EXTRACTION, PURIFICATION, CHARACTERIZATION AND BIOACTIVITIES OF POLYSACCHARIDES ISOLATED FROM SELECTED NAMIBIAN MUSHROOMS

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AMADHILA ABNER NADHIPITE

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MAIN SUPERVISOR: Prof. AHMAD CHEIKHYOUSSEF

CO-SUPERVISOR(S): Dr. NAILOKE PAULINE KADHILA

Abstract

Mushrooms are considered as one of the notable functional foods for human consumption that have been cultivated and gathered for hundreds of years. The usage of mushrooms has expanded to a wide extent not only as food but also in the area of pharmaceuticals and nutraceuticals. In particular, mushrooms have been appreciated and consumed for both their nutritional value and medicinal properties. Bioactive molecules, particularly polysaccharides (PS) from mushrooms have been the subject of intense research, taking into account their high potential for application in different sectors. Mushroom polysaccharides (MPS) are not only important as prebiotics, but possess other biological properties such as antitumor, antimicrobial, antioxidant, immunomodulatory, antiviral and hypoglycemic effects. Despite this interest, no one to the best of my knowledge has studied the prebiotic properties of the Namibian wild edible mushrooms namely, *Kalaharituber pfeilii* and *Termitomyces schimperi*. Hence, this study is a preliminary attempt to elucidate the bioactivities of *K. pfeilii* and *T. schimperi* PS for potential application in functional food and nutraceuticals. Water and alkali PS extracts of *K. pfeilii* and *T. schimperi* were extracted and purified using Freimund's method. The PS were chemically analysed by thin layer chromatography (TLC) and Fourier transform infra-red for monomeric sugar composition and functional groups determination. Additionally, the PS's total carbohydrate and total phenolic content were evaluated using Phenol-Sulfuric acid method. Their antioxidant activity was evaluated using DPPH assay as well as by reducing power assay. Furthermore, the PS's prebiotic activity was determined by the ability to lower the medium pH and increasing biomass of three probiotic strains: *Lactobacillus plantarum* ATCC 8014, *Lactobacillus acidophilus* ATCC 4356 and *Enterobacter aerogenes*. *K. pfeilii* hot water extracts had the highest contents of total carbohydrates / reducing sugars with 261.8 µg/200µL. In

terms of monosaccharide composition, alkali and water extracts of *T. schimperi* were mostly composed of glucose, fructose and minor proportions of sucrose. Both *T. schimperi* and *K. pfeilii*'s alkali and water PS showed good scavenging activities against DPPH radical in a dose dependant manner in all concentrations studies. The scavenging ratio at high concentration of KPA and KPW were 48.1% and 41.5% respectively. Whereas, the scavenging ratio of TSA and TSW were 52.1% and 56.3% respectively. TSA, KPW and TSW showed their ability to act as carbohydrate source for the studied bacteria. This research may be an important first step for the design of PS with bioactivities that may be used in new health therapeutics and/or incorporation in functional foods or dietary supplements to infer health benefits and used as complementary or alternative medicine resource.

List of Publication(s)/Conference(s) proceedings

Conference Presentation and posters

Amadhila, A.N., Cheikhyoussef, A., Kadhila, N.P. (2019). Mushroom polysaccharides from *Kalaharituber pfeilii* and *Termitomyces schimperi* reveal antioxidant and prebiotic functions. The 3rd Multi/Interdisciplinary Research Conference, Windhoek, Namibia.

Amadhila, A.N., Cheikhyoussef, A., Kadhila, N.P. (2018). Polysaccharides from fruiting bodies of *Kalaharituber pfeilii* and *Termitomyces schimperi*: Structural characterization; antioxidant and immunostimulation activities. The National Student Research Symposium, Windhoek, Namibia.

Amadhila, A.N., Cheikhyoussef, A., Kadhila, P.N. (2018). Immunostimulation, antioxidative and prebiotic properties of polysaccharides extracts of the edible mushrooms *Kalaharituber pfeilii* and *Termitomyces schimperi*. The 6th University of Namibia Annual Science Conference, Windhoek, Namibia.

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List of Abbreviations and/or Acronyms

Caco-2	Epithelial intestinal cell lines
TNF-α	Tumour Necrosis Factor alpha
IL	Interleukin
IFN-γ	Interferon gamma
NMR	Nuclear Mass Resonance
GC–MS	Gas-Chromatography Mas-Spectrometry
FT-IR	Fourier Transform Infra-Red
LPS	Lipopolysaccharides
D-Glc*p*	D-glucopyranosyl
µg	micrograms
v/v	volume per volume
w/w	weight per weight
KBr	Potassium bromide
M	Molar
Mg	Magnesium
mM	Milli-molar
U mL^{-1}	Units per millilitre
DPPH	2,2-diphenyl-1-picrylhydrazyl
ELISA	Enzyme linked immunosorbent assay
mL	Millilitre
β	Beta
α	alpha
%	Percent
µM	Micro-Molar
NaOH	Sodium hydroxide
$NaBH_4$	Sodium borohydride
HO^{-}	Hydroxyl radical
HO^{2-}	Hydroperoxyl radical
O^{2-}	Superoxide anion radical
$^1O^{2-}$	Singlet oxygen radical
RO^{-}	Alkoxy radical

ROO^{-}	Peroxyl radical
NO^{-}	Nitric oxide radical
H^2O^2	Hydrogen peroxide
HOCl	Hypochlorous acid
DNA	Deoxyribonucleic acid
MWCO	Molecular weight cut-off
TSW	Termitomyces schimperi hot water extract polysaccharide
TSA	Termitomyces schimperi alkali extract polysaccharide
KPW	Kalaharituber pfeilii hot water extract polysaccharide
KPA	Kalaharituber pfeilii hot water extract polysaccharide
TLC	Thin Layer Chromatography
GAE/g	Gallic acid equivalence per gram
mg/ml	milligram per millilitre
$NADP^{+}$	Nicotinamide adenine dinucleotide phosphate
$FeSO_4$	Iron sulphate

Throughout this paper we use the terms ‘β-glucans’ and ‘polysaccharides’ interchangeably.

Acknowledgements

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Last but not least, I would like to thank my family: my parents Armas Mekeliwa and Beata Amadhila, my siblings: Toivo, Maija, Jesaya and little Tega, as well as my lovely Aunt Elizabeth for supporting me spiritually throughout my life.

Dedication

"We all have dreams. But in order to make those dreams come into reality, it takes an awful lot of determination, dedication, self-discipline and effort" – Jesse Owens

This thesis is dedicated to:

My grandma Saima Ikumanene, greatest teacher who taught me the purpose of life,

My great parents, who never stops giving of themselves in countless ways,

The University of Namibia, my second magnificent home,

My little sister Beata Amadhila, who is my daily reminder of all that is good in this world.

Mtambo, the highly treasured love of my life. Thanks for the patience and support.

All the people in my life who touch my heart,

I dedicate this research.

"Not that we are adequate in ourselves to consider anything as coming from ourselves, but our adequacy is from GOD." – ***2 Corinthians 3:5***

Declaration

I, **Abner Amadhila**, hereby declare that this study is my own work and is a true reflection of my research, and that this work, or any part thereof has not been submitted for a degree at any other institution.

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Name of Student Signature Date

1. CHAPTER 1 INTRODUCTION

1.1. Background of the study

The new tendency of health conscious consumers has led to a trend in the use of functional foods as complementary or alternative medicine. According to Su et al., (2013), edible mushrooms have become an attractive option for functional food or as a source for the development of pharmaceuticals and nutraceuticals. According to Wasser (2014), mushrooms and fungi are thought to possess approximately 130 medicinal functions. Hence, scientists have observed that a great variety of active molecules can be obtained and identified from mushrooms' fruiting-body, cultured mycelium and cultured broth (Wasser, 2011). In particular, many polysaccharides have been isolated from mushrooms recently and they have been the subject of intense research, taking into view their high potential for application in different sectors (Ruthes et al., 2016). They have emerged as an important class of bioactive natural products in the biochemical and medical areas due to their specific biological activities.

Mushroom polysaccharides are not only important as prebiotics but have immense other biological properties such as antitumor, hepatoprotective, antimicrobial, antioxidant, immunomodulatory, antinociceptive, antiviral and hypoglycemic effects (Wasser, 2002; Ferreira et al., 2015; Singdevsachan et al., 2016; Su, et al., 2013). One of the main biological activities explored by researchers studying mushroom polysaccharides, either in its purified or unpurified form, is the immunomodulation activity. Ruthes et al. (2016), stated that vast research have demonstrated the ability of mushroom polysaccharide to modulate key components of the immune system. Immunomodulating activity of polysaccharides from mushrooms is mainly due to the inducing secretion of pro-inflammatory cytokines, such as tumour-necrosis factor (TNF-α), interleukin IL-1, IL-6, IL-8, IL-12, and interferon IFN-γ (Xu et al., 2012;

Khil'chenko et al., 2011). In several reports, human enterocyte-like Caco-2 cells have been used for *in-vitro* studies to elucidate the immunomodulation mechanisms of polysaccharides (Belguesmia et al., 2016). Different mushrooms produce different types of polysaccharides that can be either water soluble or water insoluble such as chitin, hemicellulose, α- and β-glucans, mannans, xylans and galactans (Singdevsachan, et al., 2016). Considering the complexity of these macromolecules and the variability of their monosaccharide composition, Ruthes et al. (2016), indicated that advanced technologies such as Nuclear Magnetic Resonance (NMR), Gas Chromatography Mass Spectrometry (GC–MS), Fourier Transform Infra-Red (FT-IR), amongst other analyses are essential to determine the purity and chemical structure of the polysaccharides, and to avoid misinterpretation of the data and/or determination of chimera molecules. Based on that context this study aimed to extract and purify fractions of polysaccharides from Kalahari truffles *Kalaharituber pfeilii* and *Termitomyces schimperi* mushrooms. Additionally, the study employed Fourier Transform Infra-Red (FT-IR) spectroscopy to analyse their preliminary physiochemical and biochemical characteristics, and lastly examine their antioxidant activities as well as their immunomodulatory activity, particularly on the ability to induce cytokines secretion, their cytotoxicity towards Caco-2 cells and antioxidant activities.

1.2. Statement of the problem

Despite the fact that mushroom polysaccharides are known to possess a variety of therapeutic benefits, the study on their chemical structures still require more attention, especially in the fields of purification and chemical characterization. According to Radzki et al. (2016), a number of studies revealed that mushroom polysaccharides vary in their chemical structure in terms of molecular weight, glycosidic bond conformation, branching, tertiary conformation or sugar

composition. This is particularly important because the chemical structure of mushroom polysaccharides affect their biological activity.

From the Namibian mushrooms point of view, systematic studies on the extraction and purification of immunostimulatory polysaccharides are generally lacking. In addition to the scarce information in this regard, a study by Shikongo, et al. (2012), on the mycochemical characterization of the mushroom components was insufficient, as it focussed mainly on *Ganoderma* only with a focus generally on phytochemical characteristics. The lack of sufficient isolation, purification, and structural characterization has prevented the establishment of a broader range of structure–function relationships. Also, several immunological studies have been done only with non-purified polysaccharide-rich extracts. This way, the presence of other compounds, like polyphenols (Ebringerová et al. 2008), proteins (Zhao et al. 2012), or contaminants like lipopolysaccharides (LPS) (Schepetkin & Quinn, 2006), could affect the measured activity. The presence of mixtures of different polysaccharides in the same sample can also mask or interfere with the immunostimulatory activity of individual components (Smiderle et al., 2011). Lastly, to the best of my knowledge, there is no available report yet on the bioactivities of polysaccharides from Kalahari truffles *Kalaharituber pfeilii* and *Termitomyces schimperi* mushrooms. Based on that context there is paucity in knowledge on the polysaccharides from Namibian mushrooms regarding their biological activities. Therefore this research aims to contribute in filling these gaps.

1.3. Objectives of the study

The specific objectives of this study are:

a) to extract and purify the polysaccharides from edible Namibian mushrooms with potential application in functional food and nutraceutical, using Freimund's extraction method.

b) to perform physicochemical and structural characterization of the polysaccharides using Fourier transform-infrared (FT-IR) analysis to establish structural-function relationships.
c) to test the biological activities i.e. prebiotic, antimicrobial and antioxidant effects to ascertain the correlation between polysaccharides and their biological activities.

1.4. Hypotheses of the study

Under the assumption that Namibian mushrooms are genetically diverse, this study research hypothesizes. H_R: The indigenous Namibian mushrooms produce structurally different polysaccharides. Also the indigenous Namibian mushroom polysaccharide extracts have potential antioxidant activities and immunomodulatory properties.

1.5. Significance of the study

A better understanding of the different polysaccharide components of Namibian mushrooms would be a starting point for the government to formulate disciplines for exploitation of these mushrooms at a national level. This research will attempt to contribute to the systematization of the already available information concerning the structure–function relationships of immunostimulatory polysaccharides. This is an important first step for the design of polysaccharides with immunostimulatory activity properties that may be used in new health therapeutics or incorporation in functional foods, with health benefits. This will in turn bring attention to the locally produced health supplements. The locally made mushroom polysaccharide products can then be marketed and even exported, thus making mushrooms another source of income for the country. Lastly, results and findings obtained in the study will be published as scholarly articles and academic outputs such as thesis and journal articles. Thus providing useful information to researchers and health-pharmaceuticals

that are looking for novel application and new uses for the production of new probiotic products.

1.6. Limitation of the study

The mushroom species used in this study namely, Kalahari truffles (*K. pfeilii*) and *Termitomyces schimperi* mushrooms could only be available at certain periods of the season particularly during and immediately after the rainy season. For this reason, the researcher would ensure adequate samples collection during this shortest period of time.

1.7. Delimitation

The variation in mushrooms namely: Kalahari truffles (*K. pfeilii*) and *T. schimperi*, collected from different areas may not have similar bioactivities.

2. CHAPTER 2: LITERATURE REVIEW

2.1. Introduction

First and foremost “by the term mushrooms we refer to the definition of Chang and Miles (1992): a macrofungus with a distinctive fruiting body which can be either hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand” (Wasser, 2002). For millenia, mushroooms have been valued as an important source of edible, functional food and complementary or alternative medicine resource by humankind (Synytsya et al., 2009; Wasser, 2002). Recently, health conscious consumers do not merely intend food to satiate hunger and supply nutrients but also bring improvement in health (Singdevsachan, et al., 2016). Currently mushroom-derived-substances (polysaccharides) with antitumor, antioxidant and immunomodulating properties are used as dietary suppliments or drugs (Synytsya et al., 2009; Ruthes et al., 2016). As stated earlier, mushroom polysaccharides are not only important as prebiotics but have immense other biological properties such as antitumor, hepatoprotective, antimicrobial, antioxidant, immunomodulatory, antinociceptive, antiviral and hypoglycemic effects (Ferreira, et al., 2015; Singdevsachan, et al., 2016). For these reasons, plenty of new research has been published in the past 15 years on the isolation chemical characterisation and biological activities of polysaccharides and other extracts from mushrooms. In addition, the study on their chemical structures, especially in the fields of purification and chemical characterization is a neglected area of research. Our knowledge of Namibian mushroom’s polysaccharide bioactivities is largely based on very limited data (unpublished data). The aim of this research was therefore a preliminary attempt to elucidate the bioactivities of *K. pfeilii* and *T. schimperi* PS for potential application in functional food and nutraceuticals as well as prove if these biomacromolecules

have potential prebiotic activity. Therofore this review of literature intends to document information described up to date about edible mushroom polysaccharides, polysaccharide structures.

2.2. Edible mushrooms

In the literature, edible mushrooms have been used to refer to epigeous macrofungi that can be consumed as food (Zhu, et al., 2015). Due to their appealing nutritional value and distinctive aroma and taste, they have been highly valued in many cultures, (Rathore, et al., 2017) and in Namibia this is not an exception (Trappe et al., 2014; Mshigeni, 2001). According to Wasser (2002), the number of mushroom species on Earth is estimated to be 140,000, out of which only about 10% are known. Meanwhile, of those approximately 14,000 species that are known today, only about 50% are considered to possess varying degrees of edibility. Figure 1 below portrays some of the common edible mushrooms, more than 2,000 are safe, and about 700 species are known to possess significant pharmacological properties (Reshetnikov et al., 2001). The nutritional value of edible mushrooms is well documented in the literature. For example, in their analysis, Thatoi and Singdevsachan, (2014) underline that mushrooms are highly nutritive with high content of carbohydrate, proteins, vitamins, minerals, fibers and low/no calories and cholesterol. Additionally, a remarkable feature of mushroom is that they are known to contain a very large variety of biomolecules with high nutritional and medicinal value (Roncero-Ramos & Delgado-Andrade, 2017).

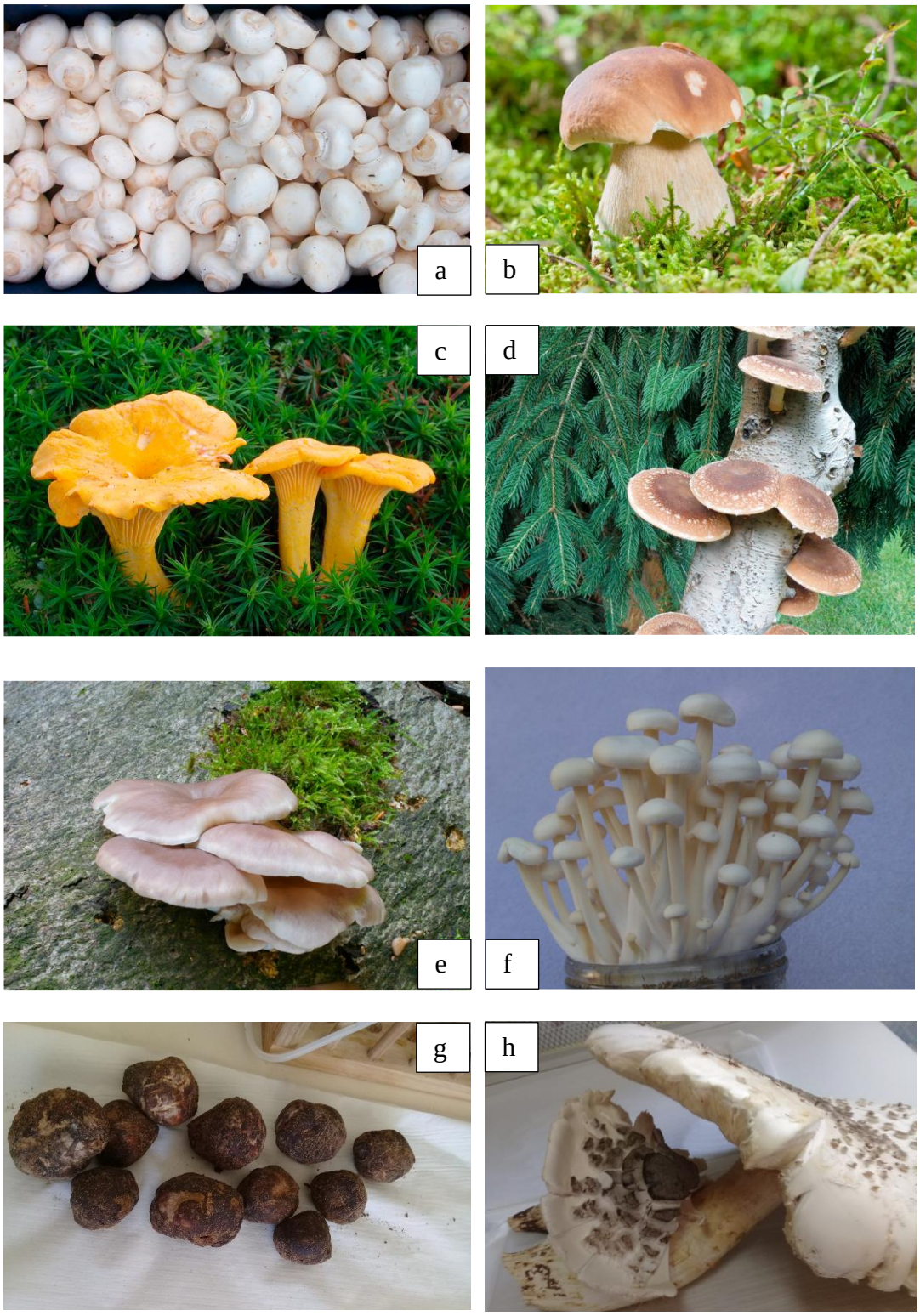


Figure 1: Some of the most common edible mushrooms worldwide and in Namibia **a)** *Agaricus bisporus* **b)** *Boletus edulis* **c)** *Cantharellus cibarius* **d)** *Lentinula edodes* **e)** *Pleurotus ostreatus* **f)** *Flammulina velutipes* (Source: *(Kalac, 2016)*) **g)** *Kalaharituber pfeilii* **h)** *Termitomyces schimperi* (Source: This work)

A considerable number of sources highlighted the fact that these delicacies are a rich sources of complete proteins, containing all the essential amino acids and fiber, mineral elements, and very little fat (Rathore et al., 2017; Friedman, 2016; Yan et al., 2017). Similarly, Stachowiak and Reguła, (2012) elaborated that mushroom also encompass vitamins B1, B2, D2 and C, as well as micro- and macroelements (K, Mg, P, Zn, Fe and Cu) (Kalac, 2009; El Enshasy, et al., 2013). The usual major mineral elements contents reported in mushrooms are given in Table 1.

There are a couple of research studies in support of the fact that polysaccharides from common edible mushrooms such as *Pleurotus* spp. (Synytsya et al.,2009), *Lentinus edodes* and *Agaricus bisporus* (Kozarski et al., 2011) mushrooms have been successfully used as nutraceuticals. However, there has been little discussion on Namibian's *Termitomyces schimperi* and *Kalaharituber pfeilii* bioactivities (unpublished data).

Table 1: Usual content (mg 100 g^{-1} dry matter) of major mineral elements in edible mushrooms

Element	Usual content
Sodium	10–40
Potassium	2000–4000
Calcium	20–100
Magnesium	80–180
Phosphorus	500–1000
Sulfur	100–300
Chlorine	100–600

Source: (Kalac, 2016)

2.3. Polysaccharides

In their review paper, Ruthes et al., (2015) maintains that most carbohydrates found in nature occur as polysaccharides, polymers of medium to high molecular weight. The term polysaccharide tends to be used to refer to a kind of natural macromolecular polymer, which is usually composed of more than 10 monosaccharides through glycosidic linkages in linear or branched chains, with a molecular weight of tens of thousands or even millions (Xie et al., 2016; Nelson & Cox, 2006). Nelson and Cox (2006), further explained that polysaccharides, also called glycans, differ from each other in the identity of their recurring monosaccharide units, in the length of their chains, in the types of bonds linking the units, and in the degree of branching. Polysaccharides play many vital roles in the natural world being used as storage, structural or protective materials. Additionally, polysaccharides have been identified as essential macromolecules in the life activities, and play important roles in storage, support, cell–cell communication, cell adhesion, and molecular recognition in the immune system (Dwek, 1996).

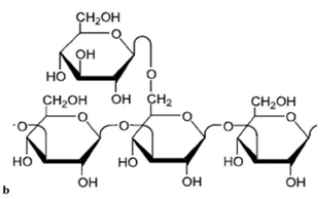
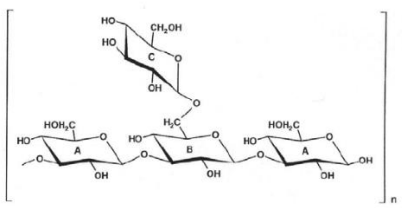
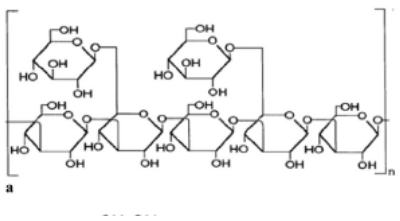
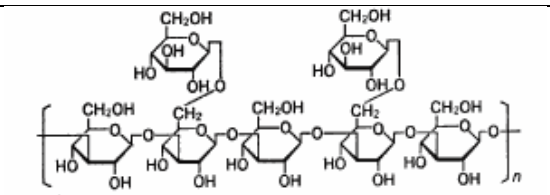
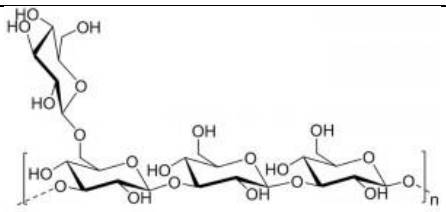
2.3.1. Mushrooms as important sources of bioactive polysaccharides

Zhu et al., (2015) underlines that polysaccharides are usually the main bioactive constituents of edible mushrooms (fungi) and the effective components of dietary supplements. Yu et al., (2018) further emphasised that polysaccharides are widely considered one of the most potent and common compounds derived from mushrooms, and exhibiting numerous health benefits.

Nowadays, several types of immunostimulatory polysaccharides can be found in literature. An increasing number of studies have analyzed that the most bioactive polysaccharides from mushroom sources exist as linear and branched glucans with varying types of glycosidic linkages such as (1-3), (1-4), (1-6)-β-glucans and (1-3)-α-glucans (Synytsya et al., 2009; Kozarski et al., 2012; Wang et al., 2013).

Singdevsachan et al., (2016) stated that different mushrooms produce different types of polysaccharides that can be either water soluble or water insoluble. Table 2 below portrays a few of many important mushroom polysaccharides found in the literature.

Table 2: Structural features of β-glucans from different mushroom sources.

Source	**Polysaccharide**	**Structural features**
Common pelit gill (*Schizophyllum commune)*	Schizophyllan	 Schizophyllan is a 1→3, 1→6 β- linkage. (Klaus, et al., 2011).
Oyster mushroom (*Pleurotus ostreats*)	Pleuran	 A polymer of glucose linked by 1→3, 1→6 β-linkage. It has been reported that glucose molecules are also linked by (1→4) and (1→6) β-linkages (Palacios, et al., 2012)
Shiitake (*Lentinus edodes*)	Lentinan	 Highly branched glucan containing mainly 1, 3 and 1, 6 linkages (Brauer, et al., 2010)
Rainbow conk (*Coriolus versicolor*)	Krestin	 This β-glucan is characterized by a diverse molecular weight ranging from 10-100 kDa (Sun, et al., 2014)
Maitake (*Grifola frondosa*)	Grifolan	 A polymer of glucose molecules: β-D-(1-3)-linked glucan backbone with a single β-D-(1-6)-linked glucopyranosyl residue branched at C-6 on every third residue. Its structure is similar to schizophyllan (Zhu, et al., 2015).

A growing body of literature has found out that the most common monosaccharides detected in mushrooms polysaccharides were glucose, galactose, fructose, xylose, mannose, fucose, rhamnose, arabinose, trehalose and mannitol (Valverde et al., 2015; Ruthes et al., 2016). Amongst the well known edible mushrooms, *Pleurotus eryngii* and *L. edodes* contain ribose, xylose, fructose, mannose, glucose and trehalose (Singdevsachan et al., 2016). Despite being of different chemical compositions, Nie, et al. (2018, *a*) reported that most mushroom polysaccharides belong to the group of β-glucans. In spite of that, studies on *Termitomyces schimperii* and *Kalaharituber pfeilii* monosacharride compositions are generally lacking in literature.

The ability of bioactive polysaccharides to modulate so many important immune cells may be due to the structural diversity and variability that these macromolecules show. Polysaccharides, that are particularly essential for the enhancement of immunity and modulation of defensive responses of humans, are components of mushrooms' hyphae, mostly glucans.

2.3.2. Polysaccharide (β-Glucans) structures as medicinal compounds

Synytsya and Novák (2013) elucidated that glucans are D-glucopyranosyl (D-Glc*p*) based polysaccharides (homoglucans) which, depending on their monosaccharide residues anomeric structure, they can be α-D-glucans, β-D-glucans, and mixed α, β-D-glucans. Most studies have published that the most occurring polysaccharides of mushrooms are glucans, some of which are linked by β-(1-3), (1-6) glycosidic bonds and α-(1-3) glycosidic bonds, most of which are true heteroglycans (Singdevsachan et al., 2016; Ruthes et al., 2015; Synytsya & Novák, 2013). β-(1-3)-D-Glucans may promote the immunostimulatory activity, as demonstrated in several cellular and *in vivo* assays. According to Ferreira et al., (2015) this activity was associated with the formation of triple helix conformations.

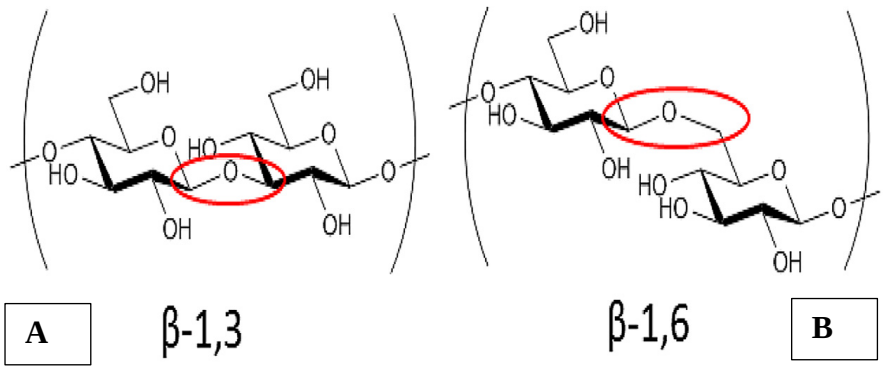


Figure 2 (A & B): Structure of b-glucan linkages *Source: (Rathore, et al., 2017).*

2.3.3. Preparation, extraction and purification of polysaccharides

Isolation of polysaccharides is an important process. There have been numerous research on the isolation technology of polysaccharides from various fungi in recent years. Zhu et al. (2015) warned that the general nature of the extraction procedure has a profound effect on the molecular weight (MW) of β-glucan, which in turn affects its functional behaviour. Nonetheless, Ruthes et al., (2015) affirms that the isolation of polysaccharides is commonly performed in aqueous solvents. According to Dalonso et al., (2015) the extraction methodologies are based on the solubility of *β*-glucan in hot water and in alkaline solutions. However, Chen et al., (2016) stated that the extraction yield of polysaccharides from fungal material depends on the different natural samples, as well as different parameters, such as extraction temperature, extraction pressure, extraction time, and ratio of water volume to raw material.

2.3.3.1. Hot-water extraction

A recent review of literature on this matter found out that water extraction is the most widely used conventional method for extracting polysaccharide, due to its efficiency in obtaining polysaccharides and its low cost (Miao et al., 2011; Chen et al., 2016; Meng et al., 2017). According to Li et al., (2013) employing this method would normally first require the removal of lipid component and other low-molecular

weight substances from the raw material using 75%-95% (v/v) ethanol. The residue is subsequently dried and then extracted with boiling water several times (Ruthes et al., 2015). The residue can then be separated from the hot water extract by centrifugation.

2.3.3.2. Alkaline extraction

Studies have reported that by employing this method, following hot water extraction a dilute alkali solution is used to extract the remaining polysaccharides in the obtained residue. The residue from aqueous extraction may then be subsequently extracted with aqueous basic solutions usually NaOH or KOH, (2% w/v) at 100℃, but these conditions could vary (Ruthes et al., 2013; Maity et al., 2014). Again, the residue is separated by centrifugation and the supernatant gives rise to the alkaline extract. In, a study by Freimund et al., (2004) extraction with basic aqueous solutions frequently is done using $NaBH_4$ to protect reducing end-units avoiding degradation of polysaccharide chains.

2.3.3.3. Purification methods

Separation and purification of polysaccharide are one of the most imperative steps before structure analysis. Several studies for instance those of Schepetkin and Quinn (2006), Ebringerová et al., (2008) as well as Zhao et al., (2012) have warned that the presence of other compounds, like polyphenols, proteins, or contaminants like lipopolysaccharides (LPS), could affect the measured activity. Therefore studies have suggested that following polysaccharide extraction, samples could be presented to several purification steps to get rid of additional substances such as proteins, phenolic compounds, monosaccharides, amino acids and other related molecules (Shi, 2016). Proteins can be removed by precipitation with trichloroacetic acid (TCA) (20%, w/v), by treatment with the enzyme protease at 40◦C for 1 h (pH 7.5),

using Sevag method as described by Staub, (1965) in (Ruthes et al., 2015), or by treatment with phenolic reagent. Moreover, several purification steps must be carried out in order to obtain pure polysaccharide fractions. Shi (2016), documented that the most common purification process used to obtain pure D-glucan fractions includes freeze–thawing, closed dialysis and ultrafiltration, treatment with Fehling solution column fractionation etc.

2.4. Physicochemical and structural characterization of polysaccharides

Researchers have always perceived the importance of clarifying the relationship between chemical structure, the chain conformations of polysaccharides, and their beneficial activities. In particular, Ferreira et al., (2015)'s assumptions on structure-function relations of polysaccharides are well grounded and plausible when they justified that chemical steps are important steps for purification and identification of factual structure–function relationships. However, polysaccharides derived from various sources are usually composed of different monosaccharide monomers, and most of them have hyper branched structures (Ruthes et al., 2016). It is generally accepted that to characterize the chemical structure and chain conformation of a polysaccharide is not an easy work, advises Xu et al. (2014). Therefore to determine the structure of glucans from different resources, a series of analytical methods have been developed and well documented in (Nie et al., 2018, *b*). In the last few years, much information on mushroom polysaccharide structural characterization has become available. According to the literature, the chemical structure of mushroom polysaccharides can be analysed by Fourier transform infrared spectroscopy (FT-IR) (Ruthes et al., 2015; Li et al., 2017; Liu et al., 2018), nuclear magnetic resonance spectroscopy (NMR) (Das et al., 2008; Manna et al., 2015; Yuan et al., 2018), gas chromatography-mass spectrometer (GC-MS) (Peng et al., 2010; de Jesus et al.,

2018), and high-performance liquid chromatography (HPLC) (Chen & Xue, 2018; Wang et al., 2018).

2.5. Importance of mushrooms as nutraceuticals and functional food

Nowadays, food is not just a source for the accumulation of energy and nutrients but also as a means of promoting health. Hippocrates advocated this 2400 years ago when he echoed: "Let your food be your medicine and medicine be your food" (Yeung et al., 2018). In literature, the term nutraceutical has a connotation of, "a food (part of food) that provides medical or health benefits, including the prevention and/or treatment of a disease" (Shekhar et al., 2014). According to Shekhar et al., (2014) the term nutraceutical was derived from the merging of "nutrition" and "pharmaceutical" by Stephen DeFelice (chairman and founder of the Foundation for Innovation in Medicine) in 1989. On the other hand, the phrase "functional food" has been applied to foods and drinks that are enhanced with particular nutrients or substances that have the potential to positively influence health and provide additional physiological benefits above the basic nutritional value (Pang et al., 2012). Moreover, Delgado et al. (2010) elucidated that functional foods provide physiological or metabolic benefits by boosting the immune system and working against diseases and degenerative disorders. The general differences between functional foods, nutraceuticals, food supplements and medicines are summarized in table 3 below.

Table 3: Main differences between functional foods, nutraceuticals, food supplements and medicines

	Functional food	Nutraceutical	Food supplement	Medicines
Form	Food	Pill, tablet, capsule, syrup.	Pill, tablet, capsule, syrup.	Pill, tablet, capsule, syrup, injectable, etc.
Consumption	Consumed as part of the normal diet.	Daily consumed, (usually for a period of time) once they are food constituents/extracts.	Usually during a certain period of time.	Controlled doses according to medical prescription and for a predetermined period of time.
Purpose	Exert a health or physiological effect; Improvement of the state of health and wellbeing and/or reduction of the risk of disease.	Promote well-being through the prevention and/or treatment of diseases and/or disorders.	Typically consumed to ensure the intake of certain ingredient(s) (e.g., vitamins, minerals, amino acids); They may also help to reduce the risk of disease.	Pharmacological purpose; To treat a specific disease.

Experts have always considered mushrooms as one of the notable functional foods for human consumption for hundreds of years in Asian countries like China and Japan. Recently, Valverde et al. (2015) are in agreement with this notion that the usage of mushrooms has expanded up to a wider extent not only as food, but also in the area of pharmaceuticals, nutraceuticals and cosmeceuticals for mankind. Many researchers have documented that edible mushrooms are a source of variety of nutraceutical compounds such as polysaccharides (β-glucans) (Ruthes et al, 2015), dietary fibres, terpenes, peptides, glycoproteins, alcohols, mineral elements, unsaturated fatty acids, antioxidants like phenolic compounds, tocopherols, ascorbic acid etc. (Rathore et al., 2017; Ma et al., 2018). Scientists focusing on the innovative functional foods or health protecting drugs production by the utilization of fruit

bodies, mycelia and the extracts obtained from mushrooms have become a common and effective direction.

The therapeutic effects of mushroom polysaccharides particularly β-glucans are innumerable, ranging from general health to specific therapeutic benefits. A number of studies from *in vitro* and *in vivo* experiments have shown some biological properties of mushroom β-glucans. There are a couple of research studies in support of the fact that active components from *Pleurotus* spp. (Synytsya et al., 2009), *Ganoderma lucidum* (Shi et al., 2013; Pan et al., 2013), *Lentinus edodes* and *Agaricus bisporus* mushroom (Kozarski et al., 2011) have been successfully used as nutraceuticals. The therapeutic and neutraceutical exploration of mushrooms gives the impression that they are the next generation food, not only in providing quality protein but also in curing deadly diseases like cancers, tumors and nervous disorders.

2.6. Bioactive properties of mushrooms that confer them the "status" of functional foods

2.6.1. Antioxidant properties

In the biochemistry field, free radicals are defined as "any chemical species capable of independent existence that contains one or more unpaired electrons" (Valko et al., 2007; Sanchez, 2017). According to Kurutas, (2016) reactive oxygen species (ROS) and reactive nitrogen species (RNS) are free radicals which are allied with the oxygen atom (O) or their equivalents and have stronger reactivity with other molecules, rather than with O_2. Based on that context, reactive molecules such as superoxide anion (O_2^-), hydroxyl radical (OH), hydroxyl ion (OH^-), nitric oxide (NO) and hydrogen peroxide (H_2O_2) are all free radicals (Sanchez, 2017) (Table 4). Oxidation is essential to many organisms for the production of energy to fuel biological processes. Taofiq, et al. (2016) explained that the body has mechanisms to balance ROS production and neutralization through its intrinsic antioxidant pool

(glutathione peroxidase, catalase and superoxide dismutase). It is well documented that ROS are often overproduced under pathological conditions, consequently resulting in free radicals beyond a limit in the body that alters the antioxidant balance resulting in oxidative stress (Sadowska-Bartosz & Bartosz, 2014; Poprac et al., 2017). The resultant oxidative stress caused by an imbalanced metabolism and an excess of reactive oxygen species (ROS) culminates into a range of disorders (Seifried et al., 2017) i.e. metabolic disease, heart disease, severe neural disorders such as Alzheimer's and Parkinson's (Barnham et al., 2004), premature aging (Finkel & Holbrook, 2000; Sadowska-Bartosz & Bartosz, 2014) and some cancers (Gorrini et al., 2013).

There is a vast amount of literature discussing the generation and effects of reactive oxygen species in biological systems, both in relation to the damage they cause and their involvement in cell regulatory and signalling pathways. In the human body (as in other aerobic organisms), free radicals and other reactive species are continually being produced during normal cellular metabolism (Sanchez, 2017). Halliwell and Gutteridge (2015), discoursed that oxidation may occur by "accident" (e.g., the leakage of electrons from the mitochondrial electron transport chain) or with some required purposes, such as involvement in cell signalling and homeostasis processes or defence against pathogens. Contrastingly, Cederbaum et al. (2009) argued that ROS are not only generated internally, in the organism, but also through various external sources like ultraviolet light, ionizing radiation, chemotherapeutics, and environmental toxins. As illustrated in figure 3 below, free radicals have different causes and different cellular targets, namely proteins, carbohydrates, lipids and nucleic acids

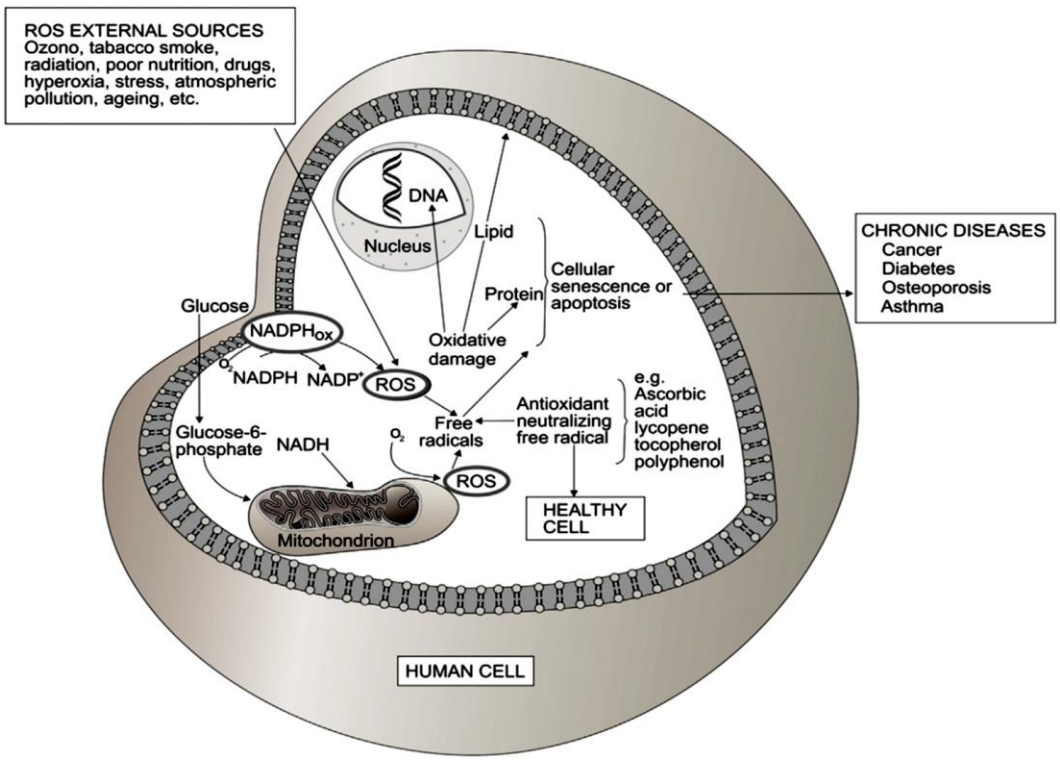


Figure 3: Main causes for the overproduction of free radicals, probable cellular targets and main consequences (chronic diseases) in human health associated with oxidative stress. (Illustration based on Sanchez, 2017).

Table 4: Reactive Oxygen Species (Guo, 2009)

Species	**Common Name**	**Half-life (37℃)**
$HO^{\bullet}$	Hydroxyl radical	1 nanosecond
$HO_2^{\bullet}$	Hydroperoxyl radical	unstable
$O_2^{\bullet -}$	Superoxide anion radical	enzymatic
$^1O_2^{\bullet}$	Singlet oxygen radical	1 microsecond
$RO^{\bullet}$	Alkoxy radical	1 microsecond
$ROO^{\bullet}$	Peroxyl radical	7 seconds
$NO^{\bullet}$	Nitric oxide radical	1-10 seconds
H_2O_2	Hydrogen peroxide	Stable
HOCl	Hypochlorous acid	Stable

R = lipid, for example linoleate

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Ferreira, et al. (2009) elucidated that antioxidants terminate the oxidation chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. Although, Ferreira, et al. (2009) clarified that cellular antioxidant defence mechanisms neutralizes most of the free radicals. Kurutas (2016) emphasized that the maintenance of the equilibrium between the free radicals and the antioxidant defences is essential for the normal function of an aerobic organism. Hence, the chemistry of free radicals and antioxidants is based on an equilibrium between both. Based on that perspective it is therefore important to maintain the balance of the antioxidants in the humans' diets to reduce the oxidative damage.

Apart from the endogenous antioxidant defence mechanisms of an organism, Carocho et al., (2018) maintained that dietary intake is another very important source of antioxidants and may contribute to oxidative homeostasis. It has been reported that antioxidant supplements or antioxidant-containing foods may be used to help an organism to reduce oxidative damage as well as protect food quality by preventing oxidative deterioration (Ferreira et al., 2009; Guo 2009; Sanchez, 2017). Therefore, diets with high level of natural antioxidants and free radical scavengers are recommended. In view of that, most researchers and physicians have recommended that the regular intake of natural antioxidants contribute to protection against cancer, cardiovascular disease, diabetes, and other age-related diseases by reducing oxidative stress (Seifried et al., 2017).

There are many different categories of natural food antioxidants, and, among them, some of the most important groups are described by (Carocho et al., 2018). In their review of antioxidants, Ferreira et al., (2009) categorised that the major classes of compounds with antioxidant activity are: vitamins (vitamin C and vitamin E), carotenoids (carotenes and xanthophylls) and polyphenols (flavonoids, phenolic

acids, lignans and stilbenes). Fruits and vegetables, including mushrooms are commonly known sources of such components. In particular, Friedman, (2016) reported that β-glucans, also have antioxidant properties, prevent DNA damage and reduce the level of carcinogenic metabolite.

A thorough review of the extensive literature on antioxidants in wild-growing mushroom species has been performed by Ferreira et al. (2009). The antioxidant activity of various food items, including mushrooms, has been quantified by various parameters, particularly free radicals scavenging activity, power reduction, metal chelating effects, inhibition of lipid peroxidation, and the identification of antioxidant compounds (Carocho & Ferreira, 2013; Oroian & Escriche, 2015; Zeng & Zhu, 2018). Numerous studies have demonstrated that mushroom polysaccharides have showed numerous antioxidant activities like free radical scavenging activity, superoxide radical scavenging, reducing properties, lipid peroxidation inhibition, and also improves the activity of antioxidant enzymes (Moon & Shibamoto, 2009; Sanchez, 2017; Kalaras et al., 2017). Liu et al., (2014) studied the antioxidant activity of the β-glucan extracted from Jinqian mushroom. The β-glucan exhibited the high DPPH radical scavenging activities of 89.84 % at 5 mg/ml and ABTS radical scavenging activities 63.96% at 5 mg/ml, moderate superoxide radical and hydroxyl radical scavenging activities, low reducing power and Fe^{2+} chelating activities.

2.6.2. Immunomodulatory properties

2.6.2.1. Overview of the immune system.

The immune system, which is composed of innate immunity and adaptive immunity, plays important roles in eliminating pathogens and foreign substances. It is composed of a wide variety of elements and complex biological processes. Based upon the literature, the major difference between innate immune responses and adaptive responses is that innate responses do not strengthen upon repeated exposure and therefore do not confer immunity (there is no memory function) (Engelkirk & Duben-Engelkirk, 2015). In addition, they elucidated that innate responses are less specific in terms of pathogen recognition. Consequently, whereas innate responses recognize classes of pathogens (eg, gram-negative bacteria) through toll-like receptors (TLRs), lymphocytes, which are part of the adaptive immune system, exhibit exquisite specificity for epitopes of individual pathogens (eg, influenza virus) (Engelkirk & Duben-Engelkirk, 2015).

On the other hand, the innate branch of the immune system includes both soluble factors and cells. Soluble factors include complement system proteins such as cytokines, which are activated through a proteolytic cascade and mediate phagocytosis, control inflammation, and interact with antibodies; interferons (INF) α/β, which limit viral infection; and antimicrobial peptides like defensins, which limit bacterial growth (Engelkirk & Duben-Engelkirk, 2015; Ballow & Nelson, 1997). Engelkirk and Duben-Engelkirk, (2015) further elucidated that cytokines in particular, are chemical mediators that are released from many different types of cells in the human body that empower cells to communicate with each other. Immunologists usually have classified cytokines as proinflammatory (IL-1, IL-2, IL-6, IL-12, IL-18, IFN-γ, and TNF-α), anti-inflammatory (IL-4 and IL-13), or immunosuppressive (IL-10, TGF-β) based on their activity (Ballow & Nelson, 1997).

2.6.2.2. Immunostimulation

Immunomodulators are key components in the modern health and wellness industries. Reflecting the fact that the immune system is the first barrier for disease prevention, immunostimulation is regarded as one of the important body's defense strategies for preventing and fighting infections, inflammatory diseases, and cancer (Ballow & Nelson, 1997). These are agents that stimulate the immune system through induction or activation of immune system components or mediators. They enhance resistance against infection, allergy, cancer, and autoimmunity (El Enshasy & Hatti-Kaul, 2013).

For the improvement of immune function, immunostimulating remedies and nutraceuticals may be of particular interest, mostly in persons with immunocompromised systems such as the elderly. Stimulation of the innate immune system with immunomodulators can increase host resistance to unforeseen pathogenic threats (Ballow & Nelson, 1997). Thus, a number of innate system immunomodulators have been identified, including cytokines, substances isolated from microorganisms and fungi (Wasser, 2002), and substances isolated from plants (Paulsen, 2001).

2.6.2.3. Mushroom polysaccharides as immunomodulators

According to El Enshasy and Hatti-Kaul, (2013) about 50 known mushrooms harbor immune-regulating organic compounds of highly diversified molecular weight and structure. Numerous dietary polysaccharides, particularly glucans, appear to elicit diverse immunomodulatory effects in numerous animal tissues, including the blood and GI tract (de Kivit, 2011). Liu et al., (2016) are in agreement that polysaccharides and polysaccharide−protein complexes originating from edible and medicinal mushrooms have attracted the most attention as immunomodulators as a result of their high effectiveness and nontoxicity.

As stated by Canton, et al. (2013) activation of the immune system by polysaccharides is thought to be arbitrated primarily through their recognition by specific receptors that will determine the resulting response. One of the mechanisms that mushroom polysaccharides exhibit is their high-affinity binding to the immune cell surface receptors with pattern recognition receptors (PRRs) as pathogen-associated molecular patterns (Stachowiak & Reguła, 2012). Thus, β-glucans activate proliferation and maturation of immune cells, stimulate activation of macrophages, T-helper and natural killer (NK) cells and other effector cells which induce the host's immune system. This is well illustrated in Figure 4 below (Akramiene et al., 2007). Several studies indicated the immunomodulation activity of mushroom polysaccharides is owing to the increased cytokine production (Ferreira et al., 2015; Leung et al., 2006). β-Glucans were found to influence the production of both pro- as well as anti-inflammatory cytokines (Luo, et al., 2008). Due to these appealing characteristics it is such that Savelkoul, (2013) reported that polysaccharides may decrease asthenia symptoms, caused by the tumor necrosis factor (TNF) and anemia.

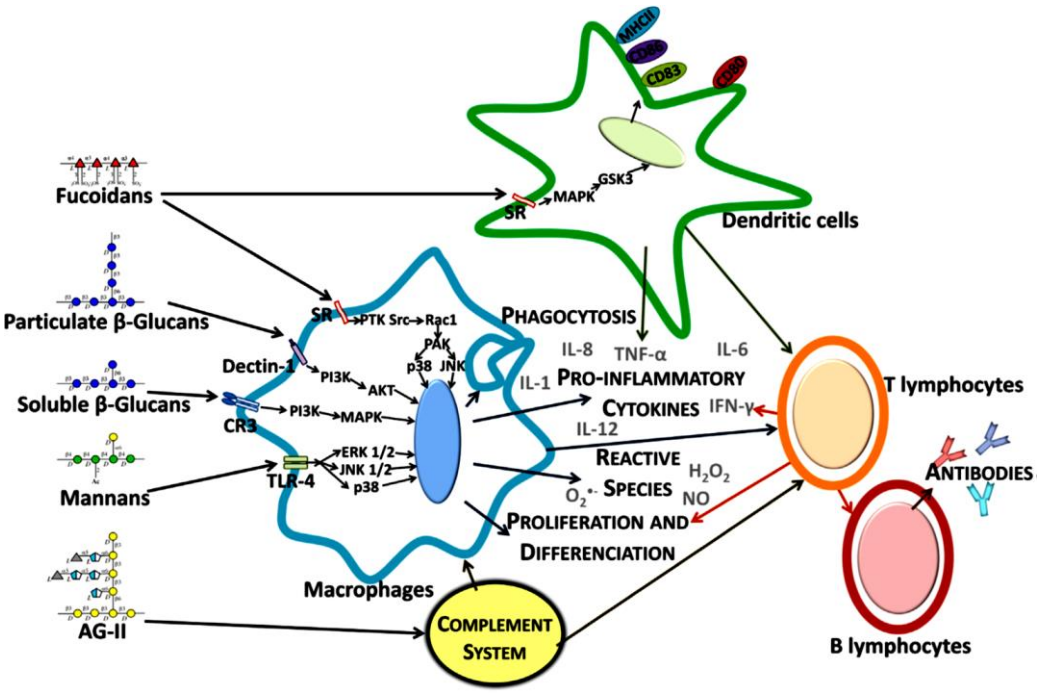


Figure 4: Illustration of immune system activation by immunostimulatory polysaccharides after interaction and trigger of several molecular/cellular events (Ferreira, et al., 2015).

In their groundbreaking review paper, Kozarski et al. (2011) elucidated that polysaccharides, that are particularly essential for the enhancement of immunity and modulation of defensive responses of humans, are components of mushrooms' hyphae, including glucans, chitin and chitosans (partially acetylated chitin forms). Based on that context the identification of mushroom polysaccharides that are capable of stimulating components of innate or acquired immunity may be of potential benefit for cancer treatment. They can be used to improve immune responses. The possibilities for their application include functional foods, food supplements or via parental administration, as a medicine.

Nowadays, several types of immunostimulatory polysaccharides can be found in the literature as tabulated in Table 5 below. However, the lack of sufficient isolation, purification, and structural characterization has prevented the establishment of a

broader range of structure-function relationships particulary in Namibian *T. schimperi* and *K. pfeilii* mushrooms. Furthermore, several immunological studies have been done only with non-purified polysaccharide-rich extracts. This way, the presence of other compounds, like polyphenols (Ebringerová et al., 2008), proteins (Zhao et al., 2012), or contaminants like lipopolysaccharides (LPS) (Schepetkin & Quinn, 2006), could affect the measured activity.

Table 5: Some of the well documented mushroom polysaccharides and their immunomodulatory activities

Source	**Polysaccharide**	**Immunomodulatory activity**
Common pelit gill (*Schizophyllum commune)*	Schizophyllan	Activation of T cell, increase interleukin, and TNF-α production (Hobbs, 2005).
Oyster mushroom (*Pleurotus ostreats*)	Pleuran	Induce IL-4 and IFN- ϒ production (El Enshasy, 2012)
Shiitake (*Lentinus edodes*)	Lentinan	Induces non-specific cytotoxicity in macrophage and enhance cytokine production (Bisen, 2010)
Caterpillar fungus *Cordyceps sinensis*	Cordiceptin	Increase in IL-5 induction with decrease in IL-4 and IL-17 (Chen, 2013)
Maitake (*Grifola frondosa*)	Grifolan	Macrophage activation, induction of IL-1, IL-6, and TNF-α secretion (Yang, et al., 2007)

2.6.3. Mushrooms polysaccharides as a potential source of prebiotics

Nowadays, consumers have become increasingly aware of the necessity to maintain their health through nutrition, and of the role of the gut flora in health and disease. Changes in the composition of the gut flora are often associated with disease and may, in some cases, be the cause of disease. In particular, Gibson and Roberfroid (2005), published that there is an increase in the incidence of illnesses that may be caused by a deficient or compromised microflora, such as gastrointestinal tract (GIT) infections, irritable bowel syndrome, inflammatory bowel disease, antibiotic-induced diarrhoea, and certain cancers (e.g. colorectal cancer). The term prebiotic is generally understood to mean a nondigestible food component that provides nutritional support to noble microbes existing inside the gut and stimulating their growth to revamp health complaints of humans or animals (Aida, et al., 2009). Similarly, The international scientific association for probiotics and prebiotics (ISAPP) defines prebiotics as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth or activity of one or a limited number of bacteria in the colon" (Gibson et al., 2017). Therefore, the proven importance of gut microbiota in the human health has inspired the search for new strategies that could positively manipulate the gut microbiota composition to ameliorate chronic diseases and symptoms (Yahfoufi et al., 2018).

Accumulating scientific evidence has suggested that dietary prebiotics, representing a group of non-digestible plant foods, polyphenols, fibers, and polysaccharides, are able to selectively promote the growth of beneficial bacteria and positively affect the host physiology (Holscher, 2017). According to Aida et al. (2009), mushrooms seem to be potential candidates for prebiotics as they contains carbohydrates like chitin, hemicellulose, β and α-glucans, mannans, xylans and galactans. Possible beneficial effects of prebiotics include the control of intestinal transit and bowel habits by

decreasing pH values and decreasing the amount of long chain fatty acids in bowels, and a reduction in the risk of obesity, atherosclerosis, type 2 diabetes, and allergies, although their effectiveness in humans remains controversial (Roberfroid, 2007; Rastall & Gibson, 2015; Singdevsachan et al., 2016; Ambalam et al., 2016; Choque-Delgado & Tamashiro, 2018).

Furthermore, it has been reported that prebiotics can modulate the immune response directly or indirectly by changing the balance of intestinal microbial population or by producing microbial compounds, such as short-chain fatty acids (SCFA) (Martinez-Gutierrez et al., 2017) . In their review, Watzl et al. (2005) in clarification published that the effects of prebiotics on immune functioning may be due to their impact on the gut microbiota and the generation of short-chain fatty acids (SCFAs) by binding to SCFA receptors. As a functional food component, prebiotics are conceptually intermediate between foods and drugs, and widely used prebiotics include fructans, inulin, fructooligossacharides (FOS), galactogliosaccharides (GOS), and xyloogliosaccharides (XOS)

Potential and known mechanisms of probiotics have been described by O'Toole and Cooney (2008) for their beneficial effects (Figure 5) which include (i) impact of prebiotics upon the composition of the microbiota apparently by competing with them for substrate availability; (ii) alteration of the dynamics of carbohydrate utilization by individual microbiota components; (iii) production of vitamins and their enhanced availability which may modulate the microbiota; (iv) effect of probiotics bacteria on the general microbiota probably by direct antagonism; (v) increase in competitive exclusion for binding sites of pathogens; (vi) improvement in barrier function of epithelial cell; (vii) reduction of inflammation, thus altering intestinal properties for colonization and persistence within; (viii) stimulation of innate immune response.

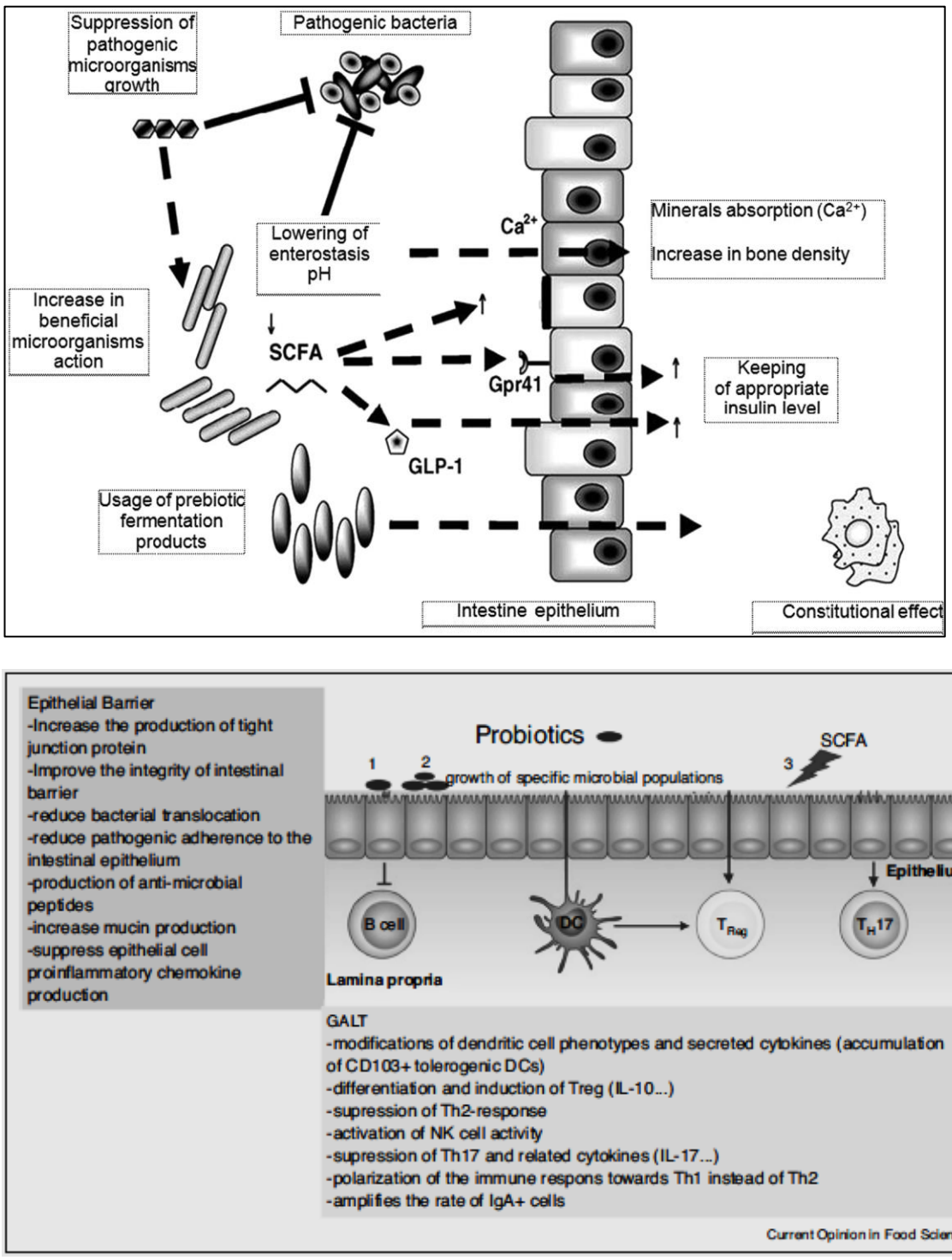


Figure 5: Mechanism of action of probiotics. Probiotics modulate the immune response directly (1), and/or by changing the microbial population of the microbiota (2) and/or by modifying the levels of microbial metabolites (i.e. SCFA) (3). *(Yahfoufi, et al., 2018)*

2.6.3.1. Prebiotics studies

Most recently extensive studies have proven that prebiotics can promote the growth of beneficial bacteria (e.g. *Bifidobacterium*, *Lactobacillus*), and metabolize in the large intestine into lactic acid and short-chain fatty acid (SCFA) for which the host's physiology, particularly gastrointestinal health can be improved (Zaporozhets et al., 2014; Zheng et al., 2018; Wang et al., 2019).

A study conducted by Vinolo, et al. (2011) found out that anaerobic bacterial fermentation of prebiotics produces mainly SCFAs which can modulate the expression of genes responsible for cytokines' production in the tissue resulting in the modulation of cytokines 'production by leukocytes such as TNF-a, IL-2, IL-6 and IL-100 (Vinolo et al., 2011). Several mushroom polysaccharides like pleuran, lentinan, schizophyllan, β and α- glucans, mannans, xylans, galactans, chitin, inulin and hemicelluloses can be credited to promising prebiotic effects (Aida et al., 2009). Pleuran from oyster (*Pleurotus ostreatus*) mushrooms and lentinan from Shiitake (*Lentinus edodes*) mushrooms are currently the most frequently used β-glucans as prebiotics. Both of them show positive effects on the intestines.

2.7. Selected Namibian mushrooms

2.7.1. *Kalaharituber pfeilii*

Kalaharituber pfeilii is also known as the Kalahari truffle. The name *Kalaharituber* means "truffle of the Kalahari" and "*pfeilii*" which was given in honour of Count Pfeil, who was known as the original collector of the species (Ferdman et al., 2005). Previously it has been misidentified as *Terfezia boudieri* Chatin, *Terfezia cleveryi* Chatin and *Terfezia pinoyi* Maire. It was identified as *Terfezia pfeilii* until molecular evidence proved otherwise and it was assigned to a new genus, *Kalaharituber* (Ferdman et al., 2005). Truffles are hypogenous mushrooms that grow exclusively

underground (10 – 30 cm below ground), taxonomically they belong to the Ascomycota (Wijayawardene et al., 2017) and are ecologically recognized as ectomycorrhizal fungi (Hall et al., 2003). Trappe et al., (2008) has pointed out that truffles grow as a symbiotic ectomycorrhizae (ECM) on the roots of a wide variety of plants (Fig 6), both gymnosperms and angiosperms in temperate, boreal, and sub-tropical forests. *Kalaharituber pfeilii* (Hennings) Trappe & Kagan-Zur (formerly *Terfezia pfeilii*) (Ferdman et al., 2005), is an edible mycorrhizal fungus that thrives in the Kalahari desert of Southern Africa, and is commonly known in English as the Kalahari truffle (Fig 6), (Adeleke & Dames, 2014).

According to Trappe, et al. (2008)

> "Native terms for the Kalahari truffle include *mahupu* or *n'xaba*; *dcoodcoò*; *kuutse* (both in Khoisan); *omatumbula* (in Bantu and Oshidonga, Mshigeni 2001); *hawan* or *haban* (in Khoe and Khoekhoegowab,); and *n/abba* (in Nama,)"

Figure 6: Left: Morphology of truffles. **Right**: Sliced *Kalaharituber* truffles showing the pale brown outer peridium and interior whitish pockets of spore–bearing tissue separated by pale brownish veins.

Man has hunted truffles and wild mushrooms since ancient times and they have been treated as a food due to their unique deliciousness and flavour (Wasser, 2011). The nutritional value of truffles and wild mushrooms such as being low calories, high

amounts of vegetable protein, and specifically chitin are well-documented (Kalac, 2016). Despite this long history of human use, there are still many unanswered questions concerning the physicochemical characteristics particularly their polysaccharides as well evaluation of the immunomodulatory properties.

Research on the biology of *K. pfeilii* has been focused on determining the possible host plants and potential role players such as Mycorrhization helper bacteria (Adeleke & Dames, 2014), with little attention being paid to physico-chemical characterization and biological activities.

2.7.2. *Termitomyces schimperi*

Termitophilous fungi (termite's mushroom) are a monophyletic group of tropical gilled mushrooms belonging to the genus *Termitomyces*. The genus *Termitomyces* R. Heim includes a group of paleotropical and edible mushrooms (Hsieh & Ju, 2018) sharing ecological and morphological features, the most noticeable of which being the obligate symbiotic association with termites belonging to the subfamily *Macrotermitinae* (Hsieh & Ju, 2018). About 30 species of *Termitomyces* has been documented globally. However, *Termitomyces schimperi*, *T. sagittiformis*, and *T. reticulatus* are the common consumed species of *Termitomyces* in Namibia (Shikongo et al., 2012).

The taste, attractiveness, high protein content and medicinal value of *T. schimperi* make it very popular and highly consumed. It was first documented by Heim (1942) for a group of termitophilous agarics. The '*Omajowa*' or 'Termitenpilz' which grows in groups around the bases of tall termite mounds. In Namibia the giant mushroom is now known to grow at Omaruru, Okahandja, Otjiwarongo, Grootfontein, Tsumkwe, east of Windhoek and in the south-eastern part of the Etosha National Park (Grahl, 2012).

The name '*Omajowa*' is used by the Herero and whereas the Owambo peoples of Namibia refers to it as '*Owoya*' for this termitophilic fungus, and the German-speaking people refer to it as *'Termitenpilz'* (NP Kadhila, pers. comm.). However, all agree that it is a much-appreciated delicacy with very good flavour. *T. schimperi* (Pat.) R. Heim in particular, when immature shows a sub globose, hemispherical or sub hemispherical pileus (Essouman, et al., 2017) most often still attached to the stipe by the veil as illustrated in Figure 7 below.

Figure 7: *T. schimperi* fruiting body portraying a hemispherical pileus still attached to the stipe.

Generally, much work has not been done on this mushroom in the area of chemical characterization, antioxidant and immunostimulation. Phytosterols, such as ergosterol and ergosterol derivatives, have been isolated, characterized and purified from *Termitomyces schimperi* (Essouman, et al., 2017). The fungi species and their growth conditions determine the level of ergosterol. Ergosterol levels are not constant throughout the growth stages of the mushroom (Aanen, et al., 2007; Essouman, et al., 2017). Laccase enzyme with optimum activity at 70℃ has been isolated and purified from *Termitomyces schimperi* (Haileka, 2015).

3. CHAPTER THREE: RESEARCH METHODS

3.1. Samples of mushroom fruit bodies

The fruiting body of *Termitomyces schimperi* was bought from market vendors near Wilhelmstal farm. This place is situated in Erongo region, Namibia, its geographical coordinates are 21°54′0″S, 16°19′0″E. Meanwhile the fruiting body of *Kalaharituber pfeilii* was bought from hawker women in Okashana village near Omuthiya. This place is situated in Oshikoto region, Namibia, its geographical coordinates are 18.3991° S, 16.6144° E.

3.2. Research reagents and materials

Ascorbic acid, trifluoroacetic acid (99 %), potassium bromide, 2,2-diphenyl-1-picry-hydrazyl radical (DPPH), potassium ferricyanide (98%), ferric chloride, trifluoroacetic acid (TFA), polyoxyethylene sorbitan monolaurate (Tween 20), sodium sulfide, ferrous chloride, H_2SO_4, 2-propanol, acetone, lactic acid, dimethyl sulfoxide (DMSO), Monosaccharide standards (D-Glucose (D-Glc), arabinose, xylose, mannose, galactose) were obtained from the Carbohydrate kit supplied by Sigma. Sodium borohydride were purchased from Sigma Chemical Co. (St. Louis, MO, USA.) Ascorbic acid (99%), TLC silica gel 60 F_{254} plates, absolute methanol (Methanol Optigrade) were obtained from Merck Co. (Darmstadt, Germany). Seamless cellulose dialysis membranes (MWCO 12400) were from Sigma-Aldrich, Germany.

3.3. Sample preparation, polysaccharides extraction and purification

Our experimental set up was practically more or less the same as proposed by Synytsya, et al. (2009). Following sample collection, separately, each of the mushroom species were brush-cleaned, thrice washed with distilled water, cut into smaller slices and sun dried until constant mass was attained. The mushroom samples were homogenized into fine powder, the instrument utilised was a sterilized mortar and pestle. In order to remove lipids, lipoprotein, phenols and smaller molecules the homogenates were washed thrice with 80% (w/w) ethanol, followed by three times washing with distilled water and then extracted with boiling water for 4 hours. The polysaccharides were separated from the residue by precipitation with 95% cold ethanol.

In an effort to purify the polysaccharides, extracts were incubated with α-amylase from *Bacillus* sp. (1:500 v/v) at pH 7 for 30 minutes, this was done in order to remove α-glucans (Synytsya, et al., 2009). Sevag reagent (chloroform/butanol 4:1, v/v) was applied to deproteinize, the procedure used is as depicted in Shi, et al. (2013). The deproteinized supernatants were further dialyzed to remove lower molecular weight compounds such as oligosaccharides and protein of lower molecular weight (Dialysis tubing, high retention seamless cellulose tubing, MWCO 12400. Sigma Co. St. Louis, MO, USA) and finally concentrated by lyophilisation (Christ) to yield water soluble fractions. The insoluble parts were extracted with 1 M solution of sodium hydroxide containing 0.05% of sodium borohydride. The supernatants were adjusted in the same way as the water extracts supernatants to yield alkali soluble polysaccharides.

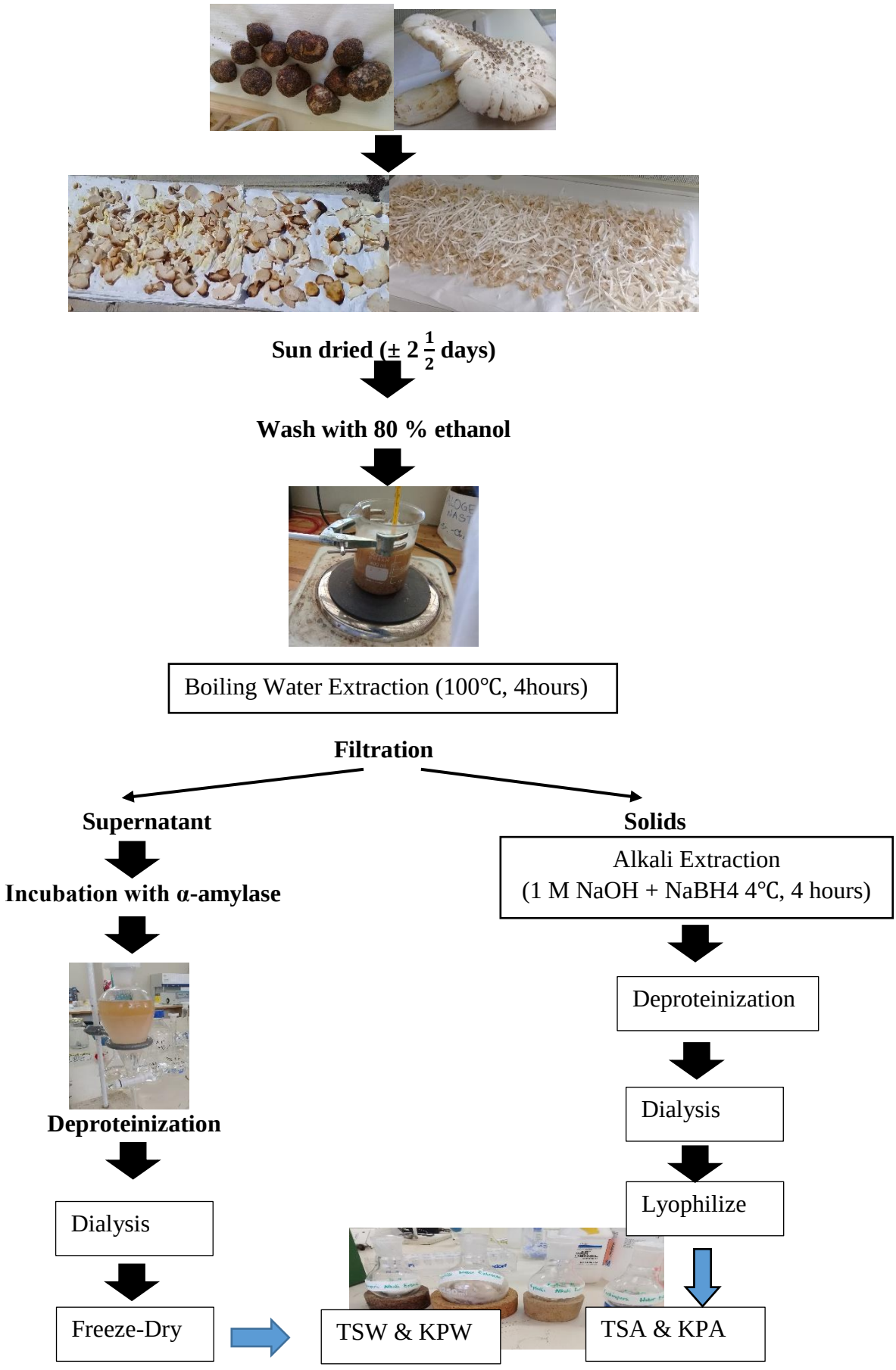


Figure 8: Flow diagram for the samples preparation for PS extraction and purification.

3.4. Physico chemical characterization

3.4.1. Determination of polysaccharides content

The procedure used proceeded in a similar way as indicated by Du Bois, et al. (1956) and cited in (Kozarski, et al., 2011). Firstly, glucose standards with concentrations of 40 – 200 µg per 200 µl were prepared by transferring respective amount of glucose from the standard glucose (D-*Glc* 99.5%, Sigma, USA) solution (1mg/ml) and adjusting it to a total volume of 200 µl by adding distilled water. Secondly, the mushroom polysaccharides extract powders were diluted to a final concentration of 200 µg in a total volume of 200 µl. After the set up, 0.2 ml of 5 % phenol solution was added to all the tubes. This was immediately followed by the addition of 1 ml of concentrated Sulphuric acid to each tube and mixed throughly. After 10 minutes, the contents of the tubes were mixed again and placed in a water bath set at 25–30 ℃ for 20 minutes. Lastly, the absorbance was measured and recorded using a Spectrophotometer (UV mini-1240, Genesy, Kyoto, Japan), at the wavelength of 490 nm. A standard curve of absorbance at 490 nm on "Y" axis versus concentration of glucose in µg/200µl on "X" axis was plotted using Microsoft Excel (Microsoft Office 2013, Microsoft, USA). The value "x" of polysaccharides total sugar concentration was extrapolated from graph corresponding to the OD reading of the test samples. This method was chosen because it is the most feasible and reliable, most economic way amongst the quantitative assays for carbohydrate estimation.

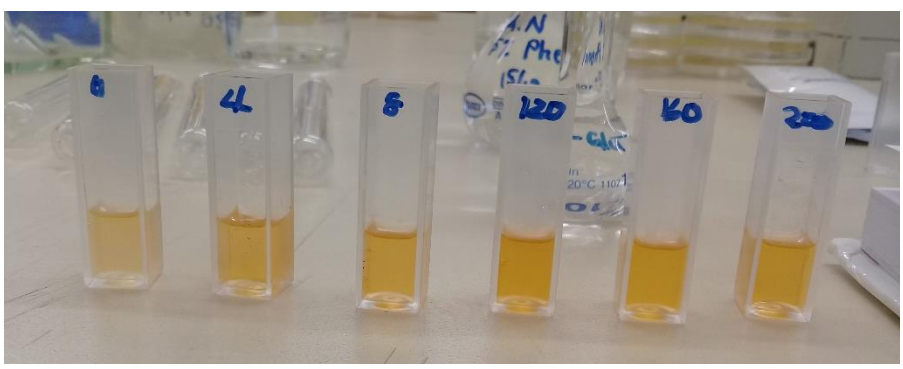


Figure 9: Phenol-sulphuric method of polysaccharide content determination.

3.4.2. TLC of sugar components

To determine the monosaccharide compositions of the polysaccharides, the extracts were screened by thin layer chromatography (TLC) using TLC silica gel 60 F_{254} plates (Merck, Darmstadt, Germany). The set up used bears a close resemblance to the one proposed by Zhou, et al., (2016) with some alterations based on Kozarski, et al. (2012).

Briefly, the polysaccharide extracts were first hydrolysed as follows: 10 mg of samples was dissolved with 2 ml trifluoroacetic acid (TFA) (2 mol/l) at 100℃ for 6 hours in a dryer. After that, the samples were cooled down by nitrogen until they were totally dry. 6 ml of 99% methanol was added to eliminate TFA, afterwards dissolved in ultrapure water for further study. The silica gel (n-butanol: ethyl acetate: pyridine: acetic acid: distilled water (4:4:1:5:1 v/v/v/v/v)) plates (5cm × 20 cm) were used to study the polysaccharides hydrolysates and the mixture of different monosaccharides standards (arabinose, rhamnose, xylose, mannose, galactose, glucose (5 mg/ml for each) (Sigma Aldrich, Germany). Monosaccharide spots were detected after spraying aniline–phthalic acid at 105℃ for 5 minutes.

The following modification were made based on Kozarski, et al., (2012): Instead of (n-butanol: ethyl acetate: pyridine: acetic acid: distilled water (4:4:1:5:1 v/v/v/v/v), the running solvents used in their respective proportion were: acetone: water (90:10 v/v) and 2-propanol: acetone: 0.1 M lactic acid (4:4:2 v/v/v). Diphenylamine-aniline-phosphate reagent (Bailey & Bourne, 1960) and 10% sulfuric acid in ethanol (v/v) was used to spray colour sugar compounds at 105◦C for 5 minutes, instead of the aniline–phthalic acid spraying reagent stipulated by Zhou, et al., (2016).

3.4.3. FT-IR analysis

In order to determine the functional group of the extracted polysaccharides, FT-IR spectra was recorded for each sample. FT-IR spectra of polysaccharides were obtained using a Fourier transform infrared spectrometer (Shimadzu, Japan). The polysaccharides (1mg) were ground with 100mg KBr powder, pressed into pellets, and then scanned for FT-IR measurement in the frequency range of 400–4000 cm^{-1} (Dilna et al., 2015).

3.5. In vitro antioxidant activity assays

3.5.1. Total phenolic contents

The total phenol content (TPC) was determined by the method described by Stoilova, et al., (2007), using Folin-Ciocalteu reagent. Concisely, one ml of extract or standard solution (Gallic acid) (of different concentrations 0.01 – 0.05 mg/ml) were added to a mixture of 10 ml deionised water and 1.0 ml of Folin-Ciocalteu phenol reagent. After 5 minutes, 2.0 ml of 20% sodium carbonate was added to the mixture. After 1 hour of incubation at room temperature in darkness, the absorbance was measured at 750 nm. The TPC was then computed from the linear regression equation of the standard curve and from this equation, the concentration of gallic acid was determined for each extract and converted to mg of gallic acid equivalents/g of dry extract (mg GAE/g).

3.5.2. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

DPPH assay is based on the premise that a hydrogen donor is an antioxidant. This colorimetric assay uses the DPPH radical, which changes from purple to yellow in the presence of antioxidants, and is widely used as a preliminary study (Moon and Shibamoto, 2009). The free-radical scavenging capability of the polysaccharides was analysed by employing the DPPH test according to a modified Blois method as

described in Wang, et al. (2013). L-Ascorbic acid was used as reference material. Briefly, 0.2 mL of methanol (MeOH) and 0.3 mL of various concentrations (50-800 µg/ml) of sample in MeOH were mixed in a 10ml test tube. DPPH (2.5 ml of 75 µM in MeOH) was added to achieve a final volume of 3 ml. The solution was kept at room temperature for 30 minutes, and the absorbance at 517 nm was measured using a spectrophotometer (UV mini-1240, Genesy, Kyoto, Japan). The DPPH scavenging effect (ϕ%) was calculated using the following formula:

$$\phi\% = 1 - \frac{A\ sample}{A\ control} \times 100$$

Where $A_{control}$ is the absorption of DPPH without sample or Vitamin C and A_{sample} is the absorption with sample or vitamin C.

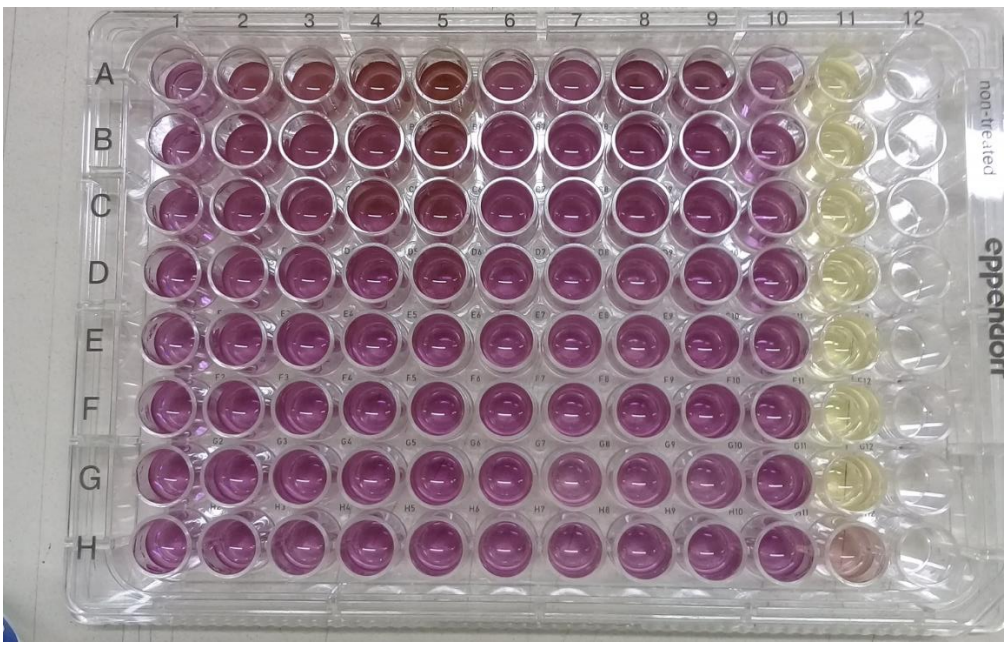


Figure 10: DPPH antioxidant assay.

3.5.3. Reducing power assay

The reducing power of the polysaccharides extracts was measured according to the method of Yen and Chen (1995) with slight modifications according to (Shi, 2016). Briefly, an aliquot of each sample (1 mL), with different concentrations, was mixed with 1 mL of phosphate buffer (200 mM, pH 6.6) followed by 1 mL of 1% potassium

ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated for 20 min in a water bath at 50℃. After this incubation, 1 mL of 1% trichloroacetic acid (TCA) was added, followed by centrifugation at 6000 g for 10 min. The supernatant (2 mL) was mixed with 2 mL of distilled water and 0.4 mL of 0.1% ferric chloride ($FeCl_3$), then the absorbance was measured at 700 nm against a blank in the spectrophotometer. A higher spectrophotometric absorbance meant a higher reducing power activity.

3.6. Measuring prebiotic activity *in vitro*

This analysis was conducted to simulate the digestion of the mushroom polysaccharides extracts by mimicking the gastrointestinal tract conditions and therefore evaluating the main effects of the extracts on selected gut microbes. The samples were treated according to Synytsya, et al. (2009), with slight adjustments. The strains selected for tests were *Lactobacillus plantarum* ATCC 8014, *Lactobacillus acidophilus* ATCC 4356 and *Enterobacter aerogenes*. These strains were supplied by Microbiologics Inc. from a collection of probiotic strains. Cultivation tests were performed at 37 ℃ in the medium based on MRS (Oxoid) commonly used for lactic acid bacteria cultivation. The medium comprised of (g/L) peptone 10.0; meat extract 8.0; yeast extract 4.0; dipotassium hydrogen phosphate 2.0; sodium acetate trihydrate 5.0; triammonium citrate 2.0; magnesium sulfate heptahydrate 0.2; manganous sulfate tetrahydrate 0.05 (de Man, et al., 1960).

The determination of the growth of test strains in the presence of crude mushroom polysaccharides was performed in U-shaped 96 well-plates. MRS broth media (200 µl) without glucose was supplemented with 1.5% polysaccharide inoculated with 20 µl of *L. plantarum* ATCC 8014, *L. acidophilus* ATCC 4356 and *E. aerogenes* (according to the McFarland standards, 0.5 contains 1.50×10^8 CFU/ml), and the plates were incubated for 72 hours. The absorbance was measured after 0, 17, 24, 48 and 72 hours of incubation using a reader at a wavelength of 550 nm, the pH value of

all samples was measured using a pH meter (Schott AG, Mainz, Germany) in the beginning (0 hours) and end (72 hours) of the experiment. Two controls were used: a negative control containing MRS broth without glucose supplemented by polysaccharide and a positive control containing MRS broth with glucose and the *Lactobacillus* strain. The absorbance readings for each sample were compared to that of the negative control (Nowak, et al., 2017).

3.7. Measuring antimicrobial activity

The antibacterial activity of the polysaccharides was studied by the method of paper disc diffusion assay (Zhu, et al., 2012). This method was chosen because it is fully compliant with international norms/standards as described by the National Committee for Clinical Laboratory Standard, (2001). *Escherichia coli* ATCC 33849, *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* (4b) ATCC 13932 were used as indicator strains and inoculated in Nutrient Broth medium. The bacteria were obtained from the cultures collection of the Biological Science Department of University of Namibia. With the inoculum solution (100 µL) about 10^6 CFU/mL of each kind of microorganism smeared on the Muller-Hinton agar medium, a filter disc with the polysaccharide solution (500 µg/mL) was placed in the middle of the medium. Sterilized physiological saline was used as a negative control, whereas ampicillin (Mastidiscs, Mast Group. Merseyside, UK) was used a positive control. After the medium was incubated for 24 h at 37℃, the inhibition zones were measured.

3.8. Data Analysis

All measurements in the experiments for antioxidant and immunomodulating activities were carried out in triplicates. The results were reported as mean ± standard error of the mean. For antioxidant activities, Students-T test was used for the comparison of the mean values, significant difference at $p = 0.05$. Statistical analysis

were done using Statistics Package for Social Science (SPSS, version 23). The correlation coefficient, R^2, between polysaccharides and antioxidants activities was determined using MS Excel (Microsoft Office 2013 Professional).

3.9. Research Ethics

Ethical clearance was obtained from the University Research and Ethics Committee (UREG) and Centre for Postgraduate Studies (CPGS). Instead of sample collection, the fruiting body samples of *Termitomyces schimperi* were purchased from market vendors. Lastly, no human trials and samples were involved in this study.

4. CHAPTER FOUR: RESULTS

4.1. Extraction and purification of polysaccharides

The polysaccharides extracted from *Kalaharituber pfeilii* were denoted with the acronym KPW (for hot water extracts) and KPA (for alkali extracts). On the other hand, the acronym TSW stands for polysaccharides from *Termitomyces schimperi*'s hot water extract and TSA denotes polysaccharides from *T. schimperi*'s alkali extract. The hot-water extract polysaccharides from both *T. schimperi* and *K. pfeilii* were deeper in brownish colour than the alkali extracts (Figure 11). In particular, KPW had more brownish coloured polysaccharides than TSW, this may be due to general natural colour of the truffles as seen earlier in Figure 6. This study also suggest that phenolic compounds, proteins and other small compounds may have contributed to the pigmentation of the polysaccharides. It could be observed that dialysis process removed part of the phenolic compounds and led to depigmentation/ lightening of the brownish colour of the polysaccharides.

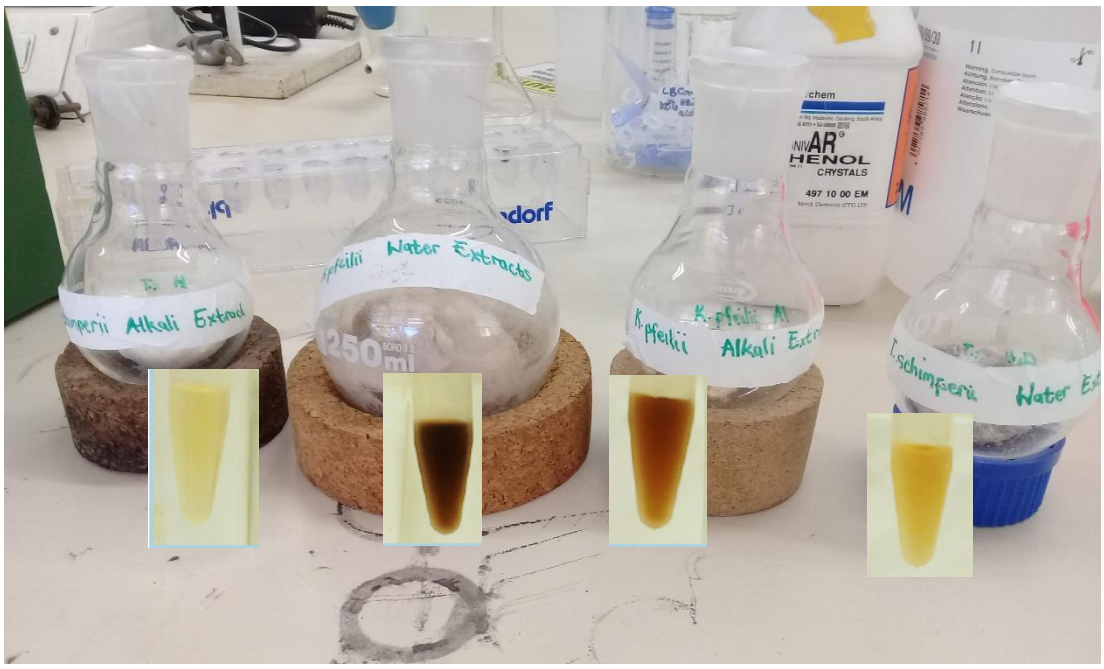


Figure 11: Appearance of the polysaccharides extracted from *K. pfeilii* and *T. schimperii* (From L-R) TSA, KPW, KPA and TSW.

4.2. Polysaccharides content

The method used was a colorimetric test for sugars and polysaccharides, where an orange colour was produced when carbohydrates were treated with a phenol/sulphuric acid solution. The intensity of this colouration is directly proportional to the amount of sugar present in these extracts. The carbohydrate content of the EPS from each polysaccharide was evaluated against a series of D-glucose standards. A calibration curve was plotted using the absorbance readings at 540 nm of the D-glucose standards (1 mL, 40 – 200 µg ml^{-1}) as shown in Figure 12.

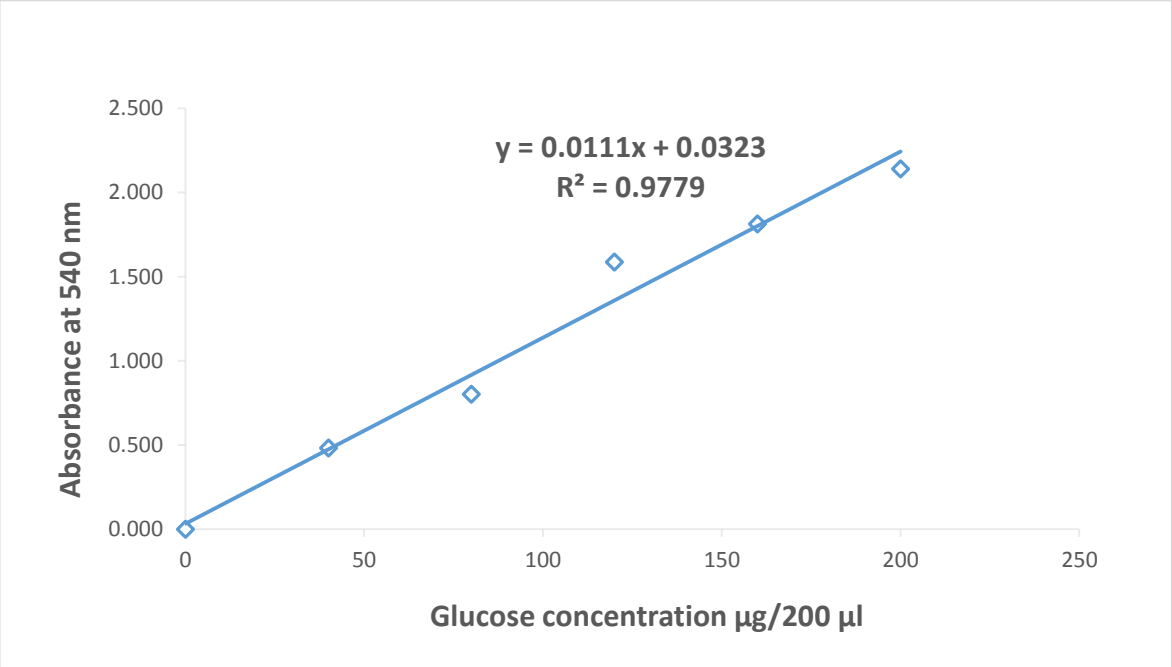


Figure 12: Calibration Curve for D-glucose Standard using Phenol/Sulphuric Acid Assay

The absorbance measurements for each concentration of D-glucose standard showed satisfactory correlation, R^2 = 0.9779. The trendline shows a slight positive bias, both the upper and lower 95 % confidence limits are positive values, therefore the bias is significant. The equation of the trendline was used to calculate the carbohydrate content of each polysaccharide; the results are shown in Table 6.

Table 6: Results for the Carbohydrate Analysis of PS from mushroom.

Samples name	**Absorbance at 540 nm**			**Calculated amount of carbohydrate (µg/200 µl)**			
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	**Final amount Carbohydrate (µg/200 µl)**
K. pfeilii							
KPW	2.874	2.795	2.898	261.83	254.71	263.99	**260.18 ± 2.80**
KPA	2.536	2.817	2.785	231.38	256.69	253.81	**247.29 ± 8.00**
T. schimperi							
TSW	1.640	1.475	1.629	150.66	135.79	149.67	**145.37 ± 4.80**
TSA	2.376	2.409	2.73	216.96	219.94	248.86	**228.59 ± 10.17**

Data are means of three independent experiments (±SE). Statistical significance of the carbohydrate content test was determined by Student's t-test. Difference was considered to be statistically significant if $p<0.05$.

4.3. Monosaccharide compositions of polysaccharides

The presence of a large amount of D-glucose with smaller amounts of D-galactose and traces of D-xylose was confirmed in all the hydrolysates by TLC. It is interesting to note that glucose was dominant and it is the main constituent in all polysaccharides. KPW gave three components upon total acid hydrolysis that were identified as D-galactose, D-mannose and xylose. In all tested hydrolysates, some components were observed with slow mobility, which are recognized to be partially hydrolysed glucan. Additionally, some disaccharides were visible. No uronic acid or arabinose was found in the thin-layer chromatographs when a solvent for separation of acidic components was used. Table 7 shows the monosaccharides detected in all of the polysaccharides samples (monosaccharide standards used included arabinose, xylose, galactose, mannose and glucose).

Table 7: The monosaccharides/reducing sugars detected on TLC in all of the polysaccharides samples

PS Extracts	Monosaccharide standards used				
	arabinose	xylose	galactose	glucose	mannose
TSW	–	–	+	+	+
TSA	–	–	+	+	–
KPW	–	+	+	+	+
KPA	–	–	+	+	+

+ Detected

– Not detected.

4.4. FT-IR spectral analysis

To describe polysaccharide extracts of *T. schimperi* and *K. pfeilii*, characteristic absorption of polysaccharides was performed in the range of 4000–400 cm^{-1}. The polysaccharides' characteristic absorption peaks of KPA, KPW, TSW and TSA in the FT-IR spectrum are presented in Figure 13 and 14 below.

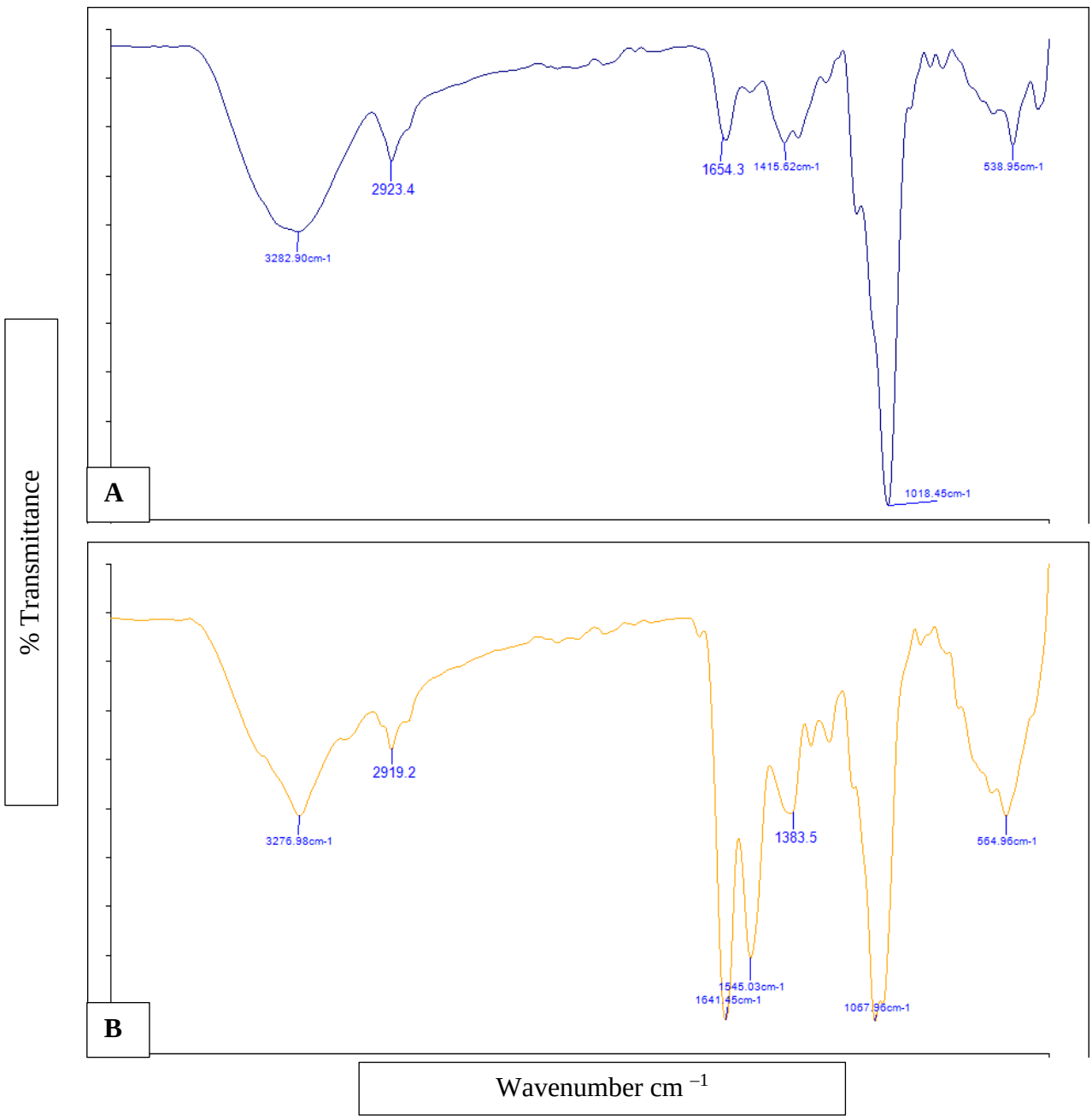


Figure 13: The FT-IR spectra of *Termitomyces* polysaccharides **A)** TSA and **B)** TSW

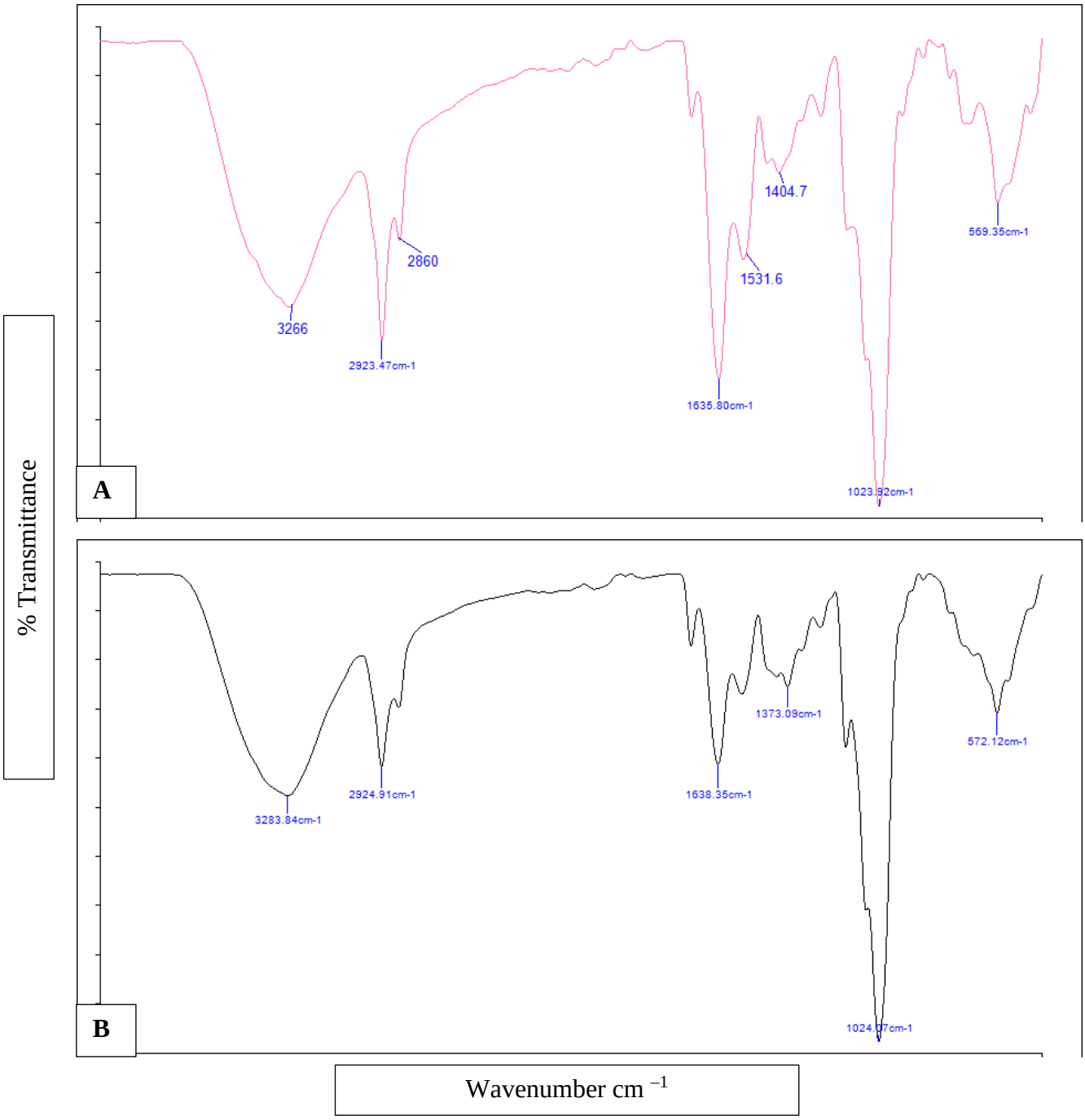


Figure 14: FT-IR spectra of *Kalaharituber* polysaccharides A) KPW and B) KPA in the range of 400–4000 cm^{-1}.

Except for *Termitomyces'* hot water extract polysaccharide TSW, the spectra of all samples were almost undistinguishable from each other. They displayed a broad, strong stretching band at 3300–3400 cm^{-1} (3282.8 cm^{-1}; 3266 cm^{-1}; 3276.9 cm^{-1} and 3282.6 cm^{-1} for KPA, KPW, TSW and TSA respectively) which corresponds to hydroxyl group (–OH) of carbohydrates.

Additionally, they also displayed a weak stretching band at around 2900 cm^{-1}, which was representative of polysaccharides. The same band mainly at the range of 2930 cm^{-1} (2924.9 cm^{-1}; 2923.4 cm^{-1}; 2919.2 cm^{-1} and 2923.4 cm^{-1} for KPA, KPW, TSW and TSA respectively) points to the suggestion that the methyl ($–CH_2$), H–C=O functional groups are present in the analysis. Additionally, the absorption peaks in the 1650 cm^{-1} region can indicate the presence of carboxylate groups (C=O) or C=C groups vibration in structures. The overlapping absorption bands between 950 and 1200 cm^{-1} region evidenced the (C–O) stretching of alcohols. It is interesting to note that spectra for TSW had extra band at 1545 cm^{-1} region that is characteristic of a small amount of protein. Additionally, peaks at 1415.6 cm^{-1} observed in TSA and 1404.7 cm^{-1} detected in KPW were indicative OH groups of phenolic compounds, which explains later good antioxidative activity in these mushroom polysaccharides.

4.5. Total phenolic content

The phenolic content of the EPS from each polysaccharide was evaluated against gallic acid. A calibration curve was plotted using the absorbance readings at 750 nm of the gallic acid standard (3 mL, 0.01 – 0.05 mg mL^{-1}, Figure15).

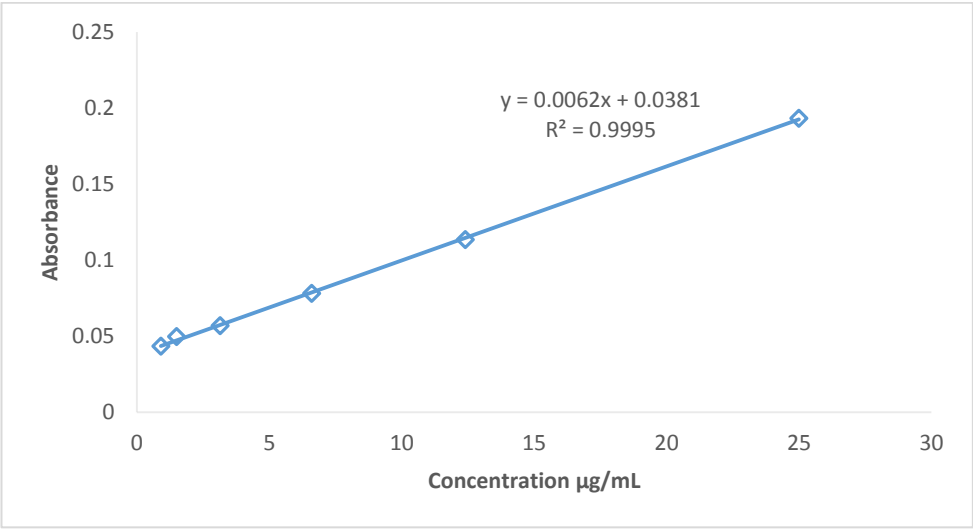


Figure 15: Standard curve of Gallic Acid

The total phenolic value was expressed as mg of gallic acid equivalents (GAE) per mg of dry weigh. Both the polysaccharide extracts of *K. pfeilii* demonstrated high phenol content but the highest estimated phenol content was observed for *T. schimperi* as 7.05 mg GAE/g in TSA, followed by 6.73 mg GAE/g in TSW, the total phenolic contents of all mushrooms are tabulated in Table 8 below.

Table 8: Total phenolic contents in *K. pfeilii* and *T. schimperi* PS extracts.

Samples name	**Absorbance at 750 nm**			**Total phenols (mg/mL)**			**Average mg GAE/g**
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	
K. pfeilii							
KPA	0.0612	0.0637	0.0621	3.73	4.13	3.87	3.91 ± 0.20
KPW	0.0599	0.0609	0.0582	3.52	3.68	3.24	3.48 ± 0.22
T. schimperi							
TSW	0.0782	0.0801	0.0811	6.47	6.77	6.94	6.73 ± 0.24
TSA	0.0834	0.0775	0.0845	7.31	6.35	7.48	7.05 ± 0.61

Data are means of three independent experiments (±SD).

4.6. Antioxidant activity assay

4.6.1. DPPH free radical scavenging activity

DPPH is considered as one of the most effective methods for evaluating the concentration of radical-scavenging materials (Oroian & Escriche, 2015). The results were calculated as the percentage of DPPH scavenged and graphically presented in Figure 16 and Figure 17. The DPPH radical scavenging activity results are shown in the figures below. It has been found that all polysaccharide extracts have significant amounts of radical scavenging activity.

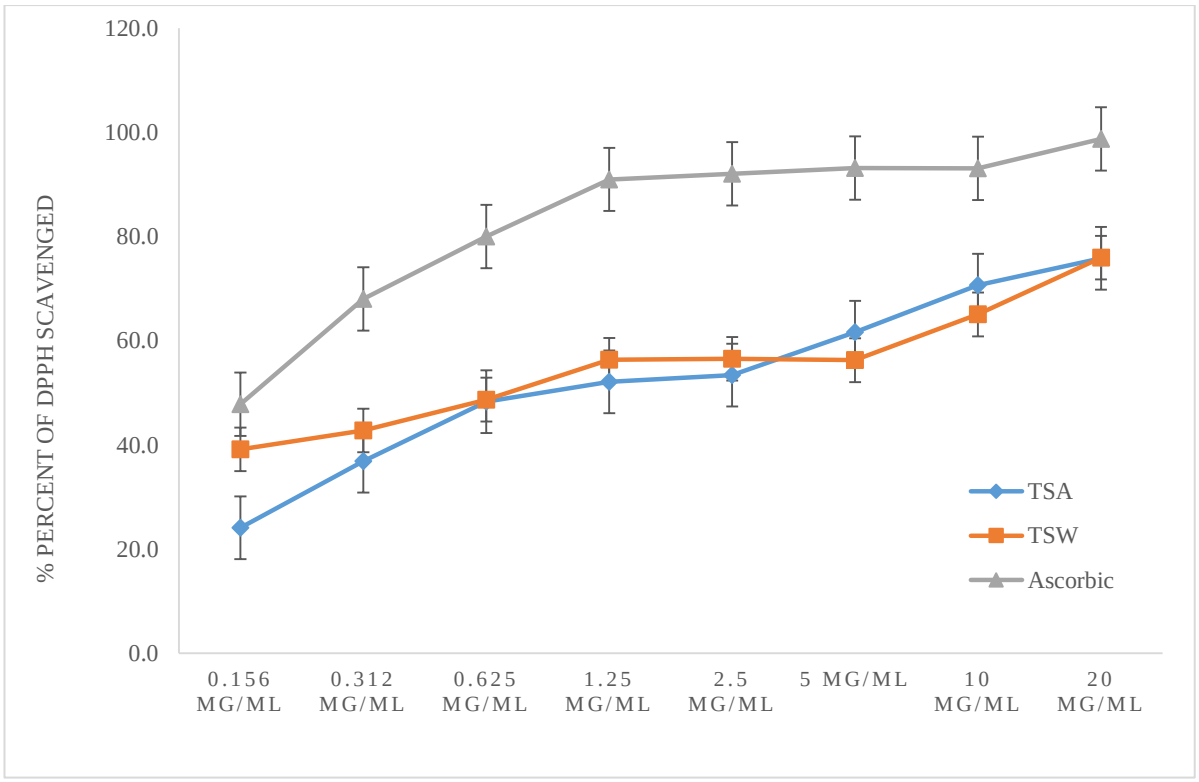


Figure 16: Antioxidant activities of TSA, TSW and ascorbic acid: DPPH radical scavenging activity.

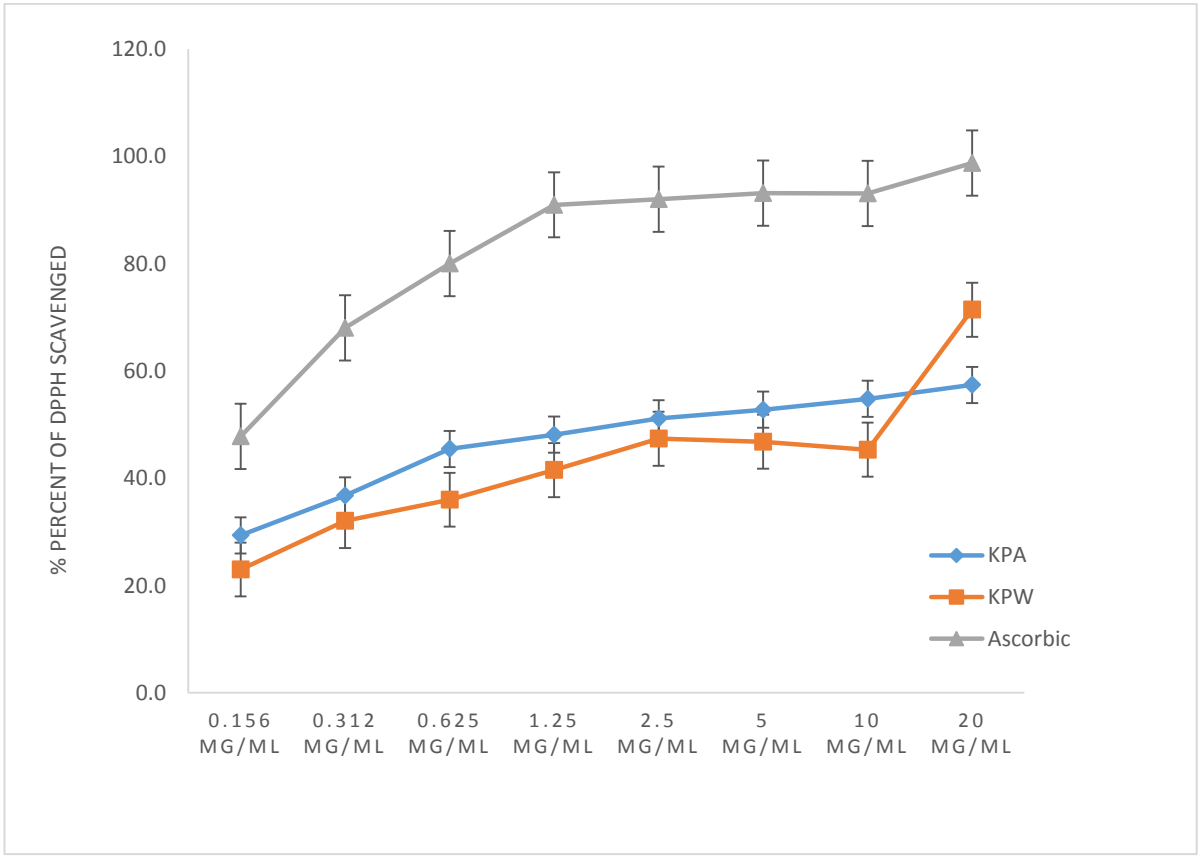


Figure 17: Antioxidant activities of KPA, KPW and ascorbic acid: DPPH radical scavenging activity.

The IC_{50} values (mg extracts/mL) is the necessary concentration of the polysaccharide that effectively reduces 50% of the initial DPPH concentration and was obtained by interpolation from the linear regression analysis. The results are shown in Table 9.

Table 9: IC_{50} values for K. pfeilii and T. schimperi EPS polysaccharide extracts.

Polysaccharide extracts	**IC_{50} mg/ml**
TSW	1.68
TSA	3.55
KPA	8.14
KPW	8.86

4.6.2. Reducing power assays

The reducing ability was measured by monitoring $Fe^{3+} \rightarrow Fe^{2+}$ conversion by the mushroom polysaccharides. The formation of Fe^{2+} is determined from the absorbance values at 700 nm (Shi, 2016), at five different concentrations. A higher spectrophotometric absorbance meant a higher reducing power activity. It can be seen that *T. schimperi* water polysaccharides (TSW) exhibited good reducing power as demonstrated in Figure 16 below. Polysaccharides from alkali extracts of *K. pfeilii* (KPA) have also showed highly significant reducing ability. The reducing power of the polysaccharides from these mushrooms can be ranked as TSW > KPA > KPW > TSA. The results of the Fe^{3+} reducing abilities of polysaccharides are presented in Table 10 below.

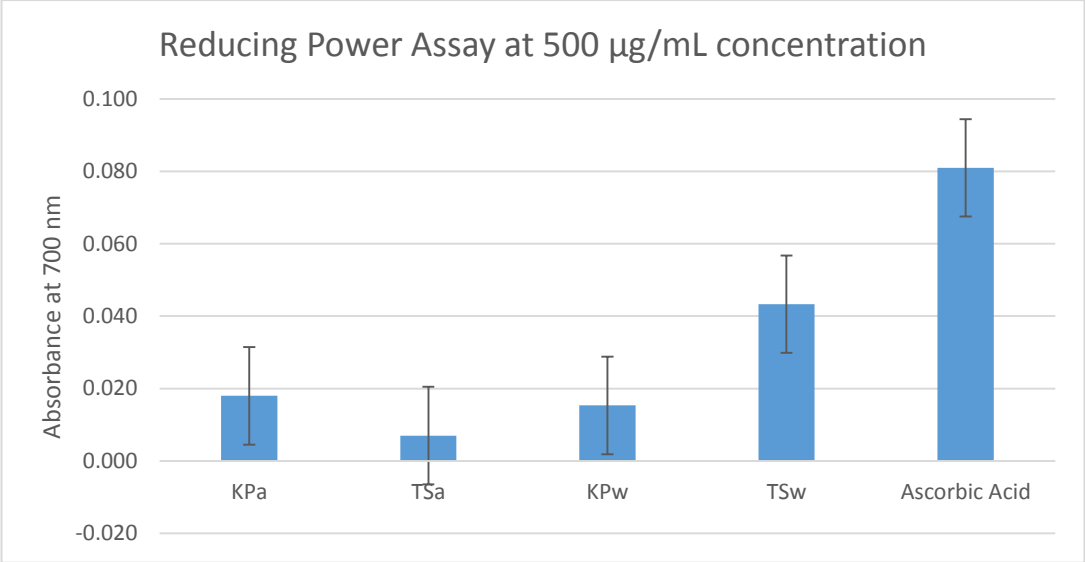


Figure 18: Reducing power assay at 500µg/mL of KPA, KPW, TSA, TSW EPS extracts and ascorbic acid:

Table 10: Reducing power antioxidant properties of polysaccharides extracts from *K. pfeilii* (KPA, KPW) and *T. schimperi* (TSA, TSW).

	Concentration			
PS Extract	**500 µg/mL**	**250 µg/mL**	**125 µg/mL**	**62.5 µg/mL**
KPA	0.018 ± 0.002	0.010 ± 0.002	0.002 ± 0.000	NA
TSA	0.007 ± 0.001	0.004 ± 0.001	NA	NA
KPW	0.015 ± 0.001	0.011 ± 0.001	0.005 ± 0.002	0.002 ± 0.002
TSW	0.043 ± 0.001	0.030 ± 0.002	0.015 ± 0.003	0.002 ± 0.000
Ascorbic Acid	0.081 ± 0.002	0.066 ± 0.003	0.043 ± 0.002	0.024 ± 0.001

NA: No absorbance, each value is expressed as the mean ± standard deviation.

4.7. Antimicrobial activity

In vitro antimicrobial activity of *T. schimperi* and *K. pfeilii* is shown in Table 11 below. A zone of inhibition greater than 7mm was considered significantly positive. Among the four polysaccharides, TSW showed the best antimicrobial activities against *E. coli, L. monocytogenes,* and *S. aureus* with inhibition zones of 10.0 mm, 11.0 mm, and 9.0 mm, respectively. It is interesting to note that *L. monocytogenes* and *S. aureus,* which are both gram positive bacteria, were the most sensitive bacteria to both PS. However, *E. coli* was the most resistant strain and the highest inhibition zone diameter was of 9 mm for TSA and TSW. In general, *T. schimperi* derived polysaccharides extracts showed more antibacterial activity in comparison to *K. pfeilii*.

Table 11: The antimicrobial activity of PS.

PS Extract	**Bacteria zone of inhibition**		
	Escherichia coli	***Listeria monocytogenes***	***Staphylococcus aureus***
KPA	6.1 ±0.10	10.0 ±0.30	10.0 ±0.40
TSA	9.0 ±0.00	11.5 ±0.20	7.0 ±1.00
KPW	7.0 ±1.00	10.0 ±2.00	11.0 ±0.41
TSW	9.0 ±0.30	11.0 ±0.40	9.0 ±0.50

Results are the average of triplicate experiments; each value is expressed as the mean ± standard deviation.

4.8. Prebiotic assay

Prebiotic potential of the polysaccharide extracts has been assessed against *L. plantarum* ATCC 8014, *L. acidophilus* ATCC 4356 and *E. aerogenes* as described in Section 3.6. The difference between the values of analysed parameters (maximum biomass concentration and acid production) measured was compared for each specific bacteria strain (Figure 20). Positive value indicates stimulating effect of extract to related growth characteristic of the selected strain, whereas a negative value portrays an inhibitory effect. From the results, it can be seen that TSA, KPW and TSW polysaccharides showed their ability to act as a carbohydrate sources for these bacteria. These extracts support probiotic bacteria biomass growth and SCFA (short chain fatty acids) production especially for *Lactobacillus* strains as indicated by decrease in pH value and increased optical density. Additionally, all the four bacterial species showed significant activity as evidenced by the decrease of pH of the growth medium (Figure19). Polysaccharide extracts from *T. schimperi* demonstrated the ability to act as better probiotics growth source than that from *K. pfeilii*. It should also be noted that alkali extracts exhibited better stimulating effects than water extracts for both *K. pfeilii* and *T. schimperi*.

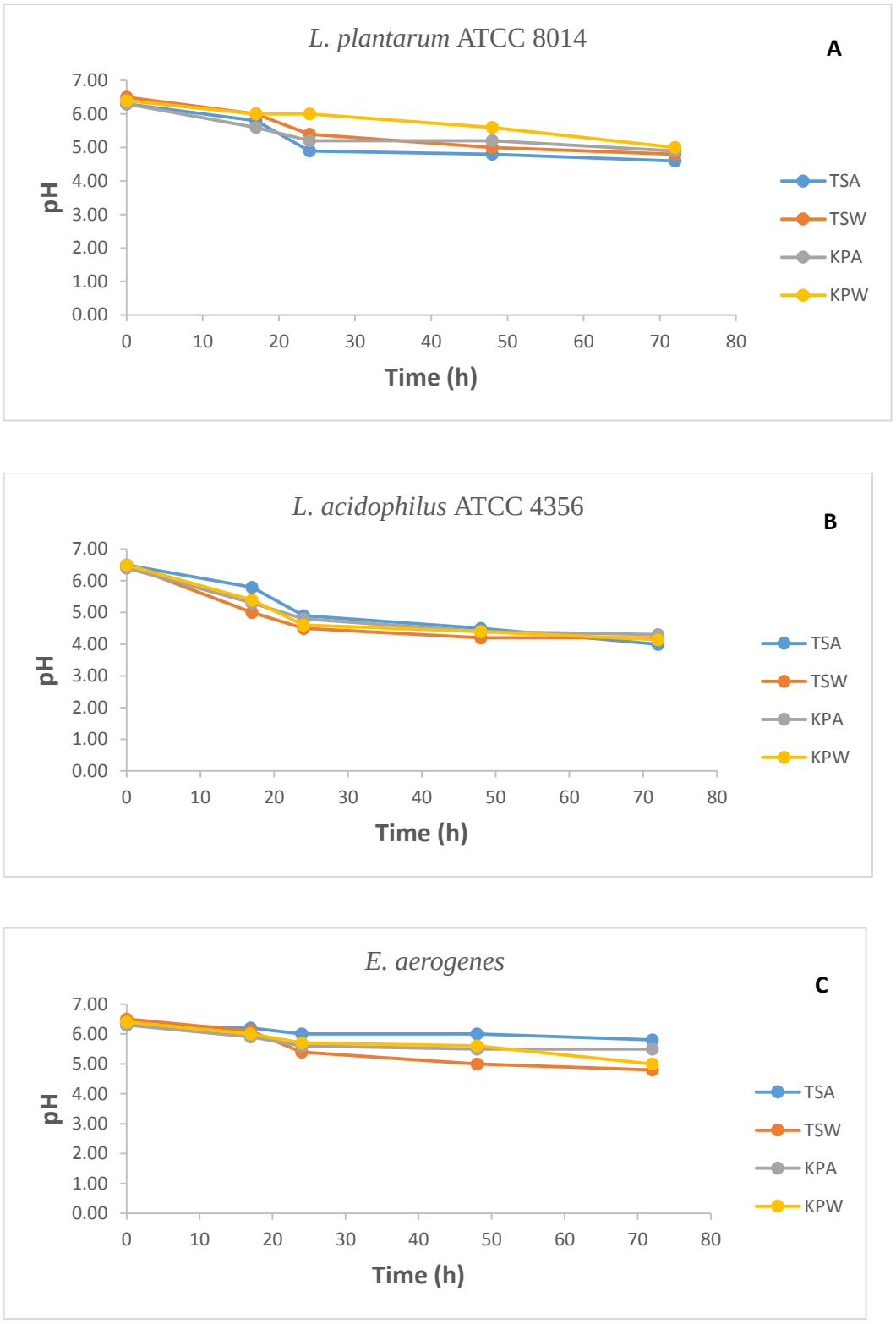


Figure 19: Determination of pH-value after 0, 17, 24, 48 and 72 hours of incubation of bacterial strains **A)** *L. plantarum* ATCC 8014, **B)** *L. acidophilus* ATCC 4356 and **C)** *E. aerogenes* with different mushrooms polysaccharide extracts (TSA- *T. schimperi* alkali extracts; TSW - *T. schimperi* water extracts; KPA – *K. pfeilii* alkali extracts and KPW – *K. pfeilii* water extracts.

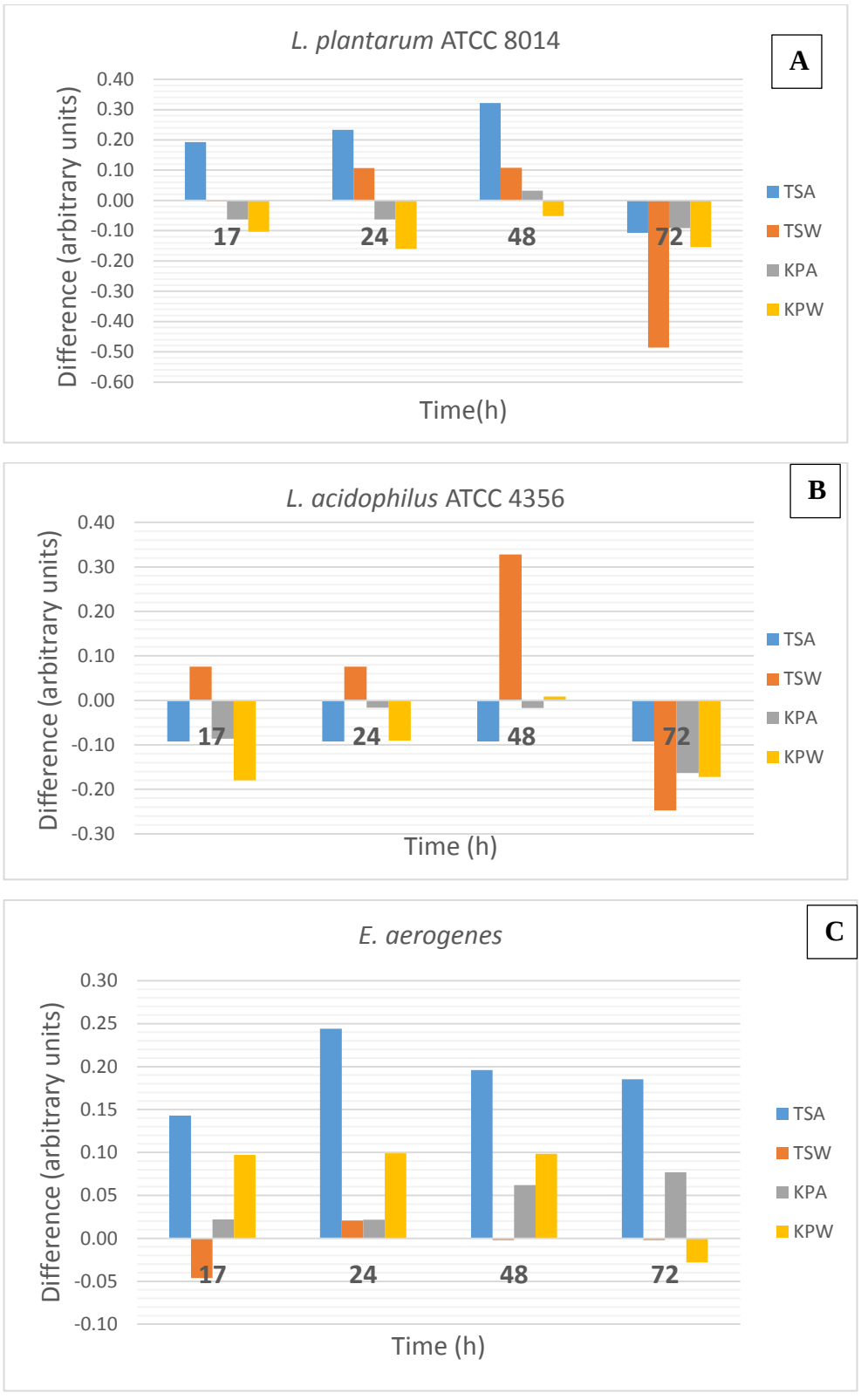


Figure 20: The plot of difference between the values of maximum biomass concentration of different strains **A)** *L. plantarum* ATCC 8014, **B)** *L. acidophilus* ATCC 4356 and **C)** *E. aerogenes* measured as optical density (OD_{550}) for the medium supplemented with different mushroom polysaccharides extracts at different times of incubation 0, 17, 24, 48 and 72 h.

5. CHAPTER FIVE: DISCUSSION

5.1. Chemical Characterization

5.1.1. Polysaccharide contents

It is clear that the polysaccharides contained a significant quantity of total sugar content. However, due to the use of different extraction methods, the total carbohydrate content found in hot-water extracts was significantly different from that found in alkali extract, even though they came from the same mushroom sample. The *Termitomyces* mushroom hot-water extracted polysaccharides (TSW) had a relatively lower total carbohydrate content (145.37 ± 4.80 µg/200 µl) than that of alkali extract (TSA) (228.59 ± 10.17 µg/200 µl) (Table 6). Additionally, statistical test has also revealed that there is a significant difference in the total sugar content between TSA and TSW (*p=0.002*, see Appendix 1A). Our results are in agreement with previous results of Chen et al. (2016). As reported by Chen et al. (2016), the evidence we found points to a suggestion that other substances were present in a considerable amount in TSW, whereas TSA was a rather pure carbohydrate. Proteins were probably bound to the soluble glucans, and the Sevag method was not able to separate them completely. These findings appear to be substantiated by Chen and Cheung, (2014) who's study of the cell wall of *Pleurotus* (fruiting bodies) showed that the polysaccharide, extracted with hot water, was composed of polysaccharides and protein complex.

In contrast, *Kalaharituber* mushroom hot-water extracted polysaccharides (KPW) had higher total carbohydrate content (260.18 µg/200 µl) than that of alkali extract (TSA) (247.29 µg/200 µl) (Table 6). However, there is no statistical difference between the alkali extracts and hot water-extracts of *Kalaharituber* polysaccharides at 95 % confidence interval *p= 0.203* (see Appendix 1B).

5.1.2. Monosaccharide compositions of polysaccharides

According to Wasser (2002), mushroom polysaccharides are present mostly as glucans with different types of glycosidic linkages, such as (1-3), (1-6)-*β*-glucans and (1-3)-*α*-glucans but also as heteroglucans. The polysaccharides can be composed by different monosaccharides assuming pyranosidic or furanosidic conformations. Monosaccharides found in mushroom polysaccharides include glucose, galactose, mannose, xylose, arabinose, fucose, ribose, and glucuronic acid (Ruthes, et al., 2016). In this study as it was qualitatively determined by TLC and portrayed in Table 7, the polysaccharides contained glucose (all extracts), xylose (only in KPW), galactose, mannose (except in TSA), and galactose as a main component or in different combinations. Our results for *Termitomyces* are barely distinguishable from that of Mondal, et al. (2006) who isolated a water-soluble heteropolysaccharide (Fig. 18) from fruiting bodies of *Termitomyces striatus* which comprised of D-glucose, D-galactose, D-mannose, and L-fucose.

In addition to that, previous studies have also isolated water-soluble heteroglucans (Fig. 21), from fruiting bodies of *Termitomyces clypeatus*, *T, heimii* consisting monomeric sugar units of D-glucose, D-galactose, D-mannose, and L-fucose as monosaccharides (Pattanayak, et al., 2015; Bhanja, et al., 2012; Manna, et al., 2015). Given that our findings are based on *T. schimperi* species the results from such analyses should consequently be treated with the utmost caution. As far as we aware this is the first study reporting on characterising monosaccharides from *K. pfeilii*. Previous research revealed that most of the truffles polysaccharide monomers were D-mannose, D-glucose, and D-galactose (Zhao, et al., 2014). In particular sugar compositions similar to KPA, containing mannose, glucose, was found in a polysaccharide isolated from *Tuber rufum* (Pattanayak, et al., 2017).

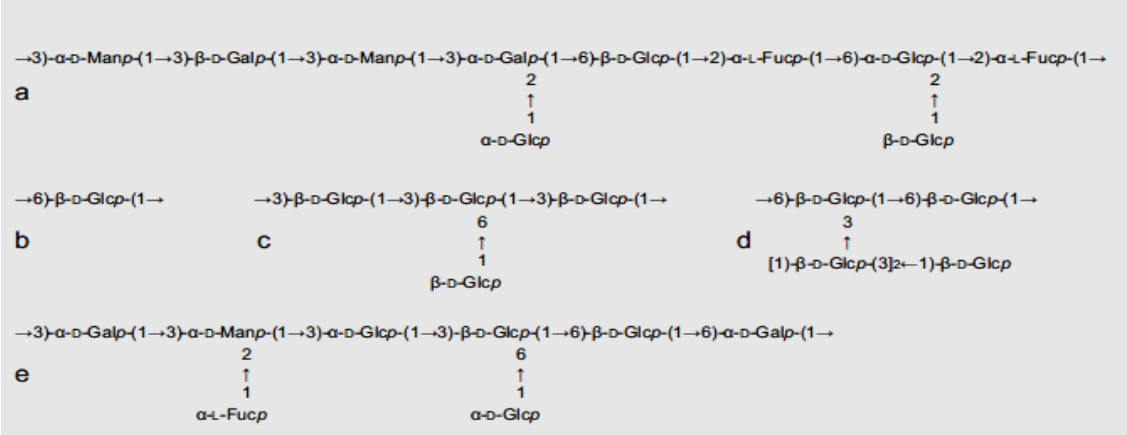


Figure 21: Chemical structures of polysaccharides isolated from the fruiting bodies of *Termitomyces* (Source: Hsieh and Ju, (2018)) of which (**e**) may be a possibility of our *T. schimperi* polysaccharide.

It can be observed that the **(e) heteropolysaccharide** comprises of monomeric sugar units (glucose, galactose and mannose) which are similar to the one detected in TSW and KPA.

5.1.3. FT-IR spectral analysis

Belton et al., (1991) highlighted the fact that Fourier transform-infrared spectroscopy has been a useful technique in tracking structural alterations in biopolymers. The spectra of corresponding mushroom polysaccharides isolated from stems of other species were similar to that in this study (Figure 13 and 14). FT-IR spectra of polysaccharides from *K. pfeilii* and *T. schimperi* demonstrated the characteristic peaks of mushroom polysaccharides (Figure 13 and 14). The polysaccharides showed high absorbance between the regions of 1200-950 cm^{-1} which according to Dilna et al. (2015) is considered the thumbprint region of polysaccharides. According to Das and Goyal (2014), the intense peak at 1030 cm^{-1} usually indicates the existence of α-(1–6) glycosidic bond. This peak was clearly observed in our polysaccharides spectra at 1018 cm^{-1}; 1023 cm^{-1}; 1024 cm^{-1} for TSA, KPA and KPW polysaccharides (Figure 13 and 14) indicating the presence of a pyranose sugar and β(1–4) glucans in our samples (Zhang, et al., 2014 ; Galichet, et al., 2001). Carbonyl groups (C=O)

showed two bands: an asymmetrical stretching band around 1650 cm^{-}). The weak C-H extension band at 2900 cm^{-1} can also point to the occurrence [1] and a weak symmetric stretching band near 1400 cm^{-1} (Rao, 1967; Casu et al., 1978of amino acid groups (Synytsya, et al., 2009). Hence, Our FT-IR result confirms that the sample comprises some proteins, which may not have been completely removed by deproteinization with Sevag reagent (Synytsya, et al., 2009). It has been observed in literature that the presence of α–glucans and β–glucans were often associated with the antioxidant capacity of the polysaccharide (Ruthes, et al., 2016). By FT-IR analysis we were able to confirm their presence in our samples.

5.1.4. Total phenol content

Phenolic compounds are particularly potent natural products with wide range of biological activities, including antioxidant, anti-inflammatory, antimicrobial and anticancer properties (Halliwell & Gutteridge, 2006). The fact that mushroom polysaccharides contain polyphenolic compounds is well established (Klaus, et al., 2011). Polyphenolics tend to bind with polysaccharides with hydrogen bonds, hydrophobic interactions or even covalent bonds (McManus, et al., 1985; Renard, et al., 2001). According to Beara, et al., (2014), it is well established that bound polyphenolics contribute to antioxidant activity of polysaccharides and their presence is highly beneficial. Therefore, the confirmation of these compounds in the examined *K. pfeilii* and *T. schimperi* polysaccharide extracts could contribute to their numerous potential applications, apart from using them as food.

In order to evaluate the phenolic profile of the investigated extracts, the total phenolic content was determined. Total phenolic contents (Table 8) varied from slightly to moderately between the extracts, from 3.48 mg GAE/g of dry weight (*K. pfeilii* water extract (KPW) to 7.05 ± 0.61 GAE/g of dry weight of *T. schimperi* alkali extract (TSA). Generally, it could be observed that a slightly higher content of

the examined phenolic compounds was determined in extracts of *T. schimperi* than in those of *K. pfeilii*. However, it was statistically deduced by the Students T-test that there is indeed a significant difference (p = 0.009) between phenolic contents of the two mushroom samples as portrayed in Appendix 2.

This depicts that *T. schimperi* may have better antioxidant potential when compared to *K. pfeilii,* which illustrates that it may have commercial application for their use as food additives in several industries. Variations in phenolic content between the two solvent extracts may be due to the variable solubility of plant phenolics in methanol and ethanol. Variation of total phenolics contents in the examined extracts of the same species may also be attributed to different extraction conditions applied (extraction method, particle size, solvent type, solvent concentration, etc.), which is known to affect a phenolic profile quality (Yim, et al., 2009). Our findings appear to be well substantiated by Islam et al. (2016), who reported that the total phenolic content of 43 mushroom species ranged from 26.21 to 0.19 mg GAE/g. Furthermore our results share a number of similarities with Gursoy et al., (2009) whose study found that the TPC of seven mushroom species ranged from 25.38 ± 0.70 mg GAE/g to 12.36 ± 1.21 mg GAE/g.

These findings indicate that our Namibian mushrooms are an important source of phenolic compounds. However, to the best of our knowledge, this is the first comprehensive analysis of phenolic compounds in *K. pfeilii* and *T. schimperi*, which could be a valuable basis for further approval of using it as food with health benefits. It is plausible that one limitation might have influenced the results obtained. Namely, the Folin–Ciocalteu assay is highly sensitive for polyphenols, flavonoids, and tannins. However, the presence of some easily oxidizable substances, including sugars and amino acids, could cause interference.

5.2. Antioxidant activity assays

Dietary antioxidants have a dual role: they can prevent food oxidation, in particular lipid oxidation, and at the same time, increase the antioxidant intake from diet. In organism, these exogenous antioxidants can manifest a wide variety of actions, including inhibition of oxidising enzymes, chelation of transition metals, transfer of hydrogen or a single electron to radicals, singlet oxygen deactivation, or enzymatic detoxification of reactive oxygen species (Beara, et al., 2014). Common result of these actions are the protection against degenerative diseases, for example, atherosclerosis, cancer, diabetes, rheumatoid arthritis and inflammatory diseases, which are caused by the increased level of reactive radical species (Prior, Wu, & Schaich, 2005). Therefore, in order to extensively characterize antioxidant potential of the truffle extracts examined, different antioxidant assays were applied and the results are presented in Section 4.6.

5.2.1. DPPH free radical scavenging activity

The results of both DPPH scavenging experiments by spectrophotometric measurement indicated the possession of strong scavenging of DPPH radicals by *K. pfeilii* and *T. schimperi* in concentration dependent manner. Phenolics present as detected in section 4.5 in both *K. pfeilii* and *T. schimperi* possess hydroxyl groups that cause the quenching of DPPH radicals. A possible mechanism may be attributed to the hydrogen atom donating ability of the hydroxyl group of polysaccharides to free radicals to terminate the radical chain reaction by converting radical into stable atoms or molecules (Halliwell & Gutteridge, 2015). It has been reported that the chemical properties of polysaccharides such as molecular weight, monosaccharides composition, linkage and conformation may also affect the antioxidative actions (Chen, et al., 2012). Correlation plots were developed in order to reveal the

relationship between the antioxidant activity and total phenolic content of the polysaccharide extracts.

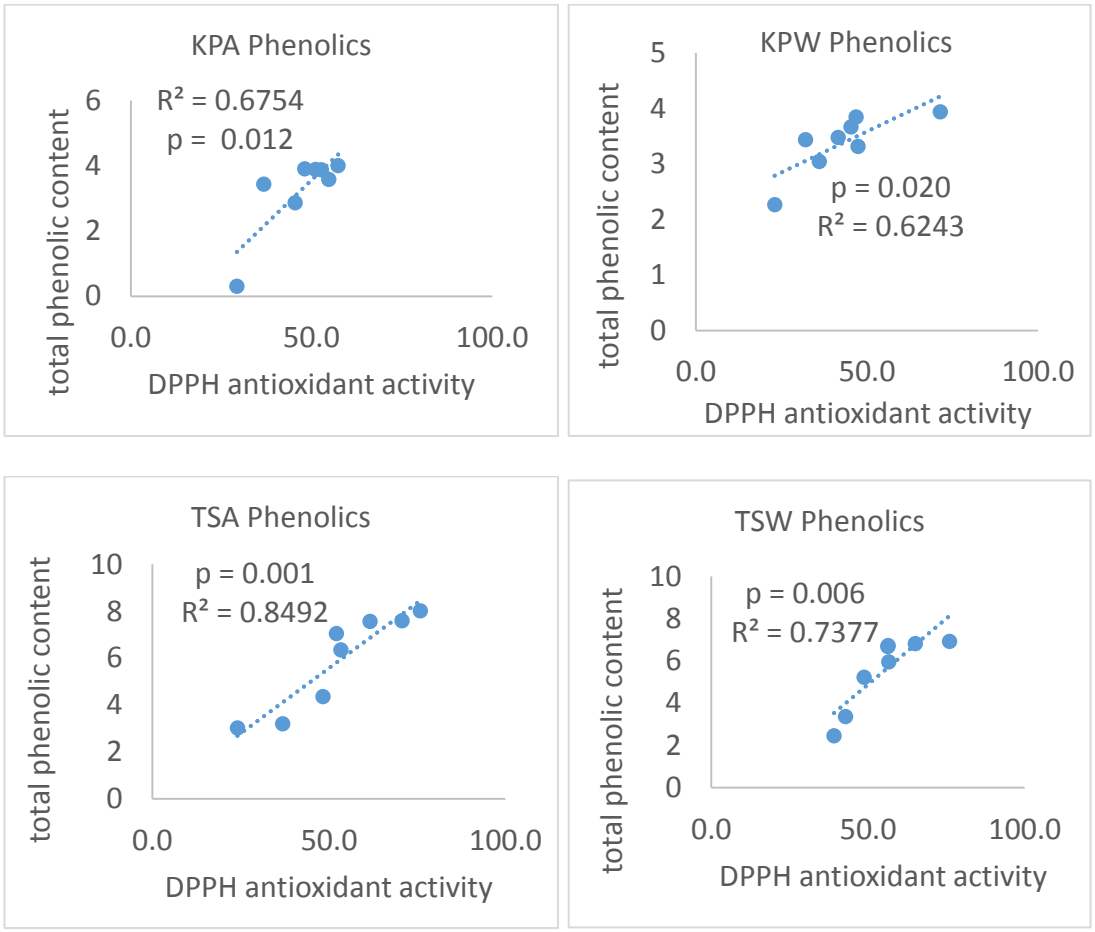


Figure 22: Correlation of DPPH antioxidant activity to total phenolic content of polysaccharide fractions from *T. schimperi* (TSA & TSW) and *K. pfeilii* (KPW & KPA). Linear regression coefficient (R^2) and significance values (*p*) are indicated.

*Significant effect at $p < 0.05$

According to the trend lines and linear regression coefficients (R^2), TSA was significantly positively correlated to the phenolic content (R^2=0.85, p<0.05). Both KPA, KPW and TSW were moderately positively correlated to the phenolic content. The results implies that the total phenolic contents are important contributors to the antioxidant activities of the water soluble water extracts of the mushrooms and this observation is in agreement with the literature (Puttaraju, et al., 2006).

Puttaraju, et al., (2006) compared the antioxidative activities of water extract and methanolic extract from fruiting bodies of 23 species of edible mushrooms, among which *T. heimii, T. microcarpus, T. mammiformis, T. tylerianus,* and *T. schimperi* were included. Additionally, Woldegiorgis et al. (2014) also reported that the major phenolic acid present in the fruiting bodies of the three *Termitomyces* species that they studied was gallic acid, with caffeic acid, chlorogenic acid, pcoumaric acid, ferulic acid, and p-hydroxybenzoic acid in less quantities contributed significantly to the antioxidant activities of the water soluble water extracts of the mushrooms.

5.2.2. Reducing power assays

Fe^{3+} reducing power of the polysaccharides extracts were also measured as part of evaluating the antioxidant potential of the mushroom polysaccharides. The results of concentration dependant reducing power of the extracts are presented in Table 10. The extracts from TSW & KPA showed significant reducing ability. These activities of edible mushroom polysaccharides may be linked to the presence of reducing sugars (Table 7) such as galactose and glucose (Selvendran & Isherwood, 1967). It should be noted that all the active polysaccharides studied here contain large quantities of galactose and glucose. A correlation of reducing power antioxidant activities of the selected mushroom polysaccharide extracts and their carbohydrate contents is presented in Figure 21 below.

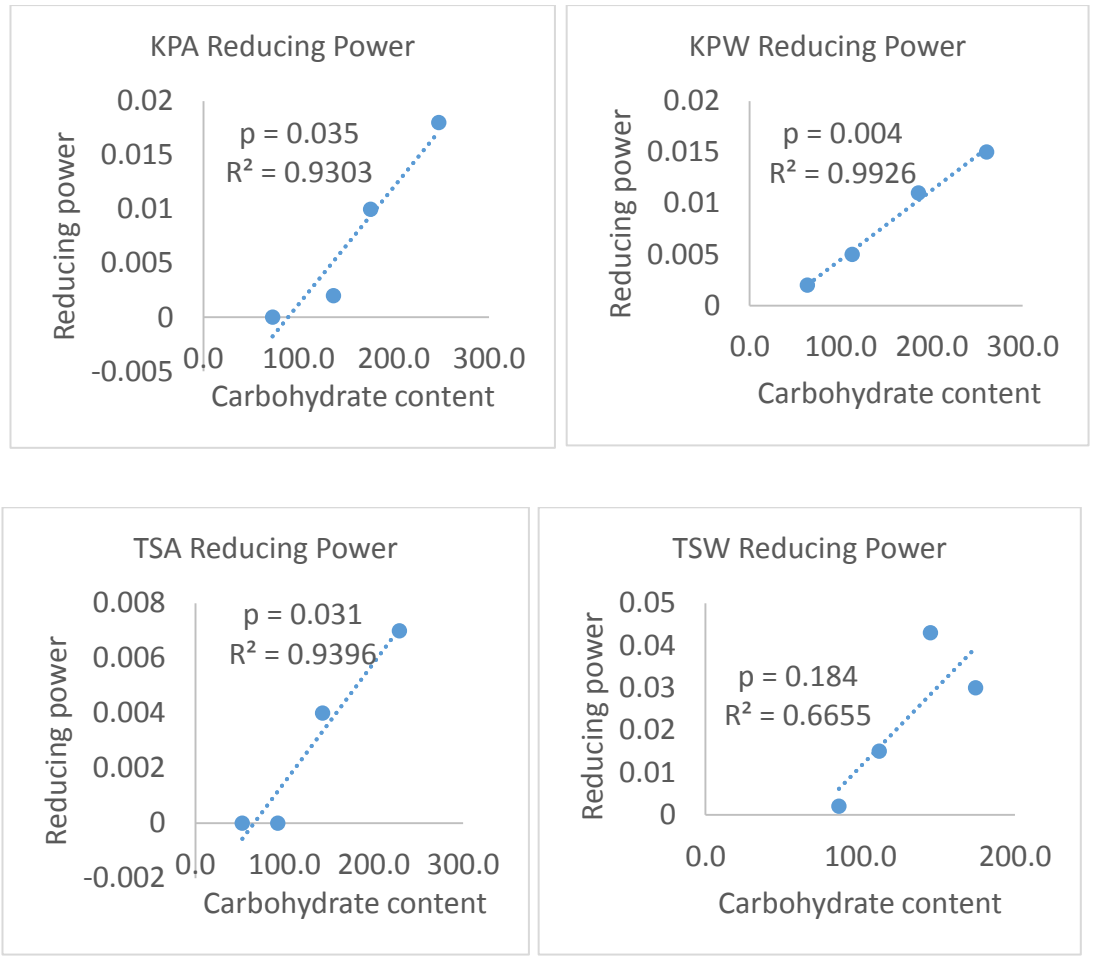


Figure 23: Correlation of reducing power antioxidant activity to carbohydrate content of polysaccharide fractions from *T. schimperi* (TSA & TSW) and *K. pfeilii* (KPW & KPA). Linear regression coefficient (R^2) and significance values (*p*) are indicated.

*Significant effect at $p < 0.05$

Both KPA, KPW and TSA were strongly positively correlated to carbohydrate content of mushrooms both with linear regression coefficients (R^2) above 0.9. TSW was not significantly correlated to the carbohydrate content (R^2=0.66, $p>0.05$). According to previous studies, glucans in the α-form have shown a strong correlation to the reducing power of polysaccharides from four well-known medicinal mushrooms including *Ganoderma applanatum*, *G. lucidum*, *L. edodes* and *T. versicolor* (Kozarski et al., 2012). Our results suggested that polysaccharides extracts

from *T. schimperi* and *K. pfeilii* possesses antioxidant activity and could be explored as a potential antioxidant.

5.3. Antimicrobial activity

L. monocytogenes and *S. aureus*, which were inhibited by the mushroom polysaccharides, are Gram-positive strains, while the only Gram negative strains used in the study *E.coli*, was resistant to the mushroom extracts. It is well documented in literature about the susceptibility of Gram positive bacteria to mushroom polysaccharide extracts, hence our study agrees with previous studies (Barros, et al., 2007; Venturini, et al., 2008).

It has been reported that inhibition provided by the phenolic compounds (Section 4.5) was greater than that of the antibiotics used for the treatment of infection promoted by *S. aureus* (Alves, et al., 2012). Based on our FTIR results (Figure 13 and 14) the presence of carboxylic acid (COOH), hydroxyls (OH), groups in the polysaccharide extracts appears to be important in relation to the anti-*S. aureus* activity, which may elucidates good antibacterial activity of TSW polysaccharide in this study.

Other possible antibacterial mechanisms of polysaccharides in previous studies would have provided some clues. However, to the best of my knowledge, this is the first study to reveal that polysaccharides isolated from *T. schimperi* and *K. pfeilii* have antibiotic activity against bacteria. Thus, the antibacterial mechanism as well as other biochemical mechanisms supporting this bioactivity remain puzzling. TSA had the weakest inhibitory activity of the four polysaccharides. This can be attributed to the fact that the disc diffusion method is very dependent on the diffusion ability of the test substances (Ren, et al., 2014). Hence, it is likely that the polysaccharides used in this study had high molecular weights, which diffuse poorly into agar.

5.4. Prebiotic activities

The current study provides first evidence of the prebiotic potential of *T. schimperi* and *K. pfeilii* polysaccharides. As described in Section 4.6. TSA, KPW and TSW showed their ability to act as a carbohydrate sources for these bacteria. All the four bacterial species showed significant activity as evidenced by the decrease in pH of the growth medium as a function of incubation time (Fig. 19) indicating fermentation of the polysaccharides. The decrease in pH is the result of the initially high carbohydrate availability (Table 6), which results in saccharolytic fermentation and subsequent formation of SCFAs and lactate acid, thus lowering the pH (Holscher, 2017). Prebiotic activity of the polysaccharides was also demonstrated by monitoring the turbidity (optical density) of the medium during these experiments and the results have shown a clear increase in turbidity as a function of incubation time (Fig. 20) reflecting increased numbers of bacteria. The differential growth rates promoted by all the polysaccharide extracts reflect differential mechanisms underlying each probiotic strain, something that requires further and extensive investigation. These results indicate that polysaccharides displayed significant prebiotic activities. TSA showed better prebiotic activity than KPW, TSW and KPA with all species of probiotic bacteria tested in this research.

This is the first such determination for these mushroom species. In more recent studies, previous data revealed that other mushrooms were able to promote the growth and viability of probiotic bacteria. Different extracts of *Pleurotus ostreatus* and *Pleurotus eryngii* were also studied, and their prebiotic potential was revealed by Synytsya et al. (2009). *Pholiota nameko* extracts were found to increase the growth of *L. acidophilus* (Rodrigues, et al., 2017). Furthermore, the polysaccharide fraction from *G. lucidum* revealed the potential for the stimulation of the growth of both *Lactobacillus* sp. and *Bifidobacterium* sp. (Yamin, et al., 2012). Crude

polysaccharides from *C. cibarius* were tested for their potential stimulation for the growth of the reference strain of *L. acidophilus* and then two clinical strains of *L. rhamnosus* in order to determine their potential use as prebiotic compounds (Nowacka-Jechalke, et al., 2018).

Up till now, non-digestible carbohydrates isolated from mushroom are universally acknowledged as prebiotics (Delgado-Fernandez, et al., 2019). The end-products of prebiotics fermentation by probiotics, such as acetic, propionic and butyric acids, have been demonstrated to play important roles in immune-modulation, lipid metabolism regulation and integrity improvement of colon epithelial barrier, except serving as energy sources (Rastall & Gibson, 2015). SCFAs produced from fermentable carbohydrates are reported to acidize the luminal pH of colon and cecum (Gibson & Roberfroid, 2005), which in turn affects the SCFAs production. In this study, after fermentation, pH of culture medium was significantly decreased which can be attributed to the increased concentration of lactic acid and SCFAs. Lowered large bowel pH as a consequence of SCFAs production by *Lactobacillus* fermentation with mostly TSA and KPW polysaccharides could benefit large bowel health through undesirable pathogens proliferation inhibition, microbial enzymes' activity modification, and protection effects against carcinogenesis.

Prebiotics show several nutritional characteristics. They can be incorporated into food to enhance the growth of microflora in the GI tract thereby improving the quality of foods. They can be formulated either as a powder or syrup and marketed as supplements or incorporated into food products (e.g., yogurts and breads). Prebiotic functional food products includes biscuit, cakes, sauces, breads, pasta, snack foods, dietetic products, frozen yoghurt, custard, desserts, table top sweetener, candies, soft drinks, fruit juices, lactic acid bacteria drinks, coffee and beverages (Douglas & Sanders, 2008).

6. CHAPTER 6: CONCLUSION

Overall, this investigation has fulfilled a comprehensive experimental study on the isolation, structural characterization and antioxidant activities of *K. pfeilii* and *T. schimperi* polysaccharides. Our results suggest that *T. schimperi* mushroom polysaccharide are very good source of naturally-derived antioxidant and potential functional food ingredient. Based on the FTIR spectra it can be concluded that all four polysaccharides showed absorption bands characteristic for the configuration of both glycosidic linkages, i.e. α- and β types. Polysaccharides from *T. schimperi* showed significant radical scavenging and iron reducing power activities indicating that these polysaccharide extracts have great antioxidant potential. Scavenging ratio of KPA and KPW were 69.1% and 51.5% whereas, the scavenging ratio at of TSA and TSW were 72.1% and 57.3% respectively. This work has brought to our attention that these underutilised mushrooms have great scavenging activities and that this factor is concentration dependent, mainly when dealing with lower polysaccharides concentrations. The antioxidative capacity demonstrated by this fact can be one of the major way in which these polysaccharides can help maintain the oxidative equilibrium in our systems. It is also interesting to note that monosaccharides such as galactose, glucose, mannose and arabinose are most likely to be responsible for the antioxidant activities. SCFAs and modulated large bowel environment pH will benefit the host's well-being and health. As displayed in figure 19, the polysaccharides caused a decrease in pH up to 4.14 in *L. acidophilus*. The polysaccharides displayed good prebiotic activities thus, are potential candidates as functional ingredients to improve human health and functional applications through regulating gut flora. The results and findings from the project can provide useful references and insights for developing effective strategies and processes for

extraction, isolation and purification of polysaccharides from mushrooms, and investigate their properties-bioactivity relationships.

7. CHAPTER 7: RECOMMENDATIONS

Further studies on these polysaccharides may provide mechanistic insight, e.g. how they influence the balance of human gut flora. In the future, we will investigate the specific target on the bacteria and also how the mushroom polysaccharides can act on the intestinal mucosa. More probiotic strains such as *Bifidobacteria* need to be included for further confirmation for the prebiotic action. Furthermore we also recommend that further analytical techniques employing NMR, Saccharide Mapping, determination of their molecule weights and their structure would be necessary to deeply understand the structure-function relationship. Different isolation techniques also need to be evaluated in order to isolate them in pure form. Lastly, the mechanisms involved in antimicrobial activity of polysaccharides are also worthy of further investigation.

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APPENDICES

1. Student's t-test on the differences of sugar contents obtained from (A) Termitomyces and (B) Kalaharituber polysaccharides.

A)

```
DATASET ACTIVATE DataSet1.
T-TEST GROUPS=Groups(1 2)
  /MISSING=ANALYSIS
  /VARIABLES=Ternitomyces
  /CRITERIA=CI(.95).
```

T-Test

```
[DataSet1]
```

Group Statistics

	Groups	N	Mean	Std. Deviation	Std. Error Mean
Ternitomyces	1.00	3	145.3727	8.31102	4.79837
	2.00	3	228.5857	17.61745	10.17144

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Ternitomyces	Equal variances assumed	3.437	.137	-7.399	4	.002	-83.21300	11.24644	-114.43814	-51.98786
	Equal variances not assumed			-7.399	2.848	.006	-83.21300	11.24644	-120.10807	-46.31793

B)

```
DATASET ACTIVATE DataSet0.
T-TEST GROUPS=Groups(1 2)
  /MISSING=ANALYSIS
  /VARIABLES=Kalaharituber
  /CRITERIA=CI(.95).
```

T-Test

Group Statistics

	Groups	N	Mean	Std. Deviation	Std. Error Mean
Kalaharituber	1.00	3	260.1773	4.85499	2.80303
	2.00	3	247.2943	13.85912	8.00157

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Kalaharituber	Equal variances assumed	5.425	.080	1.520	4	.203	12.88300	8.47833	-10.65661	36.42261
	Equal variances not assumed			1.520	2.484	.244	12.88300	8.47833	-17.56788	43.33388

2. Student's t-test on the differences of phenolic contents between Termitomyces and Kalaharituber polysaccharides.

```
T-TEST GROUPS=Group(1 2)
  /MISSING=ANALYSIS
  /VARIABLES=Samples
  /CRITERIA=CI(.95).
```

T-Test

Group Statistics

	Group	N	Mean	Std. Deviation	Std. Error Mean
Samples	K_pfeilii	2	6.8900	.22627	.16000
	T_schimperi	2	3.6950	.30406	.21500

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Samples	Equal variances assumed	.	.	11.922	2	.007	3.19500	.26800	2.04188	4.34812
	Equal variances not assumed			11.922	1.848	.009	3.19500	.26800	1.94579	4.44421

3. Correlation of DPPH antioxidant activity to phenolic content of polysaccharide fractions from T. schimperi (TSA & TSW) and K. pfeilii (KPW & KPA).

A) TSW

Regression

Variables Entered/Removed[a]

Model	Variables Entered	Variables Removed	Method
1	TSW_Total_Phenolics[b]	.	Enter

a. Dependent Variable: TSW_DPPH_Activity

b. All requested variables entered.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.859[a]	.738	.695	6.55015	.738	16.928	1	6	.006

a. Predictors: (Constant), TSW_Total_Phenolics

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	726.308	1	726.308	16.928	.006[b]
	Residual	257.427	6	42.904		
	Total	983.735	7			

a. Dependent Variable: TSW_DPPH_Activity

b. Predictors: (Constant), TSW_Total_Phenolics

B) TSA

Regression

Variables Entered/Removed[a]

Model	Variables Entered	Variables Removed	Method
1	TSA_Total_Phenolics[b]	.	Enter

a. Dependent Variable: TSA_DPPH_Activity

b. All requested variables entered.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.922[a]	.849	.824	7.13204	.849	33.799	1	6	.001

a. Predictors: (Constant), TSA_Total_Phenolics

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	1719.223	1	1719.223	33.799	.001[b]
	Residual	305.196	6	50.866		
	Total	2024.419	7			

a. Dependent Variable: TSA_DPPH_Activity

b. Predictors: (Constant), TSA_Total_Phenolics

C) KPW

Regression

Variables Entered/Removed[a]

Model	Variables Entered	Variables Removed	Method
1	KPW_Total_Phenolics[b]	.	Enter

a. Dependent Variable: KPW_DPPH_Activity

b. All requested variables entered.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.789[a]	.623	.560	9.43680	.623	9.921	1	6	.020

a. Predictors: (Constant), KPW_Total_Phenolics

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	883.536	1	883.536	9.921	.020[b]
	Residual	534.319	6	89.053		
	Total	1417.855	7			

a. Dependent Variable: KPW_DPPH_Activity

b. Predictors: (Constant), KPW_Total_Phenolics

D) KPA

Regression

[DataSet2]

Variables Entered/Removed[a]

Model	Variables Entered	Variables Removed	Method
1	KPA_Total_Phenolics[b]	.	Enter

a. Dependent Variable: KPA_DPPH_Activity

b. All requested variables entered.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.822[a]	.676	.622	5.88133	.676	12.509	1	6	.012

a. Predictors: (Constant), KPA_Total_Phenolics

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	432.694	1	432.694	12.509	.012[b]
	Residual	207.541	6	34.590		
	Total	640.235	7			

a. Dependent Variable: KPA_DPPH_Activity

b. Predictors: (Constant), KPA_Total_Phenolics

4. Correlation of reducing power antioxidant activity to carbohydrate content of polysaccharide fractions from *T. schimperi* and *K. pfeilii*

A) KPA

Regression

Variables Entered/Removed[a]

Model	Variables Entered	Variables Removed	Method
1	KPA_Reducing_Power[b]	.	Enter

a. Dependent Variable: KPA_Total_Carbohydrate

b. All requested variables entered.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.965[a]	.930	.895	23.6486401

a. Predictors: (Constant), KPA_Reducing_Power

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	14921.144	1	14921.144	26.680	.035[b]
	Residual	1118.516	2	559.258		
	Total	16039.660	3			

a. Dependent Variable: KPA_Total_Carbohydrate

b. Predictors: (Constant), KPA_Reducing_Power

B) KPW

Regression

Variables Entered/Removed[a]

Model	Variables Entered	Variables Removed	Method
1	KPW_Reducing_Power[b]	.	Enter

a. Dependent Variable: KPW_Total_Carbohydrate

b. All requested variables entered.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.996[a]	.993	.989	9.0968766

a. Predictors: (Constant), KPW_Reducing_Power

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	22045.504	1	22045.504	266.401	.004[b]
	Residual	165.506	2	82.753		
	Total	22211.010	3			

a. Dependent Variable: KPW_Total_Carbohydrate

b. Predictors: (Constant), KPW_Reducing_Power

C) TSA

Regression

Variables Entered/Removed[a]

Model	Variables Entered	Variables Removed	Method
1	TSA_Reducing_Power[b]	.	Enter

a. Dependent Variable: TSA_Total_Carbohydrate

b. All requested variables entered.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.969[a]	.940	.909	22.8546754

a. Predictors: (Constant), TSA_Reducing_Power

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	16244.295	1	16244.295	31.099	.031[b]
	Residual	1044.672	2	522.336		
	Total	17288.968	3			

a. Dependent Variable: TSA_Total_Carbohydrate

b. Predictors: (Constant), TSA_Reducing_Power

D) TSW

Regression

Variables Entered/Removed[a]

Model	Variables Entered	Variables Removed	Method
1	TSW_Reducing_Power[b]	.	Enter

a. Dependent Variable: TSW_Total_Carbohydrate

b. All requested variables entered.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.816[a]	.666	.499	27.2630928

a. Predictors: (Constant), TSW_Reducing_Power

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	2961.948	1	2961.948	3.985	.184[b]
	Residual	1486.552	2	743.276		
	Total	4448.500	3			

a. Dependent Variable: TSW_Total_Carbohydrate

b. Predictors: (Constant), TSW_Reducing_Power