POLYSACCHARIDES-RICH EXTRACT FROM FRUITING BODY OF MUSHROOM (*PLEUROTUS PULMONARIUS)* AS A POTENTIAL SOURCE OF PREBIOTIC

LEOW KIM TECK

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LEOW KIM TECK

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LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA	analysis of variance
AOAC	Association of Official Analytical Chemists
CFU/mL	colony forming units per mililitre
CD	Crohn's disease
MRS	de Man, Rogosa, Sharpe
^{o}C	degree Celsius
DP	degree of polymerization
Td	doubling time
Eq	equation
ETE	ethanol treated extract
FOS	fructo-oligosaccharides
GOS	galacto-oligosaccharides
GI	gastrointestinal
g	gram
HPLC	high-performance liquid chromatography
h	hour
L	litre
μ_m	maximum growth rate
µg	microgram
µL	microliter
µm	micrometer
mM	millimolar
mg	milligram
mL	milliliter
min	minute
M	molar
PRE	mushroom polysaccharide rich extract
MWSP	mushroom water-soluble polysaccharides
nm	nanometer
M_n	number average molecular weight
$OD_{600\ nm}$	optical density at 600 nm
MBG	partially pure yeast β-glucan from Megazyme
%	percentage
FBG	pure yeast β-glucan from Sigma-Aldrich
v/v	ratio of volume per volume
w/v	ratio of weight per volume
w/w	ratio of weight per weight
rpm	revolutions per minute
SCFAs	short-chain fatty acids
µ	specific growth rate
x g	times gravity
M_w	weight average molecular weight

EKSTRAK KAYA POLISAKARIDA DARIPADA STRUKTUR BADAN CENDAWAN (*PLEUROTUS PULMONARIUS)* SEBAGAI SUMBER BERPOTENSI PREBIOTIK

ABSTRAK

Prebiotik berkemampuan mengubahsuai komposisi mikrobiota usus berfaedah demi pencegahan penyakit dan pengubahsuaian sistem imunisasi perumah. Potensi prebiotik ekstrak kaya polisakarida (EKP) daripada *Pleurotus pulmonarius* (sejenis cendawan tiram) adalah tidak diketahui. Kajian dijalankan untuk menentukan sifat-sifat fizikokimia dan sifat prebiotik EKP, dan menilai hubungan sinbiotik antara EKP dengan *Lactobacillus acidophilus* FTDC3871 (La3871) dan antara EKP dengan *Lactobacillus bulgaricus* FTDC1511 (Lb1511). EKP didapati mengandungi 71.9% polisakarida yang tediri daripada D-galaktosa dan D-glukosa pada kadar molar 1.4:1.0. Peratusan polisakarida bukan glukan, β-glukan dan α-1,4-ikatan glukan adalah 40.6%, 23.5% dan 7.8% masing-masing. Komponen-komponen bukan polisakarida adalah lembapan (5.84%), protein (1.94%), gula penurun (0.2%), abu dan lain-lain (20.84%). M_w, M_n dan indeks polidispersiti (M_w/M_n) bagi polisakarida cendawan yang telah ditulinkan adalah 5.86 x 10^5 Da, 4.23 x 10^5 Da dan 1.385 masing-masing. EKP cendawan didapati rintang terhadap hidrolisis asid dalam perut *in vitro.* Apabila dihidrolisis dengan jus pankreas pada pH 6.0, 7.0, dan 8.0, julat peratusan hidrolisis bagi EKP adalah 10.3%-13.8%. Daripada 12 strain bacteria asid laktik yang diuji, petumbuhan 5 strain (La3871, Lb1511, *L. casei* FTCC0442, *L. plantarum* FTCC0350 dan *Bifidobacterium longum* FTDC8133) pada EKP adalah lebih baik daripada media kosong, dan setanding dengan media yang telah ditambah

dengan inulin. Skor aktiviti prebiotik yang positif diperolehi apabila La3871 (+0.16), Lb1511 (+0.14), *Bifidobacterium longum* FTDC8133 (+0.12) dan *Lactobacillus plantarum* FTCC0350 (+0.07) ditumbuh bersama EKP masing-masing. Berbanding dengan Lb1511, La3871 berupaya menapai EKP dengan lebih cekap. Apabila La3871 dan Lb1511 masing-masing ditumbuh bersama EKP untuk tempoh selama 24 jam, kadar asimilasi EKP, penggunaan EKP, penurunan nilai pH, dan penghasilan jumlah asid organik adalah (0.28 mg per mL per jam, 0.11 mg per mL per jam), (26.3% dan 15.5%), (0.19-0.37 dan 0.10-0.27), dan (33.81 mM dan 18.27 mM) masing-masing. La3871 and Lb1511 mempamerkan ciri homofermentasi dalam media yang ditambah dengan glukosa, tetapi menganjak ke ciri hererofermentasi apabila media MRS asas ditambah dengan EKP. Apabila La3871 dan Lb1511 ditumbuh bersama EKP untuk selama 12-72 jam, aktiviti enzim degradasi polisakarida dapat dikesan, dengan aktiviti spesifik masing-masing pada tahap 3.86-4.63 dan 1.09-2.95 U/mg protein. Kajian juga menunjukkan EKP perlu hadir dalam medium pertumbuhan untuk merangsang penghasilan enzim degradasi polisakarida oleh La3871 dan Lb1511. Kesimpulannya, EKP menonjolkan potensi sifat prebiotik dan kelihatan lebih cenderung untuk merangsang pertumbuhan La3871 berbanding dengan kebanyakan jenis bakteria asid laktik yang diuji.

POLYSACCHARIDES-RICH EXTRACT FROM FRUITING BODY OF MUSHROOM (*PLEUROTUS PULMONARIUS)* AS A POTENTIAL SOURCE OF PREBIOTIC

ABSTRACT

Prebiotic is capable to modulate the composition of beneficial gut microbiota for disease prevention and immunomodulation of the host. Prebiotic potential of polysaccharides-rich extract (PRE) from *Pleurotus pulmonarius* is unknown. Research was carried out to determine the physicochemical properties and prebiotic characteristics of PRE, and to assess the synbiotic relationship of PRE with *Lactobacillus acidophilus* FTDC3871 (La3871) and PRE with *Lactobacillus bulgaricus* FTDC1511 (Lb1511). The PRE contained 71.9% of polysaccharides which consisted of D-galactose and D-glucose at a molar ratio of 1.4:1.0. The percentage of non-glucan polysaccharides, β-glucan and α-1,4-linked glucan were 40.6%, 23.5% and 7.8% respectively. The non-polysaccharides components were moisture (5.48%), protein (1.94%), reducing sugars (0.2%), ash and others (20.48%). The $M_{w,}$ M_n and polydispersity index (M_w/M_n) of the purified mushroom polysaccharides was 5.86 x 10^5 Da, 4.23 x 10^5 Da and 1.385 respectively. The mushroom PRE was resistant to *in vitro* acid hydrolysis in the stomach, and when subjected to hydrolysis by pancreatic juice at pH 6.0, 7.0, and 8.0, PRE was hydrolyzed in the range of 10.3% to 13.8%. From a total of 12 strains of lactic acid bacteria tested, growth of 5 strains (La3871, Lb1511, *L. casei* FTCC0442, *L. plantarum* FTCC0350 and *Bifidobacterium longum* FTDC8133) on PRE was better than on blank medium, and also equivalent to medium added with inulin. Positive

prebiotic activity scores ($p<0.05$) were obtained when La3871 (+0.16), Lb1511 (+0.14), *Bifidobacterium longum* FTDC8133 (+0.12) and *Lactobacillus plantarum* FTCC0350 (+0.07) were cultivated on PRE. La3871 could ferment PRE more efficiently than Lb1511. When La3871 and Lb1511 were cultivated on PRE for 24 h respectively, the PRE assimilation rate, PRE utilization, pH reduction, total organic acids were (0.28 mg per mL per h, 0.11 mg per mL per h), (26.3%, 15.5%), (0.19-0.37, 0.10-0.27) and (33.81 mM, 18.27 mM) respectively. La3871 and Lb1511 demonstrated homo-fermentative characteristics in medium added with glucose, but shifted towards the hetero-fermentative fermentation when MRS basal medium was added with PRE. When La3871 and Lb1511 was incubated on PRE for 12-72 h, polysaccharides degrading enzyme activity was detected, with their specific activity at 3.86-4.63 and 1.09-2.95 U/mg protein respectively. Data also indicated that the presence of PRE in growth medium is necessary to induce production of polysaccharides degrading enzymes by La3871 and Lb1511. In conclusion, PRE exhibited potential prebiotic characteristics and seem to selectively enhance the growth of La3871 than most of lactic acid bacteria strains tested.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Colon is a highly varied colonised and metabolically active organ in the human body (Eckburg et al., 2005). In colon, the microbial populations consisted of about 10^{11}–10^{12} CFU per gram of colonic contents (Eckburg et al., 2005). The colonic environment which has a slow transit time, plentiful nutrients and an appropriate pH is condusive for bacterial growth (Cummings & Macfarlane, 1991). . The common genera in colon comprise bacteroides, eubacteria, fusobacteria, bifidobacteria, peptostreptococci, clostridia, lactobacilli and streptococci (Salminen et al., 1998). The composition and activity of the microbiota has strong impact on health and disease through its participation in pathogenesis and immune function of the host (Gibson & Roberfroid, 1995).

A preferred/healthy microbiota is mainly saccharolytic and contains substantial numbers of bifidobacteria and lactobacilli (Cummings et al., 2004). This definition is due to the fact that *Bifidobacterium* and *Lactobacillus* are not pathogens, and they are mainly carbohydrate-fermenting bacteria, unlike *Bacteroides* and clostridia which are also proteolytic and amino acid fermenting (Macfarlane et al., 2006). The products of carbohydrate fermentation, primarily short-chain fatty acids (SCFAs) are also favourable to host health, whereas those of protein breakdown and amino acid fermentation, which comprise ammonia, phenols, indoles, thiols, amines and sulphides are not (Cummings & Macfarlane, 1991). Bifidobacteria and lactobacilli are thought to play an important role in the upkeep of colonization resistance (Gibson et al., 2005), while a proportionately higher numbers of bifidobacteria, is associated with the breast-fed neonate's defence against pathogenic

microorganisms (Macfarlane et al., 2006). Various approaches have been attempted to sustain/increase substantial counts of bifidobacteria and lactobacilli in colon to endure beneficial health effects: 1) continuous consumption of foods that contain probiotic or 2) enrichment of food with prebiotics (Gomes & Malcata, 1999).

Prebiotics have been defined as 'a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health'(Gibson et al., 2010); while probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Reid et al., 2003). Prebiotics are important because of: 1) the increasing belief that a healthy intestinal microbiota is crucial for human health; 2) the evidence that prebiotics can modify the composition of the microbiota towards a more healthy profile; and 3) as an substitute to probiotics to maintain healthy gut microbiota (Macfarlane et al., 2006). To enable food or food component to act as prebiotic; Roberfroid (2007) suggested that the following criteria must be fulfilled; 1) 'resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption'; 2) 'fermentation by gut microbiota'; and 3) 'selective stimulation of the growth and/or activity of those gut microbiota that improve health and well-being'. Presently, inulin-type fructans (including inulin and fructo-oligosaccharides), trans-galactooligosaccharides (TOS) and lactulose are confirmed prebiotics (Gibson et al., 2010).

Pleurotus pulmonarius, which is also known as *Pleurotus sajor-caju (Fr.)* Singer (Chiu et al., 1998), is one kind of oyster mushrooms (*Pleurotus* spp) grown in Asia, Europe and Africa (Mandeel et al., 2005). Major producers of oyster mushroom were China, Japan, South Korea, Taiwan, Thailand, Vietnam, India and Malaysia (Mat Amin et al., 2014; Royse, 2014). The species is promising for

medicinal purposes: 1) the polysaccharides; xyloglucan and xylanprotein isolated from *P. pulmonarius* fruiting bodies display antitumor activity (Zhuang et al., 1994); 2) Polysaccharides-rich extract of *P. pulmonarius* has been reported capable to prevent weight gain in mice by encouraging lipolysis and may be appreciated in the formulation of adjuvant therapy for obesity (Kanagasabapathy et al., 2013).

Previous studies also showed that polysaccharides-rich extracts from other species of mushrooms are capable to stimulate the growth of lactic acid bacteria. Chou et al. (2013) studied the prebiotic effects of crude polysaccharides isolated from stipes of mushrooms *Lentinula edodes*, *Pleurotus eryngii*, and *Flammulina velutipes.* They concluded that a comparatively low concentration (0.1% to 0.5%) of polysaccharides from the stipes can encourage the endurance rate of probiotics namely *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium longum* subsp. *longum* throughout cold storage (4 ^{o}C for 28 days). Synytsya et al. (2009) investigated the potential prebiotic activity of specific glucans extracted from stems of *Pleurotus ostreatus* and *Pleurotus eryngii* using nine probiotic strains of *Lactobacillus*, *Bifidobacterium* and *Enterococcus*. In general, they concluded that both water-soluble fraction and alkali-soluble fractions from *P. ostreatus* and *P. eryngii* enhance growth rate, biomass and short chain fatty acids (SCFAs) production especially for *Lactobacillus* strains (Lac B and Lac C). *Bifidobacterium* strains demonstrated extract dependent effect, and *Lactobacillus* strains grew faster than *Enterococcus* strains and produced relative more SCFAs with all tested extracts (Synytsya et al., 2009).

1.2 Problem Statements

The use of foods (prebiotic) that promote a state of well-being, better health and reduction of the risk of diseases have become popular as the consumer is becoming more aware on health (Van Loo et al., 1995). Presently there are only three food ingredients that fulfill the criteria for prebiotic classification, i.e., inulin, fructo-oligosaccharides (FOS) and trans-galactooligosaccharides (TOS) (Roberfroid, 2007). The preceding statement also means that the source of scientific-proven prebiotic is very limited in the consumer market. Considering the benefits of prebiotics brought for human kind, there is a strong urge to search for new sources of prebiotics (Aida et al., 2009).

Although mushroom polysaccharides belonging to the group of β-glucan are resistant to acid hydrolysis in the stomach and remain non-digestible by human digestive enzymes (Van Loo, 2006) meets part of the prebiotic's criteria, as a potential source of prebiotic; more experiments are needed to provide data on the *in vitro* digestibility of polysaccharides sourced from *P. pulmonarius*. Previous studies have shown that polysaccharides-rich extracts from other species of mushrooms are capable to stimulate the growth of lactic acid bacteria (Chou et al., 2013); Synytsya et al. (2009), Gao et al. (2009); however, there is no data available on the effects of polysaccharides sourced from *P. pulmonarius* on the growth and activity towards gastrointestinal beneficial and pathogenic bacteria.

1.3 Significance of the Study

P. pulmonarius was widely cultivated and commonly available in Malaysia (Mat Amin et al., 2014); therefore, its fruiting body is relatively cheap for further extraction of its polysaccharides and downstream activities. This study attempted to investigate prebiotic potential of its polysaccharides scientifically in order to gauge its suitablity to be used as functional food ingredients in health-orientated foods and drinks which has much higher economic value.

It has been know that for certain species of mushrooms (*Pleurotus eryngii*, *Lentinus edodes*, and *Flammulina velutipes*); when the fresh mushrooms are harvested, the bases or stipes of mushrooms are normally cut-off (Chou et al., 2013). The cut-off bases or stipes comprise about 25% to 33% of the weight of fresh mushrooms (Chou et al., 2013). Because of the tough texture, they are not suitable for human consumption, and are normally further processed to make low-economic value animal feed and compost (Chou et al., 2013). Nevertheless, the underutilized wastes (bases or stipes) still contain high amount of polysaccharides, and previous study suggested that the mushroom waste are a potential source of novel prebiotics (Chou et al., 2013). Therefore, based on previous findings, it is anticipated that the findings from this study might also provide useful scientific data on how to optimize the use of fresh oyster mushrooms generated from mushroom farms as well as low quality or rejected fresh *P. pulmonarius* mushrooms (mainly of stalks and mushrooms of irregular dimensions and shape) found in market places.

1.4 Research Objectives

The overall objective of this study is to assess the potential of polysaccharides isolated from *P. pulmonarius* to function as prebiotic. The following were the specific objectives of the study:

1. To determine physicochemical properties of mushroom polysaccharides-rich extract that have potential *in vitro* prebiotic effects.
2. To determine the resistance of mushroom polysaccharides-rich extract towards digestive actions by human gastric and pancreatic juices.
3. To evaluate mushroom polysaccharides-rich extract on its ability to stimulate the growth and activity of beneficial and pathogenic intestinal bacteria.
4. To evaluate the effects of mushroom polysaccharides-rich extract on the growth of selected pure beneficial microflora, organic acids and polysaccharides degrading enzyme production.

CHAPTER 2

LITERATURE REVIEW

2.1 Global production of edible mushrooms

A minimum of 12,000 species of fungi that can be accepted as mushrooms, with a minimum of 2,000 species are fit for human consumption (Chang, 1999). Thirty five mushroom species have been cultivated for business purpose, with 20 of them are cultivated on an industrial scale (Chang, 1999). Mushroom is a macrofungi with a special fruiting body which is large enough to be seen and to be handpicked (Chang & Miles, 1992). This type of macrofungi is usually belong to the class of Basidiomycetes (Zhang et al., 2007). Edible mushrooms have been used as food or nutritional supplements for many decades; therefore, their safety is well founded (Singdevsachan et al., 2016).

The global production of edible mushroom is shown in **Table 2.1**(FAO, 2019). Global edible mushroom production has expanded rapidly worldwide since 2008. From 2008 to 2017, global edible mushrooms production increased from 6.8 million tonnes to 10.2 million tonnes represented an increase of 48.5%. China continued to be the leading global producer of edible mushrooms from 2008 to 2017. The most important global edible mushrooms producers were the EU, the United States, Canada, India, Japan, Australia, and Indonesia. Nations displaying encouraging rises in production comprised China, Poland, United Kingdom, Canada and India.

Agaricus bisporus (button mushroom) is the most cultivated mushroom internationally, trailed by *Pleurotus* spp, *Lentinus edodes*, *Auricula auricula*, and *Flamulina velutipes* (Royse, 2014). *Agaricus* (mostly *A. bisporus* with some *A.*

brasilensis) constitutes roughly 30% of the world's cultivated mushrooms. *Pleurotus*, with 5 to 6 cultivated species, contributes about 27% of the world's yield while *Lentinula edodes*, constitutes roughly 17%. *Auricularia* and *Flammulina* are the other two genera that contribute for 6% and 5% of the world's mushroom supply, respectively (Royse, 2014).

Table 2.1 Global production of edible mushrooms

Country	Production (Tonnes)[a]		Increase (%)
	2008	2017	
China	4,710,574	7,868,782	67.0
European Union	1,180,031	1,096,740	-7.1
Netherlands	255,000	300,000	17.6
Spain	133,548	159,018	19.1
Poland	185,000	302,916	63.7
France	138,783	99,096	-28.6
Italy	342,000	69,558	-79.7
Ireland	55,500	66,500	19.8
United Kingdom	70,200	99,652	42.0
United States of America	368,591	421,208	14.3
Canada	79,990	132,556	65.7
Japan	67,500	65,428	-3.1
India	37,000	98,246	165.5
Australia	46,808	46,326	-1.0
Indonesia	43,047	37,020	-14.0
All other	365,401	479,016	31.1
Total	6,898,942	10,245,322	48.5

(FAO, 2019)

[a]Data also include truffles

2.2 Global production of oyster mushroom (*Pleurotus* spp)

The global yield of oyster mushroom has improved rapidly since 1997. The yield of *Pleurotus* spp. improved from 876,000 tonnes to 6.29 million tonnes from 1997 to 2010 (Royse, 2014). China contributed for greatest of the production increase and constituted over 85% of the world's total yield in 2010 (Li, 2012). In Japan, Yamanaka (2011) reported that the yield of *Pleurotus* spp. improved 198% from 13,300 tonnes in 1997 to 39,600 tonnes in 2010. Major producers of oyster

mushroom in Asia were South Korea, Taiwan, Thailand, Vietnam and India (Royse, 2014). Malaysia also cultivates oyster mushroom and produced about 8,760 tonnes in 2014 for the domestic and export market (Amin et al., 2014).

The fruiting bodies of *Pleurotus* species, which is commonly known as oyster mushroom, have a typical texture and attractive flavour that makes them well accepted as culinary mushrooms internationally (Chang & Bushwell, 2008). Among the *Pleurotus* spp., *Pleurotus sajor-caju,* also known as *Pleurotus pulmonarius* (Chiu et al., 1998; Shnyreva et al., 2012), *Pleurotus ostreatus*, *Pleurotus citrinopileatus*, and *Pleurotus florida* have been recognized as frequently available edible mushrooms (Pramanik et al., 2007).

P. pulmonarius is one of *Pleurotus* spp which was cultivated in South East Asia, India, Europe and Africa (Mandeel et al., 2005). It was discovered growing naturally on succulent tissues of *Euphorbia royleans* Boiss.; in the foothills of the Himalayas (Jandaik & Kapoor, 1976). The species can yield sporophores(fruiting bodies) by artificial culture on banana leaves, peanut hull and corn leaves, mango fruits and seeds, sugarcane leaves, wheat, and rice straw (Cangy & Peerally, 1995; Thomas et al., 1998).

Oyster mushrooms is highly nutritional, and also possess some bioactive compounds such as polysaccharides, dietary fibre, ergosterol, vitamins B and minerals (Kalac, 2009). In comparison to other edible mushroom, oyster mushrooms possess several advantages: 1) oyster mushrooms are the fastest to grow (35 days) and require less preparation time and production technology (Mandeel et al., 2005), 2) their fruiting bodies are highly unaffected to pests and diseases, 3) they can be cultivated effortlessly and cheaply (Bonatti et al., 2004). Consequently, oyster mushrooms have become more important commercially (Fernandes et al., 2015).

2.3 Global production of medicinal mushrooms

There were more than 100 species of mushrooms that were popularly utilized in traditional medicine (Ooi, 2000). In this section, only some of the traditionally important and leading non-edible medicinal mushrooms in the oriental countries are described.

2.3.1 Ganoderma spp

Ganoderma is a group of wood degrading mushrooms with hard fruiting bodies(Baby et al., 2015). They belong to the kingdom of Fungi, division of Basidiomycota, class of Homobasidiomycetes, order of Aphyllophorales, family of Polyporaceae (Ganodermataceae) and genus of *Ganoderma* (Baby et al., 2015).

Taxonomic studies showed that there were more than 300 species in genus *Ganoderma*, and most of them are found in the tropical regions(Richter et al., 2015). The genus *Ganoderma* was established by Karsten (1881) with *Ganoderma lucidum* (Curtis) P. Karst. as the type species (Moncalvo & Ryvarden, 1997). *Ganoderma* species are generally not listed among edible mushrooms because their fruiting bodies are thick, bitter and tough, and do not have the fleshy texture characteristics of edible mushrooms(Jong & Birmingham, 1992). For century, the fruiting bodies of *Ganoderma* have been used as popular medicinal mushrooms especially in China, Japan and Korea to enhance longevity and health (Cao et al., 2012), as well as a functional food to prevent and treat many immunological diseases (De Silva et al., 2013).

G. lucidum is highly popular and famously known as Lingzhi in China, Reishi in Japan and Yeongji in South Korea (Baby et al., 2015). In 1997, the worldwide production of *G. lucidum* was 4,300 MT, with 3,000 MT contributed from

China. The remaining 1,300 MT were produced mainly in Korea, Taiwan, Japan, Thailand, US, Malaysia, Vietnam, Indonesia and Sri Lanka (Gao & Zhou, 2002). In 2002, *Ganoderma lucidum* worldwide production was estimated to be 4,900-5,000 MT (Lai et al., 2004).

2.3.2 Sclerotia-forming mushrooms

2.3.2(a) Lignosus rhinocerotis

Lignosus rhinocerotis (synonym for *Polyporus rhinoceros*) is a polypore that thrives in tropical forests. Among the polypores, morphology of *Lignosus* spp. is unique as the sporophore (fruiting body) comprises a cap on a central stem (which occurs in a few polypore genera) and grows from a sclerotium in the soil, rather than from wood, as is the case with most polypores (Abdullah et al., 2013). The main functions of the sclerotium is to allow the fungi to survive under unfavourable environmental conditions and to provide reserves for the fungi to germinate (Wong & Cheung, 2008).

L. rhinocerotis was distributed in Australia, Papua New Guinea, Borneo, Philippines, Indonesia, Malaysia, Sri Lanka and Vanuatu (Ryvarden & Johansen, 1980). *L. rhinocerotis* is one of the most valuable sclerotium-forming fungi in China, and its sclerotium is considered as an expensive folk medicine for the treatment of chronic hepatitis, gastric ulcers and liver cancer (Wong & Cheung, 2008). The sclerotium of *Lignosus rhinocerotis* has also long been used by native communities as a general tonic and natural remedy for ailments as well as to stave off hunger (Lee & Chang, 2007).

The main source of *Lignosus rhinocerotis* was from natural habitats; therefore, its availability was limited (Abdullah et al., 2013). Artificial cultivation

whereby fruit body and sclerotium were induced from solid-substrate fermentation on agro-residues, and liquid fermentation for the production of its mycelium had been reported successful by Abdullah et al. (2013) and Lau et al. (2011) respectively.

2.3.2(b) Poria cocos

Poria cocos Wolf, also known as *Wolfiporia cocos* (Bi et al., 1993), is called Fuling (which is the Chinese name for the sclerotium of this fungus) in China (Ooi, 2000). It is one kind of edible and popular medicinal mushroom that grows under ground on the roots of pine tree in China, Japan, Korea, and North America, and it is widely used to treat chronic gastritis, edema and emesis (Sun, 2014; Wang et al., 2016). In China, it is mainly distributed in the southern provinces such as Yunnan and Fujian (Bi et al., 1993). The sclerotium of *P. cocos* is spherical, oval or irregular in size with a diameter of 10-30 cm (Ooi, 2000). The sclerotium is rather soft when it is fresh, but it is very hard when it is dry (Bi et al., 1993). In China, the sclerotium can be collected all year round, particularly in August and September (Liu & Bau, 1980).

The sclerotium powder of *P. cocos* is tasteless(Ooi, 2000). Polysaccharide is the main important constituent of the sclerotium of *P. cocos* Wolf (Wang et al., 2016). The polysaccharides from *P. cocos* Wolf were reported to exhibit many beneficial biological activities such as anticancer, anti-inflammatory, antioxidant and antiviral activities (Sun, 2014). In Asia, *Poria cocos* is commercially available, and is commonly utilized in the manufacturing of nutraceuticals, tea supplements, cosmetics, and functional foods (Chen et al., 2010).

The sclerotia of *P. cocos* for medicinal use are primarily sourced from cultivation (Wong & Cheung, 2008). From 2011 to 2016, China exported an average

of 9,280 MT of *P. cocos* annually to 44 countries, and more than 98% of total exports of *P. cocos* were shipped to Asian countries (Chi et al., 2018).

2.4 Polysaccharides from *Pleurotus pulmonarius* and Other Mushrooms

2.4.1 Source, Primary Structure and Molecular Weight of Mushroom Polysaccharides

Bioactive polysaccharides can be sourced from fruiting body, mycelium, and sclerotium; which characterize diverse forms in the life cycle of a macrofungi (Zhang et al., 2007). Fruiting bodies of edible mushrooms like *Pleurotus sajor-caju* (Pramanik et al., 2005), *Pleurotus eryngii* (Synytsya et al., 2009), *Lentinus edodes* (Chihara, 1993), *Flammulina velutipes* (Smiderle et al., 2006), *Pleurotus tuber-regium* (Chihara, 1993), *Tremella fuciformis* (Guo et al., 2003) and *Agaricus bisporus* mushroom (Smiderle et al., 2010) have been used as a popular source to obtain polysaccharides, particularly β-glucans.

As an attempt to meet demand from health conscious consumers, polysaccharides from edible mushrooms have been extensively studied worldwide as alternatives for development of food supplements that could improve human health (Aida et al., 2009). Mushroom polysaccharides present as a structural component of fungal cell wall (Ruiz-Herrera, 1956). In general, fungal cell wall is consisted of two major kinds of polysaccharides: one is a rigid fibrillary of chitin (or cellulose) while the other one is a matrix-like β-glucan,α-glucan and glycoproteins (Ruiz-Herrera, 1956). The cell wall of mushroom contains 50−90% carbohydrates; which are primarily exist as polysaccharides or glycoproteins (Synytsya et al., 2009). The most plentiful mushroom polysaccharides are chitin, hemicelluloses, β- and α-glucans, mannans, xylans and galactans (Synytsya et al., 2009).

The primary structure of a polysaccharide is described by monosaccharide

composition, configuration of glycosidic linkages (α, β), position of glycosidic linkages and sequence of monosaccharides (Zhang et al., 2007). Different mushroom species give different kinds of polysaccharides that can be either water-soluble or water-insoluble (Ooi & Liu, 2000). Polysaccharides could comprise only glucose moiety or have proteins attached with them (Ooi & Liu, 2000). Although polysaccharides are commonly β-linked glucose (while some also have galactose and mannose); some polysaccharides are either heterogylcan or glucan-protein complexes (Ooi & Liu, 2000). Mushroom β-glucans regularly have a β-(1→3) linked glucose main chain with different branching ratios of side chains having β-(1→6) linked glucose (Wong et al., 2005), but lack of β-(1→4) linkages (Leung et al., 2006). β-glucan content in mushrooms depends on species, cultivation environments, the extent of fruiting body maturity and total dietary fibre content (Fengmei et al., 2015).

From the fruiting bodies of *P. sajor-caju* (synonymous with *P. pulmonarius*), Pramanik et al. (2007) isolated two water-soluble polysaccharides fractions (fraction I and fraction II). The molecular weights of fraction I and fraction II were approximately 240 kDa and 35 kDa respectively (Pramanik et al., 2007). The fraction I was a water-soluble glucan with (1→3), (1→6)-β-glucans and (1→2)-α-glucans linkages (Pramanik et al., 2007). The fraction II was a heteroglycan and it contains a trisaccharides repeating unit which is discovered to consist of D-glucose, D-galactose, and D-mannose in a similar molar ratio (Pramanik et al., 2005). Pramanik et al. (2005) also concluded that D-mannose is present as a non-reducing terminal (β-glycosidically linked); D-galactose as 1,6-linked residue in the main chain(α-glycosidically linked), and D-glucose as 1,2,4-linked branched-chain moiety in the polysaccharides (α-glycosidically linked) of fraction II.

The constituent and molecular weight of polysaccharide varied with different *P. pulmonarius* cultivars (Roy et al., 2008). A strain of *P. pulmonarius* named as Black Japan contains a water-soluble polysaccharide with molecular weight of 28 kDa; was found to consist of D-glucose and D-galactose in a molar ratio of 3:1 (Roy et al., 2008). Roy et al. (2008) also concluded that (1→6)-linked-α-D-glucopyranosyl moiety, (1,4,6)-linked-β-D-glucopyranosyl moiety, (1→6)-linked-β-D-glucopyranosyl moiety, and terminal D-galactopyranosyl moiety (α-glycosidically linked), are present in the polysaccharide.

In Brazil, using hot water extraction, Carbonero et al. (2012) revealed that the polysaccharides obtained from the fruiting bodies of the mushroom *Pleurotus sajor-caju (Pleurotus pulmonarius)* is a cold water soluble, gel-like glucan, which was considered as a branched structure with a (1→3)-linked-β-Glcp main-chain, substituted at O-6 by single-unit β-Glcp side-chains, on the average of two to every third residues of the backbone, with a molecular weight of 975 kDa. In Thailand, using a mixture of hot water extraction and treatment by cell wall-degrading enzymes, followed by purification via Toyopearl DEAE column and Sepharose CL-6B column chromatography, Satitmanwiwat et al. (2012) demonstrated that the polysaccharides with a molecular weight of 45 kDa isolated from the fruiting bodies of *Pleurotus sajor-caju (Pleurotus pulmonarius)* was β-(1→3)(1→6)-glucan in the native triple helical structure.

2.4.2 Extraction, Yield and Purification of Mushroom Polysaccharides

Hot water approach founded by Mizuno (1996) has been used as reliable approach to extract polysaccharides from mushrooms fruiting bodies or cultured mycelia (Pramanik et al., 2007; Pramanik et al., 2005). According to Zhang et al. (2007), the approach requires removal of low molecular weight constituents from

mushroom material with 80% (v/v) ethanol, followed by three successive extractions with water (100^{o}C, 3 h), 2% (w/v) ammonium oxalate (100^{o}C, 6 h), and 5% (w/v) sodium hydroxide (80^{o}C, 6 h).

Hot water extraction yields water-soluble polysaccharides, while extraction with alkaline solution isolates the water-insoluble polysaccharides (Zhang et al., 2007). The basic law is to damage the cell wall from external layer to the internal layer with appropriate extraction conditions (pH and temperature); and depend on the structure and water-solubility of polysaccharides, the extraction approach can be modified accordingly (Zhang et al., 2007). In hot water extraction, the conformation of the extracted β-glucan is thought to be a triple helix, whereas in alkaline extraction the extracted β-glucan has a single helical structure (Zhang et al., 2007). In alkaline extraction, the alkaline pH efficiently breaks apart the hydrogen bonds of the β-glucan triple helix resulting in β-glucan structural changes (Satitmanwiwat et al., 2012). During extraction process, the cross-linked structure of β-glucan with other unwanted polysaccharides is thought to reduce the purity and recovery yield of mushroom β-glucan (Li et al., 2006).

The yield of crude mushrooms polysaccharides varied with different species. When mushroom stipes are introduced to hot water extraction followed by trichloroacetic acid precipitation, and ethanol precipitation, the yield of crude mushroom polysaccharides for *Lentinus edodes*, *Pleurotus eryngii*, and *Flammulina velutipes* were 3.37%, 6.36%, and 5.47% respectively (weight of extracted crude polysaccharides/initial weight of stipe × 100) (Chou et al., 2013). Using hot water extraction followed by deproteinization using the Savage reagent, and ethanol precipitation, the yield of crude polysaccharides extracted from 53 Polish wild-

growing mushrooms range widely from 0.3%-22.67% (weight of extracted crude polysaccharides/weight of dried material × 100) (Nowak et al., 2018).

A mixture of treatments, such as ethanol precipitation, fractional precipitation, acidic precipitation with acetic acid, ion-exchange chromatography, size-exclusion chromatography, affinity chromatography can be used to further purify the crude polysaccharides (Zhang et al., 2007). Treatment with cell wall-degrading enzymes can also be used to remove the undesirable substances or polysaccharides (Satitmanwiwat et al., 2012). Because enzymatic treatment requires mild conditions, it can recover the β-glucan in its native form with negligible degradation (Li et al., 2006). The separation of acidic and neutral polysaccharides can be done via anion-exchange chromatography on a DEAE-cellulose column(Zhang et al., 2007). Neutral polysaccharides can be further separated into β-glucans (non-adsorbed fraction) and α-glucans (adsorbed fraction) with the assistance of size-exclusion and affinity chromatography (Zhang et al., 2007).

Stepwise precipitation or preparative size-exclusion chromatography can further fractionate polysaccharide with a broad polydispersity, resulting in polysaccharides with different molecular weights and low polydispersity (Zhang et al., 2007).

Chemical and enzymatic treatments of mushroom materials has resulted in different degree of degradation to the fibre materials, particularly cell wall polysaccharides (Cheung & Lee, 1998). Different degree of degradation may contribute to different chemical and physical characteristics of the desirable polysaccharides which will eventually affect their the properties and efficacy when used in food applications that have impact on human health (Satitmanwiwat et al., 2012). Therefore, to minimise undesirable changes to polysaccharides structure

resulting from harsh processing conditions, the selection of appropriate extraction and fractionation procedure should carefully consider the polysaccharide composition of the original material as well as their molecular weight, branching degree and pattern of branches (Zhang et al., 2007).

2.4.3 Digestibility of Mushroom Polysaccharides in Human

Non-starch polysaccharides vary from cellulose to β-glucan from cereal grains and fungi, are not hydrolysed by mammalian enzymes (Lovegrove et al., 2017). Although the primary structure of mushrooms polysaccharides are highly diversified, most of them are considered β-glucans (Wasser, 2002). Digestive enzymes released from the pancreas or brush border of vertebrates mammals are not able to hydrolyse β-glucosidic bonds; therefore, β-glucans are thought resistant to hydrolysis by human digestive enzymes (Van Loo, 2006). The non-digestible characteristics of mushroom polysaccharide also indicates it could be reflected as a potential source of prebiotic (Aida et al., 2009). Nevertheless, more extensive study is needed as not all polysaccharides from mushrooms are belonging to group of β-glucan.

No hydrolysis products were observed when (1→3, 1 → 4)-β-D-glucan from oat was digested at condition similar to human stomach (37^{o}C and pH 1) during the 5 h or 12 h period (Johansson et al., 2006). Nowak et al. (2018) probed the digestibility of crude water-soluble polysaccharide isolated from fruiting bodies of 15 wild-growing mushrooms *in vitro* by treating them with simulated human gastric juice at pH 1 and pH 5 for 2 h at 37^{o}C. They concluded that the extracted crude polysaccharides are almost non-digestible to simulated human gastric juice.

To date, it is not known to what extent the polysaccharides from *P. pulmonarius* can be digested by human gastric juices and digestive enzymes.

2.4.4 Fermentation of Mushroom Polysaccharides by Human Microbiota

The major cell wall components in mushroom that contributing to promising prebiotic effects are polysaccharides such as glucan, chitin and heteropolysaccharides which are non-digestible (Singdevsachan et al., 2016). β-glucans isolated from mushrooms sclerotial such as *Pleurotus tuber-regium*, *Polyporous rhinocerus* and *Wolfiporia cocos* has been shown to be fermented by adult human colonic bacteria *in vitro*; with *W. cocos* as the most promising source of potential novel prebiotics as the species produce considerable amounts of short chain fatty acids during fermentation (Wong et al., 2005).

Yamin et al. (2012) evaluated the effect of crude polysaccharides (GLCP) and polysaccharide-fraction number 2 (PF-2) isolated from *Ganoderma lucidum* on the growth of three *Bifidobacterium* strains *in vitro*. They established that both GLCP and PF-2 increase the growth of all the bacterial strains (by 0.4-1.5 $\log_{10}$ CFU/mL) after 18 h in batch culture fermentation. Further *in vitro* fermentation trial using adult mixed bacterial population indicated that both GLCP and PF-2 sustain the growth of *Bifidobacterium spp. and Lactobacillus spp.* while discourage the growth of *Salmonella* genus.

Using infant faecal samples, Lam et al. (2018) studied the influence of oat β-glucan (18 kDa), barley β-glucans (179 kDa) and alkali-soluble β-glucan (96 kDa) extracted from *Pleurotus tuber-regium* on the growth of probiotic bacteria particularly bifidobacteria and lactobacilli in infant gut *in vitro*. In general, compared with most of the carbohydrates tested (monosaccharides, disaccharides, oligosaccharides and polysaccharides), data from the study suggest that the β-glucans from barley and from *Pleurotus tuber-regium* enable a longer period of fermentation to support the growth of infant faecal bacteria and they appear to selectively increase

the proliferation of lactobacilli for a longer period of fermentation time (>180 h). Based on the findings, it was expected that xylitol (relatively more 'selective' towards bifidobacteria) together with a mixture of FOS/GOS (which are comparatively less 'selective' towards bifidobacteria and lactobacilli) and complex high molecular weight β glucans (more 'persistent' to the selective growth of lactobacilli) might be realistic to create the next group of prebiotics in infant formula (Lam et al., 2018).

2.4.5 Fermentation of Mushroom Polysaccharides by Lactic Acid Bacteria

Synytsya et al. (2009) investigated the potential prebiotic activity of specific glucans extracted from stems of *Pleurotus ostreatus* and *Pleurotus eryngii* using nine probiotic strains of *Lactobacillus*, *Bifidobacterium* and *Enterococcus*. The water-soluble polysaccharides fraction from both *Pleurotus ostreatus* and *Pleurotus eryngii* (predominant by branched 1,3-1,6-β-D-glucan, 2200 kDa) and alkali-soluble polysaccharides fraction (predominantly by linear 1,3-α-D-glucan, 2300-2900 kDa) were tested in their study. In general, both water-soluble fraction and alkali-soluble fractions from *P. ostreatus* and *P. eryngii* enhance growth rate, biomass and short chain fatty acids (SCFAs) production especially for *Lactobacillus* strains (Lac B and Lac C). Extracts from *P. eryngii* are better growth substrate for *Lactobacillus* strains than those from *P. ostreatus*. *Bifidobacterium* strains demonstrated extract dependent effect. The growth of strain Bifi A in *P. ostreatus* extracts was better than those of *P. eryngii*, while strain Bifi B grew only with *P. eryngii* extract. Bifi C was also observed grew better with water fraction of *P. eryngii* than with those of *P. ostreatus*. *Lactobacillus* strains grew faster than *Enterococcus* strains and produced relative more SCFAs with all tested extracts. However, *Enterococcus* strains (Ent A and B) exhibited better growth in alkaline extracts from both mushroom species. Synytsya et

al. (2009) concluded that the growth characteristics of these probiotics were varied and dependent on source of extract (either water-soluble fraction or alkali-soluble fraction) and tested strains. The difference might due to the occurrence of structurally diverse polysaccharides present in the water-soluble and alkali-soluble fraction respectively (Synytsya et al., 2009).

Lili and Jianchun (2008) investigated the influence of three fractions of polysaccharides (PS I, PS II, PS III) from *Agaricus blazei* Murill on the growth of lactic acid bacteria. The polysaccharides fractions were isolated from a combination of alkaline extraction (100 ^{o}C, 5 h, pH 10) and purification via DEAE cellulose column chromatography. They concluded that all the polysaccharides fractions induced production of lactic acid by *Lactobacillus acidophilus* and the population of lactic acid bacteria are more than 10^{10} CFU/mL after 12 h of cultivation. In addition, PS I and PS II are found more effective than PS III to improve the growth of lactic acid bacteria.

Zhao and Cheung (2011) showed that β-glucan isolated from sclerotia of *Pleurotus tuber-regium* support the growth and could be fermented by individual bifidobacteria including *Bifidobacterium infantis* (JCM 1222), *B. longum* (JCM 1217), and *B. adolescentis* (JCM 1275) with *B. infantis,* experience a comparatively larger rise of bacterial count (3-4 $\log_{10}$ CFU). *B. infantis* was found to release almost twice the amount of total short-chain fatty acids (SCFAs) than the other two bifidobacteria. Compared with *B. longum* and *B. adolescentis*, *B. infantis* yielded a comparatively greater proportion of propionic and butyric acid but less acetic acid. Despite the dissimilarities in glycosidic linkages and molecular weight between mushroom sclerotial β-glucan (96 kDa) and inulin (5 kDa), both of the carbohydrates could be utilized equivalent well by pure cultures of bifidobacteria (Zhao & Cheung,

2011) . Gao et al. (2009) demonstrated that the β-glucans obtained from other mushroom sclerotia, namely *Polyporus rhinocerus* and *Poria cocos*, are effective to encourage the growth of *B. longum* and *Lactobacillus brevis* while retarding the growth of *Clostridium celatum*.

Chou et al. (2013) studied the prebiotic effects of crude polysaccharides isolated from stipes of mushrooms *Lentinula edodes*, *Pleurotus eryngii*, and *Flammulina velutipes.* They concluded that a comparatively low concentration (0.1% to 0.5%) of polysaccharides from the stipes can encourage the endurance rate of probiotics namely *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium longum* subsp. *longum* throughout cold storage (4 ^{o}C for 28 days). The polysaccharides not only synergize with the peptides and amino acids from yogurt culture to maintain probiotics populations more than 10^7 CFU/mL during cold storage, but also effective to enhance tolerance of probiotics against gastrointestinal tract *in vitro*.

The potential of crude water-soluble polysaccharide isolated from 53 species of wild-growing mushrooms to encourage the growth of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus in vitro* has been evaluated (Nowak et al., 2018). Among the 53 species investigated, majority of the species (> 60 %) contained crude water-soluble polysaccharides that could enhance the growth of *L. acidophilus* and *Lactobacillus rhamnosus* isolated from the human gastrointestinal tract *in vitro* (Nowak et al., 2018). Further analysis indicated that polysaccharides from the edible species *Macrolepiota procera* and *Sparassis crispa* showed the most promising stimulation effects towards lactobacilli growth. Therefore, the mushrooms polysaccharides have been suggested to have potential to be used as a source of nutraceuticals (Nowak et al., 2018)

2.4.6 Biological Properties of Mushroom Polysaccharides

β-glucans from mushrooms are important group of polysaccharides that capable to promote human health (Fengmei et al., 2015). A few different antitumor polysaccharide have been isolated from the fruiting body, mycelia, and culture medium of numerous mushrooms (*Ganoderma lucidum*, *Schizophyllum commune*, *Lentinus edodes*, *Trametes versicolor*, *Flammulina velutipes,* and *Inonotus obliquus*) in the United States, Russia, Japan and China (Wasser & Weis, 1999). These polysaccharides are harmless to the body and contribute an antitumor effect by stimulating numerous immune responses in the host (Wasser & Weis, 1999). Mushroom polysaccharides are effective to inhibit many types of tumors comprising Ehrlich solid cancer, Sarcoma 37, Sarcoma 180 solid cancers, Lewis lung carcinoma and Yoshida sarcoma (Wasser & Weis, 1999).

Zhang et al. (2007) suggested three mechanism by which mushroom polysaccharides contribute antitumor effect include: 1) cancer prevention by oral administration of polysaccharides isolated from medicinal mushrooms such as *Agaricus blazei* in Brazil and *Flammulina velutipes* in Japan, as shown in a study conducted by Ikekawa (2001) ; 2) the promotion of immunity against the bearing tumors, as noted by Wasser (2002); and 3) direct anti-proliferative effect of polysaccharides towards tumor lines resulting in apoptosis of tumor cells as demonstrated *in vitro* by Chen and Chang (2004) .

In the treatment of colon cancer, mushrooms β-glucans could reduce the size of xenografted colon cancer tumours through the stimulation of the immune system and direct cytotoxicity (Chen et al., 2013). In Japan, lentinan (the main chain of β-(1,3)-glucan with β-(1,6) branches) is one of the active ingredients developed from Shiitake that has been accepted as a biological response modifier for the treatment of

gastric cancer (Ina et al., 2013). The polysaccharides; xyloglucan and xylanprotein, which display antitumor activity, have been developed from *Pleurotus pulmonarius* fruiting bodies (Zhuang et al., 1994).

The β-glucan rich extract of *Pleurotus pulmonarius* was also reported capable to prevent weight gain in the mice by encouraging lipolysis and may be appreciated in the formulation of adjuvant therapy for obesity (Kanagasabapathy et al., 2013).

2.5 Prebiotic

2.5.1 Evolution of the Prebiotic Concept

The human gut microbiota is influenced by various elements such as diet, drug treatment, age, host physiology, peristalsis, in situ bacterial metabolism and local immunity (Berg, 1996). The gut microbiota not only sustain a symbiotic relationship with their host species, but also play an vital role in biological processes such as resistance against infections, nutrient consumption, host metabolism and development of the immune system (Belkaid & Hand, 2014; Brestoff & Artis, 2013). Because food supply the key nutrient sources for intestinal bacteria, diet is most likely the most important element determining the sort of intestinal microflora (Olano-Martin et al., 2002). Subject to availability of sufficient substrates, intestinal bacteria can generate metabolites (branched-chain fatty acids, short-chain fatty acids , bile acid derivatives and vitamins) that impact positively local and/or systemic host physiology (Bindels et al., 2015).

The clue to modify the intestinal microflora to enhance human health includes a wide range of therapeutic tools, from transplanting an entire fecal microflora to introducing single microorganisms or consortia of such organisms (probiotics) (Olle, 2013). An alternative approach is the supply of growth substrates for resident microorganisms to encourage desirable compositional or metabolic