



UNIVERSITI PUTRA MALAYSIA

***PREBIOTIC POTENTIAL OF SUGARCANE (*Saccharum Officinarum*
L.) AGRO INDUSTRIAL BY-PRODUCTS ON THE GROWTH OF
PROBIOTICS***

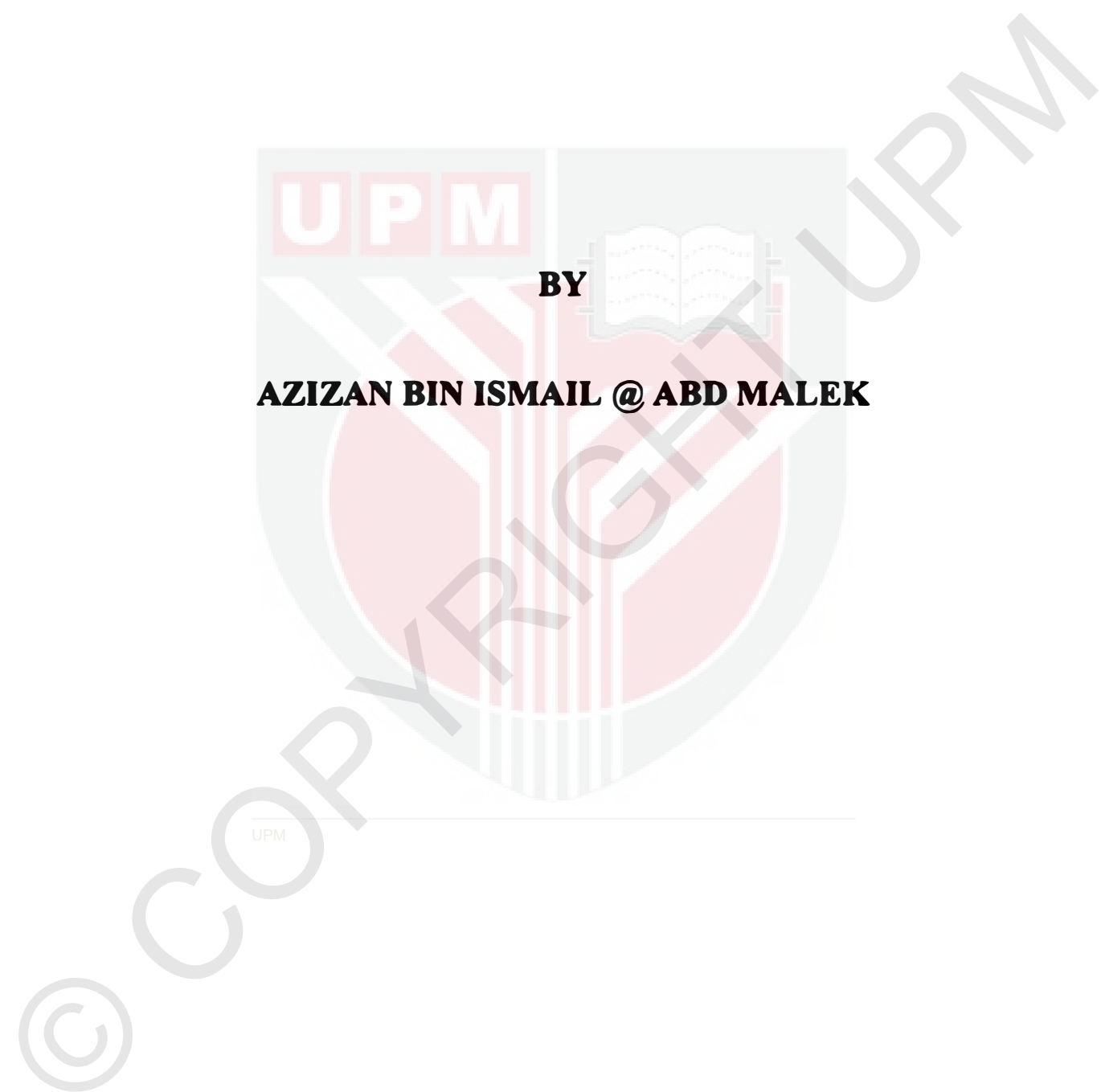
AZIZAN BIN ISMAIL @ ABD MALEK

**Ip
FPSK3 2018 30**

**PREBIOTIC POTENTIAL OF SUGARCANE (*Saccharum Officinarum L.*)
AGRO INDUSTRIAL BY-PRODUCTS ON THE GROWTH OF
PROBIOTICS**

BY

AZIZAN BIN ISMAIL @ ABD MALEK



**A project submitted as a partial fulfillment of the requirement for the degree of
Bachelor of Science (Nutrition and Community Health) from the Faculty of
Medicine and Health Sciences, Universiti Putra Malaysia**

ACKNOWLEDGEMENT

First and foremost, praises and thanks to Allah, the Almighty, for His showers of blessings throughout my research work to complete this project. This project would not have been possible done without the support of many people. I wish to express my deepest gratitude to my supervisor, Dr. Mohd Redzwan Sabran who was abundantly helpful and offered invaluable assistance, support and guidance. While in the process of completing this project, a lot of guidance and advises have been given from a number of lab instructors and demonstrators to me. They opened wide my eyes to the world of microorganisms and to understand the importance of this field. Not forgetting for my friends who always been there, especially final year student from Nutrition and Community Health course for sharing the literature and invaluable assistance. Finally, an honorable mention goes to my parents, Ismail @ Abd Malek Bin Mohd Noor and Sahrah Binti Sarthi; and all my family members for their understandings and support on me in completing this project. Your prayer for me was what sustained me thus far. Without helps of the particular that mentioned above, I would face many difficulties while doing this. Thank you very much and to those who are willing to spend their precious time to read this thesis, hopefully you will find it interesting and knowledgeable.

TABLE OF CONTENTS

	Page
TITLE	i
SIGNATURE OF SUPEVISOR	ii
ACKNOWLEDGEMENT	iii
CONTENTS	iv
LIST OF TABLES	vii
LIST OF FIGURES	ix
ABSTRACT	x
ABSTRAK	xi
CHAPTER 1 INTRODUCTION	
1.1 Research Background	1
1.2 Problem Statement	3
1.3 Significant of Research	5
1.4 Objective	6
1.4.1 General Objective	6
1.4.2 Specific Objectives	6
1.5 Hypothesis	7
CHAPTER 2 LITERATURE REVIEW	
2.1 Sugarcane	8
2.1.1 Bagasse	9
2.2 Nutritional Status of Sugarcane Bagasse	11

2.3 Prebiotic	12
2.3.1 Oligosaccharide as Prebiotic	12
2.4 Probiotic	17
2.4.1 Lactic Acid Bacteria (LAB)	19
2.5 Health Benefits of Probiotic and Prebiotic	20
2.5.1 Health Benefits of Prebiotic	21
2.5.2 Health Benefit of Probiotic	22
2.6 Prebiotic Properties of Agro Industrial By-Products	23
2.6.1 Probiotic Growth of Agro Industrial By-Products	24
2.6.2 Acidification Production of Probiotic on Agro Industrial By-Products	25
2.6.3 Prebiotic Potential: Prebiotic Activity Score on Agro Industrial By-Products	27
 CHAPTER 3 METHODOLOGY	
3.1 Substrate and Chemical	29
3.2 Propagation of Culture	29
3.3 Instruments	30
3.4 Sample Collection and Preparation	31
3.5 Experimental Design	31
3.6 Assessment of Bacterial Growth Performance	33
3.6.1 Enumeration of Probiotic Culture	34
3.6.2 Determination of Final pH of Prebiotic Samples	34
3.6.3 Bacterial Growth Parameter	35
3.7 Assessment of Prebiotic Potential: Prebiotic Activity Score	35

3.8 Statistical Analysis	36
CHAPTER 4 RESULTS AND DISCUSSION	
4.1 <i>Lactobacillus casei</i> Strain Shirota (LcS) Growth Curve	37
4.2 LcS Growth Parameters	41
4.2.1.1 The Enumeration of LcS in Log CFU/ml	42
4.2.1.2 The Final pH of Prebiotic Samples	46
4.2.1.3 The Mean Growth Rate (k) and Mean Duplication Time (g) of LcS	50
4.2.2 Prebiotic Potential: Prebiotic Activity Scores of LcS	55
CHAPTER 5 CONCLUSION, LIMITATION AND RECOMMENDATIONS	
5.1 Conclusion	59
5.2 Limitations	60
5.3 Recommendations	60
REFERENCES	62

LIST OF TABLES

TITLE	PAGE
Table 2.1 Proximate properties of sugarcane bagasse	11
Table 2.2 Prebiotic carbohydrates	14
Table 2.3 Inulin and Fructooligosaccharide content of plants commonly used in human nutrition (% of fresh weight)	15
Table 2.4 Commercial probiotic microorganisms	18
Table 2.5 Beneficial effects of prebiotic	21
Table 2.6 Probiotics and their reported effects on health	22
Table 4.1 Final fermentation (24-h) of CFU/mL counts (<i>N</i>) for the LcS employed with four different carbon sources by using One-Way ANOVA	43
Table 4.2 Final fermentation (24-h) of CFU/mL counts (<i>N</i>) for the LcS and other bacteria employed with different carbon sources in comparison with other study	45
Table 4.3 Final pH of modified prebiotic samples at the end of fermentation (24 h) for the LcS employed with the different carbon sources by using One-Way ANOVA	47
Table 4.4 Final pH (24-h) of prebiotic samples for the LcS and other bacteria employed with different carbon sources in comparison with previous study	49
Table 4.5 Fermentation kinetics, <i>k</i> (mean growth rate constant) for LcS strains employed with the different carbon	52

sources by using One-Way ANOVA

Table 4.6	Mean generation time, g (mean duplication time) for LcS strains employed with the different carbon sources by using One-Way ANOVA	52
Table 4.7	The fermentation kinetics, k (growth rate constant) and mean generation time, g (mean duplication time) LcS and other bacteria employed with different carbon sources in comparison with previous study	54
Table 4.8	Prebiotic activity score for the LcS strain employed with the different carbon sources by using One-Way ANOVA	57
Table 4.9	Prebiotic activity scores of LcS and other bacteria employed with different carbon sources in comparison with previous study	58

LIST OF FIGURES

TITLE	PAGE
Figure 2.1 Sugarcane plants with their parts	10
Figure 2.2 Chemical structures of sucrose (left), GFn-type inulin (center) and Fm-type fructo-oligosaccharides (right) G: glucose; F: fructose	16
Figure 3.1 Experimental design of prebiotic potential of sugarcane bagasse and peel	32
Figure 4.1 LcS growth curve	38
Figure 4.2 Standard growth curve of bacteria	39

ABSTRACT

PREBIOTIC POTENTIAL OF SUGARCANE (*Saccharum Officinarum L.*) AGRO INDUSTRIAL BY-PRODUCTS ON THE GROWTH OF PROBIOTICS

Azizan Bin Ismail @ Abd Malek

Sugarcane (*Saccharum Officinarum L.*) is a widespread tropical plant valued for its nutritional and sensory characteristics. Around 70% of its raw materials weight such as bagasse and peel are considered as agro-industrial by-products with high nutritional value. This study aims to assess the prebiotic potential of sugarcane agro-industrial by-products on the growth of probiotic *Lactobacillus casei* strain Shirota (LcS) using an *in vitro* experimental model. Two parameters were assessed; the growth performance of LcS (log CFU/mL, final pH, mean growth rate and mean duplication time) and the prebiotic activity score. This experiment used sugarcane bagasse and peel as samples. These samples were freeze-dried, homogenized and the total sugar content was determined by using acid hydrolysis method. Then, the customized culture media was prepared by substituting glucose with sugarcane bagasse (MRSb) and peel powder (MRSp) in the same formulation (20 g/L) of commercial MRS broth. Commercial MRS (cMRS) broth was used as a positive control. After 48 hours of fermentation, the LcS growth was significantly difference ($p < 0.023$) between the media. Further analysis indicated that the probiotic's growth in MRSp (8.60 ± 0.35 log CFU/mL) was significantly different than in the MRS broth (9.31 ± 0.28 log CFU/mL). The media's pH was reduced following the fermentation, but the final pH was not significantly different between MRSb (5.71 ± 0.03), MRSp (5.53 ± 0.70) and MRS (5.69 ± 0.11), with an exception of cMRS (4.44 ± 0.07). There was no significant difference on the mean growth rate ($p > 0.835$) and the mean duplication time ($p > 0.469$) of LcS, indicated that the probiotic growth was not affected by the source of glucose in the media. Besides, the prebiotic activity scores of MRSb (0.23 ± 0.01) and MRSp (0.18 ± 0.01) are comparable with other agro-industrial by-products reported in literature. From these findings, the sugarcane by-products are important functional ingredients for food industry due to their potential prebiotic and fermentable properties.

ABSTRAK

POTENSI PREBIOTIK TEBU (*Saccharum Officinarum L.*) BAHAN BUANGAN INDUSTRI PERTANIAN KEATAS TUMBESARAN PROBIOTIK

Azizan Bin Ismail @ Abd Malek

Tebu (*Saccharum Officinarum L.*) adalah sejenis pokok tropika bernilai dengan nutrisi dan ciri-ciri derianya. Sebanyak 70% berat pokok ini terdiri daripada hampas dan kulit dikategorikan sebagai bahan buangan industri pertanian yang mempunyai nilai nutrisi yang tinggi. Kajian ini bertujuan untuk mengkaji potensi prebiotik tebu, bahan buangan industri pertanian bagi tumbesaran probiotik *Lactobacillus casei* Strain Shirota (LcS) dengan menggunakan model eksperimen *in vitro*. Terdapat dua parameter yang telah dikaji; prestasi tumbesaran LcS (Log CFU/ml, pH akhir, purata kadar tumbesaran dan purata masa pergandaan) dan juga kiraan aktiviti prebiotik. Kajian ini menggunakan hampas dan kulit tebu sebagai sampel. Kedua-dua sampel ini telah menjalani pengeringan sejuk beku, melalui proses homogenisasi dan jumlah kandungan gula telah dikenalpasti dengan menggunakan kaedah hidrolisis asid. Selepas itu, media kultur yang telah disesuaikan disediakan dengan menggantikan glukosa dengan serbuk hampas (MRSb) dan kulit (MRSp) tebu dengan formulasi yang sama (20g/L) dengan media MRS komersial. Media MRS komersial (cMRS) digunakan sebagai kawalan positif. Selepas 48 jam proses fermentasi, terdapat perbezaan ketara pada tumbesaran LcS ($p < 0.023$) diantara media. Analisis seterusnya mendapati terdapat perbezaan ketara pada tumbesaran probiotik didalam MRSp (8.60 ± 0.35 log CFU/mL) berbanding didalam media MRS (9.31 ± 0.28 log CFU/mL). Nilai pH media mengalami penurunan semasa proses fermentasi, tetapi nilai pH akhir tidak terdapat perbezaan ketara diantara MRSb (5.71 ± 0.03), MRSp (5.53 ± 0.70) dan MRS (5.69 ± 0.11), dengan pengecualian cMRS (4.44 ± 0.07). Terdapat perbezaan yang tidak ketara pada purata tumbesaran probiotik ($p > 0.835$) dan juga purata masa pergandaan ($p > 0.469$) LcS, menunjukkan tumbesaran probiotik tidak terkesan daripada sumber glukosa di dalam media. Selain itu, kiraan aktiviti prebiotik MRSb (0.23 ± 0.01) dan MRSp (0.18 ± 0.01) adalah sebanding dengan bahan buangan industri pertanian yang lain berdasarkan rekod daripada kajian-kajian sebelumnya. Daripada keputusan kajian ini, bahan buangan tebu adalah penting sebagai bahan berfungsi untuk industri makanan disebabkan potensi prebiotik dan ciri-ciri fermentasi bahan tersebut.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Sugarcane (*Saccharum Officinarum L.*) is a widespread tropical plant valued for its nutritional and sensory characteristics. The plantation and production of sugarcane crops are highly concentrated in South America and Asia. The country with the largest area devoted to sugar and ethanol production from sugarcane is Brazil. In fact, Brazil dominates the world sugarcane production. In addition, more than 90% of sugarcane are produced by the top 20 producing countries, are concentrated in tropical and subtropical areas. For example, 53.1% of global output is produced by Central and South America countries, with an additional 36.9% from Asia and 5.3% from Africa countries. The remainder of sugar production from sugarcane is distributed with 2% or less from Caribbean region, Oceania (including Australia), North America and Europe (0.0003%) (Kaffka & Grantz, 2014).

The by-products that are generated from agro-industrial processing of sugarcane comprise mainly bagasse and peel. Sugarcane bagasse is the sugarcane stalk residues after crushing and juice extraction for sugar and alcohol industries (Leang & Saw, 2011). Some studies have investigated these agro-industrial by-products as source of bioactive phytochemicals as well as source of antioxidant (Sancho et al., 2015). Prebiotic component such as fructooligosaccharides, inulin and raffinose are naturally present in many plants. Based on the International Scientific Association for Probiotics and Prebiotics (ISAPP), Hill et al. (2014) concluded that the current ISAPP consensus panel now proposes the following definition of prebiotic: “a substrate that is selectively utilized by host microorganisms conferring a health benefit.” In these recent years, the search for new sources of prebiotic ingredients has been the subject of many studies. Plants, fruits and their agro-industrial by-products are considered matrices with potential prebiotic activities because of their high contents of particular fibres and carbohydrates (Leang & Saw, 2011). However, investigations on the prebiotic activities of sugarcane processing by-products remain scarce.

Prebiotic components promote the selective growth of beneficial probiotic bacteria in the colon with the production of different organic acids. They also exert a number of health effects in the host such as relief of constipation, improvement of minerals and other nutrients absorption, reducing the incidence of colon cancer and strengthening of immune system (Tuohy, Probert, Smejkal & Gibson, 2003). Besides, there are three main organic acids produced by the probiotic bacteria namely acetic acid, propionic acid and butyric acid. Organic acids production by the probiotic bacteria will benefit the colon includes the inhibition of pathogenic bacteria

(Gibson, Probert, Van Loo & Roberfroid, 2004). Moreover, Liu et al., (2016) has stated that *Lactobacillus* species are the most abundant among probiotic, and they have been intensively studied during the past decades for their probiotic properties. Same with other probiotics, *Lactobacillus* ferment selectively prebiotic ingredients and allow specific changes in the composition and activity of the intestinal microbiota (Huang et al., 2015).

1.2 Problem Statement

Non-digestible oligosaccharides and polysaccharides usually exhibited prebiotic properties and fructooligosaccharides, galactooligosaccharides, inulin, resistant starch and lactulose are examples non-digestible oligosaccharides derived from various source of fruits and vegetables (Thammarutwasik et al., 2009). In fact, fructooligosaccharides, galactooligosaccharides and inulin are established prebiotic and they are available commercially. Combining prebiotic and probiotic into single products produces synbiotic (Donkor et al., 2007; Sah et al., 2016). Many industries and scientific community are seen to explore and create new prebiotic ingredients with added functionalities. For examples, fibre-rich fraction from fruits, vegetables and cereals residue have commercial importance, sustainability and health benefits. Some studies have sought to enhance the growth of probiotic bacteria by fortifying the fibre-rich fractions from herbs, cereals, banana, passion, or apple processing by-products (Espírito Santo et al., 2012).

Sugarcane bagasse consist approximately 50% cellulose and 25% each of hemicelluloses and lignin. It also contains about 50% α -cellulose, 30% pentosans,

and 2.4% ash. Bagasse promotes numerous advantages in comparison to other crop residues for usage in bioconversion processes using microbial cultures. In many years, a large amount of micro-organisms including bacteria, yeast, and fungi have been used for cultivation on bagasse. But, filamentous fungi, especially basidiomycetes are the preferred choice for enzyme production and protein enrichment. Besides, it also has most widely been employed in industries (Pandey, Soccol & Nigam, 2000). So there are lacking in studies involving probiotic bacteria especially *Lactobacillus* species on sugar cane bagasse that can contribute benefits for the industries, especially on the human's health.

Besides, given the constant and increasing demand for crops for human consumption, animal feed and bio fuels, the search for functional ingredients for processed foods has led researchers to explore agro industrial by-products as a new source of prebiotics. In fruits and plant processing, around 70 % of the raw material weight, such as peel and seeds, are considered waste. The correct disposal of these materials usually represents an environmental problem and is only partially resolved by using them as animal feed or composing for fertilizer. Agamuthu, Khidzir and Hamid (2009) stated that different countries apply various classifications for industrial waste which contribute adversely to water, soil and air quality. For example, 998 million tonnes of agricultural waste is produced per year globally and in Malaysia, 1.2 million tonnes of agricultural waste is disposed into landfills annually. It is estimated that 15% of the total waste generated in Asia is agro waste, with agricultural waste generation in Malaysia accounted approximately 0.122 (kg/cap/day) in 2009 and is projected to reach 0.210(kg/cap/day) by 2025 (Agamuthu, Khidzir & Hamid, 2009).

The use of agro industrial by-products with high nutritional value as new food additives or supplement has gained increasing interest because they are high-value products and their recovery may be economically attractive (Oreopoulou & Tzia, 2007; Lamsal & Faubion, 2009). In this context, considering those aspects, the aim of this study is to determine prebiotic potential of sugarcane bagasse and its peel as agro-industrial by-product on the growth of selected *Lactobacillus* species.

1.3 Significant of Research

To my best knowledge, there is lacking published study on prebiotic potential of sugarcane (*Saccharum Officinarum L.*) agro-industrial by-product on selected *Lactobacillus* species. Pandey et al. (2000) stated most of fungi are preferred choice for enzyme production and protein enrichment on sugarcane by-products. Besides, sugarcane bagasse and peel are normally used for fertilizer and cattle feed. However, the large amount of dietary fibre in sugarcane bagasse and peel make them ideal candidates for dietary fibre in health food (Sangnark & Noomhorm, 2003). More information on characteristics of prebiotic activity on sugarcane bagasse and peel are needed before a universally prebiotic activity on sugarcane bagasse could be established, and prebiotic activity-related consequences on human health could be solved. Hence, important findings from this study such as parameter of probiotic growth performance and its prebiotic activity scores could add to the body of knowledge for prebiotic activity on agro-industrial by-product. That important data from the findings can also acts as baseline data for future researches.

Apart from that, many industries only focus on the sugarcane juice for human consumption. This research will provide the industries more understanding on the prebiotic potential of sugarcane bagasse and peel for the production of functional foods. As mentioned earlier, sugarcane bagasse and peel compose of majority cellulose, hemicelluloses and lignin and these valuable compounds will provide nutrients and organic matter that are important in improving soil properties and humans' health. Besides, these by-products can be source of nutrients for plants and microbial inoculants for bio-fertilizer production as well as nutrients for growth for probiotics in human gastrointestinal tract. Basically, the usage of sugarcane by-product will result in better, eco-friendly waste management which can reduce pollution in Malaysia.

1.4 Objective

1.4.1. General objective

To study the probiotic growth parameter, its growth performance parameter and its prebiotic potential on sugarcane processing by-products.

1.4.2 Specific objectives

- 1) To determine and compare the growth of *Lactobacillus casei* strain Shirota with sugarcane bagasse and its peel as the source of carbon.
- 2) To determine and compare the growth performance of *Lactobacillus casei* strain Shirota on sugarcane bagasse and sugarcane peel.

3) To determine and compare the prebiotic potential of sugar cane bagasse and sugarcane peel.

1.5 Hypothesis

1) There is a significant difference in growth parameter of probiotic on sugarcane bagasse and sugarcane peel as the source of carbon.

2) There is a significant difference in growth performance parameter of probiotic on sugarcane bagasse and sugarcane peel.

3) There is a significant difference of prebiotic potential on sugarcane bagasse and sugarcane peel.

CHAPTER 2

LITERATURE REVIEW

2.1 Sugarcane

Sugarcane (*Saccharum Officinarum* L.) from grass family of Poaceae is a crop that has been grown in many places throughout the world. Historically, Artschwager and Brandes (1958) added that New Guinea constituted the centre of origin of noble canes. Then, these canes have migrated through three routes; a southeastwards dispersal from New Guinea via the Bismarck Archipelago to the New Hebrides, beginning about 8000 BC, migration westwards from New Guinea to Indonesia, the Philippines, Malay Peninsula, Indochina and the arc around the Bay of Bengal, beginning about 6000 BC, and secondary dispersal among the Polynesian Islands, during the period from about 500 to 1100 AD. This happened because of transportation of stem cutting by men in old ages (Artschwager & Brandes, 1958). Basically, sugarcane is a tropical grass native to Asia and it has been grown in gardens for 4000 years. *S. Officinarum* is a product of interbreeding four species of *Saccharum* genus. It is also a giant, robust and sugary plant. The methods

for manufacturing sugar from sugarcane were developed in India by Christopher Columbus (400 BC) and over 75% of world's sugar comes from sugarcane (Baucum, Rice & Schueneman, 2006).

2.1.1 Bagasse

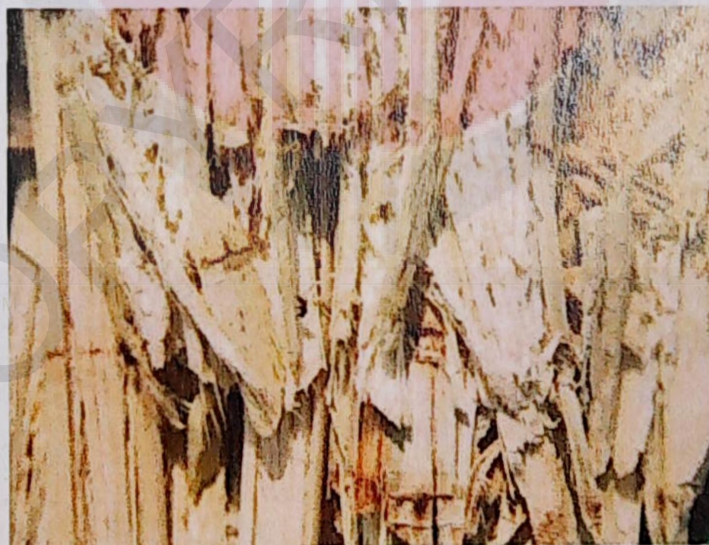
Bagasse is a fibrous material from plant (figure: 1 c) that remains once all of the juices have been squeezed from sugarcane stalk. Bagasse (figure 1: c) consists of majority stalk fibres (figure 1: a). It also contains leaves (figure 1: b) and other biomass components that were brought by harvest trucks or railcars. Bagasse can be understood as half water and half dry matter (plant material) (Pandey et al., 2000). In some places, bagasse is used as fuel for the mills. The mill burns bagasse for generating electricity (Baucum et al., 2006). Sugarcane bagasse consists of 50% cellulose and 25% each of hemicellulose and lignin. Specifically, it contains about 50% of α -cellulose, 30% pentosans and 2.4% ash. As it has low amount of ash content, it offers numerous advantages in comparison to other crops residues. One of them is for usage in bioconversion processes using microbial cultures (Pandey, Soccol & Nigam, 2000).



a) Sugarcane stalk



b) Sugarcane plant with leaves



c) Sugarcane bagasse

Figure 2.1. Sugarcane plants with their parts.

(Source: Google, 2018)

2.2 Nutritional Status of Sugarcane Bagasse

Many studies have been conducted on the proximate properties of sugar cane bagasse and these also include the Malaysian sugarcane bagasse. The proximate properties of sugarcane bagasse varied according to countries. The effects of species, location as well as climate affected the proximate properties of sugarcane bagasse. Besides, the functional properties of the sugarcane should be different because they are strongly associated with the proximate properties (Leang & Saw, 2014).

Table 2.1: Proximate properties of sugarcane bagasse.

Proximate properties		Percentage (%)
Moisture content	-Fresh bagasse	63.0±0.81
	Vacuum-packed powder	9.4±0.05
Protein		1.7±0.10
Crude fat		2.0±0.00
Crude fibre		30.4±0.05
Ash		1.01±0.09
Crude carbohydrate		55.4±0.61

Adapted from Leang and Saw (2014).

From findings by Leang and Saw (2014), the authors concluded that the Malaysian yellow cane bagasse has relatively low crude fat (2.0 percent), high carbohydrate content (55.4 percent), high crude fibre (30.4 percent), high total dietary fibre (79.5 g/100g) and insoluble dietary fibre (75.7 g/100g), high oil holding capacity (9.4 g/g), and high organic molecule absorption capacity (8.3 g/g). Besides,

they also suggested that those attributes can be a cheap source of dietary fibre for food development. However, further research is needed to incorporate the bagasse into food systems and evaluate the quality of the new food products, sensory attributes, and safety for human consumption.

2.3 Prebiotic

Prebiotics are non-digestible food ingredients that positively affect the host by selectively stimulating the growth and activity of one or a limited number of bacteria in the gastrointestinal tract and exert antagonistic effects to foodborne pathogens such as *Salmonella sp.* or *Escheria coli*, limiting their proliferation (Diaz-Vela et al., 2013). The gastrointestinal tract is mainly populated with anaerobe bacteria. Gibson (2004), reported that prebiotic can withstand acid hydrolysis in the stomach and move to the large intestine without being absorbed by the small intestine. Hence, prebiotic can stimulate the growth of indigenous microflora in the large intestine. Currently an array of prebiotic with various origin has been classified into two types; those occurring naturally in plants such as banana and asparagus and those synthesised by physical, chemical or enzymatic method (Courtois, 2009).

2.3.1 Oligosaccharide as prebiotic

Oligosaccharides are low molecular weight carbohydrates, containing between 3 and 10 sugar moieties, intermediate in nature between simple sugars and polysaccharides (Weijers et al., 2008). Many oligosaccharides are resistant to digestion and absorption by human digestive system. This occurs because of the

anomeric C atom (C1 or C2) of the monosaccharide units has a configuration making the osidic bind non-susceptible to the hydrolytic activity of human digestive enzymes (Roberfroid & Salvin, 2000). These oligosaccharides are termed as non-digestible oligosaccharide (NDO). NDO possess important physicochemical and physiological properties, thus have been accorded as prebiotics. Rastall (2011) reviewed the existing carbohydrates marketed as prebiotic and classified them as shown in Table 2.2. These NDOs are different in their chain length, monosaccharide composition, degree of branching and purity (Sako et al., 1999).

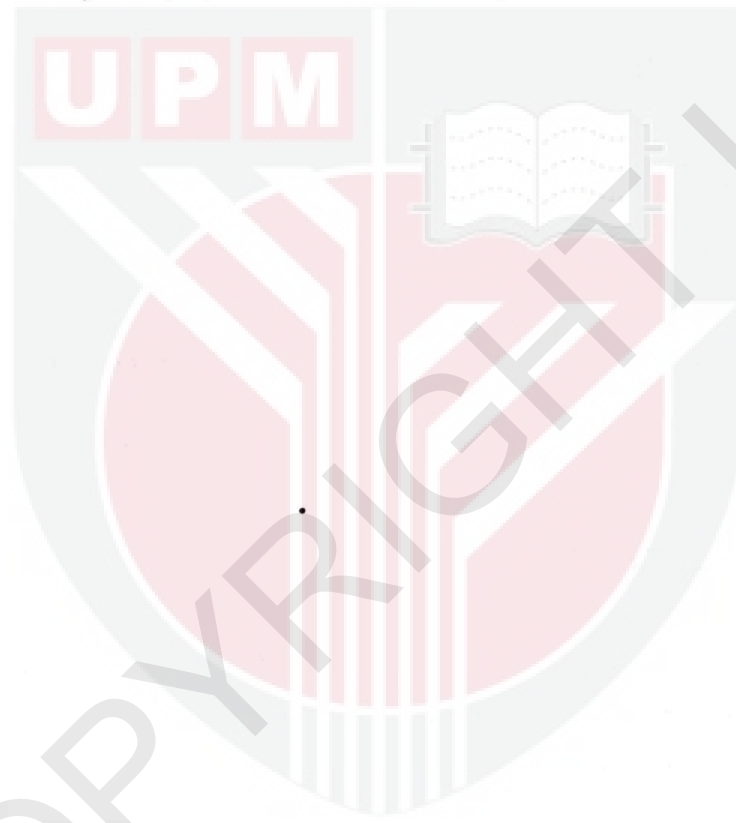


Table 2.2: Prebiotic carbohydrates

Category	Compound
^a Established Prebiotics	Inulin
	Fructooligosaccharides
	Galactooligosaccharides
	Lactulose
^b Emerging Prebiotics	Isomaltooligosaccharides
	Soybean oligosaccharides
	Resistant starch
	Xylooligosaccharides
	Polydextrose
^c Experimental Prebiotics	Gentioliogosaccharides
	Glucooligosaccharides
	Pectic oligosaccharides

a: Prebiotics established in more than one well-designed human trial with modern microbiological method; b: Carbohydrates marketed as prebiotics but with limited human data; c: Carbohydrates under investigation for their prebiotic potential but with no human data

Adapted from Van Loo (2004).

As shown in Table 2.2, inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS) and lactulose are recognised as established prebiotics. Inulin and FOS are present in many regularly consumed vegetables, fruits, cereals, including onion, garlic, chicory and artichoke (Van Loo, 2004) and the most important sources of inulin or FOS commercially available on the industrial market is either synthesised from sucrose or extracted from chicory roots. FOS is composed of three to ten monosaccharides units joined by α -glycosidic bonds (1→2) between

terminal fructose and glucose. The degree of polymerisation (DP), defined by the number of monosaccharide units, is used to define and classify FOS (DP < 10) and inulin (DP = 2 to 70) (De Leenheer & Hoebregs, 1994).

Table 2.3: Inulin and Fructooligosaccharide content of plants commonly used in human nutrition (% of fresh weight).

Source	Edible part	Solid content	Inulin	FOS
Artichoke	Leaves-heart	14-16	3-10	<1
Banana	Fruit	24-26	0.3-0.7	0.3-0.7
Chicory	Root	20-25	15-20	5-10
Jerusalem	Tuber	19-25	16-20	10-15
Artichoke				
Onion	Bulb	6-12	2-6	2-6
Yacon	Root	13-31	3-19	3-19

Adapted from Van Loo (2004).

Figure 2.2 shows the structure of inulin and FOS (Morris, 2012). Inulin is a mixture of glu-fru_n (abbrev. GF_n) molecules with 2 < n < 60 while FOS is a subgroup of inulin which is composed of GF_n and Fru_m with 2 ≤ n, and m ≤ 10 (Frank, 2000; Niness, 1999). Inulin and FOS have been shown several times to selectively stimulate *bifidobacteria* (Campbell et al., 1997; Durieux et al., 2001; Hsu et al., 2004) and to modulate immunological processes (Seifert & Watzl, 2007). Breast-fed infants are known to harbour higher counts of *bifidobacteria* in the intestine tract compared with bottle-fed babies because of the presence of FOS in human milk (Nagendra, et al., 1995). Roberfroid (2002) had concluded that inulin and FOS

showed evidence of prebiotic activity if consumed at a level of 5- 15g/day for a few weeks.

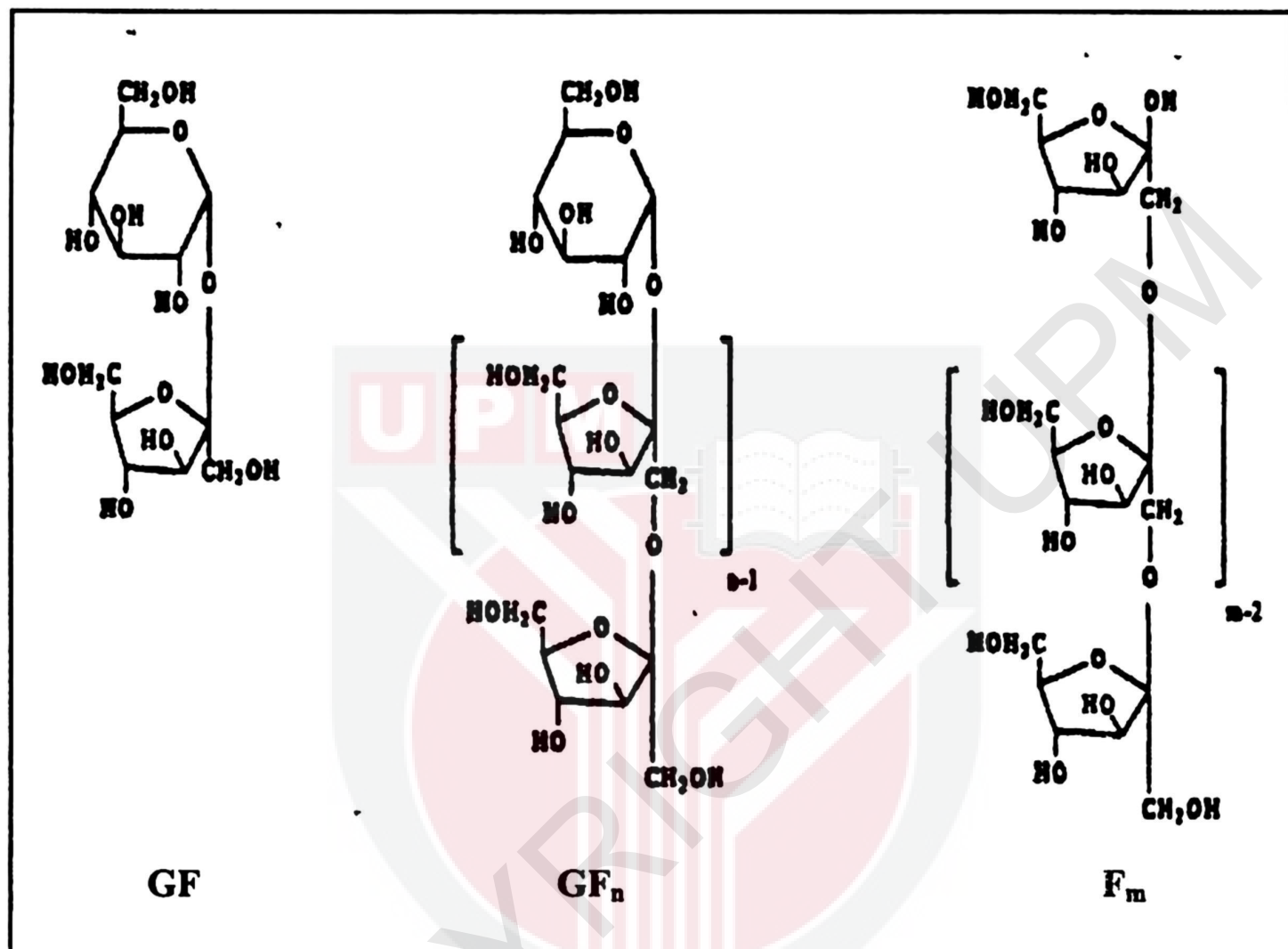


Figure 2.2. Chemical structures of sucrose (left), GF_n-type inulin (center) and F_m-type fructo-oligosaccharides (right). G: glucose; F: fructose.

(Source: Morris, 2012)

Some prebiotics can be found naturally in food products, like isomaltooligosaccharides that are found in miso and soy sauce and have been reported to increase cecal *Bifidobacterium* population by Chen et al., (2002). Lactulose is semi-synthetic disaccharide comprising galactose and fructose and widely detected in heated milk (Marconi et al., 2004). In the large intestine, lactulose stimulates the growth of selected micro-organisms; *Bifidobacterium*, *Lactobacillus*

and *Streptococcus* and inhibited potentially harmful bacteria; *Bacteroides*, *Clostridium*, *coliforms* and *Eubacterium* (Crittenden, 1999). Galactooligosaccharides (GOS) are another class of prebiotic that are manufactured and marketed in Europe as well as Japan and used widely in infant formula foods.

2.4 Probiotics

Probiotics as defined by the Food and Agriculture Organization of the United Nation (FAO) and World Health Organization (WHO) in 2006, comprise “live microorganisms which, when administered in adequate amounts, confer a health benefits on the host by influencing the composition and or metabolic activity of the flora in the gastrointestinal tract.” The predominantly selected probiotics are species of *Lactobacillus* (naturally found in human small intestine) and *Bifidobacterium* (naturally found in human large intestine) while other organisms such as probiotic yeast: *Saccharomyces boulardii* have limited uses in the food industry (Tamine et al., 2005). Table 2.4 shows lists of the commercial strains that have been used as probiotics.

Probiotics are considered beneficial if they meet the following criteria: (a) can be prepared in a viable manner and on a large scale for industrial purpose, (b) can remain viable and stable upon use or under storage, (c) can survive in intestinal ecosystem and (d) can positively affect the host (Fuller 1991; 1992).

Table 2.4: Commercial probiotic microorganisms

Microorganisms	Strain	Company (product)
<i>Bifidobacterium adolescentis</i>	ATCC 15703	-
<i>Bifidobacterium animalis</i>	Bb-12	Chr. Hansen
<i>Bifidobacterium bifidum</i>	Bb-11	Chr. Hansen
	Bb-02	
<i>Bifidobacterium lactis</i>	Lafti TM	DSM
	B94	
<i>Bifidobacterium</i>	CRL 431	-
	BB536	Morinaga Milk Industry
<i>Bifidobacterium longum</i>	SBT-2928	Snow Brand Milk
	UCC 35624	Products
		UCCork
<i>Bacillus lactis</i>	DR10	Danisco (Howaru TM)
<i>Lactobacillus bulgaris</i>	Lb12	-
<i>Lactobacillus casei</i>	Shirota	Yakult (Yakult ®)
<i>Lactobacillus casei</i>	Immunitas®	Danone ®
<i>Lactobacillus fermentum</i>	RC-14	Urex biotech
<i>Lactobacillus helveticus</i>	B02	-
<i>Lactobacillus lactis</i>	L1A	Essum AB
<i>Lactobacillus paracasei</i>	CRL 431	Chr. Hansen
	299v	
<i>Lactobacillus plantarum</i>	Lp01	Probi AB

Adapted from Krishakumar and Gordon (2001), Holm (2003), Playne et al. (2003) and Shah, (2004).

To achieve a probiotic status, microorganisms have to meet a number of criteria related to safety, functional effect, and technological properties (FAO/WHO, 2001). That is, probiotic microorganism should not be pathogenic, have no connection with diarrhoeagenic bacteria and be able to maintain genetic stability. Ouwehand et al., (1999) stated that microorganisms could be considered as functional food components if they have resistance to digestive enzyme; adhere to intestinal surface and acid and bile stable.

2.4.1 Lactic acid bacteria (LAB)

LAB are non-pathogenic, non-toxicogenic, non-motile, non-spore forming Gram-positive bacteria that may be cocci or rods. These microaerophilic bacteria are associated with the production of lactic acid from the fermentation of carbohydrates (Axelsson, 2004). This group of bacteria has diverse metabolic capacities. Lactic acid fermentation depends on the ability of LAB to produce acid, carbon dioxide, and other organic acid that inhibit the growth of other non-desirable organisms.

LAB can be divided into two groups: homofermentative and heterofermentative. The former produces only lactic acid via the glycolytic (Embden-Meyerhof) pathway, whereas the latter produces lactic acid along with alcohol, volatile compounds, and gas via the 6-phosphogluconate/phosphoketolase pathway (Axelsson, 2004). Among the major genera of LAB, *Lactobacillus* and *Bifidobacterium* are recognised as the safest for food production and consumption.

Lactobacilli represents a group of gram-positive, non-spore, long to short rods or coccobacilli, and are either micro-aerophilic or anaerobic and strictly fermentative (Hammnes & Vogel, 1995). They are more tolerant to acid than the other genera of LAB, making them important in the final phases of food fermentation. *Bifidobacteria* are non-motile, often branch, Gram-positive anaerobic bacteria. They are one of the major genera of bacteria that comprise the colon flora, which are ubiquitous and endosymbiotic inhabitants of the gastrointestinal tract, vagina and mouth of mammals and other animals. *Bifidobacteria* constitute only 5-10% of the total intestinal flora of children and adults. The numbers of *bifidobacteria* decreased with age and changes in eating habits, with the elderly has the lowest populations of *bifidobacteria* (Champagne et al., 2005).

2.5 Health benefits of probiotic and prebiotics

Prebiotics and probiotics are known to have positive effects at the nutritional and therapeutic levels. The consumption of prebiotic can significantly modulate the colon microflora by increasing the number of probiotic bacteria. In addition, short-chain fatty acid such as acetic acid, propionic acid and butyric acid are some of the fermentation end products that can lower blood ammonia levels by producing NH_4^+ from the protonation of potentially toxic ammonia and amines (Hansen, Allan-Wojtas, Jin & Paulson, 2002). As a result, mineral absorption (Ca and Mg) is enhanced, faecal weight is increased, and blood lipid levels are lowered. Furthermore, pH reduction also leads to pathogens inhibition. Current research from Hill et al (2014) indicates that prebiotic and probiotic strains live symbiotically, thereby enhancing the growth of gut microflora. Table 2.5 and 2.6 show the evidence

of available prebiotic and probiotic and their reported effects on human and animal health.

2.5.1 Health benefits of prebiotic

Table 2.5: Beneficial effects of prebiotic

Physiological effects	Health benefits
Selection of probiotic bacterial growth in large intestine (colonisation resistance)	Enhanced resistance to invading pathogens
Increased stools frequency and stool weight	Improved bowel function/Laxative effects
Non-specific stimulation of immune function	Resistance to infection
Not hydrolysed by oral micro-organisms	Anticariogenic effect
Not glycaemic	Potentially useful for diabetes
Modulation of carcinogen metabolism	Anti-colon cancer properties
Reduced synthesis of very low density lipoprotein (VLDL) cholesterol and serum triglycerides	Cardioprotective
Increased absorption of calcium and magnesium	Protection against osteoporosis

Adapted from Macfarlane and Cumming (1999), Crittenden (1999), Van Loo et al. (1999) and Roberfroid (2001).

2.5.2 Health benefit of probiotic

Table 2.6: Probiotics and their reported effects on health

Strains	Reported effects in clinical studies
<i>L. acidophilus</i> NCFM	Treatment of lactose intolerance; production of bacteriocin; lowering faecal enzyme activities
<i>L. acidophilus</i> NCFB 1748	Prevention of radiotherapy-related diarrhoea; lowering faecal enzyme activities
<i>L. casei shirota</i>	Prevention of intestinal disturbance; balancing intestinal flora; inhibition of superficial bladder cancer; lowering faecal enzyme activities
<i>L. reuteri</i>	Coloning the intestinal tract in animal studies; shortening of duration of rotavirus diarrhoea
<i>L. rhamnosus</i> GG	Antagonistic against anticarcinogenic bacteria; prevention and treatment of rotavirus diarrhoea; prevention and treatment of relapsing <i>C. difficile</i> diarrhoea; prevention of acute diarrhoea; alleviation of Crohn's disease
<i>B. lactis</i> Bb12	Balancing microflora, shortening of the duration of rotavirus
<i>B. bifidum</i>	Treatment of rotavirus diarrhoea; balancing intestinal flora
<i>Saccharamyces boulardii</i>	Prevention of antibiotic associated diarrhoea; treatment of <i>C. Difficile</i> diarrhoea

Adapted from Lee and Salminen (1995), Shah (2006) and Salminen et al. (1998).

2.6 Prebiotic properties of agro-industrial by-products

Due to the increasing demand for foods, animal feed and biofuels, the search for the functional food ingredients has led scientists to explore agro industrial by-products as a new source of dietary fibre and other nutrients. In plant processing, around 70% of the raw material weight, such seeds and peel are considered waste. Besides, the disposal of these wastes can represent environment problems. The valuable nutrients content in the agro industrial waste are lost due to the disposal of those waste and they actually can be source of nutrient for functional food (Larrauri, 1999; Oreopoulou & Tzia, 2007; Federici et al., 2009; Espírito Santo et al., 2012).

Peels are by-product of fruits and plants and they have high insoluble dietary fibre, pectin, fructooligosaccharides, as well as phenolic compounds, protein and vitamins (Nawirska & Kwasniewska, 2005; Espírito Santo et al., 2012). Moreover, the use of agro industrial by-products with high nutritional value has gained more interest as they are high-value products and their recovery may be economically attractive (Oreopoulou & Tzia, 2007; Lamsal & Faubion, 2009). Other compounds that are found from plant waste are mono-, di-, and oligosaccharides, as well as non-digestible oligosaccharides that are considered prebiotic as they can reach the colon and then ferment mainly by bifidobacteria and lactic acid bacteria, producing a positive health effects such as strengthening the immune system, relief constipation, improve mineral and nutrient absorption and also reducing the incidence of colon cancer (Gibson & Roberfroid, 1995).

2.6.1 Probiotic growth of agro industrial by-products

The prebiotic activity has been assessed in many agro industrial by-products and one of them is in pineapple waste powder. Sah, Vasiljevic, McKechnie and Donkor (2016) concluded that the net probiotic growth in 24 hours of incubation of *L. acidophilus*, *L. casei*, and *L. paracasei* in customised MRS broth containing freeze dried pineapple peel and pomace powder as a direct carbohydrate source was not substantially different ($P \geq 0.05$) from MRS broth supplemented with glucose. So, these by-products can be used as alternative of carbohydrate source for probiotics. Besides, bacterial growth in all pineapple powders tested was more than one log higher compared to the negative control, which is a customised MRS broth without carbohydrates. Espírito Santo et al. (2012) also have reported where the total dietary fibre from by-product of banana, apple or fruit passion processing increased the probiotic viability in yogurt. Other than that, the viable count of *S. thermophilus* and *L. bulgaricus* in the yogurt supplemented with pineapple powder has shown similar value in comparison to yogurts without supplementation. In fact, probiotic yogurts with pineapple waste powder had enhanced growth of probiotic species. For example, high count of *L. acidophilus*, (8.13-8.71 log cfu/g), *L. Casei* and *L. Paracasei* (8.12-8.72 log cfu/g) were seen in probiotic yogurt with pineapple powder compared than non-supplemented probiotic yogurt (7.34 and 7.83 log cfu/g) in their study (Sah et al. 2014).

In other study by Diaz-Vela, Totosa, Cruz-Guerrero and Pérez-Chabela (2013) on fermentation of added-value agro industrial by-product, they reported that *P. pentosaceus* had a significantly ($P \leq 0.05$) higher carbohydrate consumption value

than *L. rhamnosus* and *A. viridans*. Besides, carbohydrate consumption was significantly ($P > 0.05$) higher with cactus pear peel flour as the carbon source than glucose or pineapple peel flour. In addition, those carbon sources in agro industrial by-products can still enhance growth of probiotic strains, but the net growth varies based on the type of by-products. Other study by Duarte et al., (2017) reported that the viable cells count of *L. acidophilus*, *L. casei* and *L. paracasei* in broth containing glucose, fructooligosaccharides and cashew apple by-product powder during 48 hours of incubation presented positive prebiotic activity score. The cashew apple powder has shown the highest scores ($P \leq 0.05$) of viable cell count of *L. paracasei*. The purified fructooligosaccharide also presented highest prebiotic score ($P \leq 0.05$) than cashew apple powder toward the growth of *L. acidophilus* and *L. paracasei*. Importantly, this positive score provides a rational base to identify the potential of agro industrial waste to promote selective growth of specific beneficial probiotic in the presence of enteric competitors. So, these results will also add a new body of knowledge on sugar cane bagasse and peel toward probiotics.

2.6.2 Acidification production of probiotic on agro industrial by-products

Colonization and fermentation of probiotics in our colon produce short chain fatty acids (SCFAs) such as lactic, acetic, butyric and propionic acid. Basically, the production of SCFA will lower pH and benefit host in term of inhibiting pathogenic bacteria, strengthening immune system and reducing incidence of colon cancer (Duarte et al., 2017). Based on acidification parameter by Diaz-Vela et al. (2013), the kinetic parameter of acidification of *P. pentosaceus*, *A. viridans* and *L. rhamnosus* on cactus pear peel flour and pineapple peel flour were calculated and measured by

using pH meter. As the results, the maximum acidification rate values were significantly ($P < 0.005$) higher for *P. pentosaceus*, and lowest were for *E. Coli*. The highest time to reach maximum acidification was significantly ($P < 0.005$) lower for *P. pentosaceus* and *A. viridans*. Besides, the pH at that point was significantly ($P < 0.005$) lower for *L. rhamnosus*. The cactus pear peel flour fermentations resulted in significantly ($P < 0.005$) higher maximum acidification and the pH value at maximum acidification was not significantly ($P > 0.005$) different for the different carbon sources used.

Other study from Duarte et al. (2017) also has shown that the cultivation of the *Lactobacillus* strains in broth containing glucose, fructooligosaccharide and cashew apple waste product produced low pH values in cultivation media especially after 24 hours of incubation. However, no significant difference ($P > 0.05$) in pH values in the cultivation media for the same inoculated strains was observed. The observation of high viable cell counts of probiotic bacteria which reduced pH values of growth media indicates high metabolic activities in the broth inoculated with *Lactobacillus*. High bacterial metabolic activity was determined in broth containing cashew apple by-products and this is a good result that shows the survival and growth of probiotic have been associated with decrease pH and increase production of organic acid in the colon (Duarte et al., 2017).

One study showed that the pH and titratable acidity of the sample yogurts were within 4.51 – 4.48 and 1.01% - 1.07% respectively in 5 hours 45 minutes. Those results were in agreement with the various works on the addition of fibres

from fruit by-product to yogurt (Espírito Santo et al. 2012; García-Pérez et al., 2005). The pineapple powders contained dietary fibres, protein, and mineral as well as fat, which could serve as growth factors for probiotic and improved growth. Besides, improved growth has resulted in high metabolic activities and proteolysis activity (Donkor et al., 2007). Due to the findings from previous study, sugarcane bagasse and peel is expected to produce similar results.

2.6.3 Prebiotic potential: prebiotic activity score on agricultural by-products

Based on a study conducted using cactus pear peel and pineapple peel flour as prebiotic source, both cactus pear peel and pineapple peel have high prebiotic potential score ($P \leq 0.05$) for *P. pentosaceus*, and *L. rhamnosus*. It was shown on the growth of probiotics even at low concentration such as 1% of concentration of those peels. In contrast, lower score was found for *A. viridians*. There was also no significant ($P > 0.05$) difference for the carbon source, that is, the carbon sources had the same effect on lactic acid bacteria growth. This finding indicated that the chosen ingredients have a positive score if they are metabolised selectively by probiotic but not by other microorganisms, such as pathogens. Indeed, Huebner et al., (2007) stated that different prebiotic activity score is seen for different microorganisms. Fermentation of *Lactobacillus paracasei* with inulin, obtained a prebiotic activity score of 1.17, whereas with glycooligosaccharides as carbon source, the score for *L. plantarum* and *Bifidobacterium bifidum* were 0.82 and 1.24, respectively. Most of bifidobacteria with different prebiotic obtained scores in range 0.2 – 0.4. Furthermore, those values matched with findings from Huebner et al., (2007)

because *P. pentosaceus* obtained prebiotic activity scores of 0.33 and 0.32 for cactus pear peel flour and pineapple peel flour, respectively.

Besides, *P. pentosaceus* obtained higher prebiotic activity scores and higher growth and acidification parameters than the other lactic acid strains, indicating a better assimilation of the fermentable carbohydrates present in the peel flours. No difference between both cactus pear and pineapple peel flours indicated that the carbohydrates are equally available to be fermented. Positive effects were observed using these peel flours, indicating that, under the experimental conditions used, these ingredients had a positive prebiotic effect although with differences in specific growth and acidification parameters as a consequence of different structures of carbon sources (Hopkins et al., 1998; Cardelle-Cobas et al., 2011).

CHAPTER 3

METHODOLOGY

3.1 Substrate and chemicals

The chemicals and reagents used in this research were glucose (20g/L), yeast extract (6g/L), peptone from meat (30g/L), sodium acetate (8.29g/L), magnesium sulfate heptahydrate (0.2g/L), tween 80 (1.0g/L), maganese sulfate heptahydrate (0.05g/L), ammonia citrate (2g/L), disodium phosphate (2g/L) and agar powder (15g/L). These chemicals were mixed together with sterile distilled water to form De Man Rogosa and Sharpe (MRS) broth and agar. Besides, Yakult (cultured milk drink) was used as source of probiotic as well as nutrient broth and agar, MRS powder for broth (52.2g/L) and agar (68.2g/L).

3.2 Propagation of cultures

Lactobacillus casei strain Shirota (LcS) was enumerated from the Yakult drink (cultured milk drink) and the drink was purchased at a supermarket in

Putrajaya, Malaysia. In this study, standard plate count method was used as a quantitative method for determination of bacterial population, and LcS was selected as the probiotic. For isolation of LcS, 1 ml of Yakult cultured milk, which contained only LcS, was cultured on MRS agar for 48 h at 37°C. Later, a single colony of the bacteria was further grown in MRS broth for 24 h in an incubator shaker at 250 rev min⁻¹ and temperature of 37°C. The growth of LcS was monitored optically at 600 nm every 2 h for 24 h. The response of optical density (OD) was recorded and plotted using Microsoft EXCEL.

Enteropathogen strain *Escherichia coli* K12 was used as reference strain. *Escherichia coli* were reactivated in nutrient broth at 37 °C for 24 h obtaining a similar optical density.

3.3 Instruments

The instruments that was used in this experiment are freeze dryer (The VIRTIS Company, Inc Route 208, Gardiner, New York), incubator orbital shaker (716, Protech electronic), incubator (716, Protech electronic), blender 21 (Torrington Ct, USA), centrifuge machine (Universal 32R, Hettich Zentrifugen, England), weighing scale (AC Adapter, Japan) and vortex (EVM-V1000, Erla, Jayu, Korea).

3.4 Sample collection and preparation

Sugarcane stalks were collected from Kampung Sungai Ramal Dalam, Kajang Malaysia by using convenient sampling. Briefly, crushed peel and bagasse were cleaned, grinded and then dried using FD 300 freeze dryer (The Virtis Company, Inc Route 208, Gardiner, New York) and milled to fine powder. Particle of fine powder was standardized to less than 180 μm using sieves and then sterilized in UV radiation for 30 minutes. These samples were stored at $-20\text{ }^{\circ}\text{C}$ for further analysis.

3.5 Experimental design

This study used sugarcane bagasse and sugarcane peel as samples. Total available sugar was determined by using acid hydrolysis method. Then, these samples had undergone two different assessments which are bacterial growth performance and prebiotic activity score parameter. These two assessments were stated in the experimental design and the experimental design of this research was shown in figure below.

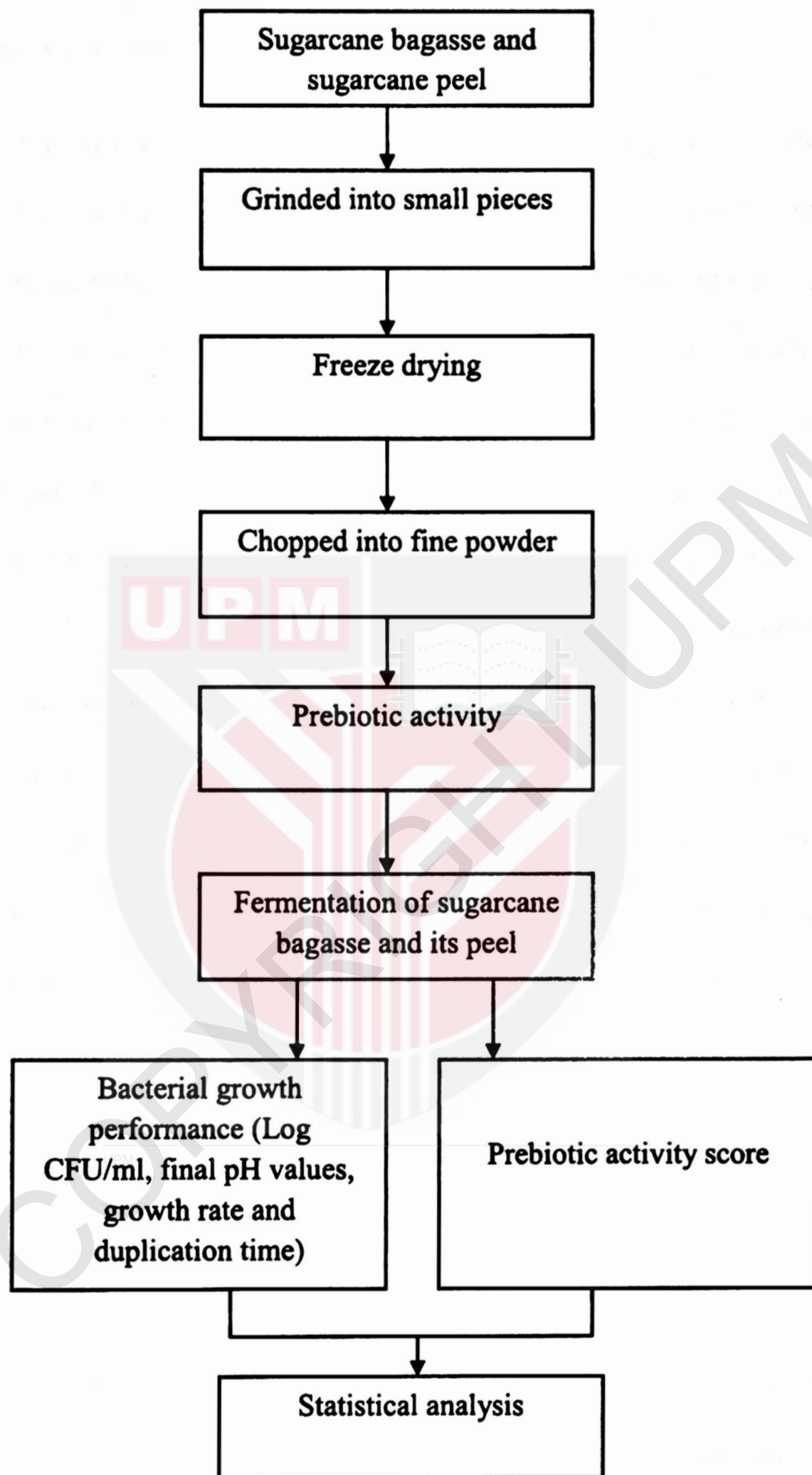


Figure 3.1. Experimental design of prebiotic potential of sugarcane bagasse and peel.

3.6 Assessment of bacterial growth performance

The bacterial growth performance was assessed through the growth of *Lactobacillus casei* with some modification. The customised culture media was prepared by substituting glucose with sugarcane bagasse and sugarcane peel powder in the same formulation of commercial MRS broth (cMRS). Customised MRS broth without glucose was prepared by mixing different ingredients in distilled water such as yeast extract (6g/L), peptone from meat (30g/L), sodium acetate (8.29g/L), magnesium sulfate heptahydrate (0.2g/L), tween 80 (1.0g/L), manganese sulfate heptahydrate (0.05g/L), ammonia citrate (2g/L) and disodium phosphate (2g/L). The pH was adjusted to 6.7 ± 0.2 at 25 °C. The sugarcane bagasse and peel, whose amount were quantified on the basis of total reducing sugar content determined by acid hydrolysis method, were mixed separately at the rate of 20 g/L to the MRS and autoclaved at 121 °C for 15 minutes. The broths (at room temperature) were aseptically inoculated with 1 % of probiotic culture ($1-2 \times 10^9$ cfu/ml) separately and incubated at 37 °C for 24 hours with agitation (120 rpm).

The bacterial colonies were enumerated on MRS agar after aerobic incubation at 37 °C for 48 h. The net growth of *Lactobacillus casei* was determined by subtracting count as log cfu/g at 0 h to that at 24 h. The experiment was then repeated using MRS broth supplemented with glucose as the positive control, sugarcane bagasse powder (MRSb), sugarcane peel powder (MRSp) and commercial MRS broth (cMRS) only as the control.

3.6.1 Enumeration of probiotic culture

Lactobacillus casei strain Shirota were enumerated by spreading 0.1 ml of the appropriate dilutions of the sample in sterile distilled water onto MRS agar plates and expressed as log cfu/g. The MRS agar plates were incubated at 37 °C in inverted position to prevent water accumulation on agars due to condensation process. The pH of the nutrient agar was adjusted to 6.2 ± 0.2 , suitable for the growth of LcS. The colonies of the bacteria were enumerated every 6 hours of incubation until 24 hours and log cfu/g was calculated to indicate the growth of the bacteria.

To determine the number of bacteria in the original sample, a formula given below was used.

$$\text{Colony forming unit } \left(\frac{\text{CFU}}{\text{ml}} \right) = \frac{(\text{number of colonies})}{(\text{Dilution} \times \text{amount plated})}$$

3.6.2 Determination of final pH of prebiotic sample

The pH of modified MRS broth samples were measured using pH 720 precision pH meter (WTW inoLab®, Weilheim, Germany) according to the AOAC official method 947.05 (Horwitz & Latimer, 2006).

3.6.3 Bacterial growth parameter

Bacterial growth parameter was determined by standard plate count in their respective culture medium, making the pertinent dilutions, incubating at 37°C for 24 hours under aerobic conditions. Mean growth rate constant (k) and mean duplication time (g) were determined according to Willey et al. (2008) equations, as follows:

$$k = \frac{\log N_{24} - \log N_0}{\log 2 \times 10}$$

$$g = \frac{1}{k}$$

Where N_{24} = CFU/ml at the end of fermentation (24 hours) and N_0 = CFU/ml at the start of fermentation (0 h).

3.7 Assessment of prebiotic potential: Prebiotic Activity Score

The prebiotic activity score was determined according to the relationship described by Huebner et al. (2007), considering the growth of each bacteria during fermentation using sugarcane bagasse or sugarcane peel and glucose as carbon source, as follows:

$$PA = LAB \frac{\Delta N \text{ prebiotic}}{\Delta N \text{ glucose}} - \text{Enteric} \frac{\Delta N \text{ prebiotic}}{\Delta N \text{ glucose}}$$

Where LAB is *Lactobacillus casei*, and Enteric is *E. coli*. According to Huebner et al. (2007), by definition, substrates with a high prebiotic activity score support good growth of the probiotic bacteria, with cellular growth comparable to that observed when grown on glucose. The cellular growth of the enteric strains grown on the prebiotics should, in theory, be very low in relation to growth on glucose.

3.8 Statistical analysis

All of the results were expressed as means $\pm$ standard deviation. One way analysis of variance (ANOVA) with post-hoc test (Tukey HSD) was used to determine the differences among these means. *P*-values < 0.05 were regarded to be significant. Statistical analysis was conducted by using IBM SPSS Statistics 24.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 *Lactobacillus casei* strain Shirota (LcS) growth curve

The growth behaviour of the probiotic *Lactobacillus casei* strain Shirota (LcS) in de Man, Rogosa and Sharpe (MRS) broth was assessed by monitoring the optical density (OD) at 600 nm absorbance for every two hours by using spectrophotometer. The standard growth curve of LcS was plotted by using Microsoft Excel 2007 and the result was presented in the Figure 4.1.

**Growth curve of *Lactobacillus casei* strain Shirota
OD600 vs time (hr)**

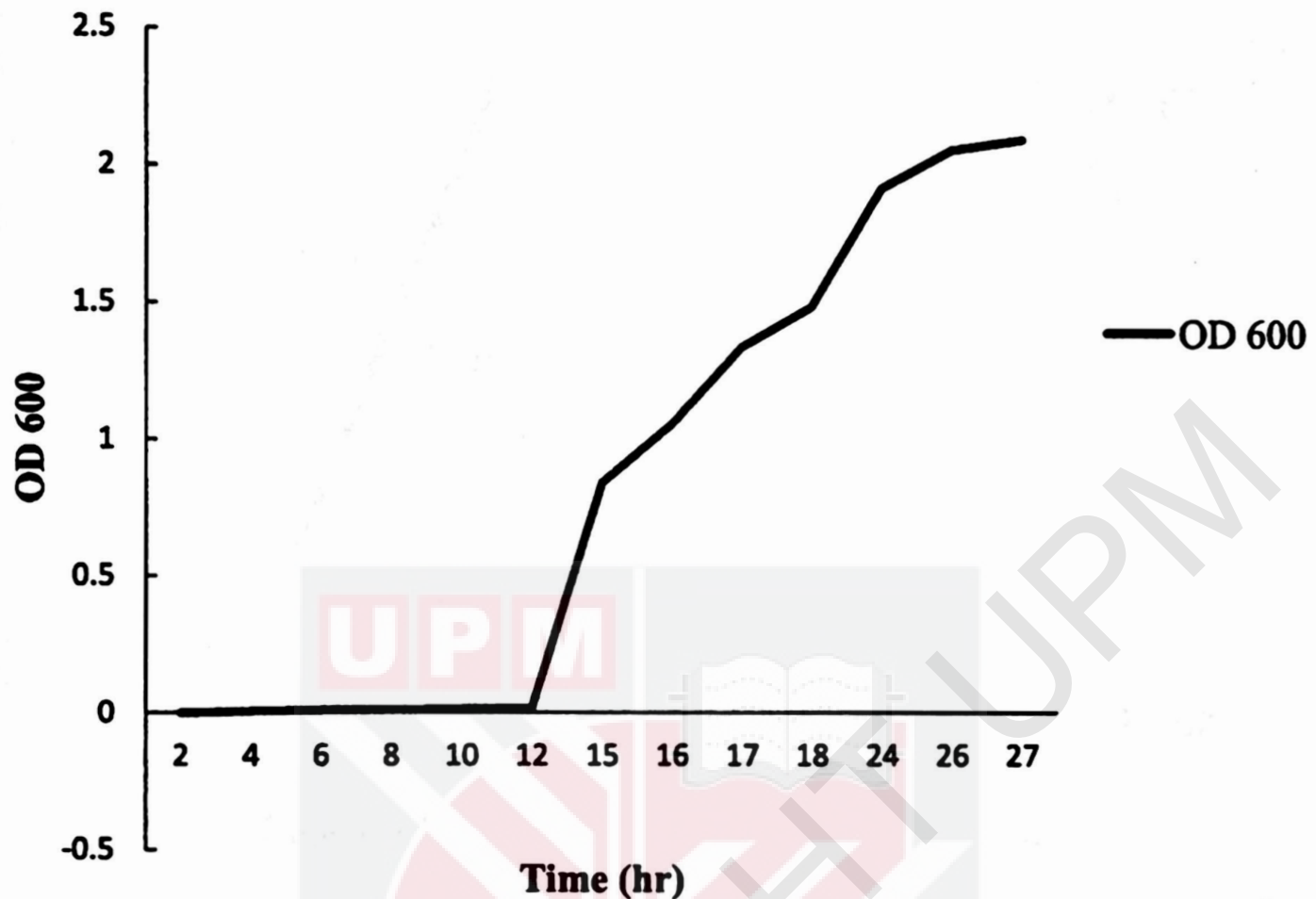


Figure 4.1. LcS growth curve

This LcS growth curve in Figure 4.1 reflects the events in LcS population when they were grown in a closed system of MRS broth culture at fixed volume. The LcS growth curve in Figure 4.1 could be fully explained by using the typical growth curve for population of cells in Figure 4.2. The typical growth curve for population of cells can be divided into several types of phases called lag phase, log phase, stationary phase and death phase as shown in Figure 4.2 below.

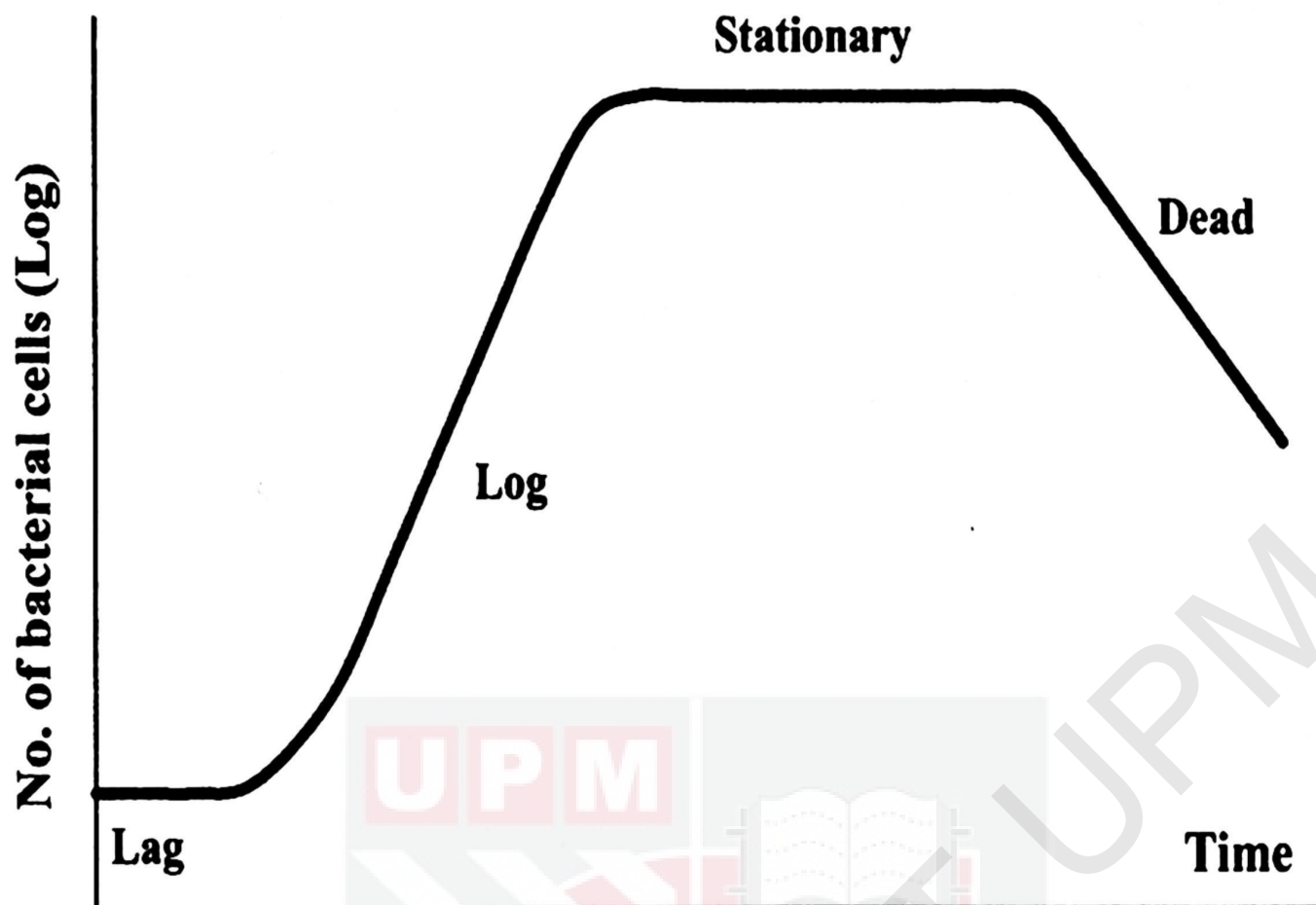


Figure 4.2. Standard growth curve of bacteria

(Source Google, 2018)

The graph in figure 4.1 shows that at time between 2 hours to 12 hours, the OD of LcS was almost 0 and the line graph shows the plateau trend. This is because when LcS population was inoculated into a fresh MRS broth, LcS do not immediately reproduce, and the population size remains constant. Based on Encyclopaedia Britannica Inc. (2018) on the growth of bacterial population, this period is named as lag phase, where the cells are metabolically active and increase only in cell size. They are also synthesizing the enzymes and factors needed for cell division and population growth under their new environmental conditions. Then the graph shows the rising OD values at time of 12 to 26 hours, with an increasing trend. This means that the population of LcS has entered the log phase or exponential phase. In this phase, bacterial cell numbers doubles during each unit time period (generation time). For example, a culture containing 1000 organisms per ml with a

generation time of 20 minutes would contain 2000 organisms per ml after 20 minutes, 4000 after 40 minutes, 8000 after 60 minutes 16,000 after 120 minutes. The generation time of most bacteria is between 20 minutes to 20 hours. *Mycobacterium tuberculosis* and *Mycobacterium leprae* has longer generation time (Encyclopaedia Britannica Inc., 2018).

When the cell number from this experiment is graphed on arithmetic coordinates as function of elapsed time, one obtains a curve with a constantly increasing slope. The rate of increase in cell number was slow initially but in later stage the cell numbers increased explosively. The rate of exponential growth varies between bacterial genera (genetic characteristics of bacteria) and is also influenced by cultural conditions (Encyclopaedia Britannica Inc., 2018). For the stationary phase, the line of graph shows plateau trend at time after 26 hours. In this phase, the number of new cells produced balances the number of cells that die, resulting in a steady state. In batch culture, exponential growth cannot occur indefinitely because the essential nutrients of the MRS culture medium are used and waste products of organisms accumulate in the environment. In stationary phase there is no net increase or decrease in cell number. The cells functions such as energy metabolism and some biosynthetic processes go on (Encyclopaedia Britannica Inc., 2018).

If incubation continues after LcS population reaches the stationary phase, the cells may start dying. The cells enter the death phase and the cell death may be due to cell lysis and this is also an exponential process but much slower than that of exponential growth. During the decline phase many cells undergo *involution*, i.e.

they assume a variety of unusual shapes. Spore forming organisms forms spore as a part of survival mechanisms.

So, according to the OD that was recorded and plotted during 24 hours of incubation (Figure 4.1), in order for the colony forming unit (CFU) to reach 10^8 , the cell must be incubated for at least 16 hours. In other words, a single colony of LcS required 16 hours growth in MRS broth in incubator shaker at 250 rev/min and temperature of 37°C to achieve 10^8 CFU (Nikbakht Nasrabadi et al., 2013). It is because at 16 hours the LcS is in the log phase and they are in rapidly growing and dividing state. Their metabolic activity increases and LcS begins DNA replication by binary fission at a constant rate. Besides, it is also important to inoculate the LcS strain from this phase so that the growth of LcS was not affected by the growth phases when different carbon sources were incorporated as prebiotic later on.

4.2 LcS growth parameters

The experiment for the confirmation of prebiotic effects were performed by evaluating two (2) parameters which are LcS growth performance (log CFU/mL, final pH, mean growth rate and mean duplication time) and prebiotic activity score related to the fermentable bacterial metabolic activities in the different cultivation media over time. As stated in Chapter 3, the customised MRS broths were prepared by substituting glucose with sugarcane bagasse and peel powder in same formulation of commercial MRS broth (20g/L). Single colony of LcS on MRS agar was inoculated in those modified MRS broths and then the broths were incubated for 24

hours at 37°C with agitation (120 rpm). All the results were recorded in section 4.2.1 and 4.2.3.

4.2.1.1 The enumeration of LcS in log CFU/mL

The enumeration of LcS grown in those modified MRS broths was determined by spreading 0.1 ml of appropriate dilutions of cultured LcS in modified MRS broths on the MRS agar. The MRS agar was then incubated for 48 hours at 37°C. The colony was counted and expressed as log CFU/ml. Ben-David and Davidson (2014) pointed that the objective of serial dilution method is to estimate the concentration (number of colonies, organisms, bacteria or viruses) of a sample by counting the number of colonies cultured from serial dilutions of the samples, and then back track the measured counts to the unknown concentration. Besides, they also defined that colony forming unit or CFU is a measure of viable bacterial or fungal cells. In direct microscopic counts (cells counting using haemocytometer) where all cells, dead and living, are counted, but CFU measures only viable cells. For convenience the results are given as CFU/ml (colony-forming units per millilitre) for liquids and CFU/g (colony-forming units per gram) for solid. CFU can be calculated using Miles and Misra method (Ben-David & Davidson, 2014); it is useful to determine the microbiological load and magnitude of infection in blood and other samples.

The viable cell counts of LcS of commercial MRS broth (MRSc) and MRS broth containing glucose (MRS), bagasse (MRSb) and peel powder (MRSp) during 24 hours of incubation are shown in Table 4.1. Interestingly, LcS strains showed significant difference ($p = 0.023$) viable cell counts in MRSb, MRSp, MRS and MRSc. Further analysis indicated that the LcS's growth in MRSp (8.60 ± 0.35 log

CFU/mL) was significantly difference than in the MRS broth ($9.31 \pm 0.28 \log$ CFU/mL). For all of the cultivation media that had been inoculated with LcS strains, it showed that the viable cell counts at the end of the incubation period were similar ($p > 0.05$) and were as high as 8.6 - 9.3 log CFU/mL.

Table 4.1: Final fermentation (24-h) of CFU/mL counts (*N*) for the LcS employed with four different carbon sources by using One-Way ANOVA.

Strains	Carbon sources	N (log CFU/ml)		
		Mean ± SD	F-value	p-value
<i>Lactobacillus casei</i>	Bagasse powder	9.17 ± 0.14 ^{ab}	5.550	0.023
Strain Shirota (LcS)	Peel powder	8.60 ± 0.35 ^a		
	Glucose	9.31 ± 0.28 ^b		
	Commercial MRS	8.84 ± 0.10 ^{ab}		
	broth			

Results are expressed as mean ± standard deviation (n=4). Values with different letter in the same column are significant ($p < 0.05$) between the carbon sources in the cultured media.

The finding of this study shows significant difference of viable cell counts (*N*) in log CFU/ml was in line with some reported previous studies. Based on Diaz-Vela et al. (2013), the final viable count (*N*) of microorganisms was significantly ($p < 0.05$) higher for two strains which are *P. pentosaceus* and lower for *E. coli* when carbon source was replaced by cactus peel flour and pineapple flour. However, there was no significant difference ($p > 0.05$) for final viable count by carbon source type. In that study, some species of microorganisms were used such as *Pediococcus pentosaceus*, *Aerococcus viridians*, *Escherichia coli* and also lactic acid bacteria

Lactobacillus rhamnosus. Those bacteria still could consume carbon source from those agricultural by-products. Table 4.2 shows comparison for final viable cell counts (N) in log CFU/ml for this study and from Diaz-Vela et al. (2013).

In other study, Sah et al. (2016) stated that the effect of pineapple waste powder (PWP) on probiotic growth was insignificant ($p > 0.05$). The viable counts of *S. thermophilus* and *L. bulgaricus* in the yogurts supplemented with PWP showed similar value in comparison to those in yogurts without supplementation. On the other hand, probiotic yogurts with pineapple waste powder experienced enhanced growth of the probiotic species-high counts of *L. acidophilus* (8.13–8.71 log CFU/g) and *L. casei*, *L. paracasei* (8.12–8.72 log CFU/g) than non-supplemented probiotic yogurts (7.34 log CFU/g and 7.83 log CFU/g, respectively). Moreover, the probiotic counts were very high compared to the minimum therapeutic count of 6 log CFU/g, if 100 g yogurt is considered as a daily serving dose (Lourens-Hattingh and Viljoen, 2001). Table 4.2 shows comparison between the viable counts of probiotic in this study and also in Sah et al. (2016) study.

Table 4.2: Final fermentation (24-h) of CFU/mL counts (*N*) for the LcS and other bacteria employed with different carbon sources in comparison with previous study.

Strains	Carbon sources	<i>N</i> (log CFU/ml)	
		Mean±SD	
<i>Lactobacillus casei</i> Strain Shirota (LcS)	Bagasse powder	9.17 ± 0.14	
	Peel powder	8.60 ± 0.35	
<i>Pediococcus pentosaceus</i>	Cactus pear flour	9.65 ± 0.25	
	Pineapple flour	9.56 ± 0.31	
<i>Aerococcus viridians</i>	Cactus pear flour	8.73 ± 0.25	
	Pineapple flour	9.33 ± 0.13	
<i>Lactobacillus rhamnosus</i>	Cactus pear flour	8.77 ± 0.15	
	Pineapple flour	9.38 ± 0.26	
<i>Escherichia coli</i>	Cactus pear flour	8.63 ± 0.24	
	Pineapple flour	8.21 ± 0.22	
<i>S. thermophilus</i>	Oven-dried PWP	9.21 ± 0.08	
	Freeze-dried PWP	9.13 ± 0.06	
<i>L. bulgaricus</i>	Oven-dried PWP	8.17 ± 0.07	
	Freeze-dried PWP	8.23 ± 0.13	
<i>L. acidophilus</i>	Oven-dried PWP	8.25 ± 0.02	
	Freeze-dried PWP	8.71 ± 0.08	
<i>L. casei</i> and <i>L. paracasei</i>	Oven-dried PWP	8.11 ± 0.03	
	Freeze-dried PWP	8.73 ± 0.08	

Adapted from Diaz-Vela et al. (2013) and Sah et al. (2016).

In parallel with previous study that portrayed in Table 4.2, by the Diaz-Vela et al. (2013) and Sah et al. (2016), respectively, it reported that comparison of final viable cell counts (N) at the end of fermentation (24 h) was almost similar with this findings. There was no significant difference ($p > 0.05$) for the viable cell counts (N) in log CFU/ml of LcS and other probiotics employed in different agro industrial by-products as carbon source. Most of the viable cell counts (N) of the probiotics were as high as 8.0 - 9.7 log CFU/mL. Clearly, it shows that almost all of the carbon sources from the agro industrial by-products that have been studied successfully promoted a favourable growth of probiotics.

4.2.1.2 The final pH of prebiotic samples

The final pH of prebiotic samples were determined by measuring the pH of modified MRS broth after cultivation of LcS in 24 hours. The initial pH values of all modified MRS broth were set at 6.70 ± 0.2 pH. The pH value plays an important role to determine whether short chain fatty acid (SCFA) were produced or not by the LcS. Basically, the colonization and fermentation of probiotics in the colon produce SCFA such as acetic, butyric, lactic and propionic acid. The production of SCFA by probiotics in the colon will reduce the pH value and this condition is helpful in inhibiting colonization of pathogenic bacteria. Besides, this condition also will enhance and strengthening immune system as preventive measures to reduce incidence of colon cancer (Rastall et al., 2005).

The cultivation of the LcS strains in MRSb, MRSp, MRS and MRSc decreased in the final pH values of cultivation media (to approximately 4.44), mainly

during the first 24 hours. The media’s pH was reduced following the fermentation, but the final pH was not significantly different ($p > 0.05$) between MRSb (5.71 ± 0.03), MRSp (5.53 ± 0.70) and MRS (5.69 ± 0.11), with an exception of MRSc (4.44 ± 0.07). The detection of high viable cell counts and decreases in final pH values of growth media during cultivation indicate the intense metabolic activities of the inoculated LcS strains in broth containing either of the added carbon sources. The intense bacterial metabolic activity in broth containing those carbon sources are related to beneficial effects to the host that have been directly associated with decreased pH and increased production of organic acids in the colon. Under these conditions, the growth of certain pathogenic bacteria is inhibited, while the growth of *Lactobacillus* and other beneficial bacteria is stimulated (Duarte et al., 2017).

Table 4.3: Final pH of modified prebiotic samples at the end of fermentation (24 h) for the LcS employed with the different carbon sources by using One-Way ANOVA.

Strain	Carbon sources	Final pH		
		Mean±SD	F-value	p-value
<i>Lactobacillus casei</i>	Bagasse powder	5.71 ± 0.03 ^b	190.08	<0.001
Strain Shirota (LcS)	Peel powder	5.53 ± 0.07 ^b		
	Glucose	5.67 ± 0.11 ^b		
	Commercial MRS	4.44 ± 0.07 ^a		
	broth			

Results are expressed as mean ± standard deviation (n=4). Values with different letter in the same column are significant ($p < 0.05$) between the carbon sources in the cultured media.

Interestingly, similar findings were reported by Duarte et al., (2017) for the study of prebiotic properties of cashew apple agro industrial by-products on different species of probiotics. The results of their findings showed that the cultivation of *Lactobacillus* strains in broth containing glucose, fructooligosaccharides or cashew apple powder resulted in a reduction of final pH values of cultivation media (to approximately 4.0), mainly during the first 24 hours. However, for the same inoculated strain, no differences ($p > 0.05$) in final pH values in the different cultivation media were observed over time. As mentioned earlier, a decrease in pH values indicated intense metabolic activities by the inoculated *Lactobacillus* strain in different carbon sources added. Table 4.4 shows the final pH (24-hours) of prebiotic samples for the LcS and other bacteria employed with different carbon sources.

Furthermore, other study from Sah et al., (2016) also concluded that the pH and titratable acidity of all inoculated yogurt samples with pineapple peel powder (PWP) as carbon source were within 4.51–4.48 and 1.01 % – 1.07 %, respectively, within 5 h 45 min. These results were in agreement with the various works on the addition of fibres from fruit by-products to yogurt (Espírito Santo et al. 2012; García-Pérez et al. 2005). The pineapple powders contained dietary fibres, proteins, and minerals as well as fats, which could have served as growth factors for probiotics and therefore improved growth. Similarly, improved growth would have resulted in high metabolic activities and hence proteolysis (Donkor et al. 2007). So this was also parallel with the finding from this study that used sugarcane bagasse and peel as carbon sources. Table 4.4 shows the final pH (24-hours) of prebiotic samples for the LcS and other bacteria employed with different carbon sources.

Table 4.4: Final pH (24-h) of prebiotic samples for the LcS and other bacteria employed with different carbon sources in comparison with previous study.

Strains	Carbon sources	Final pH
		Mean±SD
<i>Lactobacillus casei</i> Strain Shirota	Bagasse powder	5.71 ± 0.03
	Peel powder	5.53 ± 0.07
<i>Lactobacillus acidophilus</i>	Glucose	4.05 ± 0.02
	Fructooligosaccharides	4.04 ± 0.12
	Cashew apple powder	4.13 ± 0.03
<i>Lactobacillus casei</i>	Glucose	4.00 ± 0.16
	Fructooligosaccharides	4.08 ± 0.03
	Cashew apple powder	4.19 ± 0.07
<i>Lactobacillus paracasei</i>	Glucose	4.07 ± 0.05
	Fructooligosaccharides	4.11 ± 0.02
	Cashew apple powder	4.18 ± 0.01
<i>S. thermophilus</i>	Oven-dried PWP	4.51 ± 0.02
	Freeze-dried PWP	4.49 ± 0.02
<i>Lactobacillus bulgaricus</i>	Oven-dried PWP	4.50 ± 0.02
	Freeze-dried PWP	4.49 ± 0.01
<i>Lactobacillus acidophilus</i>	Oven-dried PWP	4.49 ± 0.02
	Freeze-dried PWP	4.48 ± 0.02
<i>Lactobacillus casei</i> and <i>L. paracasei</i>	Oven-dried PWP	4.48 ± 0.02
	Freeze-dried PWP	4.49 ± 0.02

Adapted from Duarte et al. (2017) and Sah et al. (2016).

In comparison with previous studies, it indicated slightly differences on the pH values of prebiotic samples added with different type of carbon sources from agro industrial by-products. This shows that most of the agro industrial by-products can be good source of nutrient for the probiotic growth. This also can be related to the production of SCFA during probiotic fermentation that cause lower pH value and this can inhibit pathogenic bacteria and also reduce incidence of colon cancer.

4.2.1.3 The mean growth rate (k) and mean duplication time (g) of LcS

For the last parameter on LcS growth performance, mean growth rate constant (k) and mean duplication time (g) were determined by using equations from Willey et al. (2008). Maier (2009) stated that during exponential growth the rate of increase of cells in the culture is proportional to the number of cells present at any particular time. There are several ways to express this concept both theoretically and mathematically. One way is to imagine that during exponential growth the number of cells increases in the geometric progression $2, 2^1, 2^2, 2^3$ until, after n divisions, the number of cells is 2^n . Besides, the time it takes for a cell division to occur is called the generation time or the doubling time. So bacterial growth rates during the phase of exponential growth, under standard nutritional conditions (culture medium, temperature, pH, etc.), define the bacterium's generation time.

In this study, there was no significant difference ($p = 0.835$) for the mean growth rate constant (k) was obtained during fermentation of LcS in MRSb (2.16 ± 0.10), MRSp (2.09 ± 0.08), MRS (2.15 ± 0.06) and MRSc (2.19 ± 0.23) after 24 hours of incubation. Also, the results shows no significant difference ($p = 0.469$) for

the mean duplication time (g) of LcS in MRSb (0.45 ± 0.02), MRSp (0.47 ± 0.02), MRS (0.45 ± 0.02) and MRSc (0.44 ± 0.04) after 24 hours of incubation at 37 °C. Similarities in the growth rate of the LcS could be attributed to same individual preferences for monosaccharides instead of carbohydrates with higher degree of polymerisation (Gopal et al., 2001). Amaretti et al. (2006) proposed several mechanisms for the use of polymeric carbohydrates, either intracellularly or extracellularly, hydrolysing monomeric components for easy assimilation. Because the monomeric composition, degree of polymerisation and type of glycosidic linkages can affect the growth of probiotic strains (Rastall et al., 2005), it is expected that the strains used consumed the monosaccharides present in each bagasse and peel powder first, but polymeric compounds such as fructooligosaccharides were also metabolised (Habibi et al., 2008). Kaplan & Hutkins (2000), using commercial fructooligosaccharides as prebiotic, reported that the use of fructooligosaccharides does not require an introduction period being as good a substrate as glucose in supporting probiotic bacteria growth. Growth with different carbon sources as the probiotic is strain-dependent and expresses additional potential for dietary and functional foods implementation (Georgieva et al., 2009). The higher reduction of acidification is related to increased carbohydrate metabolism (Ogunbanwo & Okanlawon, 2009). According to the results obtained under the particular experimental conditions, bagasse and peel powder improved metabolism because higher reduction of pH values, higher growth rates and had lower duplication time.

Table 4.5: Fermentation kinetics, k (mean growth rate constant) for LcS strains employed with the different carbon sources by using One-Way ANOVA.

Strain	Carbon sources	Mean growth rate constant (k) h		
		Mean±SD	F-value	p-value*
Lactobacillus casei	Bagasse powder	2.16±0.10	0.285	0.835
Strain Shirota (LcS)	Peel powder	2.09±0.08		
	Glucose	2.15±0.06		
	Commercial MRS	2.19±0.23		
	broth			

*p-value significant at <0.05 (n=4)

Table 4.6: Mean generation time, g (mean duplication time) for LcS strains employed with the different carbon sources by using One-Way ANOVA.

Strain	Carbon sources	Mean generation time (g) h ⁻¹		
		Mean±SD	F-value	p-value*
Lactobacillus casei	Bagasse powder	0.45±0.02	0.930	0.469
Strain Shirota (LcS)	Peel powder	0.47±0.02		
	Glucose	0.45±0.02		
	Commercial MRS	0.44±0.04		
	broth			

*p-value significant at <0.05 (n=4)

According to Diaz-Vela et al., (2013), agro industrial by-products cactus pear (*Opuntia ficus-indica* L.) peel and pineapple (*Ananas comosus*) peel as functional ingredients show comparable findings with this finding. Their study showed a significantly higher ($p < 0.05$) means growth rate constant (k) was obtained with 1.0% concentration of carbon source. Nonetheless, there was no significant ($p >$

0.05) difference for carbon source concentration for the rest of the kinetic parameters. *Pediococcus pentosaceus* presented significantly ($p < 0.05$) higher values than the other microorganisms, where *E. coli* obtained the lowest for specific growth rate (k). Conversely, the lower mean generation time (g) was significantly ($p < 0.05$) lower for *P. pentosaceus*. Glucose as carbon source resulted in significantly ($p < 0.05$) higher mean growth rate constants and significantly ($p < 0.05$) lower mean generation times. Table 4.7 shows the fermentation kinetics, k (growth rate constant) and mean generation time, g (mean duplication time) for the LcS and other bacteria employed with different carbon sources.

Table 4.7: The fermentation kinetics, k (growth rate constant) and mean generation time, g (mean duplication time) LcS and other bacteria employed with different carbon sources in comparison with previous study.

Strains	Carbon sources	Growth rate constant (k) h		Mean generation time (g) h ⁻¹	
		Mean±SD		Mean±SD	
<i>Lactobacillus casei</i> Strain Shirota (LcS)	Glucose	2.15 ± 0.06		0.45 ± 0.02	
	Bagasse powder	2.16 ± 0.10		0.45 ± 0.02	
	Peel powder	2.09 ± 0.08		0.47 ± 0.02	
<i>Pediococcus pentosaceus</i>	Glucose	1.52 ± 0.00		0.66 ± 0.00	
	Cactus peel flour	1.42 ± 0.01		0.71 ± 0.01	
	Pineapple peel flour	1.44 ± 0.01		0.76 ± 0.00	
<i>Aerococcus viridans</i>	Glucose	1.13 ± 0.02		0.90 ± 0.01	
	Cactus peel flour	0.81 ± 0.09		1.05 ± 0.16	
	Pineapple peel flour	1.07 ± 0.01		0.98 ± 0.00	
<i>Lactobacillus rhamnosus</i>	Glucose	1.36 ± 0.01		0.72 ± 0.01	
	Cactus peel flour	0.99 ± 0.01		0.86 ± 0.01	
	Pineapple peel flour	1.19 ± 0.02		0.82 ± 0.01	
<i>Escherichia coli</i>	Glucose	0.98 ± 0.16		1.25 ± 0.18	
	Cactus peel flour	0.63 ± 0.00		1.70 ± 0.01	
	Pineapple peel flour	0.62 ± 0.16		1.68 ± 0.45	

Adapted from Diaz-Vela et al. (2013).

Likewise, the similar findings was exhibited in the study of Diaz-Vela et al., (2013), it reported that there was not much different on the comparison of fermentation kinetics, k (growth rate constant) and mean generation time, g (mean duplication time). It shows that by using almost different type of agro industrial by-products as carbon source, most microorganisms including probiotic LcS have almost similar growth rate constant, k and mean generation time, g . This also indicated that the agro industrial by-products have promoted favourable growth of LcS.

4.2.2 Prebiotic Potential: Prebiotic Activity Scores of LcS

The prebiotic activity score was determined according to the relationship described by Huebner et al., (2007), considering the growth of each bacteria during fermentation using sugarcane bagasse or peel and glucose as carbon source. According to study, by definition, substrates with a high prebiotic activity score support growth of the probiotic bacteria, with cellular growth comparable to that observed when grown on glucose. The cellular growth of the enteric strains grown on the prebiotics should, in theory, be very low in relation to growth on glucose. In this study, *E. coli* was used as enteric reference strain and basically this enteric strain would have very low growth on prebiotic medium.

For the prebiotic activity score, significantly ($p < 0.05$) higher values were found for MRSc than MRSb and MRSp. There was no significant ($p > 0.05$) difference for the carbon source that is, both sugarcane bagasse and peel powder had the same effect on LcS (Table 4.8). This result indicated that the proposed

ingredients had a positive score if they were metabolised selectively by probiotics but not by other microorganisms, like pathogens. If the growth of the tested microorganism is lower than that of glucose, the scores are low or negative. Huebner et al. (2007) reported different prebiotic activity scores for different microorganisms. For example, with inulin, *Lactobacillus paracasei* obtained a prebiotic activity score of 1.17, whereas with glycooligosaccharides as carbon source, the scores for *Lactobacillus plantarum* and *Bifidobacterium bifidum* were 0.82 and 1.24, respectively. Most bifidobacteria with different prebiotics obtained scores in the range of 0.2-0.4. These values match those obtained in this work, because LcS obtained prebiotic activity scores of 0.23 and 0.18 for bagasse and peel powder, respectively. According to Huebner et al. (2007), microorganisms react differently to different sugars. This is important for symbiotic food formulation, although other fermentable sugars and microorganisms are present. In this regard, according to Huebner et al. (2007), prebiotic activity reflects the ability of a given substrate to support the growth of microorganisms compared with other microorganisms, and compared to growth on a non-prebiotic substrate, such as glucose.

In this case, LcS obtained higher prebiotic activity scores, higher growth and higher acidification parameters, indicating a better assimilation of the fermentable carbohydrates present in the bagasse and peel powder. No difference between bagasse and peel powder indicate that the carbohydrates are equally available to be fermented. Positive effects were observed using this sugarcane as agro-industrial by-products, indicating that, under the experimental conditions used; these ingredients had a positive prebiotic effect although with differences in specific growth and

acidification parameters as a consequence of different structures of carbon sources (Hopkins et al., 1998; Cardelle-Cobas et al., 2011).

Table 4.8: Prebiotic activity score for the LcS employed by using One-Way ANOVA.

Strain	Carbon Sources	Prebiotic activity score		
		Mean±SD	F-value	p-value*
<i>Latobacillus casei</i>	Bagasse powder	0.23±0.01 ^a	168.94	<0.001
Strain Shirota (LcS)	Peel powder	0.18±0.01 ^a		
	Commercial MRS broth	2.30±0.28 ^b		

Results are expressed as mean ± standard deviation (n=3). Values with different letter in the same column are significant ($p < 0.05$) between the carbon sources in the cultured media.

The growth of LcS on bagasse and peel powder as carbon source shows no significant difference ($p > 0.05$) and this indicated that s ugarcane by-products had same effect on LcS growth. This finding of this study was comparable with the previous study. According to Duarte et al. (2017) and Diaz-Vela et al. (2013) show no significant difference growth of bacteria including probiotic bacteria on different carbon sources (cashew apple peel, cactus pear peel and pineapple peel powder). It also shows that LcS and other probiotics bacteria could selectively metabolized agro industrial by-products as carbon sources. Table 4.9 shows the prebiotic activity scores for the LcS and other bacteria employed with different carbon sources.

Table 4.9: Prebiotic activity scores of LcS and other bacteria employed with different carbon sources in comparison with previous study.

Strains	Carbon sources	Prebiotic activity scores	
		Mean±SD	
<i>Lactobacillus casei</i> Strain Shirota (LcS)	Bagasse powder Peel powder	0.23 ± 0.01 0.18 ± 0.01	
<i>Lactobacillus acidophilus</i>	Cashew apple powder Fructooligosaccharide	0.89 ± 0.05 0.95 ± 0.05	
<i>Lactobacillus casei</i>	Cashew apple powder Fructooligosaccharide	0.86 ± 0.03 0.91 ± 0.02	
<i>Lactobacillus paracasei</i>	Cashew apple powder Fructooligosaccharide Inulin	0.95 ± 0.04 0.94 ± 0.03 1.17 ± 0.04	
<i>Pediococcus pentosaceus</i>	Cactus pear flour Pineapple peel flour	0.33 ± 0.03 0.32 ± 0.01	
<i>Aerococcus viridans</i>	Cactus pear flour Pineapple peel flour	-0.03 ± 0.02 0.09 ± 0.02	
<i>Lactobacillus rhamnosus</i>	Cactus pear flour Pineapple peel flour	0.19 ± 0.01 0.21 ± 0.02	
<i>Bifidobacterium bifidum</i>	Glycooligosaccharide	-1.24 ± 0.06	
<i>Lactobacillus plantarum</i>	Glycooligosaccharide	0.82 ± 0.03	

Adapted from Duarte et al. (2017) and Diaz-Vela et al. (2013).

CHAPTER 5

CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

5.1 Conclusion

The main objective of this study was to assess the prebiotic potential of sugarcane agro industrial by-products on the growth of probiotic *Lactobacillus casei* strain Shirota (LcS) using an *in vitro* experimental model. The cultivation of LcS in MRS broths containing sugarcane bagasse and peel powder as the sole of carbon source resulted in intense bacterial metabolic activities as observed by high viable cell counts in log CFU/ml, decreased in pH values and acceptable results of mean growth rate constant (k) as well as mean generation time (g) besides obtaining satisfactory prebiotic properties. The sugarcane bagasse and peel powder also presented positive prebiotic activity scores towards LcS, indicating a desirable selective fermentable activity to enteric microorganism. These findings demonstrate that the by-products generated from the sugarcane plant agro industrial processing might be sources of compound capable to promote the selective growth of probiotic LcS. Finally, these findings might stimulate the agro industrial sector to valorize the

by-products from plant, particularly from sugarcane processing as added-value ingredients to the food industry.

5.2 Limitations

This study has opened a range question, which need to be addressed in future work. Some of the limitations found in this study include lack number of strains used compared to other studies that used more than one probiotic and other microorganisms including pathogenic bacteria. Besides, there was only one concentration of sugarcane bagasse and peel powder as carbon source for the probiotic LcS which was 20g/L. Apart from that, more parameters must be conducted such as determination of organic acid and carbohydrate consumption in the prebiotic after fermentation of the probiotic LcS. Last but not least, there was insufficient number of instruments causing this study need longer time to be done.

5.3 Recommendations

The following recommendations have been made for the study of prebiotic potential of sugarcane agro industrial by-products on the growth of probiotic. For the first recommendation, different species of probiotic should be used for future study so that different effects can be seen for the fermentation of probiotics based on type of species. Besides, different concentration of carbon source can also be added in the prebiotic samples to see that maybe low or high of carbon source concentration could affect the LcS growth performance. Moreover, determination of types of organic acids could also be done so that future research about the end product of

fermentation of LcS can be studied. Predicted upon that, further research is still needed to understand the potential prebiotic properties of sugarcane bagasse and peel powder on the growth of probiotic LcS.



REFERENCES

- Amaretti, A., Tamburini, E., Bernardi, T., Pompei, A., Zanoni, S., Vaccari, G., ... & Rossi, M. (2006). Substrate preference of *Bifidobacterium adolescentis* MB 239: compared growth on single and mixed carbohydrates. *Applied microbiology and biotechnology*, 73(3), 654-662.
- Agamuthu, P., Khidzir, K. M., & Hamid, F. S. (2009). Drivers of sustainable waste management in Asia. *Waste Management & Research*, 27(7), 625-633.
- Artschwager, E., & Brandes, E. W. (1958). Sugarcane (*Saccharum officinarum* L.). *Sugarcane (Saccharum officinarum L.)*.
- Axelsson, L. (2004). Lactic Acid Bacteria: Classification and Physiology. In: Salminen, S. V., Wright, A., Ouwehand, A., (Ed.). *Lactic Acid Bacteria: Microbiological and Functional Aspects* (pp. 1-66). New York: Marcel Dekker, Inc.
- Baucum, L. E., Rice, R. W., & Schueneman, T. J. (2006). An overview of Florida sugarcane. *University of Florida, Institute of Food and Agricultural Science Extension*. Accessed January, 30, 2007.
- Ben-David, A., & Davidson, C. E. (2014). Estimation method for serial dilution experiments. *Journal of microbiological methods*, 107, 214-221.
- Campbell, J. M., Fahey, G. C., Wolf, B. W. (1997). Selected ingestible oligosaccharides affect large bowel mass, cecal and fecal short-chain fatty acid, pH and microflora in rats. *Journal of Nutrition* 127: 130-136.

- Champagne, C. P., Roy, D., Garner, N. (2005). Challenges in the addition of probiotic cultures to foods. *Critical Review Food Science Nutrition* 45(1): 61-84.
- Chen, H. L., Lu, Y. H., Lin, J., Ko, L. Y. (2000). Effects of fructooligosaccharides on bowel function and indicators of nutritional status in constipated elderly men. *Nutrition Research* 20: 1725-1733.
- Cardelle-Cobas, A., Corzo, N., Olano, A., Peláez, C., Requena, T. & Avila, M. (2011). Galactooligosaccharides derived from lactose and lactulose: influence of structure on *Lactobacillus*, *Streptococcus* and *Bifidobacterium* growth. *International Journal of Food Microbiology*, 149, 81–87.
- Courtois, J. (2009). Oligosaccharides from land plants and algae: production and application in therapeutic and biotechnology. *Current Opinion in Microbiology* 12: 261-273.
- Crittenden, R. G. (1999). Prebiotics. In: Tannock, G. W. Probiotics: A Critical Review. Wymondham: Horizon Scientific Press. Pp 141-156.
- De Leenheer, L., Hoebergs, H. (1994). Progress in the elucidation of the composition of chicory inulin. *Starch* 46: 193-196.
- Diaz-Vela, J., Totosa, A., Cruz-Guerrero, A. E., & Pérez-Chabela, M. (2013). In vitro evaluation of the fermentation of added-value agroindustrial by-products: cactus pear (*Opuntia ficus-indica* L.) peel and pineapple (*Ananas comosus*) peel as functional ingredients. *International Journal of Food Science & Technology*, 48(7), 1460-1467.

- Donkor, O. N., Nilmini, S.L., Stolic, P., Vasiljevic, T., Shah, N. P. (2007). Survival and activity of selected probiotic organisms in set-type yoghurt during cold storage. *Int Dairy J* 17(6):657-665.
- Duarte, F. N. D., Rodrigues, J. B., da Costa Lima, M., Lima, M. D. S., Pacheco, M. T. B., Pintado, M. M. E., & de Souza, E. L. (2017). Potential prebiotic properties of cashew apple (*Anacardium occidentale* L.) agro-industrial byproduct on *Lactobacillus* species. *Journal of the Science of Food and Agriculture*.
- Durieux, A., Fougnes, C., Jacobs, H., Simon, J. P. (2001). Metabolism of chicory fructooligosaccharides by bifidobacteria. *Biotechnology Letters* 23: 1523-1527.
- Espírito Santo, A. P., Cartolano, N. S., Silva, T.F, Soares, FASM., Gioielli, L. A., Perego, P., Converti, A., Oliveira, M. N. (2012). Fibers from fruit byproducts enhance probiotic viability and fatty acid profile and increase CLA content in yoghurts. *Int J Food Microbiol* 154 (3):135–144.
- FAO/ WHO. (2001). Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. Cordoba, Argentina, 1-4 October. http://ftp.fao.org/es/esn/food/probio_report_en.pdf
- FAO/ WHO. (2006). Probiotics in food. Health and nutritional properties. *FAO Food and Nutrition Paper*, Rome, Italy 85.

- Federici, F., Fava, F., Kalogerakisc, N. & Mantzavinos, D. (2009). Valorisation of agro-industrial by-products, effluents and waste: concept, opportunities and the case of olive mill wastewaters. *Journal of Chemical Technology and Biotechnology*, 84, 895- 900.
- Franck, A.M.E. (2000). Inulin and Oligofructose. In: Gibson., G., Angus, F. (Ed.). LFRA Ingredient Handbook: Prebiotics and Probiotics (pp. 1-18). Surrey Leatherhead Publishing.
- Fuller, R. (1992). Probiotics. The Scientific Basis. London, U.K: Chapman and Hall.
- García-Pérez, FJ., Lario, Y., Fernández-López, J., Sayas, E., Pérez-Alvarez, J. A., Sendra, E. (2005). Effect of orange fiber addition on yogurt color during fermentation and cold storage. *Color Res Appl* 30(6):457–463.
- Georgieva, R., Koleva, P., Nikolova, D., Yankov, D., & Danova, S. (2009). Growth parameters of probiotic strain *Lactobacillus plantarum*, isolated from traditional white cheese. *Biotechnology & Biotechnological Equipment*, 23(sup1), 861-865.
- Gibson, G. R. (2004). Prebiotic. *Journal Gastroenterology Supplement* 18(2): 287-298.
- Gibson G. R., Probert H. M., Van Loo J. A. E., and Roberfroid M. B. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr. Res. Rev.*, 17:257–259 (2004).
- Gibson, G. R., Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota-introducing the concept of prebiotics. *Journal of Nutrition* 125: 1401- 1412.

Gopal, P. K., Sullivan, P. A., & Smart, J. B. (2001). Utilisation of galacto-oligosaccharides as selective substrates for growth by lactic acid bacteria including *Bifidobacterium lactis* DR10 and *Lactobacillus rhamnosus* DR20. *International Dairy Journal*, 11(1-2), 19-25.

Growth of Bacterial Population. Retrieved April 10, 2018 from <https://www.britannica.com/science/bacteria/Growth-of-bacterial-populations#ref955457>

Habibi, Y., El-Zawawy, W. K., Ibrahim, M. M., & Dufresne, A. (2008). Processing and characterization of reinforced polyethylene composites made with lignocellulosic fibers from Egyptian agro-industrial residues. *Composites Science and Technology*, 68(7-8), 1877-1885.

Hammes, W. P., Vogel, R. F. (1995). The genus *Lactobacillus*. In: Wood, B.J.B., Holzapfel, W. H. (Ed). *The Genera of Lactic Acid Bacteria* (pp. 19-49). London: Blackie Academic & Professional.

Hansen, L. T., Allan-Wojtas, P. M., Jin, Y. L., & Paulson, A. T. (2002). Survival of Ca- alginate microencapsulated *Bifidobacterium* spp. in milk and simulated gastrointestinal conditions. *Food microbiology*, 19(1), 35-45.

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., ... & Calder, P. C. (2014). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature reviews Gastroenterology & hepatology*, 11(8), 506-514.

- Hopkins, M. J., Cummings, J. H., Macfarlane, G. T. (1998). Inter-species differences in maximum specific growth rates and cell yields of bifidobacterium cultured on oligosaccharides and other simple carbohydrate sources. *Journal of Applied Microbiology* 85: 381-388.
- Horwitz, W., Latimer, Jr. G. W. (2006). Official methods of analysis of AOAC international. AOAC International, AOAC International, Gaithersburg, Md.
- Huang C., Song P, Fan P, Hou C, Thacker P and Ma X, (2015). Dietary sodium butyrate decreases postweaning diarrhea by modulating intestinal permeability and changing the bacterial communities in weaned piglets. *J. Nutr.*, 145:2774-2780
- Huebner, J., Wehling, R.L. & Hutkins, R.W. (2007). Functional activity of commercial prebiotics. *International Dairy Journal*, 17, 770–775.
- Hsu, C. K., Liao, J. W., Chung, Y. C., Hsieh, C. P., Chan, Y.C. (2004). Xylooligosaccharides and fructooligosaccharides affect the intestinal microbiota and precancerous colonic lesion development in rats. *Journal of Nutrition* 134: 1523-1528.
- Kaffka, S. R., & Grantz, D. A. (2014). Sugar Crops. *Encyclopedia of Agriculture and Food Systems*, 5, 240–260. <https://doi.org/10.1016/B978-0-444-52512-3.00150-9>
- Lamsal, B.P. & Faubion, J.M. (2009). The beneficial use of cereal and cereal components in probiotic foods. *Food Reviews International*, 25, 103–114.

- Larrauri, J.A. (1999). New approaches in the preparation of high dietary fibre powders from fruit by-products. *Trends in Food Science and Technology*, 10, 3–8.
- Leang, Y. H., & Saw, H. Y. (2011). Proximate and functional properties of sugarcane bagasse. *Agro Food Industry Hi Tech*, 22(2), 5.
- Lee, Y. K., Salminen, S. (1995). The coming of age of probiotics. *Trends Science Technology* 6: 241-245.
- Liu H, Zhang J, Zhand S, Yang F, Thacker PA, Zhang G, Qiao S and Ma X, Oral administration of *Lactobacillus fermentum* I5007 favors intestinal development and alters the intestinal microbiota in formula-fed piglets. *J. Agricult. Food Chem.*, 62:860–866 (2016).
- Lourens-Hattingh, A., & Viljoen, B. C. (2001). Yogurt as probiotic carrier food. *International dairy journal*, 11(1-2), 1-17.
- Macfarlane, G. T., Cummings, J. H., (1999). Probiotics and prebiotics: can regulating the activities of intestinal bacteria benefit health?. *British Medical Journal* 318: 999-1003.
- Maier, R. M. (2009). Bacterial Growth. *Environmental Microbiology*, 37–54.
<https://doi.org/10.1016/B978-0-12-370519-8.00003-1>
- Marconi, E., Messiaa, M. C., Amineb, A., Mosconec, D., Vernazzad, F., Stocchild, F., Palleschic, G. (2004). Heat-treated milk differentiation by a sensitive lactulose assay. *Food Chemisty* 84(3): 447-450.

- Morris, C., Morris, G. (2012). The effect of inulin and fructooligosaccharide supplementation on the textural, rheological and sensory properties of bread and their role in weight management: A review. *Food Chemistry* 133(2): 237-248.
- Nagendra, R., Viswanatha, S., Arun Kumar, S., Krishna Murthy, B., Venkat, S. (1995). Effect of feeding milk formula containing lactulose to infants on faecal bifidobacterial flora. *Nutrition Research* 15(1): 15-24.
- Nawirska, A. & Kwasniewska, M. (2005). Dietary fibre fractions from fruit and vegetable processing waste. *Food Chemistry*, 91, 221–225.
- Nikbakht Nasrabadi, E., Jamaluddin, R., Abdul Mutalib, M. S., Khaza'ai, H., Khalesi, S., & Mohd Redzwan, S. (2013). Reduction of aflatoxin level in aflatoxin-induced rats by the activity of probiotic *Lactobacillus casei* strain Shirota. *Journal of Applied Microbiology*, 114(5), 1507–1515. <https://doi.org/10.1111/jam.12148>
- Niness K. R. (1999). Nutritional and health benefits of inulin and oligofructose. *Journal of Nutrition* 129: 1402S-1406S.
- Ogunbanwo, S. T., & Okanlawon, B. M. (2009). Influence of nutrients utilization and cultivation conditions on the production of lactic acid by homolactic fermenters. *Biotechnology*, 8(1), 107-113.
- Oreopoulou, V. & Tzia, C. (2007). Utilization of plant by-products for the recovery of proteins, dietary fibers, antioxidants, and colorants. In: *Utilization of By-Products and Treatment of Waste in the Food Industry* (edited by V. Oreopoulou & W. Russ). Pp. 209–232. New York: Springer.

- Ouwehand, A. C., Kirjavainen, P. V., Shortt, C., Salminen, S. (1999). Probiotics: Mechanisms and established effects. *Journal of International Dairy* 9: 43-52.
- Pandey, A., Soccol, C. R., Nigam, P., & Soccol, V. T. (2000). Biotechnological potential of agro-industrial residues. I: sugarcane bagasse. *Bioresource technology*, 74(1), 69-80.
- Rastall, R. A. (2011). Chapter 9. Toward Second-Generation Carbohydrate Functional Food Ingredients. In: Paeschke, T. M., Aimutis, W. R. (Ed). *Nondigestible Carbohydrates and Digestive Health* (pp. 223-225). USA: Blackwell Publishing Ltd and Institute of Food Technologies.
- Rastall, R. A., Gibson, G. R., Gill, H. S., Guarner, F., Klaenhammer, T. R., Pot, B., ... & Sanders, M. E. (2005). Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: an overview of enabling science and potential applications. *FEMS microbiology ecology*, 52(2), 145-152.
- Roberfroid, M. B. (2001). Prebiotics: preferential substrates for specific germs?. *American Journal Clinical Nutrition* 73(2): 406S-409s.
- Roberfroid, M. B. (2002). Functional food concept and its application to prebiotics. *Digestive and Liver Disease* 34(2): S105-S110.
- Roberfroid, M. B., Salvin, J. (2000). Nondigestible oligosaccharides. *Critical Review Food Science Nutrition* 40: 461-480.

- Sah, B. N. P., Vasiljevic, T., McKechnie, S., Donkor, O. N., (2016). Physicochemical, textural and rheological properties of probiotic yogurt fortified with fibre-rich pineapple peel powder during refrigerated storage. *LWT Food Sci Technol* 65:978–986.
- Sako, T., Matsumoto, K., Tanaka, R. (1999). Recent progress on research and applications of non-digestible galacto-oligosaccharides. *International Dairy Journal* 9: 69-80.
- Salminen, S., Roberfroid, M., Ramos, P., Fonden, R. (1998). Prebiotics Substrates and Lactic Acid Bacteria. In: Salminen, S., Wright, A. V. (Ed). *Lactic Acid Bacteria: Microbiology and Functional Aspects*. 2nd Ed (pp. 343-358). New York: Marcel Dekker.
- Sancho SO, Silva ARA, Dantas ANS, Magalhães TA, Lopes GS, Rodrigues S, Costa JMC, Fernandes FAN and Silva MGV, Characterization of the industrial residues of seven fruits and prospection of their potential application as food supplements. *J. Chem.*, doi: 10.1155/2015/264284 (2015).
- Sangnark, A., & Noomhorm, A. (2003). Effect of particle sizes on functional properties of dietary fibre prepared from sugarcane bagasse, *80*, 221–229.
- Seifert, S., Watzl, B. (2007). Inulin and oligofructose: Review of experiment data on immune modulation. *Journal of Nutrition* 137: 2563s-2567s.
- Shah, N. P. (2006). Probiotics and Fermented Milks. In: Chandan, R. C., White, C. H., Kilara, A., Hui, Y. H. (Ed). *Manufacturing Yogurt and Fermented Milks* (pp. 341-354). Oxford: Blackwell Publishing Ltd.

Tamime, A. Y., Saarela, M., Sondergaard, A. K., Mistry V. V., Shah, N. P. (2005). Production and Maintenance of Viability of Probiotic Microorganisms in Dairy Products. In: Tamime, A. Y. (Ed). Probiotic Dairy Products (pp. 39-72). Oxford: Blackwell Publishing.

Thammarutwasik P, Hongpattarakere T, Chantachum S, Kijroongrojana K, Itharat A, Reanmongkol W, Tewtrakul S, Ooraikul B (2009) Prebiotics – a review. *Songklanakarin J Sci Technol* 31(4):401–408

Tuohy KM, Probert HM, Smejkal CW, Gibson GR, Using probiotics and prebiotics to improve gut health. *Drug Discov. Today*, 8:692-700 (2003).

Van Loo, J. (2004). The specificity of the interaction with intestinal bacterial fermentation by prebiotics determines their physiological efficacy. *Nutrition research reviews*, 17(1), 89-98.

Weijers, C. A. G. M., Franssen, M. C. R., Visser, G. M. (2008). Glycosyltransferase-catalyzed synthesis of bioactive oligosaccharides. *Biotechnologies Advances* 26: 436-456.

Willey, J.M., Sherwood, L.M. & Woolverton, C.J. (2008). *Prescott, Harley and Klein's Microbiology*, 7th edn. Pp. 126–127. New York: McGraw Hill.

Ziemer, C. J., Gibson, G. R. (1998). An overview of probiotics, prebiotics and synbiotics in the functional food concept: Perspective and future strategies. *International Dairy Journal* 8: 473-479.