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PREBIOTIC PROPERTIES EVALUATION OF RED SEAWEED (Kappaphycus alvarezii) USING IN VITRO COLON MODEL

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By

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February, 2018

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The red seaweed *Kappaphycus alvarezii* is rich in carrageenan polysaccharides that are used in hydrocolloids industry for gelling and thickening agents in pharmaceutical, biotechnology and food application. It has been claimed that the carrageenan is not digestible by human. Present study builds on that knowledge to test the potential of red seaweed as a prebiotic through *in vitro* digestion and fermentation. The objectives of this study are to determine the digestibility of *K. alvarezii* through *in vitro* gastric and duodenal digestion, to analyze the metabolite short chain fatty acids (SCFAs) generated by the fermentation of seaweeds by using high performance liquid chromatography (HPLC) and to evaluate the fermentation selectivity of seaweeds by the human feces microbiota by using fluorescent in situ hybridization (FISH) technique. The digestibility of red seaweeds (*Kappaphycus alvarezii*) cultivated from Sabah (RSS) and Langkawi (RSL) was determined using *in vitro* gastric and duodenal digestion model that mimicking human gastrointestinal tract condition. The resulting fractions of seaweeds that resisted gastric and duodenal digestion were used as substrates for fermentation in pH-controlled batch culture system inoculated with human feces to mimic human distal colon condition. Inulin was used as positive control and samples were taken at 0, 6, 12 and 24 h of fermentation for bacterial enumeration and SCFAs analysis. Red seaweed from Sabah (RSS) showed no significant difference with inulin for its non-digestibility while red seaweed from Langkawi (RSL) showed the highest digestibility (4.55%) by *in vitro* gastric and duodenal digestion. Both RSS and RSL showed significant increase of *Bifidobacterium* sp.; from $\log_{10}$ 7.96 at 0 h to $\log_{10}$ 8.72 at 24 h, and from $\log_{10}$ 7.96 at 0 h to $\log_{10}$ 8.60 at 24 h respectively, but at 24 h both seaweeds showed no significant difference

when compared to the *Bifidobacterium* sp. count at the end of the inulin fermentation. The growth for *Clostridium* sp. in RSS (from $\log_{10}$ at 0 h 6.78 to $\log_{10}$ 7.27 at 24 h) and RSL (from $\log_{10}$ 6.78 at 0 h to $\log_{10}$ 7.24 at 24 h) also showed no significant difference in comparison to inulin (from $\log_{10}$ 6.78 at 0 h to $\log_{10}$ 7.26 at 24 h). Inulin and RSS showed significant increase in total SCFA production after 24 h of fermentation, particularly in acetate and propionate. While for RSL, the total SCFA showed significant increased at 6 h, however, the total SCFA showed no significant difference after 12 and 24 h. Overall, this data suggested that RSS have potential as a prebiotic similar to the positive control, inulin compared to RSL. This is because RSS shows more bifidogenic factor and high total SCFAs production compare to RSL.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia, sebagai memenuhi keperluan untuk Ijazah Master Sains

PENILAIAN CIRI PREBIOTIK DARIPADA RUMPAI LAUT MERAH (*Kappaphycus alvarezii*) MENGGUNAKAN KOLON MODEL *IN VITRO*

Oleh

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Rumpai laut merah *Kappaphycus alvarezii* diperkaya dengan polisakarida karagenan yang banyak dimanfaatkan dalam industri hydrokoloid sebagai pembentuk gel dan bahan pengentalan untuk industri ubat-ubatan, bioteknologi dan makanan. Karagenan telah dikatakan tidak dihadam oleh tubuh badan manusia. Kajian ini dijalankan untuk menentukan potensi rumpai laut sebagai prebiotik menggunakan kaedah penghadaman dan fermentasi secara *in vitro*. Objektif kajian ini adalah untuk menentukan penghadaman *K. alvarezii* melalui penghadaman gastrik dan duodenum secara *in vitro*, untuk menganalisis metabolit asid lemak rantaian pendek (SCFAs) hasil daripada fermentasi rumpai laut menggunakan sistem kromatografi cecair prestasi tinggi (HPLC) dan untuk menilai selektiviti fermentasi rumpai laut melalui mikrobiota najis manusia menggunakan teknik hybridasi pendaran *in situ* (FISH). Penghadaman rumpai laut merah (*Kappaphycus alvarezii*) yang ditanam di Sabah (RSS) dan Langkawi (RSL) telah diuji menggunakan model *in vitro* terdiri daripada penghadaman gastrik dan duodenum yang menyerupai keadaan saluran gastrousus manusia. Hasil rumpai laut yang berjaya bertahan dengan penghadaman gastrik dan duodenum telah digunakan sebagai substrat untuk fermentasi di dalam sistem fermentasi sesekelompok kawalan pH yang di inokulasi dengan najis manusia untuk menyerupai keadaan kolon desenden manusia. Inulin telah digunakan sebagai kontrol positif dan sampel fermentasi telah diambil pada 0, 6, 12 dan 24 jam untuk pengiraan bakteria dan analisis asid lemak rantaian pendek (SCFAs). Rumpai laut merah dari Sabah (RSS) menunjukkan tidak ada perbezaan yang signifikan dengan inulin berdasarkan sifat ketidak hadamannya manakala rumpai laut merah dari Langkawi (RSL) menunjukkan peghadaman yang paling tinggi (4.55%) melalui penghadaman gastrik dan duodenum secara *in vitro*. Kedua-dua RSS dan RSL masing-masing menunjukkan peningkatan yang signifikan untuk *Bifidobacterium* sp.; daripada $\log_{10}$ 7.96 pada 0 jam kepada $\log_{10}$ 8.72 pada 24 jam, dan daripada $\log_{10}$ 7.96 pada

0 jam kepada $\log_{10}$ 8.60 pada 24 jam, tetapi pada 24 jam kedua-duanya menunjukkan tidak ada perbezaan yang signifikan apabila dibandingkan dengan bilangan *Bifidobacterium* sp. pada akhir fermentasi inulin. Pertumbuhan untuk *Clostridium* sp. dalam RSS (daripada $\log_{10}$ pada 0 jam 6.78 kepada $\log_{10}$ 7.27 pada 24 jam) dan RSL (daripada $\log_{10}$ 6.78 pada 0 jam kepada $\log_{10}$ 7.24 pada 24 jam) juga menunjukkan tidak ada perbezaan yang signifikan apabila dibandingkan dengan inulin (daripada $\log_{10}$ 6.78 pada 0 jam kepada $\log_{10}$ 7.26 pada 24 jam). Inulin dan RSS menunjukkan peningkatan yang signifikan dalam penghasilan jumlah SCFA selepas fermentasi 24 jam, terutamanya dalam kepekatan asetat dan propionat. Manakala untuk RSL, jumlah SCFA menunjukkan peningkatan yang signifikan pada 6 jam namun jumlah SCFA menunjukkan tidak ada perbezaan yang signifikan selepas 12 dan 24 jam. Keseluruhannya, data dalam kajian ini mencadangkan bahawa substrat RSS mungkin mempunyai potensi sebagai prebiotik menyerupai kontrol positif, inulin berbanding dengan RSL. Ini adalah kerana RSS lebih menunjukkan faktor bifidogenik dan jumlah penghasilan SCFA yang tinggi berbanding dengan RSL

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AMG	Anaerobic mixture gas
cfu/g	Colony-forming units per gram
Cy3	Cyanine 3
DNA	Deoxyribonucleic acid
FISH	Fluorescent in situ hybridization
FOA	Food and agriculture organization
g	Gravity
GC	Gas chromatography
GI	Gastrointestinal
g/ml	Gram per milliliter
g/l	Gram per liter
HPLC	High performance liquid chromatography
IBD	Inflammatory bowel disease
kDa	Kilodalton
mg/ml	Milligram per milliliter
ml/min	Milliliter per minute
MT	Metric ton
mmol/l	Millimoles per liter
NADH	Nicotinamide adenine dinucleotide
qPCR	Quantitative polymerase chain reaction
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SCFA	Short chain fatty acid

U/ml	Units per milliliter
v/v	Volume per volume
w/w	Weight per weight
w/v	Weight per volume
µl/l	Microliter per liter
µm	Micrometer

CHAPTER 1

INTRODUCTION

Nowadays, consumers are becoming more aware of healthy lifestyle especially on choosing healthy food. In this sense, there has been a lot of attention given towards the concept of food with benefits on improving host health *i.e.* functional food. One particular example of functional food is prebiotic. Prebiotic is defined as a non-digestible but fermentable food ingredients that confer health benefits on the host, associated with specific changes of microbiota in the colon (Roberfroid et al., 2010). For a food ingredient to be considered as prebiotic, it must comply with some strict criterions such as; resist the digestion processes in the upper part of the gastrointestinal tract, fermented by intestinal microbiota and selectively stimulate the growth and / or activity of health promoting bacteria such as the *Bifidobacterium* and *Lactobacillus* (Roberfroid et al., 2010). These bacteria will ferment the non-digestible polysaccharides into short chain fatty acids (SCFAs) mainly acetate, butyrate and propionate that may promotes human health. The SCFAs are able to reduce the luminal pH that will inhibit the growth of enteric pathogens, affects the intestinal motility and provide energy to the host (Sarbini & Rastall, 2011).

Kappaphcyus alvarezii (K. alvarezii) is a red seaweed which are heavily cultivated in the coastal area of Malaysia is the major source for kappa carrageenan, a hydrocolloid that is used in food industry as stabilizing, thickening and gelling agent (Ranganayaki et al., 2014). *Kappaphcyus alvarezii* which is commercially known as 'cottoni' is, categorized as soluble dietary fiber due its high viscosity and gelling properties. Dietary fiber mostly is not digestible by human because the gastrointestinal tract does not have the degradation enzymes (Gómez-Ordóñez et al., 2010). Dietary fiber can be classified into insoluble and soluble. Insoluble fiber is characterized by its feces-bulking capacity while soluble fiber is characterized by its ability to form gel like substances. It is believed that soluble dietary fiber is ferment by gut microbiota and reduces the potential of colon cancer (Min et al., 2014; Raman & Doble, 2015). Therefore, with the polysaccharide content in *K. alvarezii*, it is suggested that this red seaweed is a suitable candidate for prebiotic potential because it could fulfill the first criteria of prebiotic, which is able to resist the digestion process in upper part of gastrointestinal tract. Besides that, previous studies have showed that carrageenan from red seaweed *Eucheuma cottonii* and *Chondrus crispus* were able to increase the growth of *Lactobacillus acidophilus* and *Bifidobacterium breve* population (Liu et al., 2015; Setijawati et al., 2014). Thus, for this study it is expected that *K. alvarezii* will able to mimic some prebiotic properties such as non-digestibility, SCFAs production and fermentation selectivity.

Relatively, little is known on the fermentation characteristics and potential beneficial effects of *K. alvarezii* as well as whether they were able to exhibit similar properties to the current established prebiotic such as inulin and fructo-

oligosaccharides. An *in vitro* method have been used to mimic the human large intestine condition by inoculating human feces in the anaerobic fermenter system (Coles et al., 2005). The use of human feces is non-invasive and no ethical objection (McBurney & Thompson, 1987). In this fermenter system *i.e.* the colon model, the colonic microbiota obtained from human feces will be maintained under physiological temperature, pH and anaerobic condition that mimic human large intestine. The human large intestine is metabolically active and diversely colonized (Slavin, 2013). This is due to slower transit time, favorable pH and readily available nutrients that are suitable for bacterial growth (Cummings & Macfarlane, 1991).

The study aims to determine the prebiotic potential of *K. alvarezii* from two different locations, Sabah and Langkawi through *in vitro* digestion and fermentation. The success of the investigation could recognize the candidate compound to be used for further study of prebiotic activity such as *in vivo* study in human and animal. The specific objectives of this study are:

1. To determine the digestibility of *K. alvarezii* through *in vitro* gastric and duodenal digestion.
2. To analyze the metabolite short chain fatty acids (SCFAs) generated by the fermentation of seaweeds by using high performance liquid chromatography (HPLC).
3. To evaluate the fermentation selectivity of seaweeds by the human feces microbiota by using fluorescent *in situ* hybridization (FISH) technique.

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