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Resistant starch evaluation and *in vitro* fermentation of lemantak (native sago starch), for prebiotic assessment

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Abstract

Resistant starch is the non-digestible portion of starch that reaches the colon and act as a prebiotic to stimulate the activity and growth of beneficial gut microbiota. In the present study, resistant starch content of native (*lemantak*), commercialized and retrograded sago and starch was analysed, and the *in vitro* fermentability with known probiotics were investigated. Retrograded starch was produced through two cycles of autoclaving and cooling steps. The resistant starch content of each modified starch were measured based on the method approved by AOAC 2002.02. The *in vitro* batch fermentation was carried out with inoculation of *Lactobacillus acidophilus* and *Bifidobacterium animalis* at 37°C for 24 hours in anaerobic condition. Total bacteria was enumerated at 0, 6, 12 and 24 hours. Highest resistant starch content was shown in *lemantak* (native sago starch) at 62.61%. *Lemantak* was also shown to be the most preferred fermentation substrate with the highest number of total bacterial count at all sampling hours. These findings suggest the potential of *lemantak* as a prebiotic.

Introduction

Sago and tapioca starch are two of the main locally sourced starch marketed in Malaysia. Traditionally processed sago starch, commonly known as *lemantak*, are extracted manually from the trunk of sago palm *(Metroxylon sagu),* followed by processes of washing, sedimentation and drying. Meanwhile tapioca starch are extracted from its tuber. Sago and tapioca starch are used widely in food preparation, and are often interchangeable. There is an interest in diversifying the usage of both local starches to shift consumer preference from other internationally sourced starch flour such as wheat and maize. One of new perspective in its starch utilisation is as dietary fibre through their resistant starch (RS) content.

Resistant starch is defined as the fraction of starch that was not hydrolysed by the pancreatic and brush border enzyme within the 120 minutes after consumption (Englyst *et al*., 1992). They resist upper gastrointestinal digestion and reach the colon where they might be utilized by the gut microbiota. Beneficial RS would be the starch that could selectively stimulate the growth and/or activity of one or more number of the good bacteria, which includes lactobacilli and bifidobacteria, and positively influence host health. This can be known as the prebiotic effect.

Previous study has investigated the digestibility of sago starch by known probiotic. However, there are no study conducted on *lemantak*. *Lemantak* is a type of native sago starch that are extracted through traditional processes and are often extracted on the sago plantation. They are the most common native sago starch marketed in Sarawak. On the other hand, tapioca starch was chosen because there are still limited study on its resistant starch, and even fewer study on its fermentability by probiotic.

Thus, the objective of this study is to quantify and compare resistant starch content of native and commercialized source of sago and tapioca. The native starches were then subjected to physical modification process to produce retrograded starch. The starches were then fermented with probiotics to investigate their preferential as fermentation substrate by the probiotic microorganisms.

Materials and Methods

Materials

Unless stated otherwise, all reagents and chemicals used were purchased from Sigma, Sigma laboratories (Gillingham, Dorset, UK). Commercial sago starch (Alini brand, Jakarta, Indonesia) and tapioca starch (ABC brand, Penang, Malaysia) were purchased from local market.

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Inoculum preparation

The probiotics i.e. *Lactobacillus acidophilus* and *Bifidobacterium animalis* were isolated from dietary supplement capsules (Blackmores, Singapore) using selective agar of De Man Rogosa and Sharpe (MRS) and Bifidus Selective Medium (BSM), respectively. All plates were incubated at 37°C under anaerobic conditions. After incubation for 48 h, colony count were performed. A single colony of bacterial culture from the respective agar was then transferred into MRS broth and incubated overnight at 37°C for use as inoculum.

Starch extraction

Lemantak extraction was done on site at the sago palm plantation (Sungai Talau, Mukah, Sarawak, Malaysia). Trunks that are considered to be fell-ripe were cut, and their bark were removed. The soft and fibrous pith is then rasped using bush knives. The rasped pith was then washed with groundwater and left for an hour to allow the starch sedimentation. The resulting starch is then dried under direct sun for 4 hours.

Tapioca was purchased from the local market. The skin were peeled and the flesh of the tapioca were grated. The grated tapioca were then washed thrice with tap water and left for an hour to allow the starch to sediment. The resulting starch are then dried at 40°C for 2 days, grinded using a blender and sieved through a 0.3 mm sieve (ABSS, Victoria, Australia).

Preparation of retrograded starch

The retrograded sago and tapioca starch were produced through repeated heating and cooling processes. The retrograded starches were prepared by suspending 20% of native starch in 400 ml distilled water. The suspensions were then heated in a boiling water bath for 15 minutes with stirring prior to autoclaving for 90 minutes at 121°C. The autoclaved starch suspensions were allowed to cool down to room temperature before being autoclaved again. Then, the starch suspensions were refrigerated at 4°C for 16 hours and subsequently heated at 95°C for 72 hours followed by another refrigeration at 4°C for 24 hours. The resulting starch was then rinsed with distilled water before drying at 40°C for 24 hours, grinded using a blender and sieved through a 0.25 mm sieve (ABSS, Victoria, Australia).

Determination of resistant starch content

Resistant starch portion was analysed using resistant starch assay kit (Megazyme, Wicklow, Ireland) based on the method of AOAC 2002.02 and AACC 38-40.01. First, the samples undergone digestion where the non-resistant portion of the starch were solubilised and hydrolysed to D-glucose. The digestion steps consist of incubation in a shaking water bath with pancreatic α-amylase and amyloglucosidase (AMG) for 16 hours at room temperature. The reaction was terminated by the addition of an equal volume of ethanol and the resulting RS were recovered as the pellets following centrifugation. The pellets were then dissolved in 2 M potassium hydroxide by vigorously stirring in an ice water bath over a magnetic stirrer. The solution was then neutralised with acetate buffer and the starch is quantitatively hydrolysed to glucose with AMG. The RS content of the sample was measured as D-glucose with glucose oxidase/peroxidase reagent.

In vitro *fermentation*

Fermentations were carried out in 50 ml Schott bottles for 24 hours. The bottles were placed in a jar containing GasPak Envelopes (Becton Dickinson, New Jersey, United States) at 37°C in a shaking water bath. The fermentation medium consists of 0.5% NaCl, 1% peptone, 1% test substrates and 0.5% inoculum. Samples were taken at 0, 6, 12 and 24 hours, and subsequently cultured on nutrient agar for 24 hours at 37°C after which the bacterial colonies were enumerated.

Statistical analysis

Each experimental process was done in triplicate. Statistical analysis was performed using SPSS for Windows, version 20.0. One-way analysis of variance (ANOVA) and post-hoc Tukey's test were used to determine the significant difference among the substrates.

Results and Discussion

Resistant starch content

Resistant starch is the starch fraction that can withstand upper gastrointestinal digestion in a human host. Physically or chemically altering starch composition will influence its digestibility. Retrograded starch produced in present study are a type of physically modified resistant starch. The RS content of native and commercialized starch, and retrograded starch are illustrated in Figure 1 and 2, respectively.

High percentage of RS content is attributed to the proportion of amylose and amylopectin in the starch structure (Li *et al.*, 2015; Lin *et al.*, 2016). Amylose are often linked linearly with RS content. High amount of tightly packed linear amylose resist digestion more than highly branched amylopectin

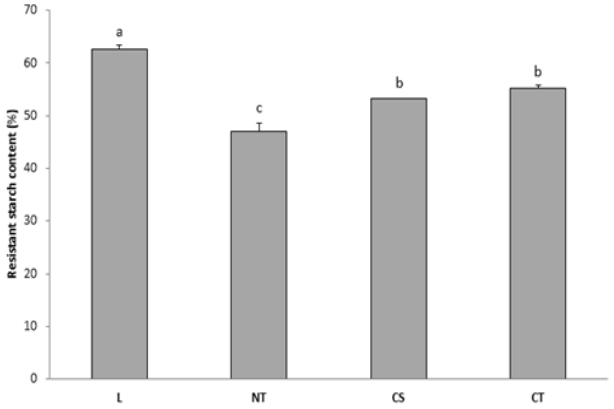

Figure 1. Resistant starch content of the native and commercialized sago and tapioca starch. (L-lemantak, NT-native tapioca starch, CS-commercialized sago starch, CT-commercialized tapioca starch). Significant differences among substrates ($P < 0.01$) were indicated with different letters above bars. Error bars represent standard deviation of the mean (n = 3).

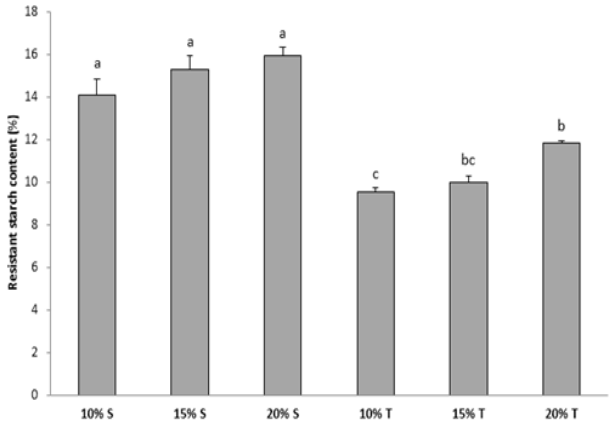

Figure 2. Resistant starch content of the retrograded sago and tapioca starch. (10% S-10% initial sago starch concentration, 15% S-15% S initial sago starch concentration, 20% S-20% initial sago starch concentration, 10% T-10% initial tapioca starch concentration, 15% T-15% initial tapioca starch concentration, 20% T-20% initial tapioca starch concentration). Significant differences among substrates ($P < 0.01$) were indicated with different letters above bars. Error bars represent standard deviation of the mean (n = 3).

(Sievert and Pomeranz, 1989; Leszczyñski, 2004; Li *et al*., 2008). Sago has a relatively low content of amylose with a significantly high portion of amylopectin (Flach, 1997). Amylose content in sago starch is in the range of 21.7 to 31% with the other 69 to 78.3% accounts largely for amylopectin. Tapioca, on the other hand, has 15 to 18% of amylose content, much lower than sago starch (Noomhorm and Tokiwa, 2006; Tongdang *et al*., 2008). Amylose to amylopectin ratio are closely linked to the different growth stages in which sago were harvested, and can also be influence by the method of starch extraction (Flach, 1997; Uthumporn *et al*., 2014).

Lemantak showed a significantly high RS content than commercialized sago and native and commercialized tapioca starches. This is significantly higher than resistant starch content of rice and maize starch at 7.98% and 46.29%, respectively (McCleary *et al.*, 2002; Mir *et al.,* 2013). Interestingly, both starches also had a significantly higher amylose content than sago and tapioca starch at the range of 29.1-33.8% and 67.4%, respectively (Mir *et al*., 2013; Maaran *et al*., 2016). This shows contradicts the pattern of positive correlation between amylose and RS content. However, several studies has showed the role of amylopectin in starch indigestibility (Eerlingen *et al*., 1994; Srichuwong *et al.,* 2005). Study with bakery products showed an increase RS content during their storage time due to amylopectin retrogradation (Eerlingen *et al.*, 1994). Native tapioca, having a lower amylose and a higher amylopectin content than sago, showed a lower amount of RS content at 47%. The higher amylopectin did not contribute to a higher RS content. Previous study on retrograded tapioca starch also showed a reduction in RS content by two times (Wronkowska *et al*., 2011).

Several studies has shown that amylopectin's structure effect on starch digestibility is dependent upon its branch-chains length distribution (Eerlingen *et al.*, 1994; Jane *et al*., 1999; Srichuwong *et al.*, 2005). Previous study has investigated the effect of amylopectin's branch-chains length of various botanical origin starches on susceptibility of the starch towards enzymatic hydrolysis. Among taro, rice, tapioca and corn starches, sago starch showed the highest resistance towards enzyme hydrolysis, and the highest distribution of high DP unit-chains (DP 25-30). Rice starch showing the highest distribution of low DP unit-chains showed highest susceptible to enzymatic hydrolysis followed by tapioca starch and corn starch (Srichuwong *et al.*, 2005). The author postulated that longer chains would make longer helices and strengthen the hydrogen bond formed between chains. Inversely, short chain will form weak and short double helices (Jane *et al*., 1999).

Commercialized sago starch showed a much lower RS content than its native counterpart, *lemantak*. This might be due to the various chemicals and/or physical processes during starch extraction and industrial processing. Commercialized starch are often bleached to obtain the white appearance to suit consumer preference (Bobrow-Strain, 2008). Chemical modification are usually used to enhance the amylose content of starch as amylose plays an integral part in starch physicochemical properties.

Table 1. Average total bacteria (log_{10} cells per ml fermentation sample) at 0, 6, 12 and 24 sampling hours (h).

h	L	NT	CS	CT	10% S	15% S	20% S	10% T	15% T	20% T
0	5.72 (0.10)^{a}	5.27 (0.08)^{bcd}	5.67 (0.12)^{a}	5.53 (0.13)^{ab}	5.08 (0.07)^{d}	5.30 (0.00)^{bcd}	5.36 (0.07)^{bc}	5.15 (0.07)^{cd}	5.30 (0.07)^{bcd}	5.39 (0.11)^{bc}
6	7.24 (0.02)^{a*}	7.16 (0.03)^{ab*}	7.17 (0.04)^{ab*}	7.13 (0.05)^{b*}	6.66 (0.07)^{de*}	6.73 (0.03)^{cd*}	6.79 (0.00)^{c*}	6.52 (0.04)^{f*}	6.60 (0.00)^{ef*}	6.36 (0.01)^{de*}
12	7.42 (0.00)^{a*}	7.35 (0.02)^{b*}	7.40 (0.02)^{ab*}	7.36 (0.03)^{ab*}	7.08 (0.05)^{c*}	7.07 (0.00)^{c*}	7.10 (0.02)^{c*}	6.97 (0.04)^{e*}	7.00 (0.00)^{de*}	7.04 (0.00)^{cd*}
24	7.75 (0.00)^{a*}	7.70 (0.01)^{b*}	7.70 (0.01)^{b*}	7.69 (0.00)^{b*}	7.31 (0.00)^{d*}	7.37 (0.00)^{c*}	7.40 (0.00)^{c*}	7.21 (0.00)^{f*}	7.26 (0.01)^{e*}	7.37 (0.03)^{c*}

Substrates tested were coded as following example: L-lemantak, NT-native tapioca starch, CS-commercialized sago starch, CT-commercialized tapioca starch, 10% S-10% initial sago starch concentration, 15% S-15% S initial sago starch concentration, 20% S-20% initial sago starch concentration, 10% T-10% initial tapioca starch concentration, 15% T-15% initial tapioca starch concentration, 20% T-20% initial tapioca starch concentration. *Significant difference from 0 h value, $P < 0.05$. Significant differences ($P < 0.05$) among treatment as indicated with different letter from the same row of data. Standard deviation is shown in parentheses (n = 3).

However, in certain case, manufacturing processes will contribute to an increase in RS content (Koo *et al*., 2010). Commercialized cross-linked corn starch often used in bread production showed increase RS content with increasing cross-linking reagent (Koo *et al*., 2010). In the present study, commercialized tapioca starch showed a significantly higher RS content than its native starch. Nevertheless, it is important to note that different manufacturers will have different starch processing process, thus RS content will differ with each manufacturer.

All retrograded sago starch showed a significantly high RS content than retrograded tapioca starch. Amylose undergoes retrogradation much faster than amylopectin due to their linear structure (Jiamjariyatam *et al*., 2015). However, rate of retrogradation did not correlate directly towards RS formation. In the present study, RS are produce via retrogradation process to produce RS type 3. During gelatinization, the starch granules are disrupted as water is absorbed. This causes the leaching of polymer molecules amylose and amylopectin as random coils (Ashwar *et al*., 2016). Upon cooling or retrogradation, the role of amylose and amylopectin in RS formation differs (Kiatponglarp *et al*., 2015). The leached amylose rearrange into double helix structure and hydrogen bond were formed to stabilize the resultant RS, while RS is formed from amylopectin by the increase molecular entanglement in the gel network and/or helix formation in a three dimensional crystalline structure (Sajilata *et al*., 2006).

The formation of the new starch structure renders retrograded starch more resistant. However, in the present study, both retrograded sago and tapioca starch showed a decrease of RS content than their native counterpart. The differences between current study and past studies might be due to the different initial RS content of each starch. The structural structure of native sago and tapioca starch, with relatively high RS content, might also interfere with the formation of the double helix and crystalline structure in the retrograded starch. Previous study with retrograded potato starch also showed a reduction of RS (Xie *et al*., 2014). The author postulated that the retrogradation increases the swelling power of the starch, where the swelling of the amorphous region increases their susceptibility to enzymatic digestion. In another study, extruded maize starch also showed significantly reduced RS content from 48% to 1.1% in which the author suggested might be due to retrogradation process involving short amylose chain produced during the extrusion (Robin *et al*., 2016).

Bacterial enumeration

Lactobacilli and bifidobacteria are well known probiotic microorganims, and are predominant members of human gut microbiota of a healthy human being (Turroni *et al*., 2008). They are beneficial saccharolytic bacteria which produces beneficial end products from their utilization of carbohydrate, which include short chain fatty acids (Guarner and Malagelada, 2003). In the present study, we investigate the ability of native, commercialized and retrograded sago and tapioca starch in influencing the growth of the two beneficial bacteria. The data are presented in Table 1.

Native sago starch was the best substrate supporting the growth of probiotic microorganisms as exhibited by having the highest viable bacterial cell counts in every fermentation sampling hour. Commercialized sago starch also showed high bacterial numbers. This result is supported by a recent study that compares the utilization of sago starch by various probiotic bacteria. The result showed that native sago starch could not be fermented by the bacteria cultures with exception of lactobacilli and bifidobacteria, which showed higher growth

percentage in native sago starch media (Zi-Ni *et al*., 2015). Native and commercialized tapioca starch also showed capability in positively influencing the growth of both bacteria. Tapioca starch are often used in the production of lactic acid via fermentation with lactobacilli (Xiaodong *et al*., 1997; John *et al*., 2006).

All retrograded starch showed a low bacterial cell count. This contradicts previous fermentation studies with retrograded sago starch but in agreement with fermentation studies of retrograded tapioca starch (Wronkowska *et al*., 2008; Zi-Ni *et al*., 2015). Previous study compares the utilization of various native and modified starches by *Bifidobacterium animalis* and the result showed that the bacteria could utilized native tapioca starch much efficiently than retrograded tapioca starch (Wronkowska *et al.*, 2008). On the other hand, several studies has supported the efficiency of retrograded sago starch as a substrate for lactobacilli and bifidobacteria fermentation (Siew-Wai *et al*., 2010; Shima *et al*., 2012; Zi-Ni *et al*., 2015). The difference in result might be due to the different retrogradation process used. Retrograded sago starch used in this study might not have been fully retrograde, especially the amylopectin portion of the starch that requires longer time to retrograde. Other study employ the use of de-branching enzyme to break down the highly branched amylopectin to facilitate the percolation of amylopectin during gelatinization and ensure the amylopectin are fully retrograded (Zi-Ni *et al*., 2015).

It is important to note that the activity of lactobacilli and bifidobacteria to ferment non-digestible carbohydrates tends to be strain dependent as different strains possess different metabolic systems for specific carbohydrates (Sarbini and Rastall, 2011). Different origin of the same type of starch, different extraction time and process also influence physical structures of the starch and will therefore affects their susceptibility towards bacterial digestion (Zaman and Sarbini, 2016). These two factors can also explain the discrepancies between the retrogradation effects on the resistant starch content of sago and tapioca starch in the present study with previous studies, as well as the utilization of the substrate by *Lactobacillus acidophilus* and *Bifidobacterium animalis*.

Conclusion

Lemantak contain high amount of RS and are preferred as a fermentation substrate for *Lactobacillus acidophilus* and *Bifidobacterium animalis*. All native and commercialized starch showed significant high viable bacterial numbers than all retrograded starch, a pattern mirrored in RS content. This therefore suggest a positive correlation between RS content and the preference of substrate for the growth of *Lactobacillus acidophilus* and *Bifidobacterium animalis.* Present study also provides preliminary insight into RS availability in commonly consumed ingredients. Future study can be carried out with a broader subject by looking at the structure and function relationship of the native and retrograded starches. Their role in manipulating the growth and activity of probiotic microorganisms can be further understood, and latter be used as prebiotic ingredients.

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