

**PHYSICOCHEMICAL PROPERTIES OF STARCH
AND PREBIOTIC POTENTIAL OF
OLIGOSACCHARIDES FROM
*Dioscorea hispida***

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*Dioscorea hispida***

BY

NAPISAH BINTI HUSSIN

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LIST OF SYMBOLS

SYMBOL	CAPTION
α	Alpha
β	Beta
$^{\circ}$	Degree
$^{\circ}\text{C}$	Degree celcius
Λ	Lambda
μL	Microliter
μm	Micrometer
mA	Milliampere
$\%$	Percentage
Θ	Theta
ΔH	Transition enthalpy
T_c	Conclusion temperature
T_o	Onset temperature
T_p	Peak temperature
$\times g$	Times gravity

LIST OF ABBREVIATIONS

ABBREVIATION	CAPTION
AACC	American Association of Cereal Chemists
AGU	Amyloglucosidase units per gram
AMG	Amyloglucosidase
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
Bif. 12	<i>Bifidobacterium</i> sp. 12
<i>C. coli</i>	<i>Campylobacter coli</i>
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	Calcium chloride dihydrate
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$	Calcium chloride hexahydrate
<i>C. perfringens</i>	<i>Clostridium perfringens</i>
CFU	Colony forming units
CuSO_4	Copper (II) sulfate
DHOS	<i>Dioscorea hispida</i> oligosaccharides
DMSO	Dimethyl sulfoxide
DP	Degree of Polymerization
DSC	Differential Scanning Colorimetry
<i>E. coli</i>	<i>Escherichia coli</i>
Eq	Equation
FOS	Fructooligosaccharide
G	Gram
H	Hour/hours
HCl	Hydrochloric acid

HPLC	High-performance liquid chromatography
H ₂ SO ₄	Sulfuric acid
I ₂	Iodine
KCl	Potassium chloride
KI	Potassium iodide
KOH	Potassium hydroxide
K ₂ HPO ₄	Potassium hydrogen phosphate
KH ₂ PO ₄	Potassium dihydrogen phosphate
K ₂ SO ₄	Potassium sulfate
kV	Kilovolt
L	Liter
LA 2131	<i>Lactobacillus acidophilus</i> FTDC 2131
LC 393	<i>Lactobacillus casei</i> ATCC 393
LC 0442	<i>Lactobacillus casei</i> FTCC 0442
LP 0350	<i>Lactobacillus plantarum</i> FTCC 0530
M	Molarity
Mg	Milligram
MgCl ₂ .6H ₂ O	Magnesium chloride hexahydrate
MgSO ₄ .7H ₂ O	Magnesium sulfate heptahydrate
min	Minute
mL	Milliliter
MPa	Megapascal
MRSA	de Man, Rogosa, Sharpe agar
N	Nitrogen
Nm	Nanometer

NaCl	Sodium chloride
NaHCO ₃	Sodium bicarbonate
NaOH	Sodium hydroxide
Na ₂ HPO ₄ ·2H ₂ O	Sodium phosphate dibasic dehydrate
NPUN	New Pullulanase Unit Novozymes
OHC	Oil-holding capacity
PUN	Pullulanase Unit Novozymes
Rpm	Revolutions per minute
RVU	Rapid Visco Unit
SEM	Scanning electron microscopy
TRIS	Tris (hydroxymethyl) aminomethane
TS	Tryptic soy
UV-VIS	Ultraviolet-visible
v/v	Ratio of volume per volume
WCA	Wilkins Chalgren Anaerobic
WHC	Water-holding capacity
w/v	Ratio of weight per volume
w/w	Ratio of weight per weight

**SIFAT-SIFAT FIZIKOKIMIA KANJI DAN POTENSI PREBIOTIK
OLIGOSAKARIDA DARIPADA *Dioscorea hispida***

ABSTRAK

Ubi daripada sejenis tumbuhan yang jarang digunakan iaitu *D. hispida* Dennst. telah diproses bagi mendapatkan kanji dan telah diuji sifat-sifat fizikokimia. Ubi *D. hispida* mengandungi 97.95% kanji total dengan amilosa (25.27%) dan amilopektin (72.68%). Kanji *Dioscorea hispida* mengandungi protein kasar (0.42%), lemak kasar (0.23%), abu (0.47%) dan fiber kasar (0.93%). Granul *D. hispida* berbentuk polihedral dengan struktur permukaan yang tidak rata dan kasar serta berdiameter 2-4 μm . Granul *D. hispida* menunjukkan corak pembelauan sinar X jenis B, suhu puncak gelatinisasi pada 87°C dan suhu pempesanan ialah 78°C. Kuasa pembengkakan dan keterlarutan granul kanji serta kapasiti pengikatan air dan kapasiti pengikatan minyak masing-masing adalah 14.22 g/g, 5.48 g/g, 2.78 g/g dan 1.14 g/g. Larutan kanji *D. hispida* dengan kepekatan yang berbeza (5%, 10%, 15%, dan 20%) telah dihidrolisis dengan menggunakan rawatan enzim tunggal iaitu α -amilase, amiloglukosidase dan pullulanase dan rawatan campuran enzim bagi menghasilkan oligosakarida *D. hispida* (DHOS). Penghasilan maksimum DHOS (19.67 mg/g) telah diperolehi melalui hidrolisis 10% kepekatan kanji *D. hispida* dengan gabungan α -amilase (pH 5.5; 90°C) dan pullulanase (pH 5.5; 45°C) pada masa hidrolisis selama 6 jam. DHOS telah dianalisis untuk ciri-ciri prebiotik dan didapati mengandungi keupayaan rintang terhadap hidrolisis oleh jus gastrik pada pH 3-4 (88.78-99.76% residu DHOS) selama 3 jam tetapi kurang rintang terhadap hidrolisis jus gastrik pada pH 1-2. DHOS didapati kurang rintang terhadap hidrolisis oleh enzim saluran

gastrousus dan penyerapan usus di mana peratus DHOS selepas hidrolisis adalah di antara 40.62-52.04%. Bagi kajian pertumbuhan bakteria, DHOS secara positif telah mempengaruhi pertumbuhan mikroflora berfaedah dalam najis tikus (laktobasili and bifidobakteria) dan telah merencatkan pertumbuhan bakteria merbahaya (bakteroides, clostridia dan enterobakteria). Indeks prebiotik telah dikira bagi membandingkan kesan positif DHOS dengan prebiotik yang lain. Indeks prebiotik DHOS, fruktooligosakarida (FOS) dan inulin selepas 72 jam masa fermentasi adalah +7.72, +8.69 and +9.27. Asid laktik dan asid lemak rantai pendek (asid asetik dan asid butirik) juga telah dihasilkan di dalam medium fermentasi yang mengandungi DHOS. Secara keseluruhannya, aktiviti β -glucuronidase berkurang di dalam media DHOS dan FOS pada 48 jam masa fermentasi. Potensi sinbiotik bagi 23 strain kultur asli daripada genus laktobasili dan bifidobakteria telah diuji dengan menggunakan substrat DHOS. Fermentasi di dalam medium DHOS telah berjaya meningkatkan bilangan pertumbuhan *Lactobacillus plantarum* FTCC 0350 dan *Bifidobacterium bifidum* 12 berbanding dengan pertumbuhan di dalam media fermentasi yang mengandungi FOS dan inulin. Fermentasi di dalam medium DHOS juga berupaya meningkatkan pertumbuhan *Lactobacillus acidophilus* FTDC 2131, *L. casei* ATCC 393 and *L. casei* FTCC 0442. Walau bagaimanapun pertumbuhan kesemua strain tersebut di dalam medium fermentasi DHOS adalah lebih rendah daripada pertumbuhan strain di dalam media fermentasi yang mengandungi FOS dan inulin. Bagi analisis Skor Aktiviti Prebiotik, *Bifidobacterium bifidum* 12 telah mencapai skor tertinggi DHOS melawan ketiga-tiga bakteria merbahaya iaitu *Escherichia coli* (+0.47), *Campylobacter coli* (+0.61) dan *Clostridium perfringens* (+0.71) berbanding dengan prebiotik komersial yang lain (FOS dan inulin). Kesimpulannya, DHOS telah menunjukkan sesetengah ciri prebiotik dan kajian *in vivo* lanjut boleh dijalankan.

**PHYSICOCHEMICAL PROPERTIES OF STARCH AND PREBIOTIC
POTENTIAL OF OLIGOSACCHARIDES FROM *Dioscorea hispida***

ABSTRACT

The underutilised *Dioscorea hispida* Dennst. tuber was processed for its starch and was evaluated for its physicochemical properties. The tuber contained 97.95% total starch comprising of amylose (25.27%) and amylopectin (72.68%). *Dioscorea hispida* starch contained crude protein (0.42%), crude fat (0.23%), ash (0.47%) and crude fiber (0.93%). The starch granules were coarse-surfaced polyhedral shape with an average diameter of 2-4 μm . The starch exhibits B-type in X-ray diffraction pattern and had gelatinisation peak temperature of 87 °C and pasting temperature of 78 °C. The swelling power, solubility, water-holding and oil-holding capacities were 14.22 g/g, 5.48 g/g, 2.78 g/g and 1.14 g/g, respectively. Different concentrations of *D. hispida* starch (5%, 10%, 15%, and 20%) were hydrolysed by individual enzymes α -amylase, amyloglucosidase or pullulanase or combination of the enzymes to yield *D. hispida* oligosaccharides (DHOS). The maximum yield of DHOS (19.67 mg/g) was obtained from the hydrolysis of 10% (w/v) of *D. hispida* starch with combination of α -amylase (pH 5.5; 90°C) and pullulanase (pH 5.5; 45°C) for 6 h. The DHOS when analysed for prebiotic characteristics was found to be resistant to hydrolysis by gastric juice at pH 3-4 (88.78-99.76% DHOS residue) for 3 h but less resistant to hydrolysis at pH 1-2. DHOS was also found to be less resistant towards hydrolysis by gastrointestinal tract enzymes and intestinal absorption as the remaining DHOS after hydrolysis was ranging from 40.62-52.04%. In term of bacterial growth study, DHOS positively

influenced the growth of beneficial rat faecal microflora (lactobacilli and bifidobacteria) and negatively influenced the growth of detrimental bacteria (bacteroides, clostridia and enterobacteria). The prebiotic index (PI) was calculated to compare the positive effect of DHOS with other prebiotics. The PI of DHOS, fructooligosaccharides (FOS) and inulin after 72 h fermentation were +7.72, +8.69 and +9.27 respectively. Lactic acid and short chain fatty acids (SCFA) were significantly produced in the media containing DHOS. The activity of β -glucuronidase was reduced in the DHOS and FOS media at 48 h fermentation. Study on the synbiotic potential of DHOS and pure cultures from the genus *Lactobacilli* and *Bifidobacterium* was conducted for 23 bacterial strains. The fermentation in the DHOS medium by *Lactobacillus plantarum* FTCC 0350 and *Bifidobacterium bifidum* 12 showed the higher growth counts compared to those in FOS and inulin. Fermentation of DHOS also enhanced the growth of *Lactobacillus acidophilus* FTDC 2131, *L. casei* ATCC 393 and *L. casei* FTCC 0442, however, the counts in DHOS medium was lower than that in the FOS and inulin fermentation media. DHOS with *B. bifidum* 12 also exhibited the highest Prebiotic Activity Score against three detrimental bacteria namely *Escherichia coli* (+0.47), *Campylobacter coli* (+0.61) dan *Clostridium perfringens* (+0.71) as compared to the commercial prebiotics (FOS and inulin). In conclusion, DHOS exhibited several prebiotic characteristics and *in vivo* studies are worth to be explored.

CHAPTER 1

INTRODUCTION

1.1 Research Background

The human large intestine is inhabited by numerous and diverse species of microorganism, forming a dense and complex microflora gut population. Colonic microflora plays a crucial role in maintaining the health and proper track of intestinal functions which influences the physiological status of host health. The actions and functions of gut microflora have been claimed to impact the development of immune system, suppress the growth of pathogen, inhibit the growth of pathogen and regulate metabolic pathway in the host besides lowering the production of toxic compounds and pH of bowels (Sekirov et al., 2010). Thus, colonic microflora must be maintained in a balanced state constituting predominantly of health promoting bacteria, such as lactobacilli and bifidobacteria that are nonpathogenic, nonputrefactive and nontoxigenic (Crittenden, 1991). Imbalance in the gut microflora has been linked to numerous diseases including colorectal cancer and inflammatory bowel disease (Zhu et al., 2014).

Several strategies have been adapted to regulate the balance and proper density of microbial gut populations. These include the use of antibiotics which is useful to eliminate the pathogenic bacteria from the gut atmosphere but inversely provides risk of side effects as implicated in their use a prophylactic agent. Another way is by the consumption of probiotics, which refers to the direct supplementation of beneficial microorganisms such as lactobacillus and bifidobacteria. The third promising

strategy involves the use of prebiotics. Gibson & Roberfroid (1995) initially defined prebiotics as ‘a nondigestible carbohydrate that could improve a balanced intestinal microflora once orally administered as food supplement by affecting the gut microflora in the host thus, improving the host health’. A prebiotic ingredient should resist digestions in the upper gastrointestinal tract and be selectively fermented by intestinal microflora associated with beneficial effects (Gibson et al., 2004). Thus, a more recent definition has been proposed and endorsed during the experts meeting of International Scientific Association of Probiotics and Prebiotics (ISAPP), New York, USA in 2008. Prebiotic has been suggested as “a selectively fermented ingredient that influences specific changes, in the composition and/or activity of the gastrointestinal microflora, hence conferring benefit(s) upon host health” (FAO, 2007; ISAPP, 2008).

The addition of prebiotic carbohydrate into food products has now emerged especially in the dairy product sectors (Huebner et al., 2007). Today, foods are no longer operated for their taste and basic nutritional needs, but also imperative factors that improve the health of consumers. The prebiotic concept has been established in the past two decades, only three food ingredients currently fulfill the prebiotic characteristics, which are inulin-type fructans, trans-galactooligosaccharides and lactulose (Gibson et al., 2010). The demand for prebiotic food is rapidly growing and is expected to reach US\$5,545.74 million by the year 2020 (Newswire, 2015). Hence, many researchers have now interested in studying various carbohydrate sources to be claimed as prebiotics such as oligosaccharides from dragon fruit flesh (Wichienchot et al., 2010), pectic oligosaccharides from orange peel wastes (Gómez et al., 2014), oligosaccharides from coffee mannan (Asano et al., 2003) and refined

arabinoxyloligosaccharides from wheat bran (Gullón et al., 2014). In this research, oligosaccharide from *ubi gadong* was tested for its prebiotic potential.

1.2 Problem Statement

In Malaysia, *Dioscorea hispida* Dennst. is commonly known as *ubi gadong*. It is categorised as an underutilised wild tuber that can be found abundantly in the secondary forest (Hahn, 1995) in the region of Malaysia. Basically, this tuber is known as a toxic tuber, and it is harmful to be consumed without a prior detoxication process of the toxic alkaloid dioscorine (Tattiyakul et al., 2010). In certain areas of tropical and subtropical regions, this tuber is categorised as an important starchy food and is widely used as a staple food (Fauziah et al., 2016). However, nowadays this crop is rarely eaten by natives due to the time consuming and laborious effort required for its preparation (Liu et al., 2006).

Moreover, people these days are conveying into fast and modern foods. Nevertheless, due to its advantages, this insignificant tuber is now currently being promoted by the Terengganu State Government as an alternative food to support the national food security agenda. There are many researches conducted on prebiotics obtained from vegetables like onion, garlic, asparagus and okra, fruits like banana, jackfruit and coconut as well as some tubers including yam. However, no scientific evidence on oligosaccharides from *D. hispida* as prebiotic is available. Furthermore, not many studies have been performed in Malaysia on prebiotics and none has been done on this neglected tubers. Thus, this study has focused on the tuber of *D. hispida*, as it is considered as a cheap source of carbohydrate and has been adjudged to be of potential value.

1.3 Significance of the Study

Dioscorea hispida Dennst. or *ubi gadong* can presumably be regarded as Terengganu traditional food source. *Ubi gadong* contains a high amount of carbohydrate, thus leading this study to explore its value in producing oligosaccharides as a new potential food ingredient with prebiotic property. Previous research on the *D. hispida* digestive property revealed its ability to reduce the risk of obesity, diabetes and other diseases (Aprianita et al., 2009). Thus, this present study was carried out to evaluate the suitability and potential application of *D. hispida* starch as an alternative source for oligosaccharides with prebiotic property. Indirectly, this can beneficially accelerate the agriculture sector by commercially planting the *D. hispida* as well as enhancing the Malaysian population health using material from local sources.

1.4 Research Objectives

Generally, this study was aimed at determining the physicochemical properties of *ubi gadong* (*Dioscorea hispida* Dennst.) starch and evaluating the prebiotic potential of oligosaccharides produced from the starch. Therefore, this study embarked on the following specific objectives:

1. To determine the physicochemical properties of *D. hispida* starch.
2. To determine the oligosaccharides profile obtained from the enzymatic hydrolysis of *D. hispida* starch using α -amylase, amyloglucosidase and pullulanase.
3. To evaluate the resistance of *D. hispida* oligosaccharides towards digestion by gastric acidity and digestive enzymes as well as towards intestinal absorption.

4. To determine the ability of *D. hispida* oligosaccharides in stimulating the *in vitro* growth and activity of intestinal microflora from rat faeces.
5. To evaluate the *in vitro* fermentability of *D. hispida* oligosaccharides by selected probiotics (*Lactobacillus acidophilus* FTDC 2131, *Lactobacillus casei* ATCC 39, *Lactobacillus casei* FTCC 0442, *Lactobacillus plantarum* FTCC 0350, *Bifidobacterium bifidum* 12) and pathogens (*Escherichia coli* FTDC 201, *Campylobacter coli* c519, *Clostridium perfringens* c438).

CHAPTER 2

LITERATURE REVIEW

2.1 *Dioscorea hispida* Dennst. (*Ubi Gadong*)

2.1.1 Overview

Dioscorea hispida, a type of yam, is a monocotyledous tuber bearing plant, belonging to the family *Dioscoreaceae*. Other most economically important species under the genus *Dioscorea* are *D. rotundata*, *D. alata*, *D. cayenensis*, *D. bulbifera*, *D. trifida*, *D. opposita*, *D. Dumentorum* and *D. japonica* (Riley et al., 2006). *Dioscorea hispida* Dennst. is a plant tuber that is also known as bitter yam (Liu et al., 2006). It is known as *ubi gadong* or toxic tuber amongst villagers in Terengganu, Malaysia. The other common names of *D. hispida* in Malaysia include *gadog*, *gadong*, *gadong lilin*, *gadung mabuk*, *gadung*, *ubi akas*, *ubi arak*, *taring pelanduk* and *sulur gadung*. This plant is a common wild plant in secondary forest, small bushes and grows in shaded areas or near streams (Nashriyah et al., 2010). It grows in the wild and is native to temperate and tropical areas of the Southeast Asia (Thailand, Myanmar, Indonesia, Papua New Guinea and Philippines) as well as China and India (Tattiyakul et al, 2010). Although this plant survives in tropical rain forest conditions with normally low elevations of less than 500 metres, it has been reported to grow at high altitudes up to 1200 metres in the Himalayas. This herbaceous twining plant can be discerned by the physical characteristics as it is a wild creeping and climbing plant (**Figure 2.1**) that can grow up to 20 metres in height with its thorny stem (Bhandari & Kawabata, 2005). It has trifoliate leaves (**Figure 2.2**) and bulky yellow or white tubers (**Figure 2.3**) that can either grow below or above the ground.

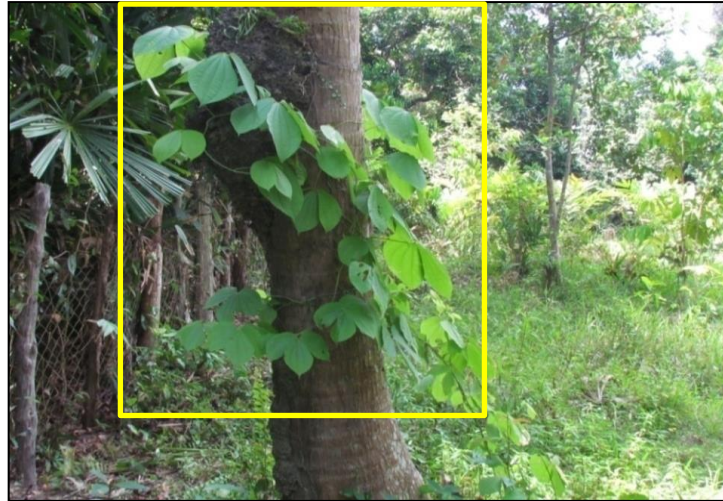


Figure 2.1 Plant of *Dioscorea hispida* (climbing plant in the yellow box)



Figure 2.2 Leaves of *Dioscorea hispida*



Figure 2.3 Tubers of *Dioscorea hispida*

****** Halved tuber is approximately 10 cm in length and 5 cm in width.

Tubers of *Dioscorea* spp. are the important foodstuffs in the tropical and subtropical regions of the world, with estimated 40 million tonnes of world production in 2003 (Bhandari & Kawabata, 2005). In Thailand, *Dioscorea* spp. is planted in the beginning of rainy season (July to August) and harvested in dry season (March to May) of the following year. In Malaysia, far from being commercialised, the *D. hispida* tuber is classified as an underutilised tuber that is only used in limited applications. Practically, the plant is not cultivated as extensively as other major carbohydrate sources but is very well known among the old Malay communities in Terengganu and Kedah for one of their traditional foods. In addition, it has long been served as a staple food for people in the rural areas in Malaysia. Like tapioca, *D. hispida* tuber is synonymous to poor villagers as their daily food as well as an important food during famine (Nashriyah et al., 2010). In Thailand, this tuber is consumed mostly after boiling, steaming and frying processes. A single *D. hispida* plant can supply up to 20 kg mature tubers which can contain up to 20 g carbohydrates per 100 g tuber on wet weight basis (Tattiyakul et al., 2006).

2.1.2 Processing of edible *Dioscorea hispida* tuber

Several studies reported that some yam species contain several toxic compounds with bitter taste and are unpalatable which can impart serious implications to health. A study by Burkill (1954) recorded that a total of 59 species from South East Asia were bitter and poisonous to be eaten raw. Scientific studies have shown that *D. hispida* is a poisonous plant as it has alkaloid dioscorine in its tuber. Alkaloids are a group of naturally occurring chemical compounds comprising mostly basic nitrogen atoms, some neutral compounds and weak acidic components (International Union of Pure and Applied Chemistry, Compendium of Chemical Terminology, 1997) by which

most of alkaloids are toxic. Several alkaloids have been isolated from *D. hispida* namely dioscorine and dihydrodioscorine. Some bitter compounds owned to this plant are saponins and sapogenins, tannins and polyphenols (Lewis, 1998). Ingestion of a large quantity of *D. hispida* tuber can result in some ill health effects such as vomiting and diarrhea if the tuber was inadequately prepared processed or eaten raw. Dioscorine can trigger fatal paralysis of the nervous system as little as 100 grams of the tuber was ingested (Swasitang et al., 2000).

Local people prepare the tubers to make them edible and non-toxic (**Table 2.1**). Detoxification is essential and many methods have been used and proposed to eliminate the toxin. Traditionally, the village elders submerge the tubers in flowing river water to remove the water soluble toxic compound prior to consumption to make it palatable. Another method is by submerging the tuber slices in a large amount of water for 5 to 7 days with daily water change before consuming the tubers. In Nepal, their local people prepare the tubers before consumption to make them palatable through different process for different species of *Dioscorea* (Bhandari & Kawabata, 2005). From all the proposed methods, the techniques have not yet scientifically proven and after traditional detoxification, the tubers are usually tested by feeding them to fish, dogs and other domestic animals. However, a protocol to automatically remove alkaloids from *D. hispida* has been successfully developed by a group of researchers from Universiti Sultan Zainal Abidin, Terengganu. It was developed based on water circling operation and using the survival of *Cyprinus carpio* fish as a detection tool for discorine removal (Hudzari et al., 2011). Kumoro et al., (2011) reported that cyanides content in the tuber can be removed by the

combination of submerging the sliced tuber in a flowing water system (flow leaching rate of $5.00 \times 10^{-5} \text{ m}^3/\text{s}$ for 1 hour) and followed by steaming at 1 hour.

Table 2.1 Processing methods of edible *Dioscorea* tubers by native people

Region	Method of processing	Authors & Claims
Australia, Papua New Guinea	Slicing followed by leaching of the sliced tubers in running water overnight	Alexander & Coursey (1969)
Malaysia	Tuber flesh is sliced and then soaked in flowing river water for 3 days or soaked in tap water for 4 days with daily water changes.	Native residents in Marang, Terengganu
Indonesia	The tubers are cut in pieces, cover with their surface covered with wood ashes for 24 hours, followed by steeping in sea water for several days, washing with fresh water, and drying. The process is repeated several times	Pambayun (2008)
	Heating and drying process	Harijono et al., (2008)

2.1.3 Future potential applications

It is expected that *D. hispida* tuber will not only be used as a poison bait for fishing and hunting but as a biopesticide too (Nashriyah et al., 2012). As man digs this tuber as food during famine, the scientists also need to discover the ethnobotanical and scientific knowledge of this toxic tuber. Apart from treating external sores of *puru* (a skin disease), it can also be further developed for cosmetics and drugs. Apparently, scientific studies have indicated that there are economic potential for *D. hispida* due to its alkaloid, saponins, sapogenins, tannins and polyphenol content. It was reported that fresh leaf and tuber of *D. hispida* at a dose of 200 mg/kg body weight exerts an anti-inflammatory effect though a reduction of 57.4% in oedema of rat paws (Murthy et al., 2011). An ethnobotanical survey in a district in India reported that eating boiled tuber could cure piles among the residents (Punithkumar et al., 2011). The focus of future research is to commercialise *D. hispida* tuber since it contains a lot of potential phytochemical compounds or phytonutrients with complex properties. A preliminary study by Napisah et al. (2011) demonstrated that this tuber possesses an anti-microbial property and Punithkumar et al. (2011) showed that the ethanolic leaf extract of this plant exhibited anticancer activity at levels from 100 to 200 mg/kg of body weight. In addition, dioscorine from *Dioscorea* species can be utilised in the pharmaceutical industry due to its analgesic effects.

Furthermore, plant saponins can cure fungal, microbial and viral infections in humans (Sodipo et al., 2000), enhance vaccine effectiveness, treat tumour cells especially lung and blood cancer, as well as can lower blood cholesterol level (Barakat et al., 1993). It is known that saponin can kill cancer cells without affecting the normal cells, by binding to the cholesterol on the cancer cell membrane followed

by interference of the cancerous cell growth and division (Ryan & Shattuck, 1994). Meanwhile, flavonoids acts as antioxidant, provides protection from allergies, inflammations, free radicals, platelet aggregation, microbes, ulcers, hepatoxins, viruses and tumours (Liu et al., 2006). The presence of phenol in *Dioscorea* species can act as anti-inflammatory, anti-clotting, antioxidant, immune enhancer and hormone modulator. Ascorbic acid which is known as a powerful antioxidant is also available in a high amount (8-10 mg/100 gm of fresh *Dioscorea* tuber) and promotes iron absorption in the intestine, protects against infection, neutralises blood toxins as well as mediates wound healing.

A diet rich in fruit and vegetables has been associated with a decreased incidence of coronary heart disease and colon cancer (Van Loo et al., 1995). Since some components of fruits and vegetables can act as substrates for bacteria residing in the human colon, diet may directly influence the composition and activity of intestinal microflora (Backhed et al., 2005). Preliminary available data suggest that the use of prebiotics may increase the resistance to intestinal and extraintestinal pathogens by modulating the immune system, which can also reduce allergic reactions. Besides, prebiotics may also improve bowel functions and have beneficial metabolic effects (Bruzze et al., 2006). Previous studies revealed several species of yam tubers possessing substantial amount of phenolic compounds (McAnuff et. al., 2005), suggesting that these tubers are a good source of dietary antioxidants. Tubers of *Dioscorea* spp., also known as yams, are important foodstuff in the tropical and subtropical regions of the world with world production in 2003 of 40 million tonnes (Bhandari & Kawabata, 2005). This cultivar is the predominant oldest tuber crop cultivated or harvested from wild in the tropical regions due to wide adaptability to

diverse agro climatic conditions throughout the world. This tuber has contributed one of the major food items for many ethnic groups in many area of habitations as a source of food due to its high starch content, calorific value and ability to cure certain ailments (Barnalli, 2015). This subterranean tuber can produce industrial starch and edible flour (Susmita & Shukla, 2014), thus, it can be assumed that it can consequently be processed to produce oligosaccharides and resistant starch. A previous study on *Dioscorea alata* starch reported that the starch was minimally digested and absorbed at a slow rate, therefore slowly releasing the products of digestion as they passed through digestive tract (Riley et al., 2006).

2.2 Starch

Plants utilise energy from sunlight and carbon dioxide from atmosphere to produce their own food, glucose, through photosynthesis process. The excess glucose is stored in the form starch, a polysaccharide of the six-carbon sugar, D-glucose, which are linked together by α -linkages regardless of the botanical sources. Starch is structurally composed of amylose and amylopectin (**Figure 2.4**). Amylose is a linear polysaccharide chain and all glucose residues are linked together by α -D-(1-4) linkages. Amylopectin does not only contains α -D-(1-4) linkages, but also comprises of α -D-(1-6) linkages, making it a highly branched molecule (Tester et al., 2004). Starch occurs in a granular form consisting of amorphous region and crystalline region where the amylose is usually located in loose pack of amorphous region while amylopectin chains are arranged in precise double helices in the crystalline region (Zhang et al., 2014).

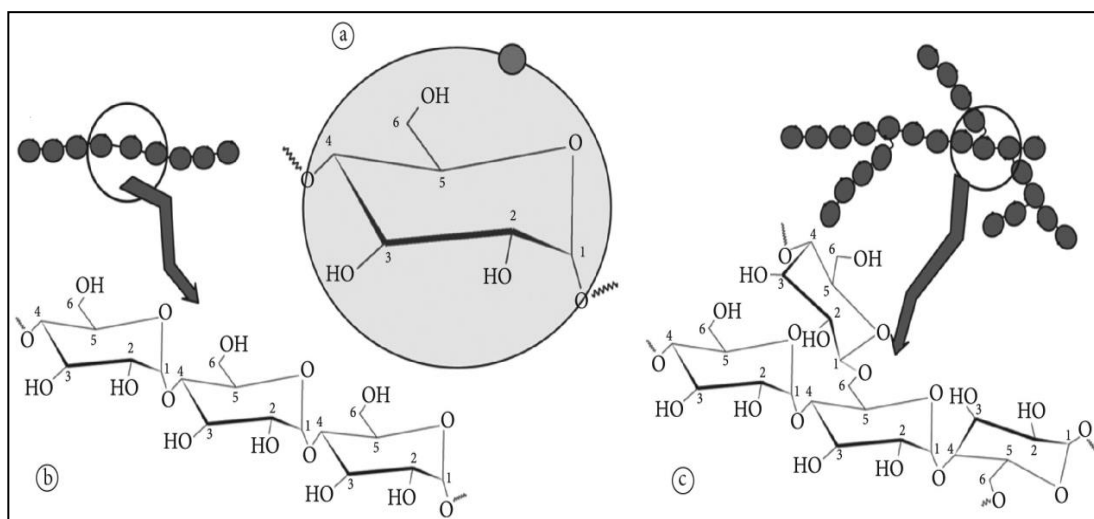


Figure 2.4 α -(1-4) and α -(1-6) glycosidic bonds of starch.

(a) Glucose unit; (b) Amylose structure; (c) amylopectin structure.
(Adapted from Pérez & Bertoft, 2010).

2.2.1 Starch Digestion in Human

Starch is the main energy intake of human daily life. The starch digestion process starts in the mouth once the starchy foods are digested. The saliva enzyme known as salivary α -amylase is responsible for hydrolysing starch into disaccharides (Lunn & Buttriss, 2007). Salivary α -amylase digests starch efficiently; however, since starch remains in the mouth for a short period of time, starch hydrolysis occurs for a lesser time than in the gut. In addition, salivary α -amylase is inactivated by the acidic condition in the stomach when starch passes down the esophagus and reaches the stomach (Singh et al., 2010). Gastric juice has a pH range of 2-4 making an extreme acidic condition for starch hydrolysis in the human stomach (Wichienchot et al., 2010). Pepsin is activated by this acidic condition to digest protein (Perera et al., 2010). Although starch cannot be hydrolysed by pepsin, strong acid namely hydrochloric acid from gastric juice can hydrolyse the 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 comprises of sodium bicarbonate and digestive enzymes. Sodium bicarbonate neutralises the acidic starchy food so that pancreatic α -amylase can further hydrolyse the starchy food in the small intestine of human. This enzyme specifically hydrolyses α -1,4 linkages of starch polymers producing mainly maltose, maltotriose and maltotetraose from amylose chains as well as dextrans or branched oligosaccharides from amylopectin chains (Singh et al., 2010). The saccharides are further hydrolysed to glucose by brush border enzymes since only glucose can be absorbed through the small intestine into human body. Starch that has escaped from digestion by human enzymes in the small intestine will pass into the colon. In the colon, bacterial enzymes favour the degradation of nondigestible carbohydrate through a process called fermentation (Perera et al., 2010). Human body does not produce digestive enzymes in the colon (Boisen & Eggum, 1991). The bacterial fermentation is further discussed in **Section 2.5**.

2.2.2 Enzymatic Hydrolysis of Starch

Starch has become a very important biopolymer used in many industries. Expanding starch functionality can be achieved through chemical or enzymatic modifications. The most important enzymatic starch processings are the production of cyclodextrins and a mixture of simpler carbohydrates, which possess different compositions and properties. These mainly contribute to the texture, colour stability and flavour of the final product, while also remaining economical. The main starch hydrolysis products are maltodextrins, high maltose syrups, maltose, glucose syrups and dextrose, which are used in food and pharmaceutical industries (**Table 2.2**).

Table 2.2 Types of nutritive sweetener

Nutritive sweeteners	DE*	Class
Maltodextrins	20	Maltooligosaccharides
High maltose syrup	20-50	Maltose, glucose, maltooligosaccharides
Maltose	53	Disaccharide
Glucose syrup	20-95	Glucose, maltose, maltooligosaccharides
High fructose syrup	-	Fructose, glucose
Dextrose	100	Glucose

*DE, Dextrose Equivalent. DE is a measure of the amount of reducing sugars present in a sugar product, expressed as a percentage on a dry basis relative to dextrose.

Starch hydrolysis involves three steps that are gelatinization, liquefaction and saccharification (Baks et al., 2008).

2.2.2(a) Gelatinisation

Gelatinisation involves the melting of starch granules in an aqueous medium under heating. In water, granule swelling increases with temperature and leads to the transfer of water in the suspension to water associated with starch components known as amylose and amylopectin. Once the starch is gelatinised, insoluble granules are disrupted, resulting in a loss of molecular organisation, thus causing the semi-crystalline nature of granules to become totally amorphous and the starch to be easily penetrated by several enzymes especially amylases (Tester et al., 2004). This process leads to an increase of viscosity and starch solubilisation as a result of the loss of granular structure and semi-crystalline structure. This process of

gelatinisation is also marked by the loss of birefringence (Douzals et al., 1996). The gelatinisation process involves several stages of granule expansion when progressively heated in excess liquid. First, it is progressively hydrated and then the double helices are broken due to hydrogen bonds rupture. After that, the crystalline regions are converted into amorphous regions where at this moment, the granules continue to absorb water and swell. When the granules are excessively swollen, the granular form will be lost and result in gelation and/or solubilisation.

2.2.2(b) Liquefaction

Liquefaction is a process of dextrinisation of gelatinised starch. During liquefaction, the slurry of reaction mixture is reduced and the gelatinised starch is partially hydrolysed, forming a product with a dextrose equivalent that varies between 15 and 30 (Baks et al., 2008).

2.2.2(c) Saccharification

When saccharification takes place, the partially hydrolysed starch chains are broken down into several molecules namely glucose, maltose, maltotriose and some higher oligomers (oligosaccharides). The dextrose equivalent varies between 40 and 98 according to the enzymes applied (Baks et al., 2008).

2.2.3 Starch degrading enzymes

2.2.3(a) α -amylase

α -amylase (1,4- α -D-glucocanohydrolase, E.C.3.2.1.1) is often used for the enzymatic hydrolysis of starch. α -amylase catalyses the cleavage of α -1,4-glycosidic bonds in the inner region of the molecule, hence causing a rapid decrease in substrate

molecular weight and viscosity. The activity and stability of α -amylase are affected by temperature, pressure, pH, substrate concentration and additives (Fitter et al., 2001). The enzymatic starch hydrolysis with α -amylase produces a mixture of branched α -dextrins, short linear oligosaccharides, maltose and glucose (Maarelet al., 2002). Branched dextrins can be further cleaved to short linear oligosaccharides by isoamylases and pullulanases. Besides, α -amylases are used in several industrial processes that take place under diverse physical and chemical conditions. Thus, for each individual application, the enzyme which best meets the particular demands of the process is desirable. High thermostability is sometimes desired since elevated temperatures improve starch gelatinisation, decrease media viscosity, accelerate catalytic reactions and decrease the risks of bacterial contamination. An additional benefit of high-temperature catalysis is the inactivation of enzymes originated from food materials that give rise to undesirable reactions during processing.

The most thermostable α -amylase currently used in biotechnological processes is produced by *Bacillus licheniformis*. It remains active for several hours at temperatures over 90°C under conditions of industrial starch hydrolysis (Synowiecki, 2007). Together with other starch-degrading enzymes (pullulanases), α -amylase are included in the family of glycosyl hydrolases (Henrissat & Bairoch, 1996) characterised by a (A/B)₈-barrel conformation. The structural and functional aspects of α -amylases have been reviewed by Nielsen & Borchert (2000) as well as MacGregor et al., (2001). The enzyme contains a characteristic substrate binding cleft that can accommodate between four to ten glucose units of the substrate molecule. According to Synowiecki (2007), each binding site has an affinity to only one glucose unit of the carbohydrate chain. However, the interaction of

oligosaccharides with several binding sites create a multipoint linkage resulting in the correct arrangement of long substrate molecules towards the catalytic site. Differences in the number of substrate binding sites and location of catalytic regions determine substrate specificity, length of oligosaccharide fragments released after hydrolysis and carbohydrate profile of the final product. Substrate binding is not sufficient for catalysis when all the glucose residues of the engaged oligosaccharides chain fall outside the catalytic region. The end products of α -amylase action are oligosaccharides with varying lengths with an alpha-configuration and alpha-limit dextrins, constituted of branched oligosaccharides (Maarel et al., 2002). The addition of pullulanase imitates the branched fragmentation during the hydrolysis process. Pullulanase is the debranching starch converting-enzyme that specifically hydrolyses an α -1-6 chain of polysaccharides (Israilides et al., 1999). This enzyme degrades amylopectin and results in long linear polysaccharides. The main degradation products are maltose and maltotriose. Increasing the substrate concentration during enzymatic hydrolysis will yield a higher glucose amount.

2.2.3(b) Amyloglucosidase

Amyloglucosidase (AMG) is a carbohydrase (EC 3.2.1.3, glucan 1,4- α -glucosidase), capable of hydrolysing (1,4)- α -D-glucosidic and (1,6)- α -D-glucosidic linkages at the non-reducing ends of glucose polymers (Kumar et al., 2012). The enzymatic action yields glucose mainly from amylose and amylopectin. AMG activity is often used in research to measure the degree of starch gelatinisation and starch accessibility (Guerrieri et al., 1997). AMG is also used as a processing aid in many industries; brewing, alcoholic beverages and pastry-making (Kumar et al., 2012). However, the industrial use of amyloglucosidase is impaired by the limited glucose yields due to

slow hydrolysis of α -(1,6)-glucosidic bonds in starch. The optimum conditions for AMG activity have been widely studied and are generally in the range of 40–60°C with pH from 4.5–5.0 (Kumar et al., 2012). Little has been reported on AMG activity at low temperatures though studies have shown that some enzymes are active at these low temperatures. Various microorganisms have been reported for the extracellular production of amyloglucosidase (Pamboukian et al., 1999).

Most frequently used fermentation techniques for the biosynthesis of amyloglucosidase enzyme are solid state and submerged fermentations (Imran et al., 2012), that have been assumed to act predominantly on the products of α -amylase digestion by rapidly converting them to glucose, thus removing the inhibitory effects of maltose on amylase activity during long digestion (although it should be remembered that maltose is not a very potent inhibitor of α -amylase) (Warren et al., 2012). Amyloglucosidase is also capable of hydrolysing α -(1-6) linkages that cannot be attacked by α -amylase, removing limit dextrins and allowing the completion of starch digestion, as is the case *in vivo* where brush border enzymes undertake the same function (Diaz-Sotomayor et al., 2013).

2.2.3(c) Pullulanase

Pullulanase (EC 3.2.1.41) is known by various names such as α -dextrin 6-glucanohydrolase, pullulan-6-glucanohydrolase and amylopectin 6-glucanohydrolase. It can be widely found in bacteria, yeasts, fungi, plants and animals. The common bacterial pullulanase producers are *Bacillus acidopullulyticus*, *Klebsiella planticola* (Kunamneni & Singh, 2006), *Bacillus deramificans* (Nair et al., 2010), thermophilic *Bacillus* sp. AN-7 (Zareian et al., 2010), *Bacillus cereus* FDA-

13 (Rudiger et al., 1995) and *Geobacillus stearothermophilus* (Kim et al., 1996). Microbial pullulanase attracts more interest due to its specific action on α -1, 6-linkages in pullulan, a linear α -glucan consisting maltotriosyl units connected by 1,6- α -bonds. In general, this enzyme can hydrolyse the α -1,6-D-glucosidic linkages in pullulan and amylopectin. Furthermore, it improves the saccharification of starch to produce glucose, maltose and maltooligosaccharides using glucoamylase, β -amylase or α -amylase.

The use of pullulanase is essential for an efficient starch processing in food industries. The efficiency of conversion of starch and glycogen is increased by adding pullulanase in the medium containing amyloglucosidase. The glucose produced by hydrolysis can be used for producing high fructose syrup or as a feedstock for microbial fermentation. Besides, pullulanase is used for the determination of glycogen structure and mechanism of actions of enzymes that synthesise glycogen (Kuroiwa et al., 2005). Pullulanases are classified into groups based on the substrate specificities and the end reaction products. Pullulanase Type I is also known as true pullulanase that is able to proficiently hydrolyse the α -1,6-glycosidic bonds in pullulan and branched polysaccharides (Roy et al., 2003), releasing panose and isopanose and is mostly active on cyclodextrins (Leemhuis, 2003). In contrast, it does not hydrolyse either α -1,4-linkages in glucans and amylose or α -1,6-linkages in glycogen and panose. Pullulanase Type II, also called amylopullulanases, is prominent in starch processing industry due to its specific debranching capacity of hydrolysing either α -1,6 or α -1,4-glycosidic linkages. This enzyme debranches pullulan and gives maltotriose as final product as well as attacks α -(1,4) bonds in starch, amylose and amylopectin (Hobel, 2004).

Both pullulanase Type I and Type II attack α -1,6-glucosidic linkages in pullulan, producing maltotriose while these enzymes are unable to degrade cyclodextrin (Matzke et al., 2000). Theoretically, pullulanase Type I and pullulanase Type II have no action on starch because they have their specific binding site where these enzymes can precisely recognise the structural differences between α -1,4 and α -1,6 glucosidic linkages. Thus, they are rather widely applied in the structural analysis of oligo- and polysaccharides (Niehaus, 1999). Meanwhile, pullulanase Type III has the ability to attack α -(1,6) as well as α -(1,4)-glucosidic linkages in pullulan leading to the formation of a mixture of maltotriose, panose, maltose and glucose (Niehaus et al., 1999). Besides, pullulanase Type III is also able to degrade starch, amylose and amylopectin, forming maltotriose and maltose as the main products.

2.3 Gastrointestinal Microflora

Human gastrointestinal tract as displayed in **Figure 2.5** comprises different section that varies in number of bacteria colonised from 10^3 CFU/g until 10^{11} CFU/g in the stomach, small intestine and large intestine (colon) (Sanders et al., 2007). There are more than 400 species of bacteria constituting the intestinal microflora, but only 40 species have been found to be the major colonies (O'Grady & Gibson, 2005). The bacteria that inhabit the stomach, the first part of small intestine and duodenum are lesser than those inhabiting in the colon. This is due to the presence of strong acidic condition in the stomach that makes it less a favourable condition for bacterial colonisation (Sanders et al., 2007). Most of bacteria reside in this colon due to the desirable conditions including longer transit time, near to neutral pH and adequate nutrient source (O'Grady & Gibson 2005). Hence, most of the bacterial metabolic activities, that could exert significant influences on host health, occur in the colon

compared with that in the small intestine. Most of colonic microflora are strict anaerobe that predominantly include bacteroides, bifidobacteria, eubacteria, clostridia, peptostreptococci, peptococci and ruminocci (Salminen et al., 1998). Nevertheless, the most significant bacteria from the list are bacteroides and bifidobacteria, which composed of 30% and 25% of the total anaerobic counts, respectively (Salminen et al., 1998).

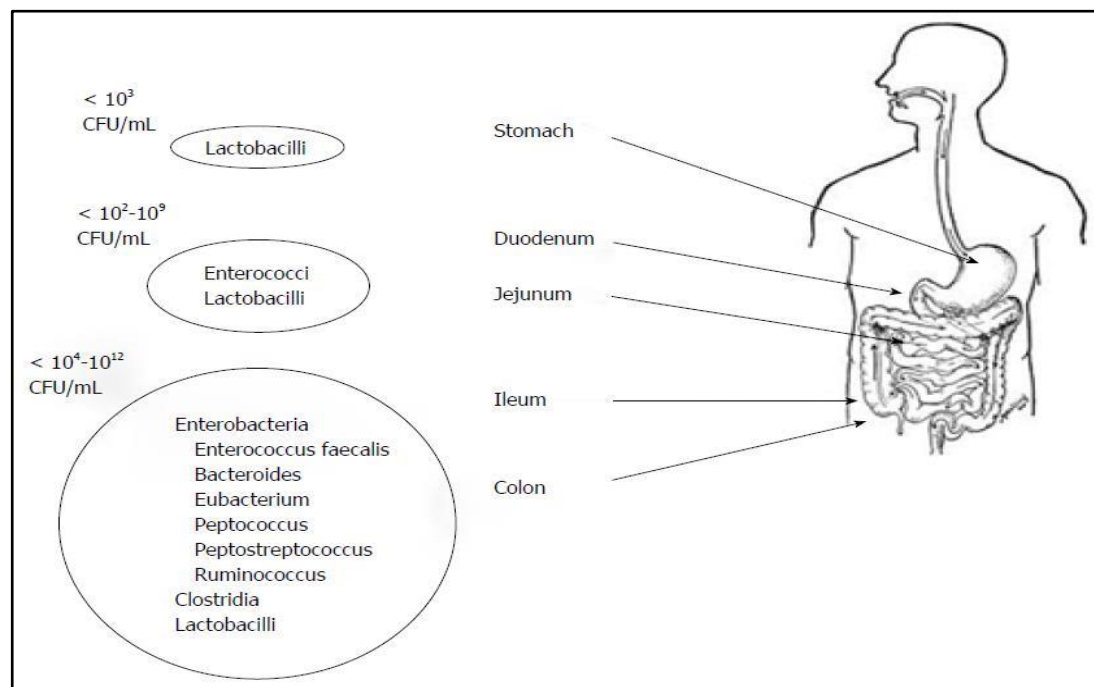


Figure 2.5 Bacterial composition and density along the proximal to distal segments of the gastrointestinal tract. (Adapted from Russo et al., 2014)

Nevertheless, the most significant bacteria from the list are bacteroides and bifidobacteria, which made up 30% and 25% of the total anaerobic counts, respectively (Salminen et al., 1998). The number of anaerobic bacteria is larger than that of aerobic bacteria by a factor of 100 to 1. These include lactobacilli, enterococci, streptococci and enterobacteriaceae (Russo et al, 2014). Colonic microflora derives energy for growth from the fermentation of dietary components and endogenous mucins (Hughes et al., 2000). Components of dietary origin include

non-digestible carbohydrates such as resistant starch, non-starch polysaccharides, oligosaccharides and sugar alcohols as well as undigested proteins that passage into the colon (Cummings & Englyst, 1991). Fermentation of carbohydrates categorised as saccharolytic fermentation produces acetic, propionic and butyric acid as the main short chain fatty acids as well as gases such as CO₂, CH₄ and H₂ (Bernalier-Donadille, 2010). These short chain fatty acids can exert several beneficial influences on host health. In contrast to carbohydrate fermentation, protein fermentation (proteolytic fermentation) produces metabolites that are potentially harmful to the host such as ammonia, amines and phenolic compounds (Bernalier-Donadille, 2010). Short chain fatty acids and branched chain fatty acids are also the end products of proteolytic fermentation (Bernalier-Donadille, 2010). Some of the bowel diseases include colorectal cancer and ulcerative colitis, which are probably linked to the excessive protein fermentation in the colon (Windey et al., 2012).

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 such as bacteroides and clostridia can perform both saccharolytic and proteolytic fermentations. Colonic microflora can be divided into beneficial or detrimental bacteria. Their influence on host health is based on their metabolic activities and fermentation end products (Gibson et al., 2010). Bacteria with saccharolytic fermentation are beneficial, whereas those having a proteolytic or both types of fermentation are either less beneficial or even detrimental (Gibson et al., 2010). Health promoting effects include impeding the growth of detrimental bacteria, improving the digestion and absorption of essential nutrients, synthesising vitamins and stimulating the immune functions,