed for all RS substrates and the concentration of acetate was higher than that of propionate and butyrate. Other researchers also gave relatively similar results as a result of the fermentation of RS (Mandalari *et al*., 2008). For the RS from native and phosphorylated sago starch substrates, the concentration of acetate, propionate and butyrate acids tends to increase up to 24 hrs on incubation and then decrease, while RS from cross-linked sago starch and inulin is relatively stable up to 48 hrs.

The SCFA produced in the present experiment indicated that the massive amounts of RS were fermented and potential as a substrate in the fermentation to improve health along with an increase in the number of bifidobacteria and lactobacilli. RS is thought to be the most significant contributor to large intestinal SCFA production (Bird *et al*., 2000). As proposed by Mandalari *et al*. (2008), increased SCFA concentration correlates with the increasing number of bifidobacteria and lactobacilli. The concentration of SCFA in the gut contents at a concentration above 80 mmol/L is thought to play a substantial role in maintaining colonic integrity (Bird *et al*., 2000).

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3.3 pH value

The results showed that the pH of RS from native and phosphorylated sago starch obtained at 24 hrs of incubation was lower than those at 0 hr, but increased at 48 hrs of incubation ($P < 0.01$), for RS from native and phosphorylated sago starches (Table 3). As it has been stated before, at the same conditions decreased SCFA concentration, results in an increased pH. For the RS from cross-linked starch and inulin substrates, the pH values acquired at 24 and 48 hrs of incubation were lower than RS from native and phosphorylated sago starches.

Table 3. Initial pH and pH change after 12, 24, and 48 hrs of *in vitro* fermentation of RS from native (RS NSS), phosphorylated (RS MSP), cross-linked sago starches (RS DSP), and inulin using human faecal microflora.

Substrate	Initial pH	pH after incubation *		
		12 hrs	24 hrs	48 hrs
RS NSS	6.95	6.90^{a}	4.12^{cd}	6.90^{a}
RS MSP	6.92	5.75^{b}	4.45^{c}	6.80^{a}
RS DSP	6.88	4.26^{cd}	3.55^{e}	3.99^{cde}
Inulin	6.9	4.35^{c}	3.79^{de}	4.00^{cde}

Values are presented as mean. Values with different superscripts within the same column statistically significantly different by Tukey's test ($\alpha = 0.05$).

The decrease in pH is caused by the SCFA produced during fermentation. These results are in line as proposed by Letellier *et al.* (2000) that the FOS fermentation produces a lower pH colon. The increased SCFA production decreases the luminal pH (Leu *et al.*, 2005; Sajilata *et al*., 2006), which results in reduced solubility of free bile acids (Grubben *et al*., 2001). This drop in pH decreased the production of secondary bile acids, which have potential tumour-promoting activity (Grubben *et al*., 2001).

Fermentation of RS supports the growth of *Bifidobacteria* and *Lactobacillus*, and leads to an increase in the concentration of SCFA as a consequence of lowering pH. The low intestinal pH and the high counts of bifidobacteria and lactic acid microflora should inhibit the proliferation of pathogenic bacteria (Bird *et al.*, 2000; Leu *et al.*, 2005), increase mineral bioavailability, and also the dissociation of alkaline compounds with toxic or carcinogenic potential and so inhibits their absorption (Bird *et al.*, 2000).

These results are in line as proposed by Rathore *et al.* (2012) that the pH obtained at 24 hrs of incubation lower. Wheat fermentation with two probiotics strain *Lactobacillus plantarum* (NCIMB 8826) and *L. acidophilus* (NCIMB 8821) at an incubation temperature of 37°C shows that pH decreases from 6.32 to 3.5, due to significant amounts of lactic acid were produced. Madhukumar and Muralikrishna (2010) proposed that the decrease in pH due to the production of SCFA indicated arabinoxylooligosaccharide fermentation with *Bifidobacterium adolescentis* NDRI 236 at 37°C for 48 hrs.

The relative tolerance of lactic acid bacteria to acidic end products is due to the pH within the cytoplasm of fermenting lactic acid bacteria (LAB) remaining more alkaline than the medium surrounding the cells (Hutkins and Nannen, 1993; Wang *et al*., 2018). To maintain the cytoplasmic pH to be more alkaline cells must have a barrier to the flow of protons. This barrier is a generally cytoplasmic membrane. The difference in susceptibility to the cytoplasmic membrane of bacteria under acidic conditions determines the tolerance to low pH. Siegumfeldt *et al.* (2000), proposed that dynamic changes in the pH values of several LABs within the decrease of extracellular pH and therefore, not generate large proton gradients. These can cause translocation of protons by using much energy. Moreover, it can lead to the accumulation of anions and organic acids in the cytosol that is toxic to cells.

4. Conclusion

RS from native, phosphorylated, and cross-linked sago starches indicated the potential novelty prebiotic. Prebiotics should not stimulate the growth of pathogenic bacteria such as toxin-producing clostridia, proteolytic Bacteroides and toxigenic *Escherichia coli*. As a result, a "healthier" gut microbiota composition is achieved by the consumption of prebiotics. The IP and SFCA values resulting from the assay indicated that RS had been potential to modify the microflora correctly assumed to promote health. Products such as SCFA may reflect RS effects and indicate microbial activity. RS from cross-linked sago starch showed prebiotic potential similar to inulin.

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