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POTENTIAL OF ACACIA GUM AS A PREBIOTIC INGREDIENT THROUGH In Vitro STUDIES USING HUMAN FAECAL MICROBIOTA

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy.

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Chair : Shahrul Razid Sarbini, PhD
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There is a growing consensus of acacia gum, it is currently marketed as functional food to improve overall human health particularly the gut. This natural product of Acacia tree is the branched polysaccharide gummy exudate made up of complex biopolymer of arabinose and galactose monosaccharides. It is widely used in African countries and in the Middle East as a traditional medicine. Recently, the consumption of acacia gum has been related to potential health benefits. In terms of its potential prebiotics properties, this may be particular relevant to treat stomach disease. Thus, in this research commercially available acacia gum was investigated in vitro to assess its prebiotic potential. The studied acacia gum was from two different species; *Acacia senegal* and *A. seyal*. The experiment employed an *in vitro* batch culture using colon model to mimic the distal part of human large intestine. This batch culture system allows rapid response in monitoring performance of acacia gum fermented by beneficial colonic microbes such as *bifidobacteria* and *lactobacilli*. Inoculation of gut microbiota from human faeces into the model start the fermentation system, continuously administered with automated pH-controller maintained at 37ºC. Samples taken at 0, 6, 12, and 24 hours of incubation were brought for short chain fatty acids (SCFA) analysis using HPLC and bacterial enumeration via fluorescent *in situ* hybridization (FISH) methods. Experiment were repeated four times with fructo-oligosaccharides (FOS) as control. Results showed that acacia gum significantly promoting *Bifidobacteria* proliferation at the end of fermentation to the same extend benefit of that in FOS while significantly inhibit the growth of pathogenic *Clostridium histolyticum* group which can be an important target group commonly associate with gut dysbiosis. The metabolites analysed for acetate, propionate, and butyrate showed a similar trend as FOS ($p > 0.05$). Further evaluation of AG was observed in the independent enrichment culture study of acacia gum. Five bacteria strains were isolated ought to be gum-fermenting bacteria of which based on 16s RNA sequence result matched to *Escherichia fergusonii*. Here, isolated *Escherichia fergusonii* shows butyrate producing activity while fermenting AG. This effects may have related to the

lowering pH regards to organic acid production. Finally, further investigation to evaluate the isolates and probiotic bacteria associated in a co-culture environment. The symbiotic combination led to increase acetate and overall SCFA production. This effect was more apparent than with the isolates, or probiotic alone treatments. In monoculture of probiotic, it showed that *Bifidobacteria longum* alone does not strive in acacia gum medium whereas co-culture of probiotics and isolated strain showed otherwise. In conclusion, the potential of AG as a prebiotic has been explored. Furthermore, a conceptual relationship has been developed during the co-culture fermentation. These products could show great potential against gut dysbiosis, as acacia gum could promote native gut-probiotics, through the modulation of microbial population and SCFA production especially butyrate, therefore lead to improve overall human health.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

POTENSI GAM AKASIA SEBAGAI RAMUAN PREBIOTIK MELALUI PENILAIAN *In Vitro* MIKROBIOTA TINJA MANUSIA.

Oleh

MUHAMAD HANIF BIN RAWI

Mei 2020

Pengerusi : Shahrul Razid Sarbini, PhD
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Kini masyarakat boleh mendapatkan gam akasia yang diperakui ramai sebagai makanan berfungsi untuk kesihatan menyeluruh termasuk usus manusia. Produk semula jadi ini adalah rembesan gam pokok Akasia dan merupakan polisakarida bercabang yang terdiri daripada biopolimer kompleks monosakarida, arabinosa dan galaktosa. Sejak dahulu lagi, gam akasia telah digunakan secara meluas di negara Afrika dan di timur tengah sebagai ubat tradisional. Kini, penggunaannya adalah berkaitan dengan pemakanan untuk manfaat kesihatan. Dari segi potensinya sebagai prebiotik, ia khusus untuk merawat penyakit perut. Penyelidikan ini bertujuan untuk menilai gam akasia komersial ini sebagai sumber prebiotik menerusi kajian model kolon *in vitro*. Gam akasia yang digunakan adalah dari dua spesies iaitu *Acacia senegal* dan *Acacia seyal*. Eksperimen ini mengguna pakai sistem kultur kumpulan *in vitro* menerusi model kolon untuk meniru bahagian usus besar (distal) manusia. Sistem kultur kumpulan ini membolehkan maklum balas yang segera dalam memantau prestasi gam akasia yang ditapai oleh mikrob kolon bermanfaat seperti *bifidobakteria* dan *laktobasili*. Sistem penapaian ini dimulakan dengan inokulasi mikrobiota dari tinja manusia ke dalam model usus. Sistem ini dilengkapi dengan kawalan pH automatik dan dikekalkan pada suhu 37ºC. Sampel yang diambil pada tempoh 0, 6, 12, dan 24 jam inkubasi, diproses untuk analisa kandungan asid lemak rantaian pendek (SCFA) menggunakan sistem HPLC dan perubahan populasi bakteria melalui penghibridan *in situ* pendafluor (FISH). Eksperimen diulang sebanyak empat kali dan frukto-oligosakarida (FOS) diguna sebagai kawalan. Hasil kajian menunjukkan gam akasia menggalakkan pertumbuhan *Bifidobakteria* dengan ketara seperti dalam FOS, dalam masa yang sama menghalang pertumbuhan patogen *Clostridium histolyticum,* yang mana kumpulan sasaran ini sering dikaitkan dengan punca penyakit berkaitan usus. SCFA (asetat, propionat, dan butirat) yang dianalisis menunjukkan kadar pengeluaran yang sama seperti FOS ($p > 0.05$). Penilaian gam akasia selanjutnya diperhatikan melalui kajian kultur gam akasia yang berasingan. Menerusi kajian ini, lima bakteria dipencil mempunyai keupayaan untuk menapai

gam akasia. Bakteria tersebut adalah dikenal pasti daripada spesies *Escherichia fergusonii*. Di sini, *Escherichia fergusonii* yang dipencil menunjukkan aktiviti kadar penghasilan butirat yang tinggi semasa proses penapaian gam akasia. Kesan ini boleh dikaitkan dengan penurunan pH hasil pengeluaran asid organik. Akhirnya, siasatan lanjut dibuat untuk menilai *Escherichia fergusonii* dan probiotik dalam kaedah kokultur. Gabungan kokultur menunjukkan peningkatan asetat dan SCFA secara menyeluruh. Kesan tersebut adalah lebih ketara berbanding dengan rawatan *Escherichia fergusonii*, atau probiotik secara berasingan. Berdasarkan monokultur probiotik, *Bifidobacteria longum* secara sendiri tidak berupaya menggunakan gam akasia manakala melalui kokultur probiotik bersama *Escherichia fergusonii* menunjukkan keupayaan yang positif. Kesimpulannya, potensi gam akasia sebagai prebiotik telah diterokai. Tambahan pula, satu konsep hubungan telah ditunjukkan semasa kokultur. Produk ini boleh menunjukkan potensi yang besar terhadap kesihatan usus, Kesimpulannya, gam akasia dapat menggalakkan aktiviti probiotik, melalui modifikasi populasi mikrob dan penghasilan SCFA, sekali gus dapat meningkatkan kesihatan manusia secara menyeluruh.

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Dedicated to my dearest mother,

This dissertation is in memory of my mother, who inspired me to come to Sarawak and pursue PhD. She would always cheer me up during those tough days. We shared so many laughs and tears together until our family lost her after my second year. I devoted her fortitude of seven years of difficult battle against kidney terminal disease. Her sufferings were very great at times of which would not worthy to be compared with mine. It would take the whole universe to rattle her stoicism, it was edifying for me being around her. Although today she would not be able to read my dissertation, I know she would be very proud of her son making such a big accomplishment!

I certify that a Thesis Examination Committee has met on 5 May 2020 to conduct the final examination of Muhamad Hanif bin Rawi on his thesis entitled "Potential of Acacia Gum as a Prebiotic Ingredient Through *In Vitro* Studies using Human Faecal Microbiota" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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Declaration by graduate student

I hereby confirm that:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

SCFA	Short Chain Fatty Acid
FOS	Fructo-Oligosaccharides
MW	Molecular Weight
HPLC	High Performance Liquid Chromatography
FISH	Fluorescence *in situ* Hybridization
DP	Degree of Polymerisation
LAB	Lactic Acid Bacteria
BCFA	Branched Chain Fatty Acid

CHAPTER 1

INTRODUCTION

1.1 Study Background

Acacia gum (AG) is a soluble secretion found on the trunks and branches of *Acacia senegal* and *Acacia seyal* trees (Mariod *et al.*, 2018). The exudate secreted as a result of subsequent injuries onto the bark of the trees. Acacia gum is among the hydrocolloids that has been exploited in various food and non-food applications. Having exceptionally low viscosity and strong adhesive made acacia gum as the best source of soluble dietary fibre because it can boost fibre levels in foods or beverages without modifying the final viscosity of food products (Samuel *et al.*, 2018).

Most consumers may ever set their hands onto soft drinks containing acacia gum without even knowing it. As the matter of fact, more than three quarter of industrial applications of this hydrocolloid are for emulsification, encapsulation, coating, and confectionaries (Roberfroid, *et al.*, 2010). It is made up of 95% long chain complex polysaccharides, mainly from highly branched galactan polymers, side chains of galactose and/or arabinose; and rhamnose or glucuronic acid as termination residues (Nakov, 2016). With this in mind, looking at its carbohydrate-based structure and functions, acacia gum is an excellent prospect to be commercialised as prebiotic.

Prebiotic is substrate that selectively nourished the host microorganisms in returns to bring health benefits to the host. Some strict criterions needed to be met before any substrates are known as prebiotic. Numbers of test have to be performed where the propose substrate should be; resistant to digestive activity from the upper gastrointestinal gut, fermentable by colonic microbiota and selectively promoting the growth and/or activity of health promoting bacteria commonly *Bifidobacterium* and *Lactobacillus* (Roberfroid, *et al.*, 2010). These bacteria are known to ferment established prebiotic compound i.e. FOS, GOS, and inulin to produce beneficial metabolites known as short chain fatty acids (SCFA) mainly acetate, propionate and butyrate (Pourabedin, Guan, and Zhao, 2015). These SCFA may exert direct effect to host health by lowering luminal pH as most enteric pathogens does not grow well in low pH. Butyrate especially contribute towards the energy requirement by epithelial cells, thus, improving intestinal motility.

These promoting bacteria also been exploited for its good property as probiotic. Probiotic as defined by FDA and WHO (2002) is the live microorganisms which when administered in adequate amounts confer a health benefit to the host. For example, probiotic known to act via a variety of means, including modulation of immune function, association with native gut microbiota, and interacting with the host epithelial cell like gut barrier integrity and enzyme formation. *Lactobacillus*

spp. and *Bifidobacterium* spp. are the most common probiotic bacteria that have been utilized by the food industry (Min *et al.*, 2018), but others such as *Saccharomyces cerevisiae (boulardii), Enterococcus, Bacillus*, and *Escherichia* are also applied. They are mainly saccharolytic metabolism that are capable of carbohydrate utilisation. Therefore, study in the importance of the species specific among particular groups might uncover the health-promoting conditions (Calame *et al.,* 2008).

Years ago, health wellness awareness arose from weight management and superficial disease-relating concern, but now it started to move to digestive health. People nowadays are more aware on probiotic products in their daily food intake. Consumers are more than aware that food can influence health, and there is a clear relationship between the food we consume and how it affects our body.

1.2 Problem Statement

Colonic bacteria metabolised range of carbohydrate hydrolysing enzymes in the production of hydrogen, methane, carbon dioxide, SCFA (mainly acetate, propionate and butyrate), and lactate. This gut flora obtained their energy through fermentation of food supplied from the upper gut (Cherbut, 2003).

Several studies have claimed that dysbiosis, which is the imbalance and unhealthy condition of colon microbiota diversity lead to disorders such as obesity (Turnbaugh *et al.*, 2009), metabolic complications, immunity dysregulation (Kamal *et al.*, 2018), changes in energy and hormone regulation and even irregular inflammatory mechanism (Ali *et al.*, 2020). Dysbiosis in the gut can reflect changes in host systems, typically in metabolic and immune process. Thus, causing diseases such as inflammatory bowel disease (IBD) (Opstelten *et al.*, 2016), psoriatic arthritis (Scher *et al.*, 2015), type 1 and type 2 diabetes (de Goffau *et al.*, 2013; Lambeth *et al.*, 2015), atopic eczema (Wang *et al.*, 2008), coeliac disease (Manichanh *et al.*,2006; Schippa *et al.*, 2010) and arterial stiffness (Menni *et al.*, 2018).

As to counteract the issue studies showed that a healthy diet, lifestyle and habit is the key toward a good human gut. Consequently, a healthy diet is another direct key towards healthy gut diversity. Apart from having molecule rich in sugar complex, prebiotic have to be fermentable by means to escape the digestion and absorption from the upper gastrointestinal tract before progress into the large colon. The knowledge of these properties is important to evaluate efficiency of different acacia gum species and unique composition of polysaccharides as prebiotic. Over the last decades, investigation on AG for its health promoting properties were studied and shown positive effect like regulation of autoimmune disease (Kamal *et al.*, 2018), reduce duodenal inflammation in mice (Ali *et al.*, 2020), anti-ulcerogenic on gastric mucosal injury in rats (Taha *et al.*, 2020), methane gas mitigation in ruminant (Adejero *et al.*, 2019), and also topical treatment of skin lesions of kwashiorkor children (Ali, 2018).

Studies made on acacia gum showed that AG plays the role of prebiotic in the human colon due to its ability to resist human upper gastrointestinal digestion (Phillips, 1998; Kishimoto, 2006). Acacia gum is readily can be used in its natural form. Historically, acacia gum has been commonly consumed by African citizen to treat stomach relating illness. It is also claimed that AG helps in enhancing the absorption of sodium and water in the small intestine into the bloodstream which prevent diarrhoea and dehydration condition (Codipillu and Wapnir, 2004; Rehman *et al.*, 2003; Wapnir *et al.*, 1997). The fermentation of AG in the colon contributing in the production of beneficial metabolites, short chain fatty acids (SCFA) such as acetate, butyrate and propionate in major portion (Kaddam and Kaddam, 2020). The strict demand of healthy food among modern people has made acacia gum that contains large amount of dietary fibre to fulfil the requirement of healthy food.

Although prebiotic effects of AG were previously studied *in vitro* and *in vivo* including humans, but there is an inadequate evidence as some of the researches were not consistent which makes it difficult to scrutinize. Several efforts made as to incorporating AG with probiotic and/or yogurt products (Ghafarloo *et al.*, 2019; Chundakkattumalayil *et al.*, 2019; Talin *et al.*, 2018) that also studied to have promising outcomes. While there is study reported that AG fibre showed bifidogenic effects, which indicating that bifidobacteria can utilised the complex structure of AG (Cherbut *et al.*, 2003).

On the other hand, as reported by Rossi *et al.* (2005), most *Bifidobacterium* species are not capable to utilise longer chain inulin but prefer shorter chain FOS substrates. Furthermore, different observation in mixed faecal cultures as other faecal bacterial species first degrade inulin to oligomers providing preferable chain length for bifidobacterial (Rossi *et al.,* 2005). The capacity to metabolise carbohydrates relies on the availability of functional transport system and intracellular metabolic pathways of gut microbiota (Buntin *et al.,* 2017). In *Lactobacillus acidophilus* and *Bifidobacterium breve*, ABC transporters have been observed to be involved in FOS uptake (Ventura *et al.,* 2007). Therefore, this study addressing the possible pathways of AG fermentation by colonic bacteria. It was done to investigate the uncertain findings on AG fermentation by the group of *Bifidobacteria* spp. describing its capabilities.

1.3 Significance of Study

Today, acacia gum is sought after for its properties as thickener, stabiliser, emulsifier in food products and used in pharmaceutical products as a demulcent (Duke, 2018). The applications of acacia gum tracked to be found since Ancient Egyptian time. Other than for traditional medicine (pain-reliver base) and in everyday applications, Egyptians known for their embalming procedure where they perfected the mummification process using acacia gum as adhesive (Scott, *et al.*, 2004; Scott *et al.*, 2009).

Hardened acacia gum on branch is fragile, fragrance-free and generally tasteless crystals. There are only two species (out of thousands) of acacia gum that are deemed for commercial uses, *Acacia senegal* and *Acacia seyal* (Abuarra *et al.,* 2014). The former is considered the best quality, and made majority for global trade due to low tannin traces and superior emulsifier (Egadu *et al.,* 2007). While the latter produces lower grade gum, *A. seyal* gum with lower market price is being discovered with further uses and thus boosted its value. The uses of *A. seyal* in 'health beverages' as a fermentable has given it a value in its own right (William and Phillips, 2009).

In this study, both species of acacia gum were tested and evaluate for its prebiotic potential. Thus, assessment made should not limit within the perspective of industrial functions only, instead, under a fair consideration, either species could have the same potential and be a good prebiotic candidate. Although many studies have shown some difference between composition of *A. senegal* and *A. seyal* (Vanloot *et al.*, 2012), but not many of those data reporting on the prebiotic aspect of the gums (Musa *et al.*, 2017). The finding from this research can be benefit to further understand current food products, since our usual meal may have incorporated with acacia gum (E414), added as stabilizer ingredients such as in candies, chewing gums, ice cream salad-dressing, bread, cereals and cola drinks.

1.4 Aims and Objectives

This research aims to investigate the impact of commercially available acacia gum as a prebiotic source through *in vitro* colon model study. Therefore, in this study work in vitro approaches have been used to investigate digestibility of AG, changes in gut microbiota and SCFA production. The experiment includes *in vitro* digestion and fermentation of acacia gum in a custom build colon model which mimic the distal part of human colon. Moreover, investigation on the putative primary degrader of acacia gum based on enrichment culture was used to generate more input on the relationship of different microbial species in gut.

The main objectives of this study are:

a. To investigate the nutritional content and digestibility of acacia gum.
b. To determine the physiological significance of prebiotic effects of acacia gum on modulating gut microbiota and its fermentation products, short chain fatty acids in relation with the gut microbiota.
c. To isolate and identify the gum fermenting bacteria through employment of enrichment culture fermentation.
d. To demonstrate the conceptual relationship in co-culture fermentation of acacia gum between probiotic strain with isolated gum fermenting bacteria.

1.5 Scope and Limitation

At least two decades of gut research has passed, lots of the unknown before to the scientists, now becoming clearer. The realisation of the relationship is far more complex and becoming more ambiguous; what does it really mean by the gut environment of having mutualists, commensals and pathogens? Dominant microorganisms inhabit the colon are generally from the phyla *Firmicutes* and *Bacteroidetes* (Walker *et al.,* 2011). Together with the host immune system, gut microbiota contributes significantly as barrier and prevention of invasion of pathogenic bacteria in the gastrointestinal tract. We have known that the microbiota within the colon expressed no straightforward interactions with host metabolic pathway (Tremaroli and Backhead, 2012). At the most basic notion, the relationship is highly mutualistic where the host provides shelter together with the food supply, while the bacteria in return assist in digestion of complex food components apart from supplying vitamin B and K to the host. But among those advance research, many were confirmed the link between diet we consumed and changes on gut microbiota always occurred upon substrate intervention (Ríos-Covián *et al.,* 2016; David *et al.,* 2014).

The fermentation of AG resulted in bacterial proliferation (May *et al.,* 1994). The increase population of colonic bacteria thus promotes the production of beneficial metabolites, i.e. the short chain fatty acids (SCFA) that play an important role in many physiological effects. Based on the study, observation was made based on selective agar medium. During those years the best possible methods to evaluate was using culture dependent techniques. Thus, having selective media that only targeting beneficial bacteria was not sufficient to actually verify the fermentability of AG by colonic bacteria. In addition, it is reported from the study of fermentation in cecum of rats and human that AG can be break down in the colon (Ross *et al.,* 1984; Walter *et al.,* 1988; Phillips, 1998; Phillips, 1998). While this study was evaluated in vivo, there is no direct proof to claimed the findings since the parameters was only evaluate the post-administration of candidate after being fed with AG during the study. Whereas, animal study of Kishimoto *et al.,* in 2006 has found that the predominant microbes responsible for AG fermentation in and contributes in production of propionate is Prevotella ruminicola-like bacterium. While the research was investigating pooled cecal inoculum of pig shown isolated bacteria was the predominant species of the animal. In contrast, sstudied using human and porcine faecal inoculum with 2% of acacia gum managed to isolate *Bifidobacterium longum* and *Bacteroides ovatus, Bacteroides oris, Bacteroides buccae* and *Prevotella ruminicola*-like bacterium (Wyatt *et al.,* 1986). Although the research concluded those results in human faecal, it turns out that presumptive measures led to their conclusions that based only on culture plating techniques but not molecular based DNA sequencing, which only emerged in 1970s that could possibly not available at that time in their lab. Therefore, the traditional detection of many uncultured species assigned to this genus may reflect current taxonomic limitations rather than a biological signal. FISH technique has limit detection 1×10^6, enabling bacteria to be accurately enumerated above this point.

The chemical compositions of AG slightly varied based on origin, sources, climate, season, tree age and species (William and Phillips, 2009). High carbohydrate content within this complex carbohydrate serves as the basic principle to evaluate the prebiotic potential of acacia gum. Species difference of AG were taken into account to see how far will the species affecting the fermentation performance in the colon. Thus, the evaluation of SCFA production and bacteria composition changes due to input of AG as substrate are therefore the foundation for the benefits of human colon health. The limitation in simulating the interaction between host and gut microbes in a fermentation system may as well be one of the criteria condition of a fermentation system. Hence, through this work, it is possible to enhance the understanding for prebiotic applications of acacia gum in various industries particularly nutraceuticals. While FOS had been profoundly studied as prebiotic to regulate and selectively stimulate bacteria population in the colon (Tuohy *et al.,* 2001; Palframan *et al.,* 2002; Hidalgo *et al.,* 2012; Cueva *et al.,* 2013).

Despite that, this current study is the first to evaluate the prebiotic potential in one continuous flow of from the in vitro digestion to the fermentation using the colon model. Apart from adding new insights towards the old knowledge about the prebiotic potential of AG, this study also opens up the new perspectives on substrate-species relationship in the metabolism of food products. In tandem with the advance of technologies and knowledges, the study aims to look at the cross feeding feature in gut microbiota to better understand how such prebiotic products could leverage research in the applications or real-world situation.

1.6 Thesis Layout

This thesis comprises of five chapters, Chapter 1 will introduce the main subjects and a brief background on the topic of the research. Followed by Literature Review in Chapter 2 is the previous research and findings on the subject matter. Also together with the types of available methods that usually was used and can be implement for this type of study. More detail and comprehensive elaborations on the Materials and Methodologies is featured in Chapter 3. Next, in chapter 4 is the parts for Results and Discussions that subdivided into several parts according to it respective objectives. Lastly, the in chapter 5, this research concluded by highlighting the outcomes and whether the objectives were fulfilled.

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