PHYSICOCHEMICAL PROPERTIES AND PREBIOTIC POTENTIAL OF NATIVE, RESISTANT AND HCL-RESISTANT STARCHES FROM SAGO (*METROXYLON SAGU*)

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PHYSICOCHEMICAL PROPERTIES AND PREBIOTIC POTENTIAL OF NATIVE, RESISTANT AND HCL-RESISTANT STARCHES FROM SAGO (*METROXYLON SAGU*)

by

TAN ZI NI

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL / ABBREVIATION	CAPTION
α	alpha
β	beta
°	degree
°C	degree Celsius
λ	lambda
%	percentage
θ	theta
ΔH	transition enthalpy
MES	2-(*N*-Morpholino)ethanesulfonic acid
AACC	American Association of Cereal Chemists
ANOVA	analysis of variance
AOAC	Association of Official Analytical Chemists
$CaCl_2.2H_2O$	calcium chloride dihydrate
$CaCl_2.6H_2O$	calcium chloride hexahydrate
CFU/mL	colony forming units per millilitre
T_c	conclusion temperature
CuKα	copper potassium alpha
CuSO4	copper (II) sulfate
MRS	de Man, Rogosa, Sharpe
$(CH_3)_2SO$	dimethyl sulfoxide
Eq	equation
FOS	oligofructose
g	gram
HPLC	high-performance liquid chromatography
h	hour
HCl	hydrochloric acid
HCl-sago RS	hydrochloric acid treated sago resistant starch
I_2	iodine
kV	kilovolt
L	litre
$MgCl_2.6H_2O$	magnesium chloride hexahydrate
$MgSO_4.7H_2O$	magnesium sulphate heptahydrate
MPa	megapascal
µg	microgram
µL	microliter
µm	micrometer
mA	milliampere
mg	milligram
mL	millilitre

min	minute
M	molarity
nm	nanometer
OHC	oil-holding capacity
T_o	onset temperature
T_p	peak temperature
KCl	potassium chloride
KOH	potassium hydroxide
KI	potassium iodide
K_2HPO_4	potassium phosphate dibasic
KH_2PO_4	potassium phosphate monobasic
K_2SO_4	potassium sulfate
PUN	Pullulanase Unit Novo
RDS	rapidly digestible starch
v/v	ratio of volume per volume
w/v	ratio of weight per volume
w/w	ratio of weight per weight
rpm	revolutions per minute
RS	resistant starch
RS_1	resistant starch type I
RS_2	resistant starch type II
RS_3	resistant starch type III
RS_4	resistant starch type IV
RS_5	resistant starch type V
sago RS	sago resistant starch
sago RS_3	sago resistant starch type III
SEM	scanning electron microscopy
SDA	slowly digestible starch
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
$Na_2HPO_4.2H_2O$	sodium phosphate dibasic dihydrate
H_2SO_4	sulphuric acid
$\times g$	times gravity
TRIS	tris(hydroxymethyl)aminomethane
TS	tryptic soy
UV-VIS	ultraviolet-visible
WHC	water-holding capacity
WCA	Wilkins Chalgren anaerobic

SIFAT FIZIKOKIMIA DAN POTENSI PREBIOTIK KANJI ASLI, RINTANG DAN HCL-RINTANG DARIPADA SAGU (*METROXYLON SAGU*)

ABSTRAK

Kanji rintang jenis III (RS_3) telah dihasilkan daripada sagu (*Metroxylon sagu*) dan dinilai sifat fizikokimia and potensinya sebagai prebiotik. Sampel mengandungi 35.7% kanji rintang (dikenal sebagai sagu RS) telah dihasilkan apabila kanji sagu asli diautoklaf dalam air suling pada suhu 121 °C selama 1 jam, dinyahcabang dengan 20 U pullulanase per g kanji pada 60 °C selama 24 jam dan seterusnya diautoklaf sekali lagi pada 121 °C selama 1 jam sebelum disimpan pada 4 °C selama 24 jam. Seterusnya, kandungan kanji rintang meningkat sehingga 63.8% (sampel dikenal sebagai HCl-sagu RS) selepas sagu RS dihidrolisiskan dengan 0.5 M HCl pada suhu 60 °C. Granul sagu RS dan HCl-sagu RS menunjukkan corak pembelauan sinar X jenis B, suhu puncak yang tinggi (143.7 °C and 146.5 ºC, masing-masing) dan struktur permukaan yang tidak sekata dan kasar. Granul kanji sagu asli menunjukkan corak pembelauan sina X jenis C, suhu puncak 74.6 °C dan permukaan yang sekata. Keterlarutan dan kuasa pembengkakan sampel HCl-sagu RS ialah 14.9% dan 1.94 g/g, masing-masing, iaitu lebih rendah berbanding sagu RS (27.4% and 2.82 g/g, masing-masing). Sampel sagu RS and HCl-sagu RS rintang terhadap hidrolisis keasidan gastrik pada pH 1-4 selama 180 min dengan kurang daripada 0.85% dihidrolisiskan. Kedua-dua sampel juga rintang terhadap hidrolisis oleh enzim saluran gastrousus dan penyerapan usus dengan masing masing 96.8% dan 98.7% RS_3 telah dipulihkan selepas penghadaman selama 3.5 jam dan dialisis selama satu malam pada suhu 37 °C. Sagu RS dan HCl-sagu RS bertindak secara terpilih

terhadap pertumbuhan bakteria, yang mana pertumbuhan bakteria dari usus tikus (lactobacilli dan bifidobakteria) telah ditingkatkan manakala pertumbuhan bakteria perosot (bacteroides, clostridia dan enterobakteria) telah dikurangkan. Indeks prebiotik sagu RS, HCl-sagu RS, oligofruktosa dan inulin ialah +12.19, +4.75, +9.45 dan +6.82, masing-masing. Penghasilan asid butirik oleh bakteria dari usus tikus dalam media dengan sagu RS dan HCl-sagu RS adalah lebih tinggi berbanding dalam media dengan oligofruktosa dan inulin. Kedua-dua kanji rintang juga menurunkan aktiviti β-glucuronidase. Sebaliknya, kanji sagu asli menyokong pertumbuhan kedua-dua bakteria baik dan bakteria perosot. Sagu RS dan HCl-sagu RS merupakan substrat pertumbuhan yang lebih baik untuk *Lactobacillus plantarum* FTCC0350 berbanding dengan FOS dan inulin. Penghasilan asid laktik dan asetik oleh *Lactobacillus plantarum* FTCC0350 adalah lebih tinggi dalam media dengan sagu RS dan HCl-sagu RS. Kesimpulannya, sagu RS sagu dan HCl-sagu RS menunjukakan sifat prebiotik dan kedua-dua sampel ialah potensi prebiotik.

PHYSICOCHEMICAL PROPERTIES AND PREBIOTIC POTENTIAL OF NATIVE, RESISTANT AND HCL-RESISTANT STARCHES FROM SAGO (*METROXYLON SAGU*)

ABSTRACT

Resistant starch type III (RS_3) was produced from sago (*Metroxylon sagu*) and evaluated for its physicochemical properties and potential as a prebiotic. A sample with 35.7% RS_3 content (designated as sago RS) was produced when the native sago starch was suspended in distilled water, gelatinized by autoclaving at 121 °C for 1 h, followed by debranching with 20 U pullulanase per g starch at 60 °C for 24 h, autoclaved again at 121 °C for 1 h before storage at 4 °C for 24 h. RS_3 content was further increased with the treatment of sago RS with 0.5 M HCl at 60 °C (sample designated HCl-sago RS) to 63.8%. Granules of sago RS and HCl-sago RS had B-type X-ray diffraction pattern, high peak temperatures (143.7 °C and 146.5 ºC, respectively) and showed irregular and rough surface structure. While granules of native sago starch had C-type diffraction pattern, peak temperature of 74.6 °C and smooth granular surface. The solubility and the swelling power of HCl-sago RS samples were 14.9% and 1.94 g/g, respectively, which were lower than that of sago RS (27.4% and 2.82 g/g, respectively). Sago RS and HCl-sago RS samples were resistant to 180 min hydrolysis by gastric acidity at pH 1 to 4 with less than 0.85% hydrolyzed. Both samples were also resistant toward hydrolysis by gastrointestinal tract enzymes and intestinal absorption with 96.8% and 98.7% of RS_3 were recovered respectively after 3.5 h digestion and overnight dialysis at 37 °C. Sago RS and HCl-sago RS acted selectively, by increasing the growth of rat intestinal bacteria

(lactobacilli and bifidobacteria) while decreasing the growth of detrimental bacteroides, clostridia and enterobacteria. The prebiotic indexes of sago RS, HCl-sago RS, oligofructose and inulin were +12.19, +4.75, +9.45 and +6.82, respectively. Butyric acid production by rat faecal culture was higher in media with Sago RS and HCl-sago RS than with oligofructose and inulin. The activity of β-glucuronidase were reduced by sago RS and HCl-sago RS. Contrary, native sago starch supported the growth of both beneficial and detrimental bacteria. Sago RS and HCl-sago RS were the better growth substrate for *Lactobacillus plantarum* FTCC0350 as compared with FOS and inulin. Lactic and acetic acid production by *Lactobacillus plantarum* FTCC0350 was higher in media with sago RS and HCl-sago RS. In conclusion, sago RS and HCl-sago RS exhibited prebiotic characteristic and they are potential prebiotic.

CHAPTER 1

INTRODUCTION

1.1 Research Background

The human large intestine is heavily populated by numerous and diverse species of microorganism, forming a complex microflora community. Colonic microflora plays a crucial role in maintaining the proper intestinal function and this influences the host health. Colonic microflora impacts the development of immune system, inhibit the growth of pathogen and regulate metabolic pathway in the host (Sekirov et al., 2010). Hence, colonic microflora must be maintained in a balanced state with predominantly constitute of health promoting bacteria, for instance, lactobacilli and bifidobacteria. Imbalance in the composition of colonic microflora may be linked to numerous diseases such as colorectal cancer and inflammatory bowel disease (Zhu et al., 2014).

A promising strategy, whereby involving the usage of prebiotic, was introduced by Gibson and Roberfroid (1995). The authors described prebiotic as a nondigestible carbohydrate which could improve a balanced intestinal microflora once administered orally as food supplement. A prebiotic ingredient should resist towards the digestions in the upper gastrointestinal tract and be selectively fermented by intestinal microflora associated with beneficial effects (Gibson et al., 2004). The addition of prebiotic carbohydrates into food products, especially in dairy products, is emerging (Huebner et al., 2007).

Although the concept of prebiotic was established two decades ago, there are currently only three food ingredients that fulfil the prebiotic characteristic: inulin-type fructans, trans-galactooligosaccharides and lactulose (Gibson et al., 2010). The

demand for prebiotic food is growing rapidly and is expected to reach $5,545.74 million by the year of 2020 (Newswire, 2015). Hence, many researches are in the progress of studying various sources of carbohydrate to claim as prebiotic, such oligosaccharides from dragon fruit flesh (Wichienchot et al., 2010), pectic oligosaccharides from orange peel wastes (Gómez et al., 2014) and refined arabinoxylooligosaccharides from wheat bran (Gullón et al., 2014)

Resistant starch is a non-digestible carbohydrate that can withstand digestion and absorption along the upper intestinal tract and can be partially or completely fermented by gut microflora (Cummings and Englyst, 1991). The primary beneficial effects of resistant starch in reducing faecal transit time, decreasing postprandial blood glucose and inducing lipid metabolism, as well as its secondary beneficial effects as a potential prebiotic have been reviewed (Sajilata et al., 2006; Fuentes-Zaragoza et al., 2011). However, most staple food products contain less resistant starch than the recommended daily consumption, which is approximately 20 g (Baghurst et al., 2001). It was reported that per 100 g, breakfast cereals only contain less than 3.6 g of resistant starch (Alsaffar, 2011); white bread, 0.9 g (Brown, 2004); cooked white rice, 7.1 g (Vatanasuchart et al., 2009); and starchy foods, 0.2-10 g (Liljeberg, 2002). Thus, the consumption of foods added with processed resistant starch as food ingredient is suggested. Previous researches have focused on the production of resistant starch type III (RS_3) from readily accessible starch sources such as maize (Zhao and Lin, 2009), wheat, rice, and potato (Garcia-Alonso et al., 1998). Less research has been reported on the production of RS_3 from sago (*Metroxylon sagu*) except our three previous researches (Leong et al., 2007; Siew-Wai et al., 2012; Purwani et al., 2012). However, none of the resistant starch listed have been scientifically proven as prebiotic.

Sago is widely planted in Sarawak, Malaysia, covering 54,087 hectares of land (Department of Agriculture Sarawak 2015a). Sago starch is one of the major export commodities for Malaysia, with an increased output from 47,687.26 metric tons in year 2012 to 47,946.37 metric tons in 2013 (Department of Agriculture Sarawak, 2015b). Due to the fact that sago starch is abundant in Malaysia, RS_3 was produced from sago in this research. Produced sago resistant starches were evaluated for its potential as a prebiotic. Indirectly, this can beneficially accelerate the development of sago industry in Malaysia, with positive effects on agricultural economy as well as health of the Malaysian population upon consumption of RS_3 containing food.

1.2 Objectives of Research

The overall aim of this study is to evaluate resistant starch type III samples produced from sago (*Metroxylon sagu*) for its potential as a prebiotic. Therefore, this study embarks on the following specific objectives:

1. To investigate the influence of different sequential processing conditions on the resistant starch content and the functional properties of resistant starches type III produced from sago.
2. To characterize and compare the physicochemical properties of produced sago resistant starches type III with native sago starch.
3. To elucidate the resistance of native sago starch and sago resistant starches type III to gastric acidity digestion, enzymatic digestion and intestinal absorption.
4. To evaluate and compare the ability of native sago starch and sago resistant starches type III to stimulate the *in vitro* growth and activity of rat intestinal microflora with commerical prebiotics.
5. To assess the *in vitro* fermentability of native sago starch and sago resistant starches type III by selected pure cultures of lactobacilli, bifidobacteria and pathogens.

CHAPTER 2

LITERATURE REVIEW

2.1 Sago

Sago palm (*Metroxylon* spp.), which is also locally known as 'rumbia', is distributed throughout the Asia-Pacific region, mainly in Malaysia, Indonesia, Thailand and Papua New Guinea. Sago starch is normally produced from the species of *Metroxylon sagu*, *Metroxylon longispinum*, *Metroxylon sylvestre*, *Metroxylon microcanthum*, *Metroxylon rumphii* (Ahmad et al., 1999).

Starch as a source of dietary carbohydrate, is typically extracted from tuber (sweet potato), root (cassava), cereals (corn, rice) and legumes (bean) (Karim et al., 2008). However, sago starch is unique as it is the only commercial starch which derived from the stem of sago palm (Karim et al., 2008). For every unit of plantation area, sago palm can produce 3 to 4 times more starch than rice corn and wheat while 17 times more starch than cassava (Karim et al., 2008). Since sago palm can produce a relatively higher yield than other starchy crops and its ability to grow well in swampy area without much care, sago starch has a higher commercial value.

In Malaysia, sago palm is mainly planted in the state of Sarawak, with occupying over three quarters of the peat land of Sarawak and being the only plant that is able to grow well and vigorously in the swampy area (Bujang and Yusop, 2006). With the establishment of sago palm estate plantations by the Land Custody and Development Authority (or Lembaga Pembangunan dan Lindungan Tanah, PELITA) of Sarawak, the total sago palm plantation was recorded to be 54,087 ha in 2013 (Department of Agriculture Sarawak, 2015a). The sago industry in the State of Sarawak is well-established and has made sago flour one of the most important

export commodities, with a current output of 45,000 metric tons/year, with revenue expected to increase from RM36 million/year to RM2.5 billion/year in 2015 (Jackson, 2007).

Sago starch is used in composites with other starches such as cassava, potato, and corn starch in local food manufacturing (Karim et al., 2008). Several researches are also in progress to use sago starch in the production of lactic acid and bioethanol through the fermentation process (Karim et al., 2008). In this study, sago starch in the form of a resistant starch type III was used to investigate its potential as prebiotic. Once this application is recognized, additional usage of sago starch will be increased.

2.2 Starch

Through photosynthesis process, plants utilize energy from sunlight and carbon dioxide from atmosphere to produce their own food, glucose. Excessive productions of these substrates will mostly being stored in the form of polysaccharides, namely, starch. Starches are basically polysaccharides of the six-carbon sugar, D-glucose, which linked together by the α-linkages regardless of the botanical source. Essentially, starches are structurally composed of amylose and amylopectin (**Figure 2.1**). Amylose is a linear polysaccharide chain and all the glucose residues are linked together by α-D-(1-4) linkages (Tester et al., 2004). Amylopectin, not only contains α-D-(1-4) linkages, also contains α-D-(1-6) linkages which making it a highly branched molecules (Tester et al., 2004).

Starches are occurred as granular form which consist of amorphous region and crystalline region (Zhang et al., 2014). Usually, amylose chains are loosely packed in the amorphous region while amylopectin chains are arranged in precise double helices in the crystalline region (Zhang et al., 2014).

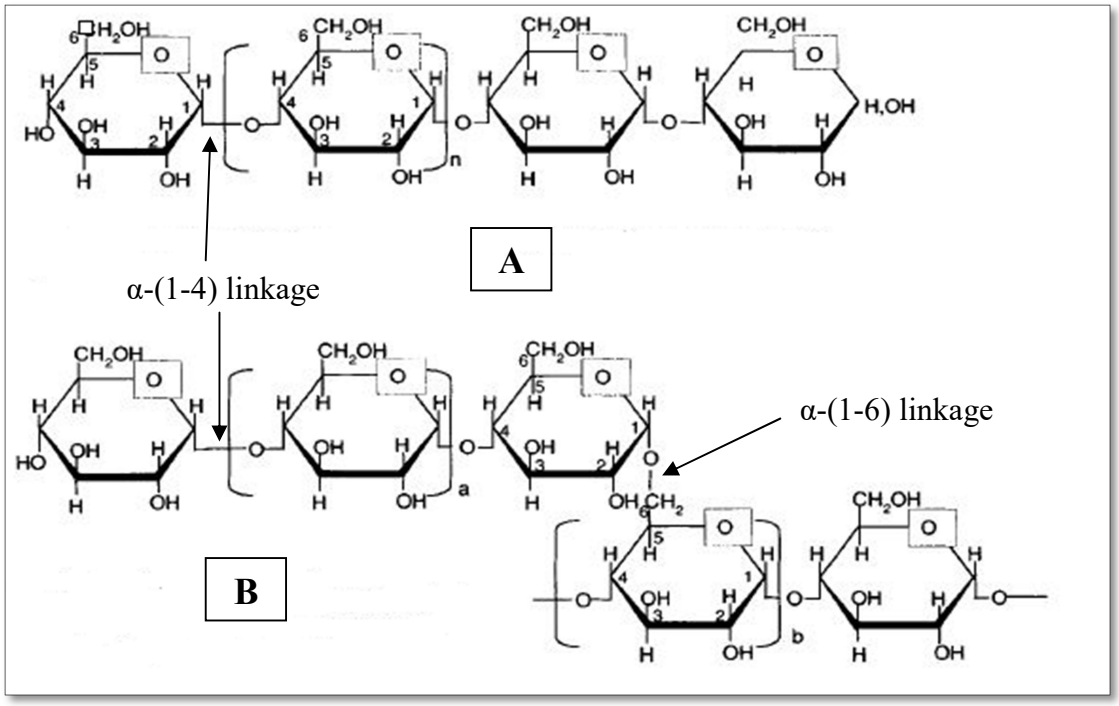

Figure 2.1: α-(1-4) and α-(1-6) glycosidic bonds of starch: (A) amylose structure; (B) amylopectin structure. (Adapted from Tester et al., 2014)

2.2.1 Starch Digestion in Human

Starch is the main energy intake of human daily. The digestion of starch initiates in the mouth once human ingests the starchy food. An enzyme in the saliva, called salivary α-amylase, is responsible in hydrolyzing starch into disaccharides (Lunn and Buttriss, 2007). This enzyme tends to digest starch efficiently but a lesser extent of hydrolysis occurs as starch remains in the mouth for a short period of time only (Singh et al., 2010). Moreover, salivary α-amylase is inactivated by the acidic condition in the stomach when starch passes down the oesophagus and reaches the stomach (Singh et al., 2010). Gastric juice that is released into it has a pH range of 2-4 making an extreme acidic condition for hydrolysis in the human stomach (Wichienchot et al., 2010). Enzyme pepsin is activated by this acidic condition to digest protein (Perara et al., 2010). Although starch cannot be hydrolyzed by this

enzyme, strong acid (hydrochloric acid) from gastric juice can hydrolyze starch (Dona et al., 2010).

While entering the small intestine, pancreatic fluid produced by the pancreas is released into the duodenum and mixes with the starchy food (Dona et al., 2010). Pancreatic fluid consists of sodium bicarbonate and digestive enzymes. Sodium bicarbonate neutralizes the acidic starchy food so that pancreatic α-amylase can further hydrolyze the polysaccharides. (Dona et al., 2010). Pancreatic fluid also consists of others digestive enzymes, such as trypsin, chymotrypsin, lipase and ribonucleases (DeSesso and Jacobson, 2001). Majority of the ingested starchy food is hydrolyzed in the small intestine of human by pancreatic α-amylase (Singh et al., 2010). This enzyme hydrolyzes the α-1,4 linkages of starch polymers specifically, producing mainly maltose, maltotriose and maltotetraose for amylose chains while dextrins or branched oligosaccharides for amylopectin chains (Singh et al., 2010). These products are further hydrolyzed to glucose by brush border enzymes as only glucose could absorb through the small intestine into the human body.

Starch that has escaped from digestion by human enzymes in the small intestine will passage into the colon. In this region, bacterial enzymes favour the degradation of nondigestible carbohydrate through a process, called fermentation (Perara et al., 2010). Human body does not produce digestive enzymes in the colon (Boisen and Eggum, 1991). The bacterial fermentation is further discussed in **Section 2.4**. Based on the nutritional properties, starches can be classified as either digestible or resistant (Sajilata et al., 2006) as summarized in **Table 2.1**. Digestible starches can be further categorized as either rapidly digestible starch (RDS) or slowly digestible starch (SDS) and these starches are completely digested in the small intestine (Sajilata et al., 2006). RDS is quickly hydrolyzed to glucose units within 20 minutes

of digestion in the small intestine (Sajilate et al., 2006). SDS is digested more slowly than RDS, but it is completely converted to glucose within 20-120 minutes of digestion in the small intestine (Sajilata et al., 2006). Resistant starch (RS) is the fraction of starch which cannot be hydrolyzed to glucose within 120 minutes of digestion in the small intestine but is fermented in the large intestine (Raigond et al., 2014).

Table 2.1: Nutritional classification of starches.

Item	Starch Fractions		
	RDS	SDS	RS (types I-V)
Digestion timeline (*in vitro*)/place	Within 20 min/mouth and small intestine	20-120 min/small intestine	>120 min/not in small intestine, main action in colon
Examples	Freshly cooked food	Native waxy maize starch, millet, legumes	Raw potato, staled bread
Amount (g per 100g dry matter)	Boiled hot potato: 65	Boiled millet: 28	Raw potato starch: 75
Main physiological property	Rapid source of energy	Slow and sustained source of energy and sustained blood glucose	Effects on gut health (e.g. prebiotic, fermentation to butyrate with hypothesized anticarcinogenic effects)
Structure	Mainly amorphous	Amorphous/crystalline	Dependent on type, mainly crystalline

(Adapted from Raigond et al., 2014)

2.2.2 Resistant Starch

2.2.2(a) Definition

EURESTA (European FLAIR Concerted Action no. 11 Physiological Implications of the Consumption of Resistant Starch in Man) had defined RS as "the total amount of starch, and the products of starch degradation that resists digestion in the small intestine of healthy people" (Asp, 1992). The definition of RS was later proposed to be "the sum of starch and starch-degradation products that, on average, reach the human large intestine" (Englyst et al., 1996).

2.2.2(b) Types of Resistant Starch

Resistant starch is classified into five different categories: RS_1, RS_2, RS_3, RS_4 and RS_5, as shown in **Table 2.2**, according to the mechanism which restricts its digestion by enzyme.

Table 2.2: Types of resistant starch and their food sources.

RS types	Description	Food sources
RS_1	Physically inaccessible starches	Whole or partly milled grains and seeds, legumes
RS_2	Ungelatinized granular starches	Raw potatoes, green bananas, some legumes, high-amylose corn
RS_3	Retrograded starches	Cooked and cooled potatoes, bread, cornflakes, food products with repeated moist heat treatment
RS_4	Chemically modified starches due to cross-linking with chemical reagents	Foods in which modified starches have been used (e.g. breads, cakes)
RS_5	Amylose-lipid complexes	Foods with high amylose content

(Adapter from Raigond et al., 2014)

RS_1 is enclosed in a non-digestible matrix, and thus it is physically inaccessible and resistant to enzymatic digestion (Haralampu, 2000). Milling and chewing enable it to be more accessible to digestion (Fuentes-Zaragoza et al., 2011). RS_2 is native starch which is not gelatinized and occurs in granular form. It is relatively dehydrate and is densely packed in a radial pattern which limits its accessibility to digestive enzyme (Sajilata et al., 2006). RS_3 is retrograded nongranular starch which formed during the cooling of cooked starch (Fuentes-Zaragoza et al., 2011). Formation of starch crystals during cooling prevents RS_3 to be digested by enzyme (Fuentes-Zaragoza et al., 2011). Detailed information on the formation of RS_3 is described in next section (**Section 2.2.2(c)**). RS_4 includes starch that has been cross-linked, esterified, or etherized with chemicals reagent to decreases their digestion by enzyme (Raigond et al., 2014). RS_5 is an amylose-lipid

complex starches that require a high gelatinization temperature (Jiang et al., 2010). It composed of linear water-insoluble polyalpha-1,4-D-glucan which is not degraded by α-amylases (Frohberg and Quanz, 2008).

Among all the resistant starches, RS_3 is preferred as a functional food due to its thermal stability high melting temperature at the range of 140 °C to 160 °C (Shamai et al., 2003). On the other hand, RS_1 and RS_2 are thermally instable, causing them to lose their functional benefits after food processing (Zhao and Lin, 2009), while the legality of RS_4 being used in food production is a major concern (Lunn and Buttriss, 2007). RS_4 needs approval for its application as food ingredient due to the fact that it is produced using chemical reagents. To date, RS_4 is a novel food which not yet approved by European Union but it is permitted in Japan (Lunn and Buttriss, 2007). The thermal stability characteristic allows food with added RS_3 to retain its functional benefits even after cooking. Research had also shown that RS_3 can be incorporated into battered food without compromising consumer acceptability (Sanz et al., 2008).

2.2.2(c) Formation of Resistant Starch Type III

Gelatinization of starch followed by rearrangement of amylose polymers, which is retrogradation, are the two general stages involve in the formation of RS_3. During gelatinization process, heating of starch suspension in excessive water raises its temperature progressively, allowing starch molecules to absorb heat energy and increasing the vibration causing the breakage of hydrogen bonds among the starch molecules (Bryksa and Yada, 2009). Meanwhile, hydrogen bonds are formed between water molecules and starch molecules, allowing water to penetrate into the starch granules to such an extent that the irreversible swelling of starch granules

occurs (Vaclavik and Christian, 2014). Swelling causes starch granules to lose their birefringence and the ordered crystalline structure. Eventually, they are disrupted, allowing polymer chains to leach out from starch granule (Vaclavik and Christian, 2014).

The starch suspension forms gel after gelatinization. While the gel is cooled, heat energy is being released out from the gel. This facilitates the formation of hydrogen bonds among the starch polymers and subsequently starch polymers re-associate from a disordered structure into a more ordered structure (Bryksa and Yada, 2009). Between the two types of starch polymers (amylose and amylopectin), amylose chains are more preferred in the process of retrogradation for the formation of RS_3 due to their linearity, which allow stronger hydrogen bond to form and causing the formation of tightly packed crystalline structure (Bryksa and Yada, 2009). This contributes to the thermally stable characteristic of RS_3, making the concept of using RS_3 as functional ingredient in processing food rational. The crystalline structure which forms from amylopectin chains during retrogradation is not tightly packed and less stable, having a melting temperature of 55 ºC to 70 ºC (Eerlingen and Delcour, 1995). This is due to the branching chains of amylopectin which restrict the formation of strong hydrogen bonds among the polymers (Eerlingen and Delcour, 1995).

Every stage of RS_3 production has its own influencing factors in addition to the starch botanical sources, ratio of amylose and amylopectin content, and the presence of other components in the starch (Sajilata et al., 2006). According to Thompson (2000), subjecting the retrograded starch to acid or enzyme hydrolysis could increase the level of RS_3.

Previous research had demonstrated that different methods used in the gelatinization of starch (autoclaving at 120 °C and boiling at 100 °C) as well as the botanical sources of starches influenced the amount of RS_3 produced (Garcia-Alonso et al., 1998). In this study, RS_3 contents produced from wheat (13.4%) and corn starches (10.6%) gelatinized by autoclaving method were significantly higher (10.4% and 9.55%, respectively) than that of which gelatinized by boiling (Garcia-Alonso et al., 1998). However, the boiling method had resulted a higher RS_3 content production (4.52%) in rice starch than that produced by autoclaving (3.22%) while the content of RS_3 produced from potato starch had no significant difference for both gelatinization methods (Garcia-Alonso et al., 1998).

One previous research showed that the concentration of amylose was positively correlated with the yield of RS_3, whereby amylomaize VII with the highest amylose content (70%) produced the highest RS_3 (21.3%) while waxy maize with the lowest amylose content (< 1%) yielded the lowest RS_3 content of 2.5% (Sievert and Pomeranz, 1989). However, amylomaize VII starch was produced from the breeding of maize crops to obtain high amylose content. Natural starches contain low amylose content of 15% to 20% (Sajilata et al., 2006), making retrogradation of amylose chains restricted as the amount of amylopectin chains are readily high. Nevertheless, debranching enzyme can be used to cleave the α-D-(1-6) linkages in amylopectin so that a mixture of long and short unit of amylose can be released (Leong et al., 2007). The increased amount of amylose can facilitates recrystallization to be occur easily (Zhao and Lin, 2009). None of the previous researches could produce resistant starch samples with 100% RS_3 content. Even for the commercial available resistant starch (**Table 2.3**), none of them contain 100% of RS_3.

Differences in processing methods such as cooking, tempering, extrusion, puffing, roasting, and flaking influence the RS_3 content of the cooked foods. Puffing of rice snack by frying and roasting, produced products with higher RS_3 content; 2.6% and 2.9%, respectively, compared with the raw product itself (Vatanasuchart et al., 2009). Extrusion cooking of high amylose starch (Hylon VII) significantly reduced the RS_3 content from 60% to 13.8% (Htoon et al., 2009).

Table 2.3: Commercially manufactured resistant starches.

Brand name of commercial RS	Type	RS[a]/TDF[b] content	Manufacturer
Hi-maize	RS_2	30-60% TDF	National Starch and Chemicals Co., USA
Crystalean	RS_3	19.2-41% RS	Opta Food Ingredients Inc., USA
Novelose 240	RS_2	47% RS	National Starch and Chemicals Co., USA
Novelose 260	RS_2	60% RS	National Starch and Chemicals Co., USA
Novelose 300	RS_3	<30% TDF	National Starch and Chemicals Co., USA
ACT* -RS_3	RS_3	53% RS	Cerestar (a Cargill company)
Fibersym HA	RS_4	>70% TDF	MGP Ingredients, Inc. (Atchison,KS) and Cargill
Fibersym 80ST	RS_4	80% TDF	MGP Ingredients, Inc. (Atchison,KS) and Cargill
Hylon VII	RS_2	23% TDF	National Starch and Chemicals Co., USA
Neo-amylose	RS_3	87 or 95% RS	Protos-Biotech. (Celanese Ventures GmbH)

[a]RS: resistant starch; [b]TDF: total dietary fibre. (Adapted from Raigond et al., 2014)

2.3 Human Gastrointestinal Microflora

Different sections of the human gastrointestinal tract (**Figure 2.2**) vary widely in the numbers of bacteria, harbouring approximately 10^3 CFU/g, 10^{6-7} CFU/g, 10^{11} CFU/g, in the stomach, small intestine and large intestine (colon), respectively (Sanders et al., 2007). There are more than 400 species of bacteria constitute the intestinal microflora but only 40 species be in the majority (O'Grady and Gibson,

2005). Lesser bacteria inhabit in the stomach and the first part of small intestine, duodenum. This is because the strong acidic condition in the stomach and the presence of pancreatic fluid and bile salt in duodenum create an unfavorable environment for the bacterial colonization (Sanders et al., 2007). Due to the desirable conditions of the colon, including a longer transit time, the near neutral pH and adequate of nutrient supply, majority of bacteria reside in this region (O'Grady and Gibson, 2005). Hence, most of the bacterial metabolic activities, which could exert significant influences on host health occur in the colon compared with that of in the small intestine.

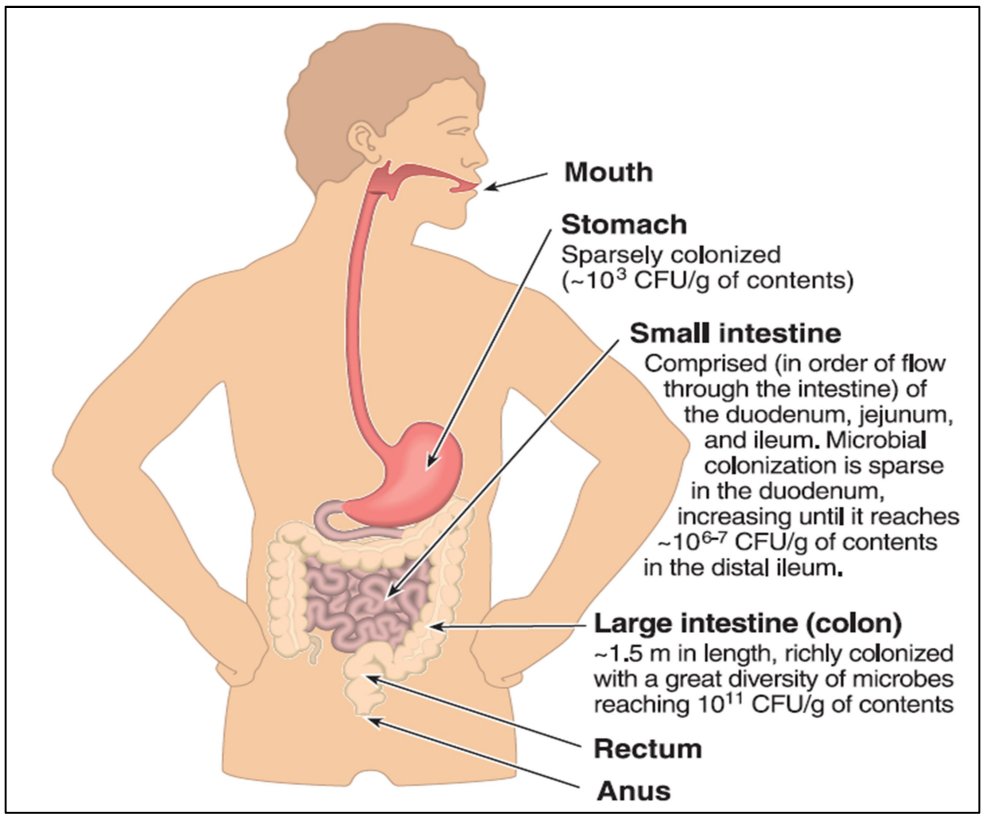

Figure 2.2: The occurrence of bacteria throughout the human gastrointestinal tract. (Adapted from Sanders et al., 2007)

Most of the colonic microflora is strict anaerobes which predominantly include bacteroides, bifidobacteria, eubacteria, clostridia, peptostreptococci, peptococci and ruminocci (Salminen et al., 1998). Of these, bacteroides and

bifidobacteria are numerically preeminent as these two groups can constitute to 30% and 25% of the total anaerobic counts, respectively (Salminen et al., 1998). Strict anaerobes outnumber facultative anaerobes by a factor of ~1000 (Rastall, 2004). The most common facultative anaerobes are lactobacilli, enterococci, streptococci and *Enterobacteriaceae* (Rastall, 2004).

Colonic microflora derives energy for growth from the fermentation of dietary components and endogenous mucins (Hughes et al., 2000). Components of dietary origin include nondigestible carbohydrates, such as resistant starch, non-starch polysaccharides, oligosaccharides and sugar alcohols as well as undigested proteins which passage into the colon (Cummings and Macfarlane, 1991).

The fermentation of carbohydrates (saccharolytic fermentation) produces acetic, propionic and butyric acid as main short chain fatty acid and gases such as CO_2, CH_4 and H_2 (Bernalier-Donadille, 2010). These short chain fatty acids could exert several beneficial influences on host health. Contrary to carbohydrate fermentation, protein fermentation (proteolytic fermentation) produces metabolites which are potentially harmful to the host, such as ammonia, amines and phenolic compounds (Bernalier-Donadille, 2010). Some of the bowel diseases, for instance, colorectal cancer and ulcerative colitis are probably linked to the excessive protein fermentation in the colon (Roberfroid et al., 2010; Windey et al., 2012). Short chain fatty acids and branched chain fatty acids are also the end products of proteolytic fermentation (Bernalier-Donadille, 2010).

The main saccharolytic bacterial groups are bacteroides, bifidobacteria, eubacteria, lactobacilli and clostridia while the main proteolytic bacterial groups are bacteroides and clostridia (Roberfroid et al., 2010). Some of the bacteria, for instance, bacteroides and clostridia could perform both saccharolytic and proteolytic

fermentation.

Colonic microflora can be divided into bacteria that having either beneficial or detrimental influences on host health owing to their metabolic activities and fermentation end products (Gibson et al., 2010). Bacteria with a saccharolytic fermentation are beneficial whereas those having a proteolytic or both types of fermentation are either less beneficial or detrimental (Gibson et al., 2010). Health promoting effects include impede the growth of detrimental bacteria, improve the digestion and absorption of essential nutrients, synthesize vitamins and stimulate the immune functions whereas detrimental effects include diarrhoea/constipation, liver damage, infections, carcinogenesis and intestinal putrefaction (Gibson and Roberfroid, 1995).

Due to the fact that colonic microflora plays a significant role in host health, their composition should be modulated. Gibson and Roberfroid (1995) had proposed that maintaining the colonic microflora in a balanced state could ideally support the health and well-being of the host. According to the authors, this "balanced microflora" concept implies that the colonic microflora must comprise high numbers of bacteria associated with health promoting effects and concomitantly low numbers of bacteria associated with harmful effects. Roberfroid (2005) mentioned that the latter groups of bacteria should keep in low numbers and do not necessarily have to be removed completely, especially for those that could exert both pathogenic and health promoting effects on host health. It is obviously shown in **Figure 2.3** that some of the bacteria such as bacteroides and *E. coli* could attribute not only pathogenic influences but also beneficial influences on host health. The most obvious health promoting bacteria are lactobacilli and bifidobacteria (**Figure 2.3**).

The colonic microflora can be modulated towards a balanced composition through the dietary approaches, which are by: 1.) ingestion of live microorganism, probiotic (**Section 2.5**); and 2.) ingestion of non-digestible food ingredient, prebiotic (**Section 2.6**) (Gibson and Roberfroid, 1995; Windey et al., 2012).

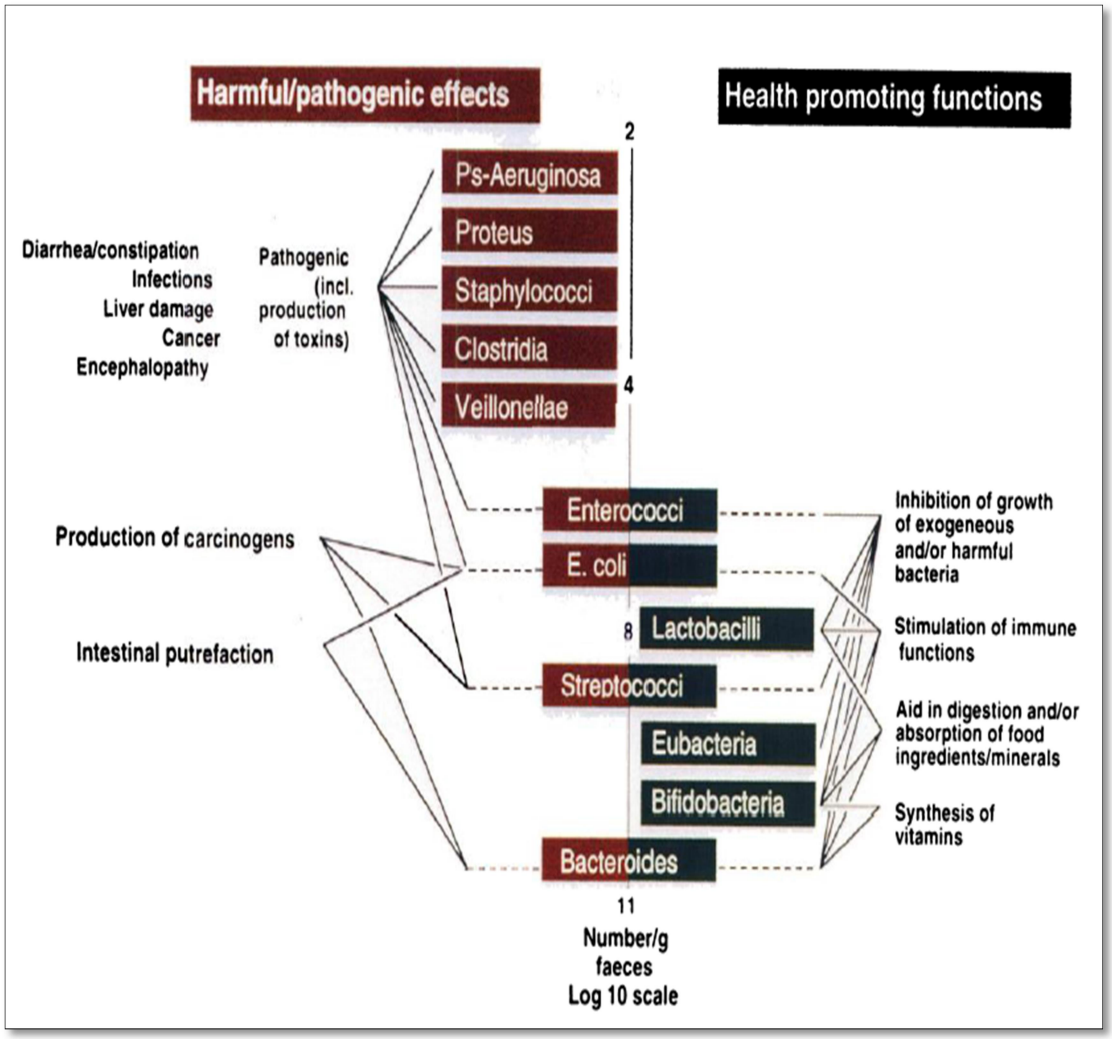

Figure 2.3: Composition and health effects of human faecal microflora.
(Adapted from Gibson and Roberfroid, 1995)

2.4 Fermentation of Starch in the Colon

As mentioned earlier in **Section 2.2.1**, starch which cannot be digested by host enzymes in the small intestine is referred as resistant starch. Once it travels into

the colon, bacteria residing in this region could ferment this nondigestible carbohydrate to derive energy for growth. Depending on the dietary intake, other nondigestible carbohydrates, such as non-starch polysaccharides, oligosaccharides, and sugar alcohol could also serve as a growth substrate (Cummings and Macfarlane, 1991). Due to the fact that most of nondigestible carbohydrates have complex structure, they must be degraded to their monomer units prior to fermentation (Bernalier-Donadille, 2010). The fermentation of carbohydrates to short chain fatty acids can be described as a two-step phenomenon (**Figure 2.4**) as follows:

1) Degradation of polysaccharides to monosaccharides

2) Fermentation of monosaccharides to short chain fatty acids

The fermentation of complex carbohydrates in the gut is a complicated process (**Figure 2.5**) which involves cross-feeding, whereby the end products from the metabolic activity of one/more bacterial species can act as a substrate to support the growth of other bacterial groups (Sarbini and Rastall, 2011). Cross-feeding occurs as bacterial species in the colon are varied in their metabolic capabilities and not all of them could initiate the carbohydrate fermentation (Gibson and Roberfroid, 1995). These metabolic interactions are indeed essential for maintaining diverse species of bacteria in the colon.

Degradation of starch to glucose in the colon is initiated by primary starch degrading bacteria that are capable of producing starch degrading enzyme. In a past research performed by Macfarlane and Englyst (1986), culture-dependent approach had been utilized to identify amylolytic bacteria by inoculating human faecal bacteria from six participants on peptone yeast agar plates supplemented with soluble starch as sole carbon. Colonies with clearing zone around (confirmed by the iodine test) were starch-degrading colonies and 120 of these amylolytic colonies were selected at

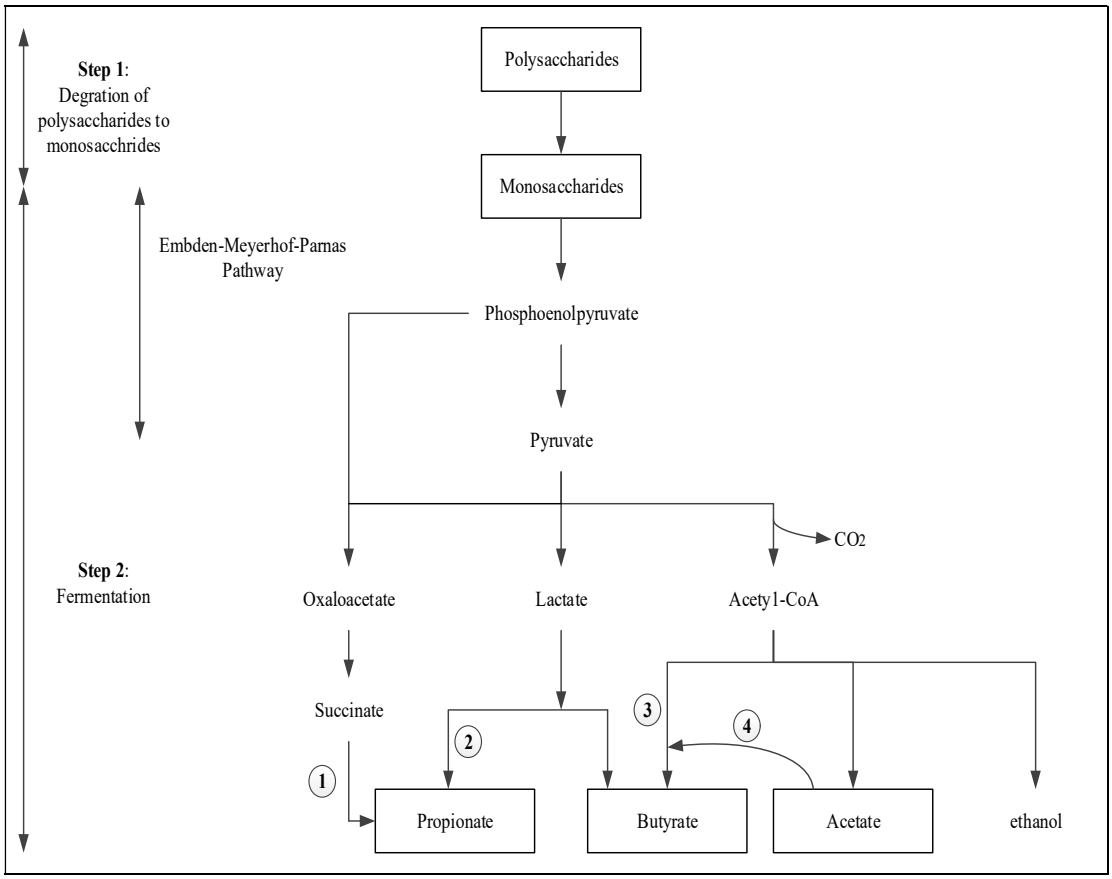

Figure 2.4: Main metabolic pathways involved in the fermentation of carbohydrate to short chain fatty acids by human intestinal microflora. 1, succinate pathway; 2, acrylate pathway; 3, butyrate kinase pathway; 4, butyryl-CoA CoA-transferase pathway. (Adapted from Bernalier-Donadille, 2010 and Louis et al., 2007 with modification)

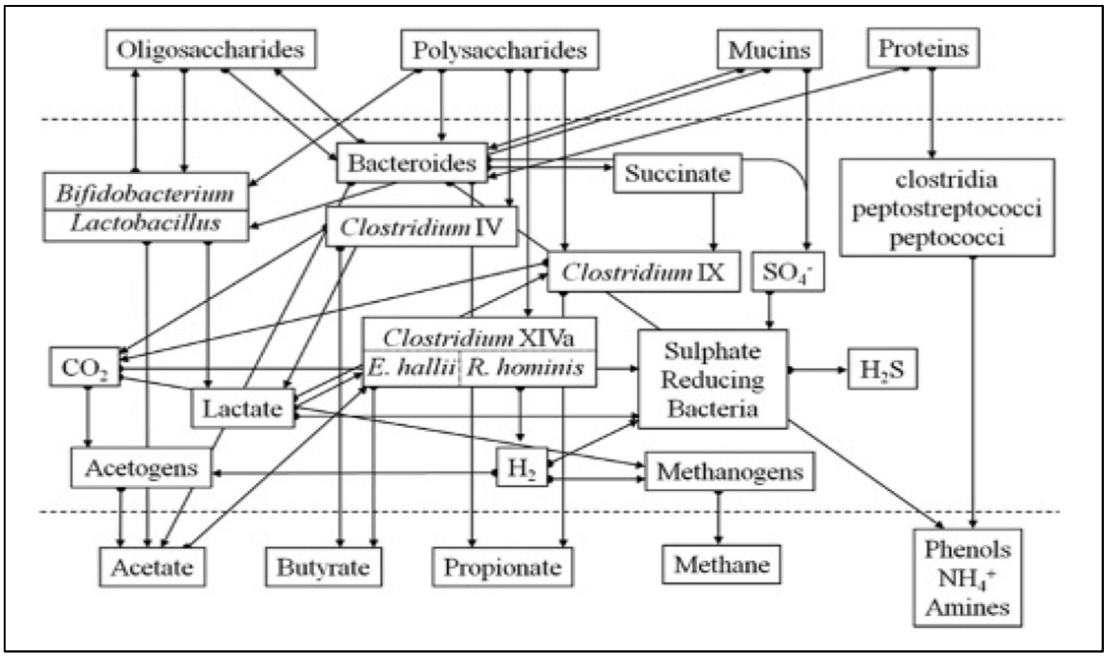

Figure 2.5: Metabolic interactions in the human colon. (Adapted from Sarbini and Rastall, 2011)

random for further characterization to the genus level. The authors reported that most of the amylolytic bacteria were from the genera of *Bifidobacterium*, *Bacteroides* and *Fusobacterium*/*Butyrivibrio*, which accounted for 58%, 18% and 10% of the total isolated amylolytic bacteria, respectively.

Recent works had applied the molecular technique based on 16S ribosomal RNA (rRNA) genes to study the colonic microbial ecology of human (Leitch et al., 2007; Abell et al., 2008; Kovatcheva-Datchary et al., 2009; Walker et al., 2011; Ze et al., 2012) . *In vitro* fermentation conducted by Leitch et al. (2007) using human faeces from four adults reported that *Ruminococcus bromii*, *Eubacterium rectale* and *Bifidobacterium* spp. accounted for 81.3% of 16S rRNA sequences recovered from high amylose corn starch (Hylon VII). Similar groups of resistant starch-fermentating bacteria were found to be involved in ^{13}C-labelled potato starch fermentation under *in vitro* conditions inoculated with human faeces from seven adults (Kovatcheva-Datchary et al., 2009). In this study, the authors suggested that *Ruminococcus bromii* was the primary starch degrader and could produce acetic acid while *Eubacterium rectale* might convert this acetic acid to butyric acid.

Data from human dietary intervention studies had also revealed that *Ruminococcus bromii* could degrade resistant starch (Abell et al., 2008; Walker et al., 2011). A study done by Abell et al. (2008), where the influence of diets (supplemented daily for 4 weeks) rich in nonstarch polysaccharides or rich in nonstarch polysaccharides and resistant starch on the composition of faecal microflora in forty-six healthy adults (16 men and 30 women with age ranged from 25 to 66 years) was examined, reported a significant increase in the level of *Ruminococcus bromii* when individuals on the diet rich in nonstarch polysaccharides and resistant starch, but not the diet rich in nonstarch polysaccharides only. Other

species of bacteria, *Faecalibacterium prausnitzii*, *Eubacterium rectale* and *Bacteroides thetaiotaomicron* also showed an increase in a number of volunteers on the diets rich in nonstarch polysaccharides and resistant starch.

In another *in vivo* study, fourteen overweight male volunteers aged between 27 to 73 years consumed diet either high in RS_3 or high in wheat bran every day for 3 weeks (Walker et al., 2011). Result from the study demonstrated that twelve volunteers showed a significant increase of approximately 4.5-fold in the level of faecal *Ruminococcaceae* in response to diet high in RS_3, whereby this group of bacteria accounted for 17% and 3.8% of the total bacteria in volunteers consuming diet supplemented with RS_3 and wheat bran, respectively. Low numbers of *Ruminococcaceae* was reported in faecal samples of the other two volunteers on RS_3 diet with more than 60% of ingested resistant starch recovered in the stool, compared with that of less than 4% recovered in the twelve volunteers. The authors mentioned that this variation in fermentation was attributed to the initial composition of gut microflora which diversed among individuals.

In vitro fermentation of resistant starches performed by Ze et al. (2012) using four strains of amylolytic bacteria (*Eubacterium rectale* A1-86^T, *Ruminococcus bromii* L2-63, *Bifidobacterium adolescentis* L2-32 and *Bacteroides thetaiotaomicron* 5482) isolated from human faeces had further demonstrated *Ruminococcus bromii* as a primary degrader of resistant starch in the human colon. In this study, co-cultural fermentation involving pairwise combination of these four amylolytic bacteria showed that *Ruminococcus bromii* could stimulate the utilization of boiled resistant starches (RS_2 and RS_3) by the other three amylolytic bacteria, even in the medium that did not promote its growth. Combinations without *Ruminococcus bromii* showed a limited ability to utilize boiled resistant starches. Besides that, for the fermentation

using human faeces provided by one of the volunteer which had shown low RS_3 fermentation and low number of *Ruminococcaceae* in the study of Walker et al. (2012), the addition of *Ruminococcus bromii* enhanced the degradation of RS_3 substantially. This incidence was not seen in the faecal fermentation of RS_3 with addition of the other three amylolytic bacteria. The authors concluded that *Ruminococcus bromii* possessed the greatest ability to initiate degradation of resistant starch among the four amylolytic bacteria tested.

Oligosaccharides released from starch are further degraded by the other gut bacteria and thus making glucose available for fermentation. Most of the colonic microflora applies the Embden-Meyerhof-Parnas pathway to form pyruvic acid from glucose (Bernalier-Donadille, 2010). Pyruvic acid, which is the key fermentation intermediate, is further converted to acetic, propionic and butyric acid as the main metabolites of carbohydrate fermentation through different pathways (**Figure 2.4**). Other intermediate metabolites are formed too, such as lactic acid, succinic acid, and, ethanol (Bernalier-Donadille, 2010).

2.5 Lactic Acid Bacteria and Bifidobacteria

Lactic acid bacteria are a group of Gram-positive, acid tolerant and non-spore forming bacteria which produce lactic acid as major end product during the carbohydrate fermentation (Reddy et al., 2008). Based on the end product from carbohydrate fermentation, lactic acid bacteria are mainly divided into two groups (**Figure 2.6**): homofermentative and heterofermentative. Homofermentative lactic acid bacteria use the Embden-Meyerhof-Parnas pathway to convert 1 mol of glucose to 2 mol of lactic acid whereas heterofermentative lactic acid bacteria utilize phosphoketolase pathway to yield 1 mol each of lactic acid, ethanol/acetic acid, and

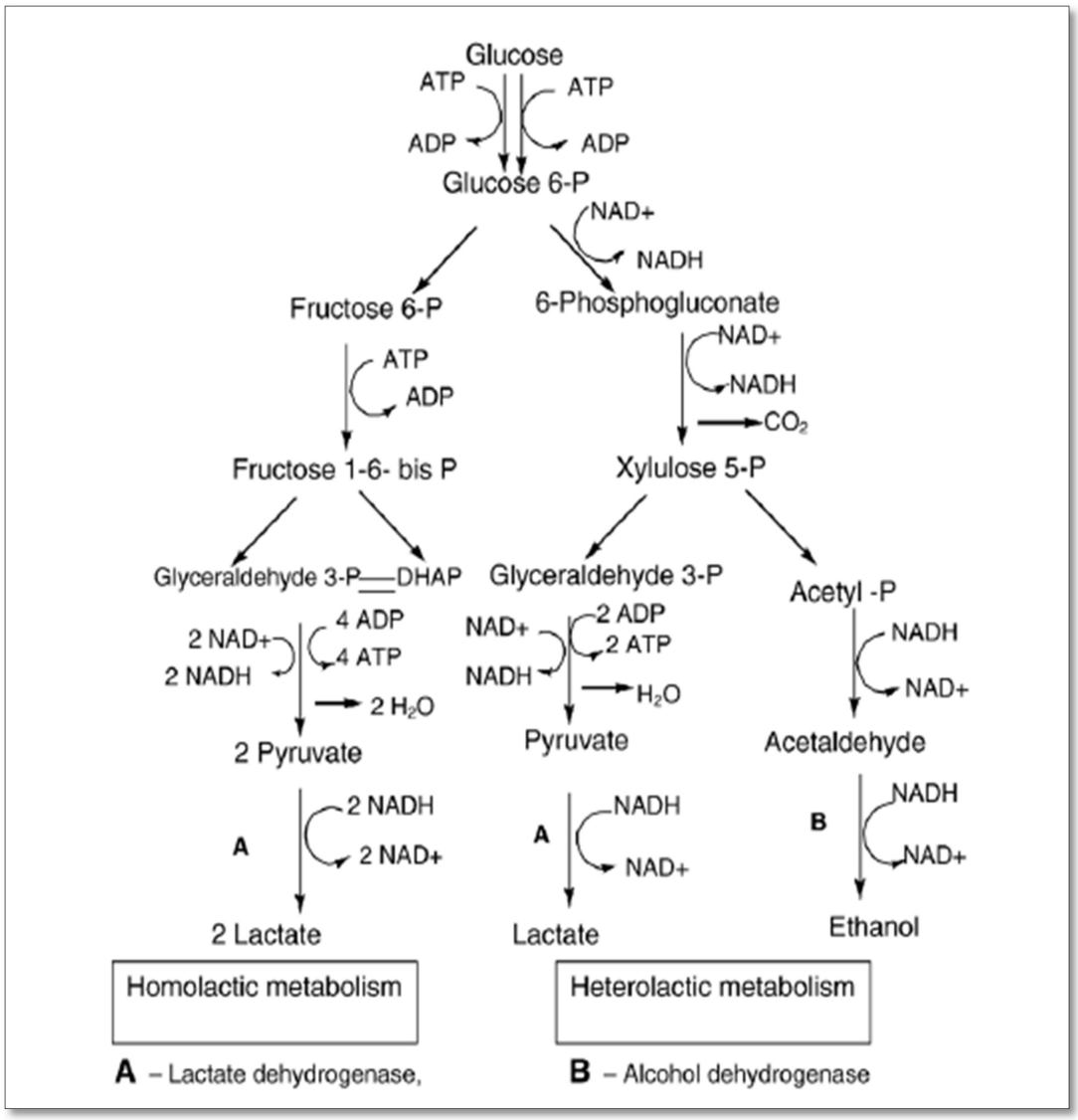

Figure 2.6: The major fermentation route of lactic acid bacteria. (Adapted from Reddy et al., 2008)

carbon dioxide (Axelsson, 2004). Commonly, lactic acid bacteria include the genera of *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus* (Rattanachaikunsopon and Phumkhachorn, 2010). Of these, lactobacilli are considered to be safe because they had have a long history of use in food industry. They are commonly used as starter culture in the production of fermented food.

Bifidobacteria are gram-positive, anaerobic, non-motile and branched rod-shaped bacteria (Ballongue, 2004). The metabolism of bifidobacteria differs from