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Chemical composition and *in vitro* bioaccessibility of bioactive compounds from different sporocarp parts of a medicinal mushroom

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ABSTRACT

Ganoderma lucidum is a well-known medicinal mushroom, both historically and currently. Driven by the ethnopharmacological prospect and the crescent body of scientific evidence that associates *G. lucidum* intake with health, the interest in its metabolites, primarily triterpenes and polysaccharides, has been further fostered. Whereas most research on medicinal mushrooms has focused on the comprehensive identification and yields of metabolites throughout their different growth phases, the distribution of those compounds along the sporocarps (fruiting bodies) in the mushroom's antler growth phase remains poorly investigated. Moreover, for the compounds in the mushroom to exert biological activities, following the ingestion they must be bioaccessible in the upper gastrointestinal tract or fermented at the colon, but no work has been dedicated to investigating the bioaccessibility of bioactive compounds from *G. lucidum.* This study aimed to directly compare the nutritional and chemical composition of the exterior skin and interior flesh of *G. lucidum* sporocarps, besides the bioaccessibility of triterpenes and prebiotic potential after digestion of both samples. Samples were provided by Käapa Biotech (Finland) and the proximate composition evaluated using Official methods. Triterpenes were extracted with hydroethanolic solution by maceration (150 rpm, 2 cycles of 1h), and Soxhlet (6 cycles), as conventional methodologies, and ultrasound-assisted extraction (UAE, 47% amplitude, 15 min), as a green alternative methodology. Samples were homogenised and subjected to *in vitro* digestion (IVD) using the INFOGEST protocol. Extracts and bioaccessible factions were analysed by HPLC-DAD-(ESI-)HRMS/MS. The prebiotic activity with and β-glucan content of the colonic residue of either sample was also investigated. Carbohydrates constituted the major component of samples, accounting for 80% of the total nutrients regardless the sporocarp part. The proximate composition of both samples was generally similar. Thirty-seven compounds were tentatively identified in both extracts and comprised lanostane-type tetracyclic triterpenes. Among the triterpenes, 16 lucidenic and 9 ganoderic acids were found, with peak 23 (lucidenic acid A, ganoderlactone B and ganolucidic acid) and peak 17 (lucidenic acid P and 7,15,?-trihydroxy-4,4,14-trimethyl-3,11-dioxochol-8-en-24-oic acid) being the major compounds in the flesh and skin. The total triterpene content was superior in the external skin in contrast to the inner flesh (2 or 3 times) , and the proportion among some individual compounds also varied. The outer part also provided a higher average bioaccessibility of these compounds when compared with the inner part (53% and 39%, respectively). On the other hand, the inner sporocarp part showed superior prebiotic activity, evidenced by rapid bacterial growth and higher optical density across all tested strains, suggesting an enhanced fermentable substrate availability. The higher β-glucan content in the inner flesh (15.43% w/w) in contrast to the outer skin (9.77% w/w) likely contributed to its higher prebiotic effect. Whereas the external skin of *G. lucidum* sporocarps presented more bioaccessible triterpenes with putative health benefits, the inner part presented greater potential of promoting the gut heath by modulating gut microbiota compared to the outer skin. This is the first time a study has examined the variations of components between different parts of *G. lucidum* sporocarps as well as their bioaccessibilities, highlighting the differential potential of each particular part. Understanding the differential composition and bioaccessibility of compounds from various parts of *G. lucidum* sporocarp can inform better utilisation strategies in functional food and nutraceutical applications, enhancing their potential health benefits.

Keywords: *Ganoderma lucidum*; triterpenes; INFOGEST; prebiotic activity; nutritional composition; LC-MS/MS

RESUMO

Ganoderma lucidum é um cogumelo medicinal bem conhecido, tanto históricamente como atualmente. Impulsionado pela perspectiva etnofarmacológica e pelo crescente corpo de evidências científicas que associam a ingestão de *G. lucidum* à saúde, o interesse pelos seus metabólitos, principalmente triterpenos e polissacarídeos, tem sido ainda mais fomentado. Embora a maioria das pesquisas sobre cogumelos medicinais tenha se concentrado na identificação abrangente e na produção de metabólitos ao longo de suas diferentes fases de crescimento, a distribuição desses compostos ao longo dos esporocarpos (corpos de frutificação) na fase de crescimento do chifre do cogumelo permanece pouco investigada. Além disso, para que os compostos do cogumelo exerçam atividades biológicas, após a ingestão devem ser bioacessíveis no trato gastrointestinal superior ou fermentados no cólon, mas nenhum trabalho foi dedicado a investigar a bioacessibilidade dos compostos bioactivos de *G. lucidum*. Este estudo teve como objetivo comparar diretamente a composição nutricional e química da parte externa e da polpa interna de esporocarpos de *G. lucidum*, além da bioacessibilidade de triterpenos e potencial prebiótico após digestão de ambas as amostras. As amostras foram fornecidas pela Käapa Biotech (Filândia) e a composição centesimal avaliada utilizando métodos oficiais. Os triterpenos foram extraídos com solução hidroetanólica por maceração (150 rpm, 2 ciclos de 1h) e Soxhlet (6 ciclos), como metodologias convencionais, e extração assistida por ultrassom (UAE, amplitude de 47%, 15 min), como metodologia alternativa verde. As amostras foram homogeneizadas e submetidas à digestão *in vitro* (IVD) utilizando o protocolo INFOGEST. Extratos e frações bioacessíveis foram analisados por HPLC-DAD-(ESI-)HRMS/MS. A atividade prebiótica e o conteúdo de β-glucano do resíduo colónico das amostras também foram analisados. Os carboidratos constituíram o principal componente das amostras, corespondendo 80% do total de nutrientes, independentemente da parte do esporocarpo. A composição centesimal de ambas as amostras foi geralmente semelhante. Trinta e sete compostos foram provisoriamente identificados em ambas as partes e compreendiam triterpenos tetracíclicos do tipo lanostano. Entre os triterpenos, foram encontrados 16 ácidos lucidênicos e 9 ganodéricos, sendo o pico 23 (ácido lucidénico A, ganoderlactona B e ácido ganolucídico) e pico 17 (ácido lucidénico P e ácido 7,15,?-trihidroxi-4,4,14-trimetil-3,11-dioxocol-8-en-24-oico) os principais compostos na polpa e na pele. O conteúdo total de triterpenos foi superior na casca externa em contraste com a polpa interna (2 a 3 vezes mais), e a proporção entre alguns compostos individuais também variou. A parte externa também proporcionou maior bioacessibilidade média desses compostos quando comparada com a parte interna (53% e 39%, respectivamente). Por outro lado, a parte interna do esporocarpo apresentou atividade prebiótica superior, evidenciada pelo rápido crescimento bacteriano e maior densidade óptica em todas as cepas testadas, sugerindo uma maior disponibilidade de substrato fermentável. O maior teor de β-glucano na polpa interna (15,43% p/p) em contraste com a casca externa (9,77% p/p) provavelmente contribuiu para seu maior efeito prebiótico. Enquanto a parte externa dos esporocarpos de *G. lucidum* apresentou mais triterpenos bioacessíveis com supostos benefícios à saúde, a parte interna apresentou maior potencial de promoção da saúde intestinal através da modulação da microbiota intestinal em comparação com a pele externa. Esta é a primeira vez que um estudo examinou as variações de componentes entre diferentes partes dos esporocarpos de *G. lucidum*, bem como suas bioacessibilidades, destacando o potencial diferencial de cada parte específica. A compreensão da composição diferencial e da bioacessibilidade de compostos de várias partes do esporocarpo de *G. lucidum* pode suscitar melhores estratégias de utilização em alimentos funcionais e aplicações nutracêuticas, aumentando seus potenciais benefícios à saúde.

Palavras-chave: *Ganoderma lucidum*; triterpenos; INFOGEST; atividade prébiótica; Composição nutricional; LC-MS/MS

LIST OF ABBREVIATIONS AND ACRONYMS

µg	Microgram
ABTS	(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
Acetyl-COA	Acetyl Coenzyma A
AOAC	Association of Official Analytical Chemists
BF	Bioaccessible phase
CMGO	Colonic material from outer sporocarp
CMGI	Colonic material from inner sporocarp
Da	Danton
DAD	Diode Array Detector
DMAPP	Dimethylallyl diphosphate
DPPH$^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl radical
dw	Dry weight
EC50	Half-maximal effective concentration
ESI	Electrospray ionization
EtOH	Ethanol
EU	European Union
FPP	Farnesyl diphosphate
FT-IR	Fourier-transform infrared spectroscopy
fw	Fresh weight
g	Gram
GAs	Ganoderic Acids
GAE	Gallic Acid Equivalent
GC-FID	Gas chromatography with flame ionization detector
GC-MS	Gas chromatography coupled to mass spectrometry
GI	Ganoderma inner sporocarp part
GO	Ganoderma outer sporocarp part
GPP	Geranyl diphosphate
GGPP	Geranylgeranyl diphosphate
H_2O	Water
HPGFC	High-Performance Gel Filtration Chromatography
HPLC	High-Performance Liquid Chromatography
HPLC-DAD-MSn	High-Performance Liquid Chromatography coupled to Diode Array Detector and tandem Mass Spectrometry
IPP	Isopentyl Pyrophosphate
LC-MS/MS	Liquid Chromatography with tandem Mass Spectrometry
MAC	Maceration

MAE	Microwave-assisted extraction
MeOH	Methanol
MEP	2-C-Methyl-D-Erythritol 4-Pphosphate
mg	Milligram
mg/g	Milligram to gram ratio
MIC	Minimum Inhibitory Concentration
min	Minutes
mL	milliliter
MS	Mass spectrometry
m/z	Mass to charge ratio
MVA	Mevalonic Acid Pathway
nc	Not calculated
nd	Not detected
NMR	Nuclear Magnetic Resonance
nq	Not quantified
PDA	Potato Dextrose Agar
PUFA	Polyunsaturated Fatty Acids
RDI	Reference Daily Intake
rpm	Rotations per minute
Rt	Retention time
SD	Standard deviation
SIF	Simulated Intestinal Fluid
SGF	Simulated Gastric Fluid
SPE	Solid-Phase Extraction
SPME	Solid-Phase Microextraction
SSF	Simulated Salivary Fluid
TCM	Traditional Chinese Medicine
tr	Traces
UAE	Ultrasound-Assisted Extraction
UV-Vis	Ultraviolet-Visible light
v/v	Volume to volume ratio
(% v/v)	Percentage of volume ratio
VOC	Volatile Organic Compounds
w/v	Weight to volume ratio
(% w/v)	Percentage of weight to volume ratio
(% w/w)	Percentage of weight ratio

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1. INTRODUCTION

Ganoderma lucidum is a notable medicinal mushroom, historically and currently. Known as "*Leng zhi*" in China and "*Reishi*" in Japan, this mushroom has been extensively used over 2,000 years in Traditional Medicine Systems to prevent and ameliorate several health conditions such as tumors, hepatitis, immune deficiencies, among others (Zhou et al., 2015; Bulam et al., 2019). Since then, *G. lucidum* has been typically cultivated more because of its pharmacological properties rather than its nutritional ones, unlike other edible mushrooms (Wachtel-Galor et al., 2011). The whole sporocarp of *G. lucidum* is popularly commercialized in its dried form that can be directly added to food, encapsulated, or used to prepare traditional brews like teas, or as dried extracts also in capsules.

Recent research efforts have been directed towards providing scientific evidence for the traditional use of *G. lucidum* and understand how its primary or secondary metabolites may contribute to improve health. For instance, *Ganoderma*'s triterpene fraction has been associated with anticancer and cytotoxic, antioxidant, neuroprotective, and anti-inflammatory activities (Bishop et al., 2015; Hasnat et al., 2015; Ahmad, 2020; Kou et al., 2021; Kolniak-Ostek et al., 2022).

Those investigations linking the mushroom intake with health outcomes have further fostered the interest in factors modulating the profile or production yields of *G. lucidum* secondary metabolites, particularly for the mushroom sporocarp (Ding et al., 2023). While large attention has been given to the comprehensive identification and recovery yields of metabolites from medicinal mushrooms throughout their growth phases (Bishop et al., 2015), the distribution of those compounds along the sporocarps in antler growth phase remains poorly investigated. The knowledge on the chemical composition of a specific sample is also of interest as it is the first step to improve the understanding of their functional potential and to guide the development of any formulation. Therefore, this project first focused on determining and comparing the nutritional and triterpene composition in the exterior skin and interior flesh of *G. lucidum* fruiting bodies.

In addition, the chemical characterization to improve the knowledge on the natural sources and the production of *G. lucidum*-based functional extracts and formulations to meet the crescent market demand depend both on the efficient extraction of these compounds, a key process that must consider the quality of the final extract without losing sight of sustainability and economic aspects. In this sense, this study compared the chemical composition of the exterior

skin and interior flesh of *G. lucidum* sporocarps subjected to different conventional and green extraction procedures.

Studying the chemical composition of a food or dietary supplement that is a source of bioactive compounds is indeed of primary importance, but more is needed to predict its health benefits (Rodrigues et al., 2022). For a dietary compound to benefit human health, it must be released from its matrix during digestion and reach the intestine in a bioaccessible form, so that it or its metabolites can be absorbed and reach target tissues, where they can promote their biological properties (Rodrigues et al., 2022). Therefore, understanding the bioaccessibility of dietary compounds in their consumption form is crucial for assessing their efficacy and potential benefits, rather than focusing only on the ingested amount and assuming no effect of the food matrix. On this behalf, the *in vitro* bioaccessibility of triterpenes from *G. lucidum* sporocarp parts was assessed in their traditional forms of consumption.

Some compounds not bioaccessible at duodenum reach the colon and can be biotransformed by bacteria, producing *in situ* or systemic effects, or even becoming bioaccessible lately at colonic level. Prior research on *G. lucidum*'s has indicated microbiota-modulating effects, related mainly to the polysaccharides present in this mushroom. In this sense, the prebiotic activity of compounds was assessed in the digestion residue of both sporocarp parts.

The general aim of this study was to chemically characterize and compare different parts (outer skin and interior flesh) of fruiting bodies of the medicinal mushroom *G. lucidum*, particularly regarding their triterpene fraction, and assess the *in vitro* bioaccessibility of these compounds. *In vitro* digestion studies produce samples that closely mimics what happens *in vivo*, improving the physiological relevance of the investigations, and associated with analytical methods of high sensitivity and selectivity such as HPLC-DAD-(ESI-)HRMS/MS may generate important information. This information may guide the consumption and development of functional preparations containing *G. lucidum*'s sporocarps and provide evidence on its health benefits from a more physiological perspective.

2. LITERATURE REVIEW

2.1 *G. LUCIDUM* AS A FOOD AND MEDICINAL MUSHROOM

2.1.1 *Ganoderma* and its cultivation through history

The term "mushroom" refers to the sporocarp or fruiting body of fungi, specifically known as the basidiome in basidiomycete fungi. It is estimated that there are 1.5 million species of mushrooms, with 70,000 identified to date. Of these, around 10,000 are distributed globally, approximately 2,000 are safe for human consumption, and nearly 300 have been associated with beneficial biological activities for human health (Ahmad, 2018).

Among mushrooms, the *Ganoderma* genus stands out for its medicinal and cultural significance. This genus includes woody mushrooms characterised by a varnished, hard surface. The most renowned species in this group is *Ganoderma lucidum*, also called "Reishi" in Japan and "Ling zhi" in China, recognized for its health benefits and commonly referred to as the “mushroom of immortality”. This mushroom has a long history of safe use in traditional Asian medicine systems, particularly in China, Japan, and Korea, where it has been employed for over 5,000 years in Traditional Chinese Medicine (TCM), for instance (Aslam, 2018).

G. lucidum is a basidiomycete fungus, classified as a polypore mushroom, with large, firm, woody and generally reddish-brown fruiting bodies with a glossy appearance that typically grow in a fan or kidney shape on the trunks of dead, deciduous trees, particularly willow, oak, eucalyptus, maple, and elm (**Figure 1**). Taxonomically, it belongs to the Kingdom Fungi, Phylum Basidiomycota, Class Agaricomycetes, Order Aphyllophorales, Family Ganodermataceae, Genus *Ganoderma*, and Species *Ganoderma lucidum*. The name *G. lucidum* was initially used for the British species and later applied to all species within the genus, which led to confusion due to the diversity within the genus. Taxonomic studies have since been conducted to improve the classification of these species (Aslam, 2018).

Traditionally, *G. lucidum* mushrooms are distinguished by the colour of their fruiting bodies and can be classified into red, black, blue, white, yellow, and violet Reishi varieties. The red Reishi, in particular, has been found to exhibit significant health-promoting biological activities (Yang et al., 2019).

Figure 1: Natural growth of the fruiting body of *G. lucidum* on a dead tree trunk (Bijalwan et al., 2020).

The life cycle of *G. lucidum* comprises five primary stages, namely the spore stage, mycelial development, the formation of the pinhead (also known as the needlehead or thin head), elongation of the stipe, and finally, the development and maturation of the fruiting body or sporocarp. These stages during *G. lucidum*'s production in growth bags can be seen in **Figure 2**. The time required for the formation of the fruiting body varies greatly depending on cultivation conditions. Different cultivation techniques introduce variations in the developmental processes of this mushroom. In traditional, artisanal production using natural one-metre-long logs inoculated with spores and partially buried, the emergence of the fruiting body typically takes 6–24 months, with maturation continuing for up to five years (Hapuarachchi et al., 2018; Bijalwan et al., 2020).

In addition to its natural growth, *G. lucidum* can be cultivated artificially for specific purposes, such as achieving greater uniformity in metabolite production. The first successful controlled cultivation was conducted in China in 1969 using spore inoculation on wooden logs or tree segments, which subsequently boosted cultivation and consumption in Asia (Atila, 2022). Today, biotechnological cultivation is also employed, often using mycelia, to rapidly produce specific secondary metabolites. For instance, submerged fermentation of mycelia has been used to enhance the production of ganoderic acids (GAs). This method involves manipulating cultivation parameters such as pH, oxygen supply, and the addition of salts like sodium or manganese, alongside altering gene expression, to improve metabolite yield (Bishop et al., 2015). In another study, polysaccharides from *G. lucidum* were obtained through submerged

fermentation and identified using high-performance gel filtration chromatography (HPGFC) and Fourier-transform infrared spectroscopy (FT-IR). These polysaccharides were also quantified via HPGFC. The main polysaccharide, designated GLP-1-1, exhibited a molecular mass of 22,014 Da and comprised glucose (92.33%), mannose (7.75%), and galactose (0.22%) (Ai-lati et al., 2017).

Commercially, the most common method of cultivation involves substrates in controlled environments. Frequently used substrates include wooden logs, short linden wood segments, tree stumps, sawdust-filled bags, and bottle-based procedures (**Figure 3**) (Hapuarachchi et al., 2018).

Figure 2: Growth stages of *G. lucidum* production in grow bags. **A**. Early stage of mycelium growth (spawn running) **B**. Completion of mycelium growth **C**. Pin head formation **D**. Early stages of fruiting body growth **E**. Harvesting stage (Jeewanthi et al., 2017).

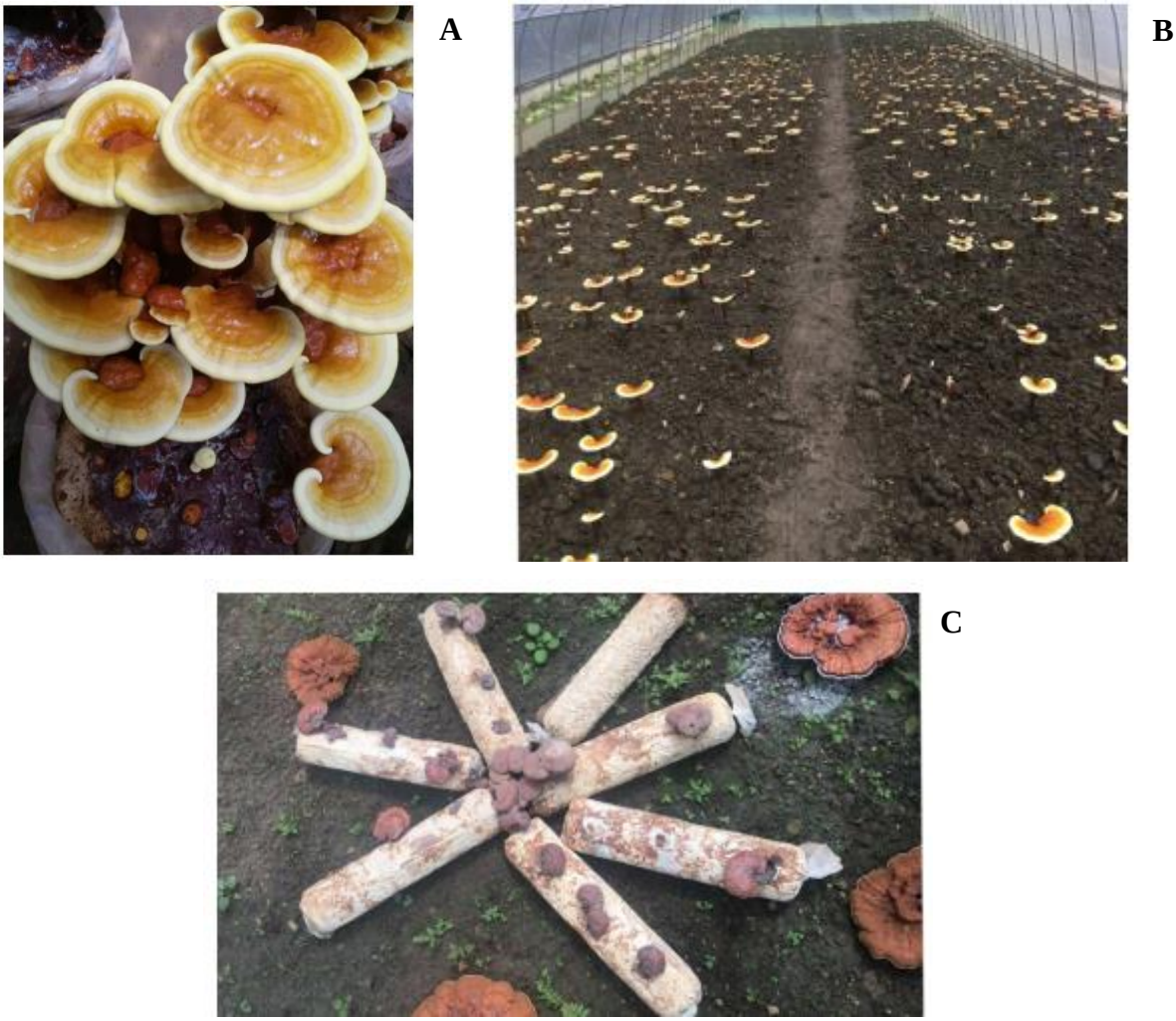


Figure 3: Production of *G. lucidum* fruiting bodies from **A.** substrate bags in greenhouses (Hapuarachchi et al., 2018) **B.** substrate bags (Sheikha, 2022) and **C.** short wooden logs in greenhouses (Hapuarachchi et al., 2018).

The consumption and traditional use of the mushroom *G. lucidum* have been practiced in various forms and for a range of purposes. In early Chinese literature, dating back to 206 BC, it was primarily described as an energy revitaliser, muscle strengthener, enhancer of vision and vital organ function, life-prolonging agent, and even as a promoter of immortality. Over time, as China evolved as a densely populated and multi-ethnic country, the methods of use and applications varied geographically and among ethnic groups. Despite these variations, a widely accepted method of consumption involves hot water extraction techniques. Additionally, although *G. lucidum* has bitter taste, it is still incorporated in the dietary habits of several Chinese ethnic groups. These communities traditionally believe that the mushroom can treat dizziness, insomnia, neurasthenia, hypertension, hypercholesterolemia, hepatitis, chronic bronchitis, silicosis, and other conditions. Notably, some uses reported by certain ethnic minorities have

been integrated into the Chinese Pharmacopoeia. These include its purported nerve-relaxing properties and its ability to alleviate cough and asthma symptoms (Bulam et al., 2019; Wang et al., 2020).

2.1.2 Chemical composition of *G. lucidum*

The chemical composition of *G. lucidum* and the *Ganoderma* genus has been studied for years, with research continually revealing new, dereplicated compounds. For instance, Xia et al. (2014) reported 316 triterpenoids in the *Ganoderma* genus. By 2018, records from 40 years of phytochemical studies indicated the identification of 431 compounds, 380 of which were triterpenoids (Ahmad, 2018). By 2023, Galappaththi et al. noted that over 600 chemical compounds had been isolated from the genus *Ganoderma*, including alkaloids, meroterpenoids, nucleobases, nucleosides, polysaccharides, proteins, steroids, and triterpenoids (Galappaththi et al., 2023).

Despite this growing number of identified compounds, almost all studies consistently report the content of macronutrients, micronutrients, secondary metabolites, and other chemical groups common to the genus. This is because the chemical composition of *G. lucidum* and the *Ganoderma* genus varies substantially based on factors such as species, strain, part of the mushroom, developmental stage, and growth or cultivation conditions.

Although there is some debate over whether *G. lucidum* can be classified as a food—mainly due to its bitterness from triterpenoids—its nutritional composition is comparable to that of traditional foods (**Table 1**) (Ahmad et al., 2021).

Table 1: Nutritional composition of *G. lucidum* fruiting body from different origins

Sample Origin	Moisture	Ash	Proteins	Lipids	Carbohydrates	Fibre
Nigerian	90	8 – 10	10 – 40	2 – 8	3 – 28	3 – 32
European	-	-	13.3	3	82.3	-
Bangladesh	10.2 – 12.6	3.9 – 6.1	27.88 – 29.86	1.9 – 2.4	37.1 – 51.6	12.2 – 14.6
Pakistani	-	2.01	15.04	0.53	82.47	54.1
Indian	-	1.8	7 – 8	3 – 5	26 – 28	59
Portuguese	-	2.40	6.72	2.50	88.4	-
Ghanaian	-	0.68 – 2.1	15.7 – 24.5	0.5 – 1.40	73.3 – 81.9	-

Nutrient content is expressed as a percentage (g nutrient/100g sample) Source: (Ahmad et al., 2021).

In terms of macronutrients, *G. lucidum* is notably rich in polysaccharides, which, alongside triterpenoids, are considered the most important compounds in this mushroom (Cai et al. 2021; Li et al., 2023). Polysaccharides are present in larger quantities and exhibit a greater variety of chemical groups compared to other nutrients in *G. lucidum*, with over 100 distinct types identified. The highest concentrations are found in the mycelium and pores (10^5 – 10^6 Dalton, Da) compared to the fruiting body (10^3 - 10^6 Da). Major glycan types include glucans, primarily α-(1→3) glucan, α-(1→6) glucan, mannan, and galactosan linked to α- and β-glucans or other bonds. Polysaccharides containing glucose, mannose, rhamnose, and galactose are also commonly found (Wu et al., 2024;Oke et al., 2022;Benkeblia, 2016).

G. lucidum also contains proteins, the most notable being Lin zhi-8 (LZ-8), a protein with 110 amino acid residues and an acetylated amino-terminal, with a molecular mass of 12 kDa, and ganodermin, with a molecular mass of 15 kDa. Among the lipids, significant compounds include phosphatidic acid, palmitic, linoleic, oleic, stearic, and nonadecanoic acids, as well as other polyunsaturated fatty acids. Lipids are the least abundant macronutrients in *G. lucidum* compared to proteins and polysaccharides (Oke et al., 2022).

G. lucidum is also rich in micronutrients, including vitamins and minerals. It contains thiamine (B1) at approximately 3.49 mg/100 g, riboflavin (B2) 17.2 mg/100 g, niacin (B3) 61.9 mg/100 g, pyridoxine (B6) 0.71 mg/100 g, ascorbic acid (C) 32.2 mg/100 g, vitamin E, vitamin D, and β-carotene (Sheikha, 2022). Mineral content includes macroelements and trace elements, such as potassium (432 mg/100 g), phosphorus (225 mg/100 g), sulphur (129 mg/100 g), magnesium (7.95 mg/100 g), sodium (2.82 mg/100 g), and calcium (1.88 mg/100 g). Trace elements include copper (26 mg/100 g), manganese (22 mg/100 g), iron (2.22 mg/100 g), and zinc (0.7 mg/100 g) (Sheikha, 2022).

Among secondary metabolites, triterpenoids, steroids, phenolic compounds, and alkaloids are prominent. Triterpenoids are the most abundant and extensively studied compounds in the *Ganoderma* genus. They are also among the most common secondary metabolites in nature, with over 20,000 triterpenes identified. The triterpenoids of *Ganoderma* are mainly lanostane-type compounds (Xia et al., 2014). Lanostanes in *G. lucidum* typically contain 30 or 27 carbon atoms, with some having 24, usually modified at carbon 17, with 260 triterpenoids identified across the fruiting body, mycelium, and spores (Wu et al., 2024). Lanostane-type triterpenoids are metabolised through the mevalonate acid pathway (MVA) (**Figure 4** and **Figure 5**). Examples of 30-carbon triterpenoids in *G. lucidum* include ganoderic acids (GA) A, B, C, D, E, F, O, Df,

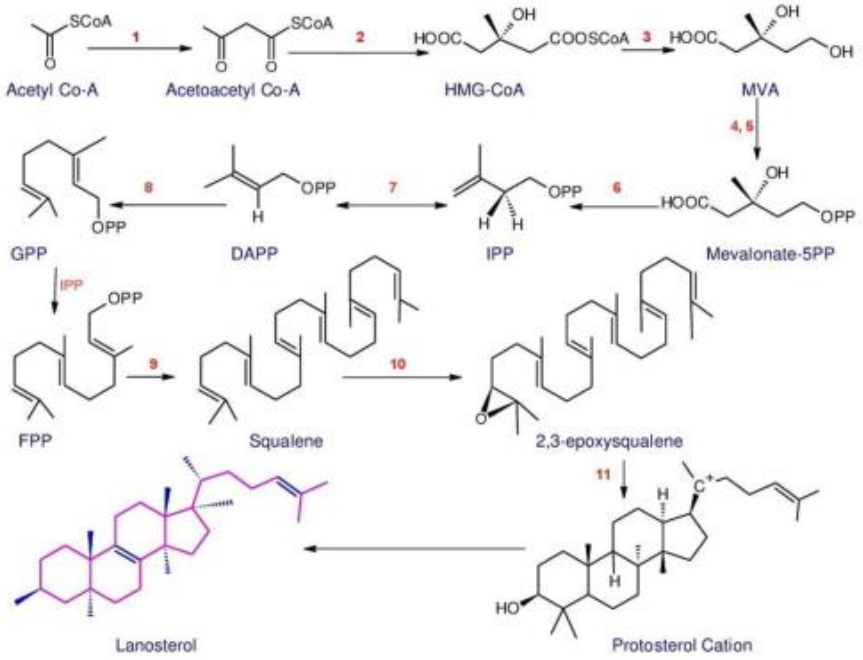


Figure 4: MVA Pathway in the biosynthesis of the lanostane-type ring. Enzymes involved: (1) acetyl-CoA acetyltransferase (AACT); (2) 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS); (3) 3-hydroxy-3-methylglutaryl-CoA reductase; (4) mevalonate kinase (MK); (5) phosphomevalonate kinase (MPK); (6) phosphomevalonate decarboxylase (MVD); (7) isopentenyl diphosphate isomerase (IDI); (8) farnesyl diphosphate synthase (FPPs); (9) squalene synthase (SQS); (10) squalene monooxygenase (SE); (11) 2,3-oxidosqualene-lanosterol cyclase (OSC) (Galappaththi et al., 2023).

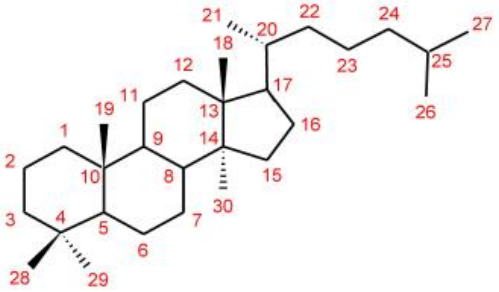
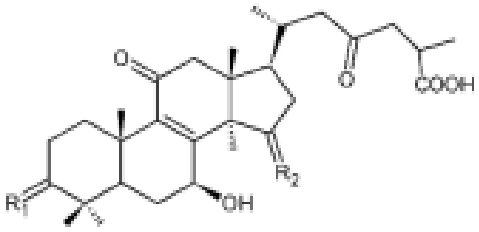


Figure 5: Chemical structure of lanostane-type triterpenes with 30 carbon atoms, the most common in *G. lucidum* (Galappaththi et al., 2023).

methyl ganoderate M, N, O, K, lucidadiol, and lucialdehydes B, C, and D (**Figure 6** shows some examples). Among the 27-carbon compounds are lucidenic acids A, B, C, D, I, J, K, L, M, N, methyl lucidate A, D, E, F, N, Ha, ethyl lucidate A, butyl lucidate A, N, P, D1, D2, E1, E2, Q, and 20-hydroxy-lucidic acids A, E, N, P, E2, among others (**Figure 7** shows some examples). Some triterpenoids with 24 carbons include lucidones A, B, C, and H (**Figure 8** shows some examples). A small number of saponins have also been isolated from the genus (Galappaththi et al., 2023).



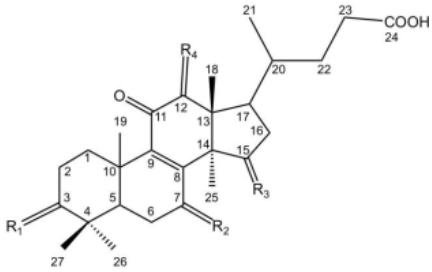
1: R_1=O, R_2=α-OH

2: R_1=β-OH, R_2=O

3: R_1=β-OH, R_2=α-OH

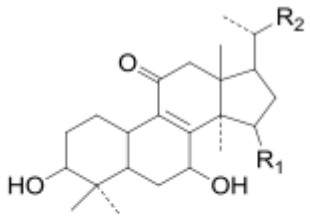
Figure 6: Ganoderic acids A (1), B (2) and C (3) (Galappaththi et al., 2023)

Basic Chemical Structure



Lucidenic Acid Type	R_1	R_2	R_3	R_4
Lucidenic acid A	R_1 = O	R_2 = -OH	R_3 = O	R_4 = H
Lucidenic acid B	R_1 = O	R_2 = -OH	R_3 = O	R_4 = -OH
Lucidenic acid C	R_1 = -OH	R_2 = -OH	R_3 = O	R_4 = -OH
Lucidenic acid D1	R_1 = O	R_2 = O	R_3 = O	R_4 = O

Figure 7: Lucidenic acids A, B, C and D1 (Zheng et al., 2023)



Cpd	R1	R2
313	=O	=O
314	=O	OH
315	α-OH	CH_2OH

Figure 8: Lucidone A (313), Lucidenol (314) and Ganosineniol A (315) (Xia et al., 2014)

Fruiting bodies contain higher concentrations of triterpenoids than mycelium or spores. One study comparing 12 mycelial samples with a fruiting body sample found that the fruiting body contained more triterpenoids, even after the mycelium had undergone cell wall disruption via oscillation and bionic extraction simulating digestion in gastrointestinal fluid (Liu et al., 2020). Another study reported that the fruiting body reaches its highest triterpenoid content, particularly of GA A, at its optimal maturation stage; levels decrease after this point (Nakagawa et al., 2018).

After triterpenoids and polysaccharides, steroids are the next most significant chemical compounds, with more than 20 sterols present, which are categorised as either ergosterol- or cholesterol-type. Spores contain more steroids than other *G. lucidum* structures. Examples of steroids in spores include ergosterol, ganodermasides A, B, C, and D; in fruiting bodies, examples include ergosta-7,22-dien-3-one, ergosta-7,22-dieno-3β-yl palmitate, and epidioxiergosta-6,22-dien-3β-ol. In mycelium, ergosta-4,7,22-trien-3,6-dione is notable (Yang et al., 2019).

Phenolic compounds in *G. lucidum* are secondary metabolites present in relatively small amounts, with limited chemical variety. Examples include protocatechuic acid (1.807 ± 0.001 mg/g), *p*-hydroxybenzoic acid (2.98 ± 0.01 mg/g), syringic acid (1.51 ± 0.01 mg/g), and a total phenolic acid content of 6.30 ± 0.01 mg/g (Taofiq et al., 2017). Alkaloids are the least abundant secondary metabolites in the *Ganoderma* genus and represent a relatively recent area of study; choline- and betaine-type alkaloids have been isolated from spores (Oke et al., 2022).

2.1.3 Biological properties associated with *G. lucidum*

Today, various biological activities of *Ganoderma lucidum* are attributed to its secondary metabolites, which are reported to have effects ranging from activity in physiological disorders to antimicrobial actions. To illustrate, several studies have linked different parts of the mushroom to specific biological activities.

One *in silico* study evaluated the antiviral and metabolic syndrome-related activity of certain secondary metabolites from *G. lucidum* and related species. The authors developed a database of 279 secondary metabolites and modelled interactions with five viral targets and 22 targets associated with metabolic syndromes. They found that among the viral targets, the HIV-1 protease enzyme showed the highest affinity, interacting with 40 lanostane-type triterpenoids. For metabolic syndrome targets, the farnesoid X receptor (FXR), which regulates carbohydrate

and lipid metabolism, was the primary target, interacting with over 62 secondary metabolites (Grienke et al., 2015).

Further studies report the antioxidant and anticancer properties of *G. lucidum.* A methanolic extract of the mushroom's fruiting body contained 28 phenolic compounds and 13 triterpenoids, exhibiting antioxidant activity at a lower concentration than resveratrol, apigenin, and ascorbic acid controls. The EC_{50} values for antioxidant activity were 51.3 ± 1.04 µg/mL (DPPH assay), 81.26 ± 1.10 µg/mL (ABTS assay), and 49.87 ± 1.58 µg/mL (FRAP assay), all statistically significant at $p \leq 0.05$. Anticancer activity, indicated by IC50 values for inhibition of cancer cell lines, were as follows: 25.38 ± 0.24 µg/mL for MDA-MB-231; 209.6 ± 0.24 µg/mL for MCF7; 235.4 ± 0.26 µg/mL for MCF7/DX; 47.90 ± 2.60 µg/mL for SW 620; 188.4 ± 0.76 µg/mL for LOVO; and 3140.9 ± 35.75 µg/mL for LOVO/DX, showing significant statistical differences at $p \leq 0.05$. These cell lines include breast cancer (MCF7, MCF7/DX, MDA-MB-231) and colorectal cancer (SW 620, LOVO, LOVO/DX) (Kolniak-Ostek et al., 2022).

An ethanolic extract from the mycelium of an Italian specimen also demonstrated antioxidant, antiproliferative, and DNA-protective activities against oxidative damage. Mass spectrometry identified 67 compounds, including phenolic compounds and triterpenoids. The EC_{50} for DPPH radical scavenging was 1.43 ± 0.17 mg/mL, and lipoxygenase inhibition was 0.25 ± 0.04 mg/mL. The antiproliferative effect of the ethanolic extract was concentration-dependent, showing significant inhibition starting at 37.5 µg/mL and reaching approximately 90% inhibition at 225 µg/mL. This effect was tested on human promonocytic U937 cell lines in the MTT assay after 48 hours. Non-denaturing Fast Halo Assay (FHA) and DNA laddering confirmed that U937 cells undergo significant apoptosis when treated with 225 µg/mL of the extract for six hours. Additionally, the extract reduced Fe^{2+}-induced DNA strand breaks by chelating Fe^{2+} ions and protected free DNA from damage through AAPH-induced oxidative stress. Researchers attribute these biological activities to the combined actions of the diverse compounds present in *G. lucidum* extracts (Saltarelli et al., 2019).

Extracts of *G. lucidum* have demonstrated notable anti-inflammatory effects. Studies on cell lines and *in vivo* models of colitis induced in laboratory rats revealed that extracts derived from a mixture of *G. lucidum* fruiting bodies and germinated brown rice suppress the production of nitric oxide (NO) and prostaglandin E2 (PgE2) in lipopolysaccharide (LPS)-stimulated macrophages in a dose-dependent manner (0.25, 0.50, and 1 mg/mL). Additionally, these extracts reduce the expression of key inflammatory biomarkers, such as cyclooxygenase-2 (COX-2),

tumour necrosis factor-alpha (TNF-α), inducible nitric oxide synthase (iNOS), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10 mRNA). They also inhibit the activation of mitogen-activated protein kinases (MAPKs), including p38, extracellular signal-regulated kinases (ERK), and c-Jun N-terminal kinase (JNK), as well as nuclear factor kappa B (NF-κB). In a DSS-induced colitis model in rats, macroscopic, biochemical, and histological evaluations showed significant regression of colonic mucosal lesions after treatment with 100 mg/kg of the extract. Immunofluorescence analysis suggested that DSS-induced nuclear translocation of NF-κB in colonic tissue was attenuated by the extract. The study identified 11 phenolic compounds in the extract and proposed that its anti-inflammatory effects are mediated through the inhibition of the MAPK and NF-κB pathways (Hasnat et al., 2015).

Sulphated polysaccharides from *G. lucidum* (GLPss58) have also shown anti-inflammatory effects by inhibiting L-selectin binding to its ligands sTyr/sLeX (IC_{50} = 13.5 µg/mL), the initial step in leukocyte-mediated inflammation. GLPss58 also inhibited pro-inflammatory cytokines TNF-α and interferon-gamma (IFN-γ), suggesting a broader inhibition of L-selectin-mediated pathways (Zhang et al., 2014).

G. lucidum extracts have been associated with improved gut functionality and lipid metabolism. An 80% ethanol extract of the mushroom's fruiting body containing five ganoderic acids (G, B, H, A, and F, all triterpenoids) showed probiotic activity and reduced markers of dyslipidaemia in high-fat diet-fed rats. After eight weeks of treatment, lipid parameters, including triglycerides (TG), total cholesterol (TC), and LDL-C, were reduced by 57.23% to 19.94%, while HDL-C levels increased compared to untreated rats. Additionally, biochemical markers such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as well as free fatty acids (FFA), decreased by up to 30.15% and 20.29%, respectively.

The extract's highest concentration of 150 mg/mL demonstrated the greatest activity. Improvements in lipid profiles were associated with modulation of gut microbiota, preventing dysbiosis and loss of microbial diversity observed in untreated rats fed a high-fat diet (Tong et al., 2023).

Studies have linked *G. lucidum* compounds to potential benefits for CNS disorders. In aged rats, extracts containing 28% triterpenoids reduced oxidative stress, inflammation, and cognitive impairments associated with ageing. In the Morris Water Maze test, cognitive abilities improved significantly with doses of 10 or 20 mg/kg. The extract reduced markers of neuronal degeneration in serum, including acetylcholinesterase, advanced glycation end products, NO,

TNF-α, and interleukin-2 (IL-2). Lipid-related markers such as TC, TG, and malondialdehyde were also lowered, while antioxidants such as total superoxide dismutase, catalase, and total antioxidant capacity were increased. Histopathological and genetic analysis revealed improvements in liver and hippocampal tissue. The extract reduced the expression of transcription factors such as FOXO4 and mTOR, increased sirtuin (SIRT1) levels in the liver, and lowered glial fibrillary acidic protein (GFAP), iNOS, PI3K, AKT, and IL-6 expression in the hippocampus, biomarkers associated with inflammation and ageing (A. Wang et al., 2020).

Another study demonstrated that *G. lucidum* fruiting body extracts (40 mg/kg) reduced anxiety and depression-like behaviours in rat offspring separated from their mothers. The treatment, conducted over three weeks, significantly reduced peripheral and cerebral levels of pro-inflammatory biomarkers such as IL-1β, IL-6, TNF-α, and IL-10, without apparent toxicity to vital organs (Mi et al., 2022).

G. lucidum has also been explored for topical health benefits. Taofiq et al. (2017) developed a cosmeceutical cream containing 50 mg of ethanolic extract per gram of base, demonstrating *in vitro* antioxidant, anti-tyrosinase, and antimicrobial activities. The EC50 values for DPPH scavenging and reducing power assays were 34 ± 10 mg/mL and 13.60 ± 0.09 mg/mL, respectively. For anti-tyrosinase activity, a L-DOPA assay reported an EC_{50} of 23.80 ± 0.03 µg/mL. The extract inhibited microbial strains, with minimum inhibitory concentrations (MICs) ranging from 100–400 mg/mL, including efficacy against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* extended-spectrum beta-lactamase (ESBL) strains (Taofiq et al., 2017).

Although *G. lucidum* contains macronutrients, micronutrients, and bioactive secondary metabolites, its potential as a nutraceutical remains underexplored. Current formulations primarily focus on the biological properties of polysaccharides and triterpenoids, with less emphasis on its nutritional value (Bishop et al., 2015).

Beyond the examples discussed, other studies associate *G. lucidum* with protective effects against renal, cardiac, and splenic injuries, primarily through anti-inflammatory and immune-modulating mechanisms. Continued research is warranted to fully harness the mushroom's diverse bioactive compounds.

2.1.4 Market overview of *G. lucidum* and its formulations

The market for *G. lucidum* is experiencing significant growth, with global sales of its products estimated to exceed $2.5 billion annually, showing an annual growth rate of 18% (Wang et al., 2020). In China, this mushroom contributes substantially to the local economy, particularly in provinces such as Yunnan, where residents collect naturally growing fruiting bodies from mountainous regions and sell them in local markets (Wang et al., 2020).

While *G. lucidum* products have gained popularity worldwide, China, Japan, and Korea remain the largest producers and suppliers of this mushroom and its formulations. Meanwhile, the United States represents the largest consumer market (Hapuarachchi et al., 2018). Despite the variety of commercially available products, including toothpaste, teas, capsules, powders, wound ointments, and cosmetic creams, there are challenges related to low reproducibility and standardisation, as well as insufficient quality control. Factors such as seasonal variations, differing soil conditions, and the developmental stage of the fruiting body used can hinder consistent quality in formulations. Therefore, there is a critical need to develop reproducible manufacturing protocols to ensure the high quality, standardisation, and safety of *G. lucidum* products (Hapuarachchi et al., 2018).

G. lucidum formulations are generally classified into three types: those based on the fruiting body, the mycelium, or spore powders (Bulam et al., 2019). **Table 2** and **Table 3** below present major companies involved in the *G. lucidum* trade and examples of products currently available in the international market.

Table 2: Alphabetical list of the largest companies involved in the commercialization of *G. lucidum* up to 2018

Company	Country of origin	Website
ALPHAY	China	http://www.alphayglobal.com
AMAX NUTRASOURCE	USA	http://www.amaxnutrasource.com
BIO-BOTANICA	USA	http://www.bio-botanica.com
BRISTOL BOTANICALS	UK	http://www.bristolbotanicals.co.uk
DXN	Malaysia	http://www.dxn2u.com
DRAGON HERBS	USA	http://www.dragonherbs.com
GANO EXCEL	Malaysia	http://www.ganoexcel.com.my
GANOLIFE	USA	http://www.ganolife.us
GUXIN	China	http://www.gubaolz.com
HANGZHOU	China	http://johnsun.en.alibaba.com

HOKKAIDO REISHI	Japan	http://www.hokkaido-reishi.com
HUAHERNHAFANG	China	http://www.hhhf.com.cn
HUACHENGBIO	China	http://www.huachengbio.com
JIABAO	China	http://www.lqjiabao.com
LINKANGSHI	China	http://www.gzlzzp.com
MUSHROOM SCIENCE	USA	http://www.mushroomscience.com
NATUREPLUS	China	http://www.gonatureplus.com
NAMMEX	Canada	http://www.nammex.com
ORGANO GOLD	Canada	http://www.organogold.com/ca-en/
RUIZHI	China	http://www.rzswkj.com
SANLIAN	China	http://www.cn-lingzhi.com
SERENIGY	USA	http://sites.google.com/site/serenigycoffeebusiness/home
SHOUXIANGU	China	http://sxg1909.com
SHUANG HOR	China	http://www.shuanghor.com.my/select_country.jsp
TOTAL LIFE CHANGES	USA	http://totallifechanges.com
XIANKELAI	China	http://www.xkl-cn.com
XI'AN GREENA BIOTECH	China	http://www.greena-bio.com
XIANPAILINGZHI	China	http://www.shinpire.com
XI' AN SOST	China	http://www.xianzhilou.com
XIANZHILOU	China	http://www.xiazhilou.com
XIAN YUENSUN	China	http://www.yuensunshine.com
XUCHANG YUANHUA	China	http://www.yamasuan.com
YUEWEI	China	http://www.gdyuewei.cn
YUNLE	China	http://www.hsyllz.com
ZHENGXIN	China	http://www.taishanlingzhi.net
ZHONGKE	China	http://www.zhogke.com

Source: (Hapuarachchi et al., 2018).

Table 3: Examples of food supplements, foods, and formulations containing *G. lucidum* or its extracts commercially available

Brand Name and Presentation or Pharmaceutical Form	Composition	Dosage	Company and Country
ZHICELL Mushroom Dietary Supplement (30 sachets)	<1g of carbohydrates, 1.25g of organic Lingzhi spores (*G. lucidum*), *Cordyceps* mycelium powder	1 sachet, twice daily, in warm water on an empty stomach	Alphay, USA
Balance Mushroom & Herbs Dietary Supplement (120 capsules)	1g of carbohydrates, 1.5g of a blend of Reishi fruiting bodies, *Lion's Mane* (*Hericium erinaceus*) fruiting body extract, *Cordyceps* hyphae, *Schisandra* fruit extract	3 capsules daily	Alphay, USA
Rich Black Lingzhi Coffee (30 sachets)	Organic coffee, extracts of organic *Lingzhi* (*G. lucidum*), organic *Maitake* (*Grifola frondosa*), organic *Shiitake* (*Lentinus edodes*), natural coffee extract, *Cordyceps* mycelium	1 sachet contains 3.68g of content, 15 calories per sachet	Alphay, USA
Organic *Ganoderma lucidum* Spore Powder (250mg - 90 capsules)	*G. lucidum* spores	2 capsules, 3 times daily with warm water. 6 capsules provide 1,500mg of organic *G. lucidum*	Organo Gold, Canada
Ganoderma lucidum Organic Powder (90 capsules, 250mg per capsule, 1 dose = 2 capsules/500mg)	Aerial parts of *G. lucidum*	2 capsules, 3 times daily. 6 capsules provide 1,500mg of organic *G. lucidum*	Organo Gold, Canada
Royal Black by Organo	Coffee from Colombia and Brazil, Reishi, *Cordyceps*, and *Lion's Mane*	1 sachet daily in 250mL of hot water, sweetened or unsweetened	Organo Gold, Canada
Reishi Capsules (*G. lucidum*, 60 capsules, 400mg)	Combination of hot water and ethanol extracts. At least 30% polysaccharides and 3% triterpenoids (ganoderic and lucidenic acids)	1 capsule daily	Bristol Botanicals Ltd., United Kingdom

Brand Name and Presentation or Pharmaceutical Form	Composition	Dosage	Company and Country
KAAPA Reishi Mushroom Tincture	Reishi fruiting body, reverse osmosis water, and 22% alcohol	1–4 half droppers per day, adjusted to user preference. Drops can be taken directly under the tongue or added to a drink	KAAPA Mushrooms, Finland

2.2 TRITERPENES

The term "terpene," proposed by Dumas in 1866, originates from the Latin word *turpentine* (*Balsamum terebinthiane*), a liquid extract derived from pine trees (Ninkuu et al., 2021). Terpenes are secondary metabolites composed of hydrocarbon units called isoprene, chemically referred to as 2-methylbuta-1,3-diene (C_5H_8). To date, over 80,000 terpenoids have been identified (Dinday & Ghosh, 2023).

Terpenes are classified based on the number of isoprene units (5C) in their molecular structure in hemiterpenes, with one isoprene unit (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), tetraterpenes (C_{40}) and polyterpenes, more than eight isoprene units (>C_{40}) (**Figure 5**) (Xavier et al., 2023).

Plants produce terpenes in response to biotic stress (e.g., pathogenic microbes, herbivorous pests, and weeds) and abiotic stress (e.g., water, temperature, and light). Volatile terpenes, such as hemi-, mono-, sesqui-, and some diterpenes, facilitate these interactions (Ninkuu et al., 2021, Boncan et al., 2020). In addition to their ecological roles, terpenes have significant applications in human industries, including food, beverages, rubber, biofuels, and pharmaceuticals. They are also investigated as potential active principles for anticancer, anti-inflammatory, antioxidant, antimicrobial, antiparasitic, antidiabetic, lipid-lowering, and cardioprotective therapies, as well as treatments for obesity and diseases of the liver, kidneys, and spleen (Tetali, 2019; Masyita et al., 2022).

Triterpenes are molecules containing six isoprene units (C_{30}). Their biosynthesis involves the "head-to-head" condensation of two farnesyl pyrophosphate (FPP) molecules, derived from the mevalonate (MVA) pathway, catalysed by the enzyme squalene synthase (SQS) to produce squalene. Squalene is subsequently oxidised to 2,3-oxidosqualene by squalene epoxidase (SQE) and undergoes cyclisation via specific oxidosqualene cyclases (OSCs), also known as triterpene synthases (tTPSs), encoded by various genes across living organisms (**Figure 4**). These reactions yield tetra- or pentacyclic structures, which serve as precursors for metabolites such as steroids, saponins, and cardiac glycosides/steroid lactones (Nagegowda & Gupta, 2020).

Triterpenes are subclassified based on their structural cyclicity into acyclic, monocyclic, bicyclic, tricyclic, tetracyclic, and pentacyclic forms. Tetracyclic and pentacyclic triterpenes are the most abundant in nature (**Figure 9**). Tetracyclic triterpenes derived from squalene cyclisation into lanostane, dammarane, protostane, cucurbitane, apotirucallane, tirucallane, euphanane, and cycloartane ring systems. Pentacyclic triterpenes are formed from bacarane, lupane, ursane,

taraxerane, multiflorane, bauarane, glutinane, friedelane, paquissanane, and taraxastane ring systems or hopane, neohopane, fernane, adianane, filicane, and gammacerane derivatives (Noushahi et al., 2022). Lanostane-type triterpenes, a subclass with 30 carbon atoms arranged in A, B, C, and D rings, are structurally similar to steroids but differ by the presence of three methyl group substitutions at C-4 and C-14 (Silva et al., 2020). *G. lucidum* predominantly contains lanostane-type triterpenoids biosynthesised via the MVA pathway. These compounds are central to the mushroom's pharmacological properties, with broad applications in health and medicine.

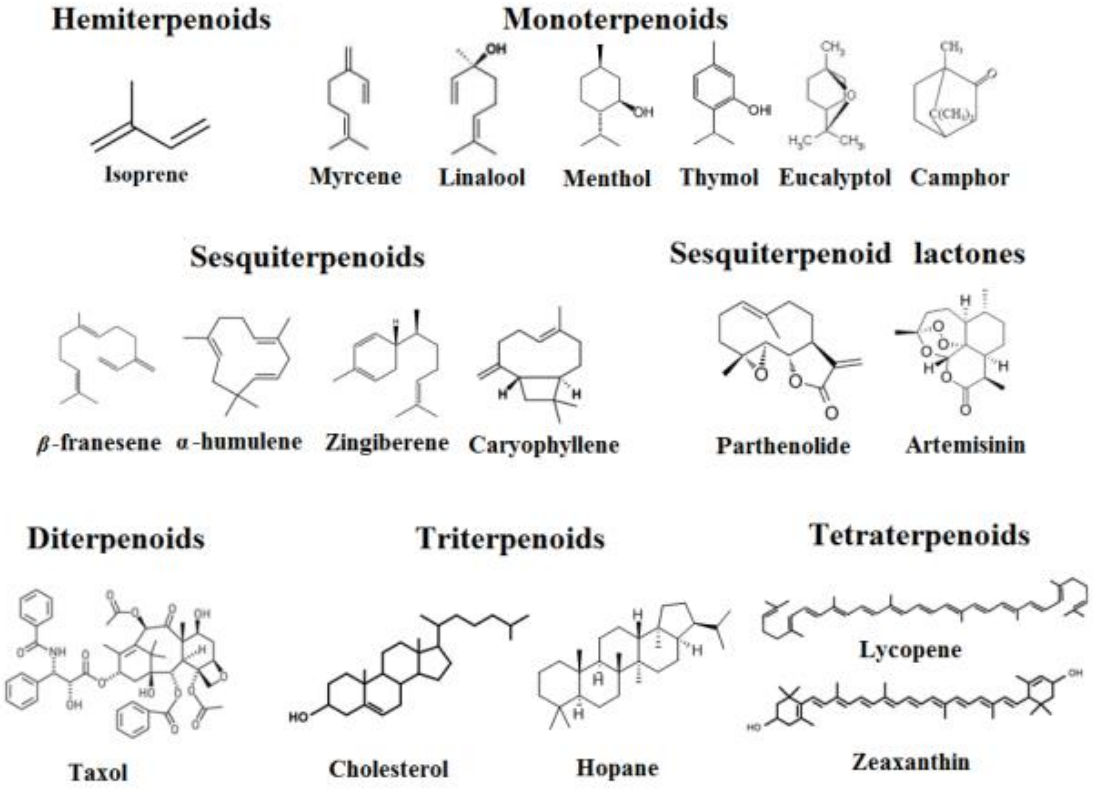


Figure 9: Examples of different terpene classes (Abdallah & Quax, 2017).

To date, most research in this scope for *G. lucidum* triterpene production research has focused on either optimization of yields from submerged fermentations throughout their different growth phases, namely mycelium, primordia, fruiting bodies and spores, or optimization of the extraction and downstream processing methods. Nakagawa et al. (2018) observed both total triterpenoid and polysaccharide contents were highest at a younger growth stage. Zhou et al. (2018) measured highest ganoderic acid C2 content in the primordial growth phase, highest ganoderic acids A, B, D, F, G and ganoderenic acid B content in the antlered growth phase and highest contents of ganodermanontriol, ganoderiol and ganoderic acids S, T and DM in the

maturing pileus phases. Ren et al. (2020) found that the mature pileus phase contained higher contents of ganoderic acids A and D whereas highest ganoderic acids B, C2, and G contents were found at bud elongation or antler growth phase. Although significant attention has been directed to the comprehensive identification of metabolites from *G. lucidum* in different growth phases, the distribution of those compounds along the sporocarp inner and outer parts in the mushroom's antler growth phase remains poorly investigated (Bishop et al., 2015).

2.3 BIOACCESSIBILITY STUDIES

Bioaccessibility measures the proportion of compounds consumed in a meal that are released from the food matrix during digestion in the luminal content and are accessible for absorption in the small intestine or biotransformation by the gut microbiota. Therefore, the bioaccessibility comprises the initial, digestion related, but critical steps for bioavailability of a given compound. Bioavailability, in its turn, refers to the amount of these compounds that are effectively absorbed and reach their target tissues, either in their intact form or as metabolites, to exert their bioactivity. Finally, bioactivity encompasses the biological changes induced by these compounds or their metabolites, resulting in measurable physiological effects in the organism. (Rodrigues et al., 2022).

The concept of bioaccessibility originates from food sciences but has gradually been adopted by other disciplines, such as pharmaceutical sciences. Secondary metabolites, nutraceuticals, vitamins, and minerals are common subjects of bioaccessibility studies across various matrices. Among secondary metabolites, phenolic compounds are the most extensively studied, with numerous investigations focusing on the antioxidant capacity of bioaccessible phenolics from herbal products, supplements, or food matrices (Lucas-González et al., 2018). In contrast, studies evaluating the bioaccessibility of triterpenes, particularly tetracyclic ones, remain very limited.

The study of bioaccessibility and bioavailability studies can be conducted with human volunteers. *In vivo* and, particularly, human models, provide more accurate and realistic results and are considered the "gold standard." However, analysing the multiple and complex processes occurring during digestion in animals and humans is technically challenging, costly, and often constrained by ethical considerations, particularly when potentially harmful substances are involved. These factors, along with the necessity to investigate of specific hypothesis and conduct screening studies with different samples has urged the development of *in vitro* models.

The first scientific article on *in vitro* digestion was published in 1954 (Lucas-González et al., 2018). Since then, researchers have developed a range of specific *in vitro* digestion models, often leading to ambiguities in results and difficulties in cross-comparisons. To address these issues, in 2014, a group of researchers from the COST network established a standardised static *in vitro* digestion protocol for adults, known as INFOGEST. This protocol aimed to provide international consensus on digestion processes and accessible methodologies for researchers in the field. INFOGEST organises the digestion process into three main phases: oral, gastric, and intestinal. At the end of the process, the chyme is separated and analysed. The entire procedure takes approximately five hours and requires strict control of various conditions, particularly pH, at each phase (**Figure 10**) (Minekus et al., 2014). In 2019, the same group of researchers published the improved INFOGEST 2.0 protocol. Brodkorb et al. (2019) presented a refined procedure addressing many of the challenges raised with the original protocol. The improved protocol divides the process into three stages: preparation, digestion, and subsequent collection, treatment, and analysis of the chyme. It also addresses some post-digestion processes, including techniques for collecting and processing chyme. Additionally, the protocol highlights quality control procedures, the need for a colonic phase in some cases, and recommended reagent brands (**Figure 11**) (Brodkorb et al., 2019). Beyond the widely adopted INFOGEST models, other *in vitro* digestion methods—static, semi-dynamic, and dynamic—are being developed. These models aim to simplify digestion processes while providing more realistic approximations of human digestion.

As reported above, phenolic compounds are the most extensively studied in *in vitro* digestion (IVD). Few studies of IVD can be found in the literature for triterpenes of *G. lucidum*. A static simulated digestion of various preparation methods for *G. lucidum* spores revealed that, in general, the amount of triterpenoids in the chyme is low, while polysaccharides dominate the digested fractions. The amount of triterpenoids retained in the undigested matrix was 9.2 ± 0.8 mg/g for spores, 24.7 ± 1.3 mg/g for spores with broken cell walls, and 32.5 ± 1.1 mg/g for ethanolic (95%) extracts of spores prepared in the same manner. In the simulated intestinal fluid (SIF), triterpenoid levels were 4.6 ± 0.3 mg/g, 5.8 ± 0.3 mg/g, and 8.3 ± 0.4 mg/g, respectively. The triterpenoid profile underwent significant alterations during digestion, with only ganoderic acids B and A identified in the chyme. The best bioaccessibility for triterpenoids was reported for the ethanol extract, which initially contained approximately 2.56 ± 0.04 g/100 g. The findings suggest that triterpenoids are strongly bound to the *G. lucidum* spore matrix (Cai et al., 2021).

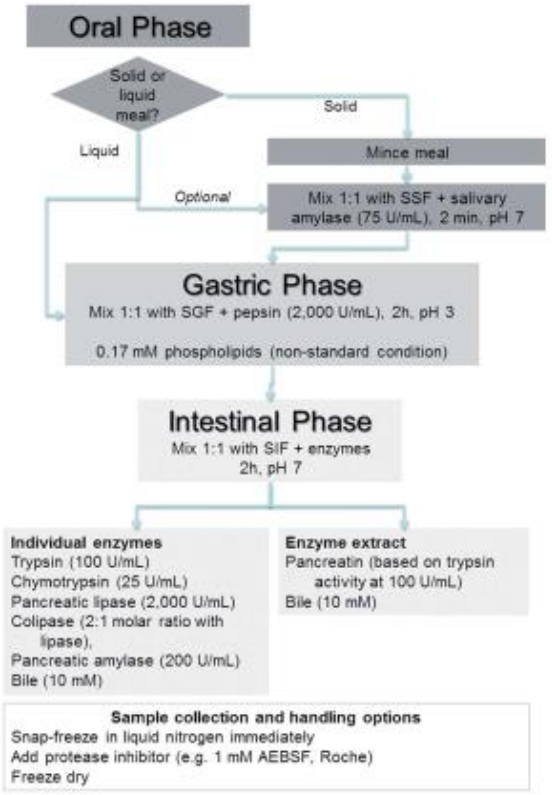


Figure 10: Schematic representation of the static *in vitro* digestion INFOGEST (Minekus et al., 2014)

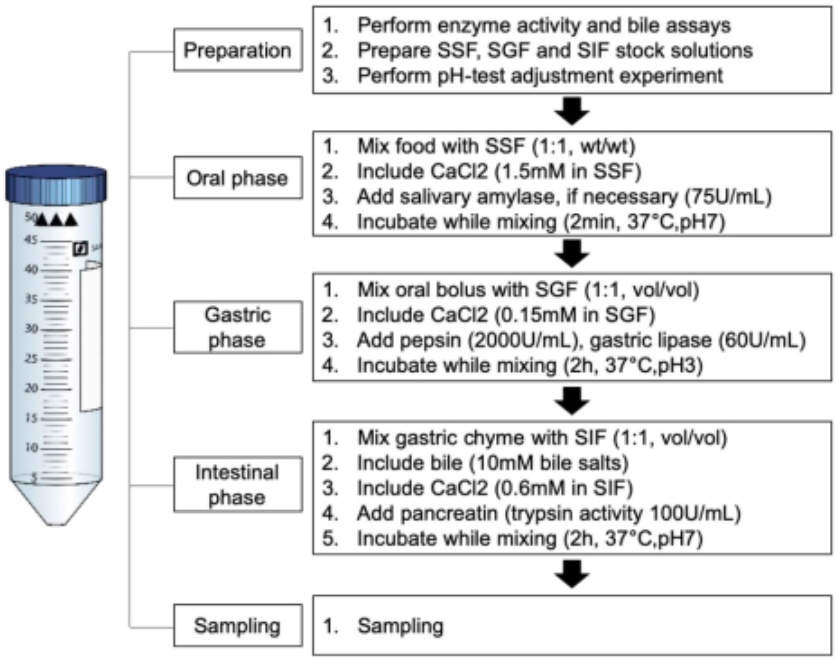


Figure 11: Static *in vitro* digestion INFOGEST 2.0. (Brodkorb et al., 2019; Li et al., 2020).

3. OBJECTIVE

3.1 MAIN OBJECTIVE

The primary objective of this study was to chemically characterize and compare different parts (outer skin and interior flesh) of fruiting bodies of the medicinal mushroom *G. lucidum*, particularly regarding their triterpene fraction, and assess the *in vitro* bioaccessibility of these compounds.

3.2 SPECIFIC OBJECTIVES

To achieve the main objective, the following steps were performed (**Figure 12**):

- **i.** Determination of the nutritional composition of *G. lucidum* sporocarp parts;
- **ii.** Extraction of triterpenes from *G. lucidum* sporocarp parts using different green and conventional extraction protocols established in the laboratory routine as an initial screening;
- **iii.** Exhaustive extraction of triterpenes of *G. lucidum* sporocarp parts with the selected extraction method for chemical characterization;
- **iv.** Separation, identification and quantification of the triterpenes in the extracts by HPLC-DAD-(ESI-)MS/MS;
- **v.** *In vitro* digestion of samples to estimate the bioaccessibility of triterpenes;
- **vi.** Determination of prebiotic potential and β-glucan content of the residue of the *in vitro* digestion of *G. lucidum* sporocarp parts;

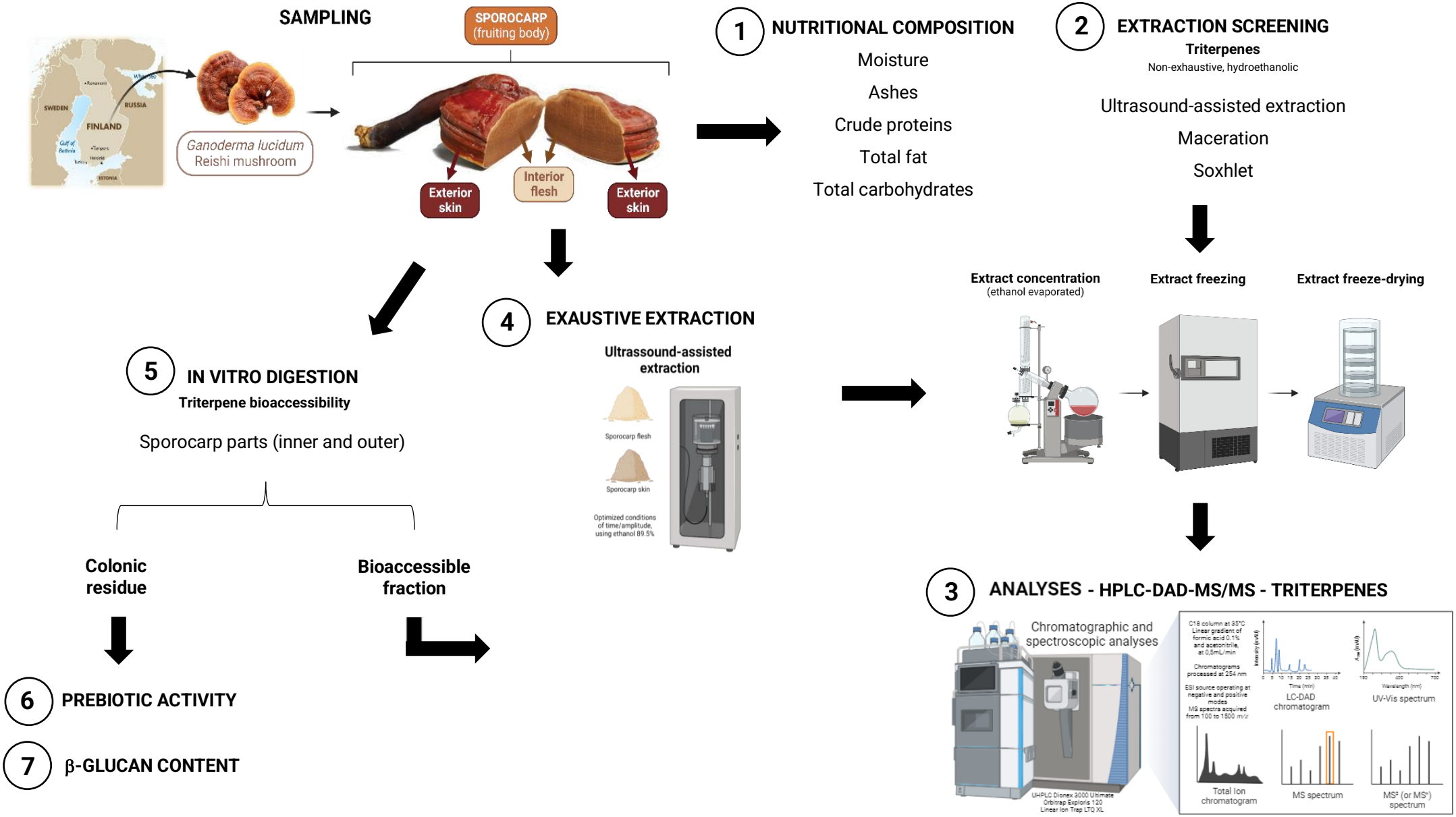


Figure 12: Overview of the developed work plan.

4. MATERIAL AND METHODS

4.1 CHEMICALS

Standard of Ganoderic acid A (purity ≥ 95%, HPLC) and analytical-grade reagents used in the *in vitro* digestion and other protocols were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzymes α-amylase (A1031), pepsin (P7125), and pancreatin (P7545), and bile extract (B8631) were also acquired from Sigma-Aldrich. Microbiology supplies for prebiotic activity including Man–Rogosa–Sharpe broth, were obtained from LiofilChem S.R.L (TE, Italy). Bacteria stains employed in the prebiotic activity assay (*Lactobacillus casei* NCTC 6375, *Lactobacillus plantarum* DMS 12028, *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* spp. lactis Bb12) were purchased from the company Probio-Tec (Denmark). β-Glucan kit (K-YBGL for yeast and mushroom) was acquired from Neogen Corporation (Lansin, MI 48912 USA) and Megazyme Ltd. (Bray, Ireland). LC-MS-grade methanol and acetonitrile and extra pure formic acid were provided by Fisher Scientific (Leicestershire, UK). Water was purified in a TGI Pure Water Systems (Greenville, SC, USA). HPLC solvents and samples were filtered through 0.45 and 0.22 μm membranes of regenerated cellulose, respectively, prior to chromatographic analysis.

4.2 FUNGI MATERIAL

G. lucidum sporocarps were cultivated in the facilities of Kaapa Biotech (Finland), company that provided the samples for this study. Batches of 10kg of the sample were collected and the exterior skin was manually separated from the interior flesh of *G. lucidum*'s fruiting bodies. These two samples were and reduced to a granular material to produce a composite sample of each part at the installations of Kaapa Biotech, before being carefully transported to the Centro de Investigação de Montanha (CIMO) - Instituto Politécnico de Bragança (IPB). Samples were then stored at room temperature, protected from light, until analyses.

4.3 NUTRITIONAL COMPOSITION

The study of nutritional composition was carried out according to the Official Methods of AOAC (1999).

4.3.1 Moisture content

Moisture was assessed according to the protocol established by the official method of analysis No. 925.45b (AOAC, 1999). An electronic moisture balance (ADAM, PMB 163, Oxford, USA) was used to weigh approximately 1.6 g of each sample, and the moisture was completely removed using infrared radiation (**Figure 13**). The percentage of moisture was determined from the difference between the initial and final mass of the sample, with the results expressed in grams of moisture per 100 grams of dry weight.

Figure 13: Moisture scale.

4.3.2 Ash content

To analyse the ash content, the procedure described in the official method of analysis number 935.42 (AOAC, 1999) was used. Samples of 280 mg were meticulously weighed into previously treated porcelain crucibles, identified and weighed. The samples were then subjected to incineration in a muffle furnace (IVYMEN, N-8L, Barcelona, Spain) at 550 °C for a period of 6 hours, until white ash was obtained, indicating complete calcination. After the process, the crucibles containing the calcined samples were transferred to a desiccator and left to cool until they reached room temperature (~25°C) and then weighed until a constant weight was obtained

(**Figure 14**). The percentage of ash was determined using the difference between the initial and final mass of the sample, with the results expressed in grams of ash per 100 grams of dry weight.

Figure 14: Ashes of the inside (top) and outside (bottom) parts of the sporocarps.

4.3.3 Total fat

The total fat content was determined according to the protocol established by the official analysis method number 989.05 (AOAC, 1999). Initially, approximately 3 g of each sample was weighed into a paper cartridge, which was then inserted into a Soxhlet-type fat extractor, using petroleum ether as the extraction solvent, at a temperature of approximately 120°C, with a cycle duration of 6 h (**Figure 15**). The results were expressed in grams of fat per 100 grams of dry weight and were calculated using the gravimetric difference between the initial mass of the sample and the residual mass of fat.

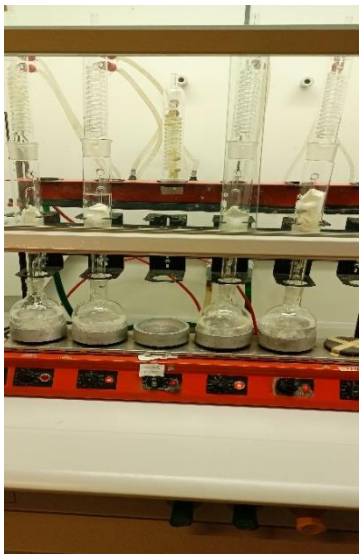


Figure 15: Total fat extraction process using Soxhlet equipment.

4.3.4 Crude protein

The crude protein content was determined using the macro-Kjeldahl method, as prescribed by the official method of analysis number 991.02 (AOAC, 1999), using a conversion factor of 4.38 to convert the nitrogen (N) content into crude protein, a nitrogen-to-protein conversion factor more specific for mushrooms (AOAC 1978; USDA 2011; Kalač 2013). For the digestion process, approximately 0.25 g of sample was weighed into Kjeldahl tubes, to which two catalyst tablets (Kjeltabs) and 15 mL of concentrated sulfuric acid (H_2SO_4) were added. The tubes were then placed in a digester block at a temperature of 420°C for 70 min. After the samples were completely digested and cooled, 25 mL of distilled water was added. Using a Kjeldahl analyser (Velp Scientifica UDK 152), NaOH was added to the tubes containing the digested sample, releasing the nitrogen in the form of NH_3, which was then collected by steam distillation in a 0.1N H_2SO_4 solution. Finally, a titration was carried out with 0.1N NaOH, using methyl red as an indicator to calculate the amount of nitrogen, which was converted to protein amount according to the equation below. The results were expressed in grams per 100 grams of dry weight.

$$Protein\ (g/100g, \%) = \%\ of\ nitrogen\ (N)\ x\ Conversion\ factor \quad (4.38)$$

4.4 EXTRACTION OF TRITERPENES FROM *G. LUCIDUM* SPOROCARP PARTS

Mushroom samples were homogenised and subjected to extraction protocols carried out in standardized conditions used in our laboratory. These included maceration (MAC), and Soxhlet (SOX), as conventional methodologies, besides ultrasound-assisted extraction (UAE), which represents a green alternative methodology. All methods used ethanol:H_2O (89.5:10.5, v/v) as extraction solvent, according to the optimized methodology described in detail elsewhere (Oludemi et al., 2018). Upon completion of each extraction procedure, ethanol was evaporated under vacuum (T < 37°C) in a rotatory evaporator (Büchi R-210, Flawil, Switzerland), and the remaining aqueous extract were frozen and freeze-dried (-55±0.5°C, 48h - Freeze Dryer Telstar LyoQuest-55, Milan, Italy).

Maceration (MAC): Aliquots of 2 g of each sample were weighted and combined with 60 mL of 89.5% ethanol, being the mixture stirred continuously at 500 rpm for 1 h at room temperature (magnetic bar stirrer Multimatic 9-N, Selecta- Barcelona, Spain). After this period,

the extract was separated by filtration (filter paper, Ø 125 mm, CMHLAB - Barcelona, Spain) and the residue re-extracted under the same conditions. The resulting extracts from the 2 cycles were combined.

Soxhlet Extraction (SOX): Samples (3.0 g) were added with 100 mL of 89.5% ethanol and the compounds were extracted by refluxing in a Soxhlet apparatus in a total of 6 cycles.

Ultrasound-Assisted Extraction (UAE): A portion of 3g of each sample was mixed with 100 mL of 89.5% ethanol and subjected to sonication for 15 min at 47% amplitude in an ultrasonic device equipped with a titanium probe (model CY-500, Optic Ivymen System, Barcelona, Spain) operating at 25 kHz. An ice bath was employed during the procedure to prevent overheating of the extract. When an exhaustive extraction was performed, the initial sample volume was reduced to 0.5 g, and the supernatant of each cycle was separated from the residue after centrifugation. At the end, all the extracts were combined (**Figure 16**).

Figure 16: UAE extracts from *G. lucidum* sporocarp parts.

4.5 Analysis of triterpenes in the extracts

Extracts from *G. lucidum* sporocarp parts were analysed in a Thermo Scientific HPLC (Dionex UltiMate 3,000 series, Thermo Fisher Scientific - San Jose, CA, USA) equipped with a diode array detector (DAD) and connected in series to an Orbitrap mass spectrometer (MS, Orbitrap Exploris 120, Thermo Fisher Scientific - San Jose, CA, USA, **Figure 17**). The triterpene-rich extracts were separated in a Spherisorb S3 ODS-2 C_{18} column (3 µm, 4.6 x 150 mm, Waters - Milford, USA) kept at 35 ºC, under a gradient of 0.1% (v/v) formic acid in ultrapure water (A) and acetonitrile (B). The mobile phase proportion (A:B, %) ranged from initial 85:15 for 5 min to 80:20 in 5 min, reaching 75:25 in 10 min, 65:35 in 10 min, and 50:50

in 10 min, finally returning in 10 min to the initial condition, kept for further 10 min for column reconditioning. The flow rate was 0.5 mL/min, and the injection volume was 10 μL. UV-Visible (UV-Vis) spectra were acquired between 180 to 700 nm and the chromatograms processed at 254nm. The HPLC eluate was analysed by high-resolution, tandem mass spectrometry, using an electrospray ion source (ESI) source operating in negative and positive modes. Full MS and MS/MS spectra were acquired in the range from 110 to 1,500 charge-to-mass ratio (*m/z*). Data acquisition and processing were conducted with the Xcalibur® software (Thermo Fisher Scientific, San Jose, CA, USA). For compound identification, elution order on the C_{18} column and characteristics of the UV-Vis and mass spectra (molecular ion ($[M-H]^-$), and MS/MS fragments) were interpreted and compared with standards, when available, literature data and libraries (NIST®, MZ Vault® and MZCloud®) available in the Freestyle® software (Thermo Fisher Scientific - San Jose, CA, USA), also employed for data processing. Quantification of triterpenes was performed using a 9-point external calibration curve of ganoderic acid A, and results were expressed as mg per g of sample ($mg.g^{-1}$, dw). Phenolic compounds in the triterpene-rich extracts were analysed using the same methodology, detected at 280, 330 and 370 nm, quantified using a calibration curve of 4-hydroxybenzoic acid and expressed as mg per g of freeze-dried extract ($mg.g^{-1}$, dw) (Rodrigues et al., 2023).

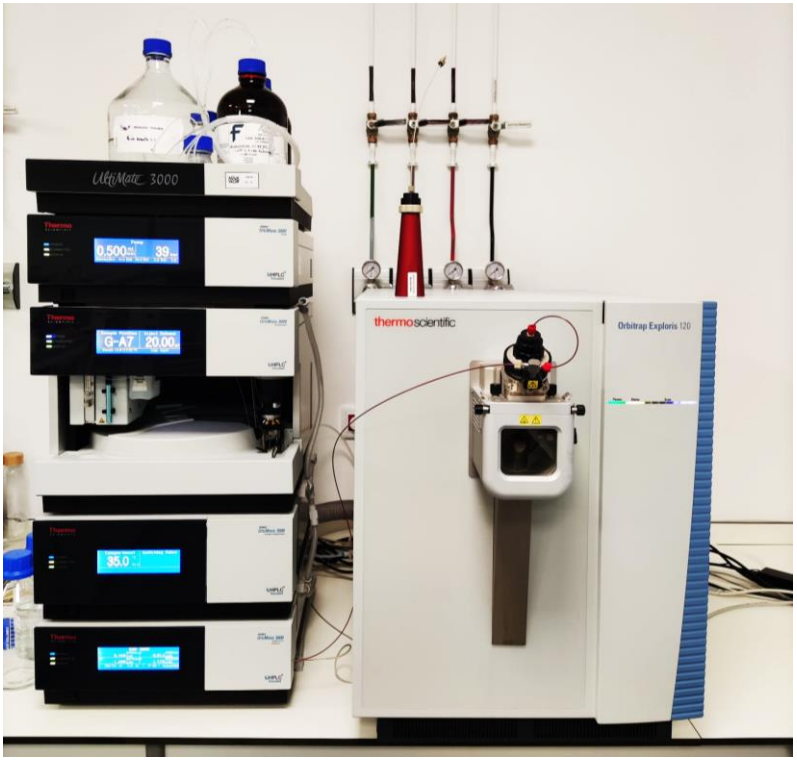


Figure 17: Thermo Scientific HPLC (Dionex UltiMate 3,000 series, Thermo Scientific - San Jose, CA, USA) equipped with a diode array detector (DAD) and connected in series to an Orbitrap mass spectrometer (MS, Exploris 120, ThermoFinnigan - San Jose, CA, USA).

4.6 *IN VITRO* BIOACCESSIBILITY OF TRITERPENES FROM *G. LUCIDUM* SPOROCARP PARTS AND THEIR TERPENE-RICH EXTRACTS

The internationally recognized protocol of *in vitro* digestion (IVD) known as INFOGEST (Minekus et al., 2014) was employed to assess the duodenal (small intestinal) bioaccessibility of compounds from *G. lucidum* samples. Specific assays of enzyme activities were carried out according to the protocols described in detail by Minekus et al. (2014) and Brodkorb et al. (2019). For the dried mushroom powder, around 2.5g of the external and internal part of the mushroom were weighed and added by ultrapure water until reaching around 25g, the samples were crushed in an ultraturax (IKA T25 digital, ULTRA TURAX) to simulate chewing for three minutes and 8,000 rpm (**Figure 18**). For each part, three replicates of approximately 5 g of the homogenized mushroom were prepared. To 5 g of the rehydrated mushroom sample or 5 mL of the brew preparation, 3.5 mL of salivary fluid (pH 7.0), 0.5 mL of α-amylase solution 1500 $U.mL^{-1}$, 25 µL of 0.3 M $CaCl_2$ and 975 µL of ultrapure water were added (**Figure 19**). The mixture was be incubated at 37 °C under stirring for 2 min. To simulate the gastric phase, 7.5 mL of simulated gastric fluid (pH 3.0), 1.6 mL of pepsin solution 25,000

Figure 18: Sporocarp sample being homogenized using an IKA digital ultraturax before IND experiments.

Figure 19: Samples in the oral phase of the *in vitro* digestion protocol.

$U.mL^{-1}$ (dissolved in SGF) and 5 µL 0.3 M $CaCl_2$ were be added. The pH of the mixture was adjusted to 3.0 and the volume made up to 20 mL with ultrapure water. The mixture was incubated at 37 °C under stirring for 2 h. To simulate intestinal conditions, 11 mL of simulate

intestinal fluid (pH 7.0), 5 mL of pancreatin solution 100 U.mL^{-1}, 40 µL of 0.3 M $CaCl_2$ and 2.5 mL bile salt solution were added. The pH of the mixture was adjusted to 7.0, and ultrapure water was added to make up the final volume to 40 mL. The mixture was further incubated for 2 h at 37 °C. After this period, the final chyme was cooled in an ice bath. The final mixture was centrifuged (12,000g, 10 min) to obtain the supernatant as the bioaccessible fraction (BF) of compounds. Both BF and the solid residue containing undigested or colonic material (CM) were stored at -80°C until analysis. Aliquots of BF were subjected to solid-phase extraction (SPE, Strata-X from Phenomenex) (**Figure 20**). The resulting extracts were dried under nitrogen flux, resuspended and analysed by HPLC following the methodology described in the item 4.5.

The bioaccessibility was calculated as the percentage of triterpenes of the non-digested mushroom (starting material) that was found in the bioaccessible fraction after the simulated digestion, according to the equation bellow:

$$Bioaccessibility\ (\%) = \left(\frac{[terpenes]bioaccessible\ fraction}{[terpenes]mushroom}\right)x100$$

Aliquots of the CM were freeze-dried, and their β-glucan content was assessed according to the protocol described in the item 4.8.

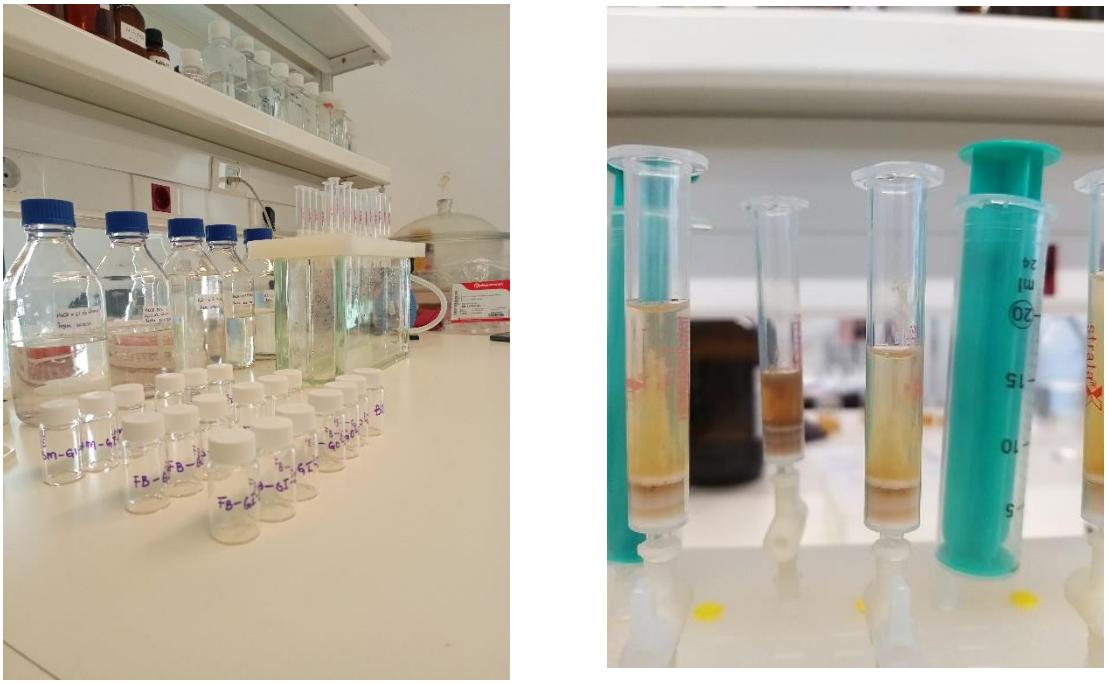


Figure 20: Solid-phase extraction of compounds from bioaccessible fractions obtained after IVD.

4.7 PREBIOTIC POTENTIAL OF THE SOLID UNDIGESTED FRACTIONS OF *G. LUCIDUM* SPOROCARP PARTS OBTAINED AFTER *IN VITRO* DIGESTION

The *in vitro* method consisted of evaluating the ability of the samples to develop four bacteria of the intestinal flora, *Lactobacillus casei* NCTC 6375, *Lactobacillus plantarum* DMS 12028, *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* spp. lactis Bb12 (Spréa et al., 2024). Prior to the assay, bacteria were grown in Man-Rogosa-Sharpe (MRS) medium containing 20 g/L of glucose under anaerobic conditions for a minimum of three days, being then transferred to a fresh broth 12 h before the experiment to reach the log growth phase of growth. For *B. animalis*, the medium was additionally supplemented with 0.05% *L*-cysteine.

The solid residue obtained after centrifugation of chymes (colonic material, CM) from IVD of both the outer (CMGO) and the inner (CMGI) sporocarp parts was freeze-dried prior to the assay. These samples and the positive controls, namely inulin and fructooligosaccharide (FOS), were suspended in a concentration of 2% (w/v) in MRS medium without glucose, pasteurized (72-75ºC, 1 min) and centrifuged (8,000 g, 3 min). The inoculum was also suspended separately in MRS medium without glucose until it reached a turbidity of 0.5 McFarland equivalent (1.5 x 10^8 CFU/mL). Subsequently, mixtures of 10 μL of the inoculum plus 2.290 μL of each sample or positive control was prepared to reach a concentration of 5×10^5 CFU/mL, and aliquots of 200 μL of these mixtures were transferred to wells of 96-well plates (U-shape bottom). Wells were all sealed with 50 μL of paraffin to maintain anaerobiosis. The MSR medium containing 2% glucose (w/v), and the MSR medium alone also served as positive and negative controls, respectively, while the suspensions of samples or positive controls in MRS broth without inoculum served as blank controls. The microplates were incubated at 37ºC for 48h, and the optical density at 620 nm was read every one hour on a microplate reader. Finally, the growth curve (optical density *vs*. time) was constructed and analysed.

4.8 β-GLUCAN CONTENT

The determination of β-glucans was carried out using the Kit K-YBGL developed for measurement of 1,3:1,6-β-D-glucans in mushroom and mycelial products, yeast and fungal preparations (**Figure 21**, McCleary & Draga, 2016). The method assesses total glucans and α-glucans, being the β-glucan content calculated from the difference between them. Briefly, glucans are hydrolysed using acid (2M H_2SO_4) and subsequently using a mixture of exo-1,3-β-

glucanase and β-glucosidase, releasing D-glucose that is measured to give the total glucan content. Then, α-glucans found in digestible carbohydrates such as glycogen, starch, sucrose, maltodextrins, and trehalose are hydrolysed by incubation with amyloglucosidase and invertase, also releasing D-glucose, which is measured along with free D-glucose to give a measure of α-glucan content. The β-glucan content is then calculated indirectly by subtracting α-glucans from total glucans.

Figure 21: K-YBGL Kit used for β-glucan estimation.

Glucose content was determined according to the kit instructions by a colorimetric assay (510 nm, Zuzi spectrophotometer model 4255/50, **Figure 22**). Samples were freeze-dried before the assay. Calculations were conducted using the spreadsheet made available by the company (K-YBGL_CALC), and the results expressed as percentage (%, w/w) of glucans in the sample.

Figure 22: Zuzi spectrophotometer model 4255/50 used during β-glucan analysis.

4.9 STATISTICAL ANALYSIS

Data was obtained in triplicate and compared by analysis of variance (ANOVA) with the comparison of means by Tukey's test, with a significance level of 5%. The means of triterpene content, polyphenol total content, nutritional content, and bioaccessibility determined either in the different parts of the mushroom or by two different extraction methods or functional preparations were compared by Student's *t*-test for two independent means. The program Statistica 7.0 was used to carry out the tests. Regression analyses were performed using Origin software version 8.0.

5. RESULTS AND DISCUSSION

5.1 NUTRITIONAL CHARACTERIZATION OF *G. LUCIDUM* SPOROCARP PARTS

The proximate composition analysis of the inner (GI) and outer (GO) parts of the *G. lucidum* fruiting body showed no substantial differences between the two samples, indicating they have comparable amounts of total macro- and micronutrients (**Table 4**).

Moisture content was slightly higher in the inner part (13.75±0.21%) compared to the outer part (11.29±0.37%), being the only component, in fresh weight (fw) basis, statistically different between the two samples. This difference could reflect the tissue structure of each part, with the inner flesh being more hydrated than the outer skin. The relatively low moisture levels observed in this study contrast with some reports in the literature, for instance, the moisture content of 47% for undried *G. lucidum* fruiting bodies (Sheik et al., 2022), and of up to 90% for Nigerian Reishi (Ahmad et al., 2021) is likely due to variations in sample drying and preparation methods.

Carbohydrates comprised the main class of macronutrients in both samples, accounting for about 80% of the total nutrients of the *G. lucidum* sporocarp, regardless the part. Such high carbohydrate levels are consistent with *G. lucidum* general findings across different geographical origins and underscore the mushroom's role as a carbohydrate-rich food source, which is possibly due to its structure and storage compounds. Ahmad et al. (2021) reported 82.3% and 73.3–81.9% of carbohydrates in European and African *G. lucidum*, while a value of 88.4% was found in *G. lucidum* from Portugal (Taofiq et al., 2017). Whereas the total amount of carbohydrates was similar across the sporocarp parts studied in the present work, this class comprise a range of molecules with varied degree of complexity, from simple sugars to highly complex polysaccharides that can be digestible or classified as insoluble or soluble fibre. So, it is still possible that the carbohydrate composition of the sporocarp parts can be distinct.

Total proteins, lipids and ashes constituted roughly 5-6%, 1-2% and 0.5-0.6% (g/100g, fw), respectively, of the proximate composition for either sample. On dry basis, this would represent approximately 5.99%, 1.17% and 0.64% of those respective nutrients in inner flesh and 6.35%, 2.45% and 0.69% of total compounds in outer skin. No studies assessing the composition of sporocarp parts of *G. lucidum* separately can be found in the literature (Ahmad et al., 2021, **Table 4**). In summary, the proximate composition of the inner and outer parts of the fruiting body studied in the present study were not so different between them and are overall in line to the composition reported in previous studies found in literature. Therefore, separating the outer and inner parts of the *G. lucidum* sporocarp only in terms of proximate composition

(total content) would not provide substantial technological benefit in terms of enhancing the recovery of macronutrients and minerals from one of them.

Table 4. Nutritional composition of *G. lucidum*'s sporocarp parts

	Proximate composition (%)				
	Carbohydrates	**Lipids**	**Proteins**	**Moisture**	**Ashes**
Inner part	79.52[a]	1.01±0.26[a]	5.17±0.14[a]	13.75±0.21[a]	0.55±0.11[a]
Outer part	80.3[a]	2.17±0.64[a]	5.63±0.23[a]	11.29±0.37[b]	0.61±0.01[a]

Data are mean ± standard deviation (g/100g fresh weight). Total carbohydrates were calculated by difference. Different superscript letters in the same column indicate significant differences ($p < 0.05$, t test).

5.2 EXTRACTION SCREENING OF TOTAL TRITERPENES FROM *G. LUCIDUM* SPOROCARP PARTS

An initial extraction screening was performed using methodologies standardized in our laboratory to select the extraction methodology able to extract most of the triterpenes of the *G. lucidum* sporocarp parts. Each protocol was performed according to their established conditions within our laboratory framework. An ethanolic solution (ethanol:water, 89.5:10.5, v/v) was employed as the extraction solvent for either method, since it has demonstrated to efficiently extract triterpenes from *G. lucidum* as investigated by Taofiq et al. (2018). Although triterpenic compounds with intermediate to low polarities have been described, the green character of ethanol was also considered along with its extraction efficiency.

Table 5 displays the total triterpene content (sum of compounds identified by HPLC-DAD) of the hydroethanolic extracts obtained from the inner and outer sporocarp parts of *G. lucidum* using dynamic maceration (MAC), Soxhlet (SOX), and UAE. Notably, the outer skin consistently presented a triterpene content of about 2 to 3 times higher ($p<0.05$) than the inner part (from 24.30±0.48 to 68.78±0.54 mg/g freeze-dried extract for maceration and UAE, respectively). For both sporocarp parts, MAC recovered the lowest amount of triterpenes.

Table 5: Total triterpenes, as assessed by HPLC-DAD, of different hydroethanolic extracts obtained from outer skin and interior flesh of *G. lucidum* sporocarps

	Total triterpenes (mg/g freeze-dried extract)		
	MAC	**SOX**	**UAE**
OUT	24.30 ± 0.48 ^{Ca}	54.01 ± 1.02 ^{Ba}	68.78 ± 0.54 ^{Aa}
IN	8.33 ± 0.12 ^{Cb}	33.14 ± 0.07 ^{Ab}	23.98 ± 0.89 ^{Bb}

Data are mean ± SD, expressed as mg/g freeze-dried extract. MAC: SOX: UAE: OUT: IN: Different uppercase letters in the same column indicate differences (ANOVA followed by Tukey's test, $p<0.05$) among the triterpene content of the three different extraction methodologies within a given sample of sporocarp, whereas different lowercase letters in the same row indicate difference (Student's t test, $p<0.05$) between the triterpene content of outer and inner sporocarp parts for a given extraction method.

Although the primary focus was the extraction of triterpenes, phenolic compounds present in the sporocarp were simultaneously extracted with the hydroethanolic solvent and present in the crude extract and were also analysed. Interestingly, in contrast to the triterpenes, the inner part of the sporocarp presented a higher total amount of polyphenols, up to 5 times the external skin depending on the extract (ranging from 15.21±0.70 to 29.32±0.67 mg/g extract dw, for maceration and Soxhlet, respectively, **Table 6**). The majority of these compounds were identified as phenolic acids.

Table 6. Total phenolic compounds, as assessed by HPLC-DAD, of different hydroethanolic extracts obtained from outer skin and interior flesh of *G. lucidum* sporocarps

	Total polyphenols (mg/g freeze-dried extract)		
	MAC	**SOX**	**UAE**
OUT	9.08 ± 0.18 ^{Ab}	4.84 ± 0.07 ^{Bb}	10.09 ± 0.01 ^{Ab}
IN	15.21 ± 0.70 ^{Ca}	29.32 ± 0.67 ^{Aa}	26.84 ± 0.31 ^{Aa}

Data are mean ± SD, expressed as mg/g freeze-dried extract. MAC: SOX: UAE: OUT: IN: Different uppercase letters in the same column indicate differences (ANOVA followed by Tukey's test, $p<0.05$) among the triterpene content of the three different extraction methodologies within a given sample of sporocarp, whereas different lowercase letters in the same row indicate difference (Student's t test, $p<0.05$) between the triterpene content of outer and inner sporocarp parts for a given extraction method.

In terms of total compound recovery, the UAE (80.70±0.01 mg/g dw) exceeded that of Soxhlet (60.07±0.76 mg/g dw) and maceration (34.05±39 mg/g dw) for the outer part, whereas for the inner part the order was Sohxlet > UAE > maceration (64.59±0.57, 51.32±0.04 and 24.11±0.80 mg/g extract dw, respectively).

Table 7. Total compounds (triterpenes and phenolic compounds), as assessed by HPLC-DAD, of different hydroethanolic extracts obtained from outer skin and interior flesh of *G. lucidum* sporocarps

	Total compounds (mg/g freeze-dried extract)		
	MAC	**SOX**	**UAE**
OUT	34.05 ± 0.39 ^{Ca}	60.07 ± 0.76 ^{Ba}	80.70 ± 0.01 ^{Aa}
IN	24.11 ± 0.80 ^{Cb}	64.59 ± 0.57 ^{Aa}	51.32 ± 0.04 ^{Bb}

Data are mean ± SD, expressed as mg/g freeze-dried extract. MAC: SOX: UAE: OUT: IN: Different uppercase letters in the same column indicate differences (ANOVA followed by Tukey's test, $p<0.05$) among the triterpene content of the three different extraction methodologies within a given sample of sporocarp, whereas different lowercase letters in the same row indicate difference (Student's t test, $p<0.05$) between the triterpene content of outer and inner sporocarp parts for a given extraction method.

Therefore, from the initial screening of the chemical composition of *G. lucidum* sporocarps (total triterpenes and total polyphenols) obtained though different extraction methods, significant differences in the distribution of bioactive compounds were found between

the outer skin and inner flesh. These findings highlight the superior triterpene content in the outer skin and the higher polyphenol concentration in the inner flesh, emphasizing the importance of targeted extraction strategies to optimize the yield of specific compounds.

The chemical characterization of natural sources and the development of natural-based products rely on the effective extraction of compounds from the natural matrix. While it is crucial to evaluate the extract's final quality in terms of composition, it is also important to ensure the sustainable approaches in both analytical and production processes. In this context, among the extraction methods evaluated, UAE demonstrated the highest efficiency in recovering total triterpenes, which was the focus of the work, besides total compounds from the outer skin, while Soxhlet extraction was most effective for the inner flesh. As UAE is regarded as a greener approach in contrast to Soxhlet, this methodology was selected for posterior extractions.

5.3 CHEMICAL CHARACTERIZATION OF TERPENE-RICH EXTRACTS FROM *G. LUCIDUM* SPOROCARP PARTS

Exhaustive triterpene-rich extracts were produced by UAE from the two parts of the *G. lucidum* sporocarp. A total of 37 compounds were tentatively identified in both extracts, comprising primarily lanostane-type tetracyclic triterpenes (**Table 8, Figure 23**). Among the triterpenes, 16 lucidenic and 9 ganoderic acids were found. Peak 23 (lucidenic acid A, ganoderlactone B and ganolucidic acid) and peak 17 (lucidenic acid P and 7,15,?-trihydroxy-4,4,14-trimethyl-3,11-dioxochol-8-en-24-oic acid) among the major compounds in the inner flesh and outer skin. It is important to note that some of these peaks represent co-elution of more than one compound.

As already mentioned, several works can be found in the literature reporting the triterpene identification and quantification in different parts of *G. lucidum*'s, and all the identified compounds were already described (Biswal et al., 2022). For instance, Oludemi et al. (2017) identified 34 compounds in whole fruiting body of *G. lucidum*. Three of them were phenolics and 31 were triterpenes. In all extraction techniques evaluated by those authors, ganoderic acid A was the most abundant triterpenes present (Oludemi et al., 2017). Interestingly, both compounds were not found in the samples of the present work. Nonetheless, to the best of our knowledge, there is no study investigating the triterpene composition in different parts of *G. lucidum* sporocarp.

Whereas the overall chemical profile (qualitative terms) was consistent across the sporocarp parts, the proportion among the individual compounds and total triterpenic content varied considerably. The total triterpene content in the UAE extract was higher for the external than to the internal sporocarp parts ($p<0.05$, 94.47±0.59 μg/mg in contrast to 19.99±0.46 μg/mg freeze-dried extract, respectively).

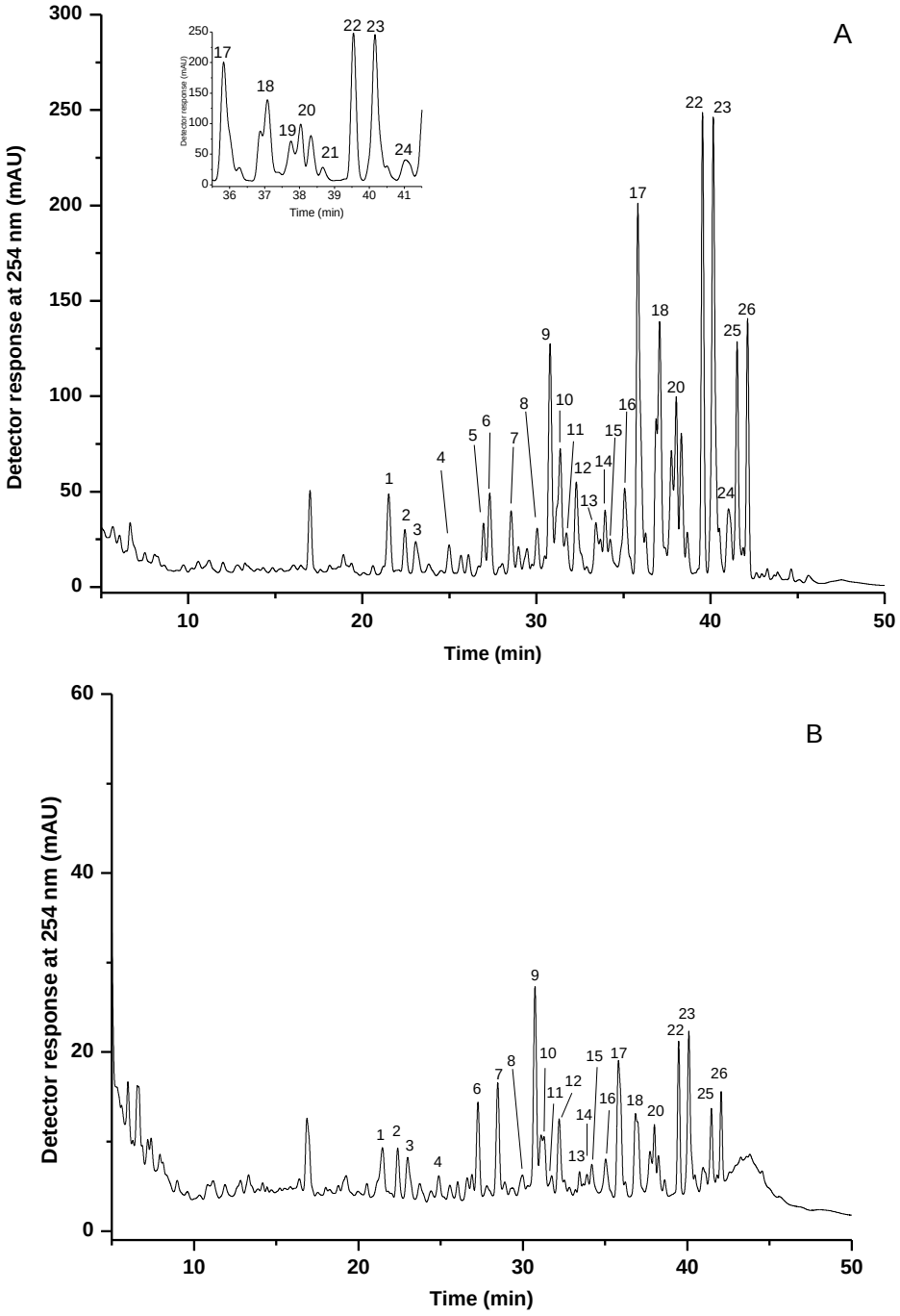


Figure 23. Representative chromatograms (254 nm) of phenolic and triterpenoid compounds from *G. lucidum* sporocarp extracts obtained by UAE. **A.** Outer skin extract; **B:** Inner flesh extract. Inset: chromatogram zoom ranging from 36.0 to 41.0 min.

Table 8. Chromatographic and spectroscopic data of compounds in the UAE extracts of sporocarp parts from *G. lucidum*

Peak [a]	Rt (min) [b]	λ max nm) [c]	Tentative identification	$[M-H]^-$ / $2[M-H]^-$ *(m/z)*	MS^2 (*m/z*)	Quantitation (μg/mg freeze-dried extract)	
						Outer	Inner
1	21.37	228, 256, 368	Lucidenic acid J	489/979	[979]: 489 [489]: 471, 459, 441, 409, 355, 343, 313, 277, 219	2.39±0.01	0.62±0.03
2	22.36	258	Not identified	473	[473]: 443, 411, 396, 371, 339, 249 [443]: 371, 339, 249	1.06±0.01	0.54±0.01
3	23.00	264	Lucidenic acid G	475	[475]: 457, 439, 303, 301	1.05±0.03	0.61±0.02
4	24.83	257	Lucidenic acid I	473	[473]: 455, 411, 381, 315, 302, 301, 299, 247	0.79±0.005	0.39±0.02
5	26.89	257	Lucidenic acid derivative	473/947	[947]: 473 [473]: 455, 443, 425, 407, 399, 381, 369, 363, 355, 320, 287, 271, 155	1.17±0.03	0.24±0.02
6	27.31	258	Lucidenic acid N	459	[459]: 441, 423, 415, 397, 371, 369, 351, 303, 301, 287, 285, 249, 207	2.06±0.01	1.03±0.04
7a	28.44	261	Lucidenic acid B	473	[473]: 455, 437, 425, 301	1.76±0.12	1.25±0.02
7b			3β,7β,15α,24-Tetrahydroxy-11,23-dioxo-5α-lanosta-8,20 E-dien-26-oic acid	531	[487]: 469, 457, 301		
8	30.03	257	Ganoderic acid C2	517/1035	[1035]: 517 [517]: 517, 499, 473, 455, 361, 303, 287, 251	1.09±0.03	0.25±0.01
9	30.33	256	Iso-ganoderic acid G	531	[531]: 513, 498, 469, 303	6.16±0.09	2.31±0.08
10	30.78	258	Dehydrolucidenic acid N	457	[457]: 457, 442, 427, 413, 397, 385, 353, 303, 249, 207, 163, 149, 109, 59	3.86±0.24	1.04±0.05
11a	31.4	257	20-Hydroxylucidenic acid F	471	[471]: 471, 441, 427, 411, 409, 383, 365, 355, 330, 317, 302, 301, 285, 193	0.91±0.013	0.17±0.01

Peak [a]	Rt (min) [b]	λ max nm) [c]	Tentative identification	[M-H]$^-$ / 2[M-H]$^-$ *(m/z)*	MS^2 (*m/z*)	Quantitation (μg/mg freeze-dried extract)	
						Outer	Inner
11b			Not identified	531	[515]: 477, 469, 454, 439, 407, 363		
12a	32.29	256	Lucidenic acid K	461	[471]: 453, 441, 301	2.41±0.04	0.85±0.02
12b			Lucidenic acid M	471	[461]: 303, 301, 287		
13	33.41	256	Ganoderic acid B	515/1031	[1031]: 515 [515]: 497, 471, 441, 426, 401, 383, 359, 341, 303, 289, 285, 249	1.08±0.04	0.18±0.01
14	33.67	257	Ganoderenic acid B	513	[513]: 513, 497, 469, 439, 400, 385, 357, 301, 303, 275, 249	1.48±0.04	0.2±0.01
15	34.23	259	Ganoderic acid AM1	513	[513]: 495, 469, 453, 439	0.77±0.03	0.28±0.02
16a	35.07	256	3,12,20-Trihydroxy-7,11,15-tricarbonyl-cholestane-8,16, 24-triene-26-acid	527	[527]: 497, 485, 467, 455, 437, 393, 300, 299	2.94±0.22	0.48±0.01
16b		258	Elfvingic acid derivative	457	[457]: 427, 413, 397, 385, 353, 249		
16c		253	Ganoderic acid δ	515/1031	[1031]: 515 [515]: 515, 497, 471, 453, 435, 359, 341, 301, 285, 261, 249		
17a	35.85	257, 261	Lucidenic acid P	517	[517]: 499, 475, 457, 439, 427, 303, 289, 287, 249	11.14±0.01	2±0.03
17b			7,15,?-Trihydroxy-4,4,14-trimethyl-3,11-dioxochol-8-en-24-oic acid	475	[457]: 439, 413, 395, 369, 301, 285, 249, 155		
18a	36.90	259	Lucidenic acid E	515/1031	[1031]: 515 [515]: 515, 497, 473, 455, 443, 411, 393, 351, 330, 303, 289, 249	8.24±0.39	1.35±0.03
18b			Elfvingic acid A	527	[527]: 509, 497, 483, 465, 453, 423, 341		
19	37.52	256	Lucidenic acid F	455	[455]: 395, 383, 301, 283, 285, 247, 163, 149	2.6±0.242	0.42±0.01

Peak [a]	Rt (min) [b]	λ max nm) [c]	Tentative identification	$[M-H]^-$ / $2[M-H]^-$ *(m/z)*	MS^2 (*m/z*)	Quantitation (μg/mg freeze-dried extract)	
						Outer	Inner
20a	37.75	256	7,15-Dihydroxy-4,4,14-trimethyl-3,11-dioxo-chol-8-en–24-oic acid	459/919	[919]: 459 [459]: 459, 441, 423, 397, 301, 300, 299, 285, 155	6.42±0.254	0.85±0.02
20b			12-Hydroxyganoderic acid D	529	[529]: 511, 481, 467		
21a	38.32	258	Lucidenic acid D	513/1027	[1027]: 513 [513]: 471, 453, 441, 427, 409, 397, 367, 355, 330, 317, 302, 149	0.7±0.046	0.12±0.01
21b			Lucidenic acid B	473/947	[947]: 473 [473]: 455, 437, 425, 303, 301, 285 [455]: 439, 425, 407, 301, 283		
21c			Ganoderic acid G	531	[513]: 469, 439, 357, 301, 247		
21d			3-*O*-acetyl-ganoderic acid H	613	[613]: 595, 571, 553, 529, 511		
22a	39.53	256	Ganoderic acid D	513	[513]: 513; 495, 477, 471, 451, 436, 423, 409, 383, 351, 301, 287, 247, 149	9.69±0.06	1.28±0.02
22b			Lucidenic acid F	455/911	[911]: 455 [455]: 425, 411, 395, 383, 351, 335, 247, 149		
23a	40.14	259	Lucidenic acid A	457/915	[915]: 457 [457]: 457, 439, 421, 395, 383, 301, 287, 261, 247, 209, 149	12.71±0.12	1.76±0.04
23b			Ganoderlactone B	453/907	[907]: 453, 409 [453]: 438, 423, 409, 381, 379, 367, 337, 301, 299, 272, 257, 245		
23c			Ganolucidic acid D	499/999	[999]: 499 [499]: 499, 457, 439, 421, 413, 395, 379, 365, 351, 301, 283, 285, 257, 155		
24	40.50	253	12-Acetoxy-ganoderic acid F	569/1139	[1139]: 569	1.94±0.12	0.34±0.01

Peak [a]	Rt (min) [b]	λ max nm) [c]	Tentative identification	[M-H]⁻ / 2[M-H]⁻ *(m/z)*	MS^2 (*m/z*)	Quantitation (μg/mg freeze-dried extract)	
						Outer	**Inner**
					[569]: 539, 527, 509, 497, 483, 465, 453, 341		
25	41.03	253, 256	12-Acetoxyl-4,4,14-trimethyl-3,7,11,15-tetraoxochol-8-en-24-oic acid	513/1027	[1027]: 513 [513]: 471, 301, 299	4.98±0.14	0.7±0.02
26	41.52	256	Lucidenic acid D2	513/1027	[1027]: 513 [513]: 471, 453, 441, 328, 301, 300, 195	5.06±0.08	0.71±0.02
					Total triterpenes	94.47±0.59[a]	19.99±0.46[b]

[a]Peaks numbered according to the chromatogram shown in Figure 19. More than one row per peak indicates coelution. [b]Retention time on C_{18} column. [c]Gradient of formic acid (0.1%) and acetonitrile. Data are mean ± standard deviation (n=3). ni: not identified. FA: formic acid adduct. Different superscript letters in the same row indicate significant differences ($p < 0.05$, t test).

5.4 BIOACCESSIBILITY OF TRITERPENES FROM *G. LUCIDUM* SPOROCARP PARTS

The inner and outer parts of *G. lucidum* sporocarp were subjected to simulated digestion to assess the compounds that were liberated and transferred from sample matrices to the aqueous intestinal media during digestion (bioaccessible fraction, BF), which would be accessible to intestinal absorption after oral consumption. On average, 52±3% of the total triterpenes were bioaccessible after the IVD of the outer part, which exceeded ($p<0.05$) the average triterpene bioaccessibility of 39±2% of its inner counterpart. In absolute values, 3.09±0.13 mg triterpenes/g sample (fw) and 0.62±0.03 mg triterpenes/g sample (fw) were bioaccessible after digestion of GO and GI, respectively.

Figure 24A and **24B** shows the comparison of the chromatographic profile of compounds in the starting material (before digestion, in black line) with the profile of compounds in the BF (coloured line) for the respective outer (GO) and inner (GI) sporocarp parts of *G. lucidum*. For either sample, it can be noticed that all the compounds in the starting material were solubilized in the bioaccessible fraction to some extent. The relative bioaccessibility of individual triterpenes in *G. lucidum*'s sporocarp parts is shown in **Figure 25**.

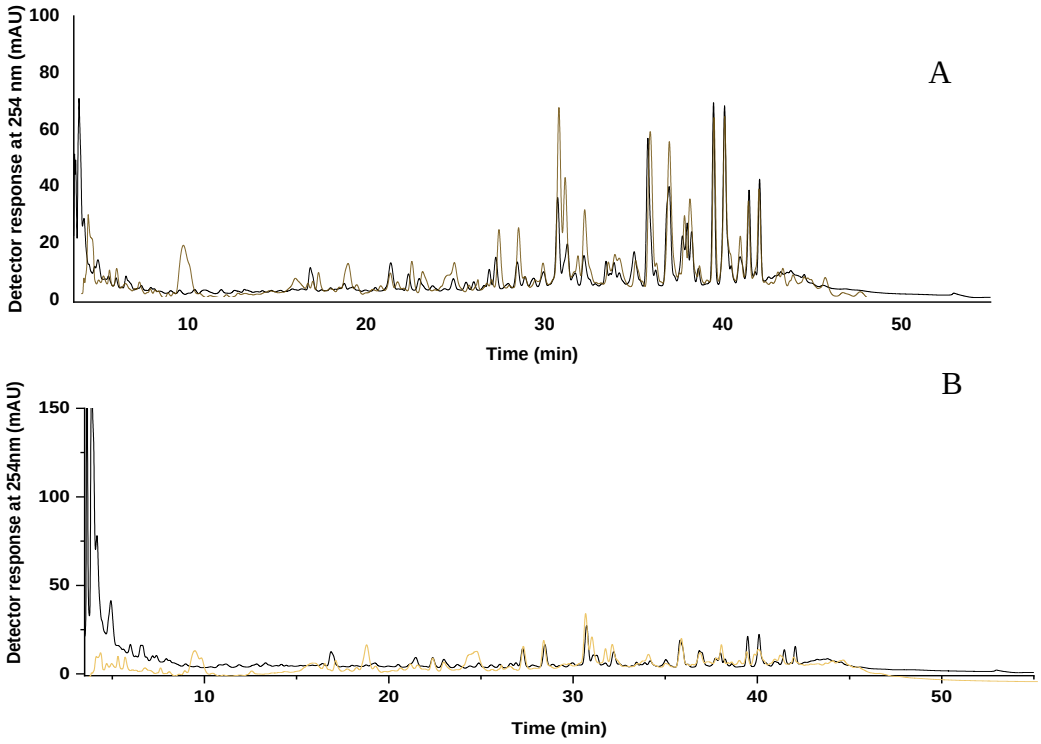


Figure 24. Superposed chromatograms, obtained by HPLC-DAD, of starting material (black line) and bioaccessible fraction (coloured line) after *in vitro* digestion of **A.** outer sporocarp part and **B.** inner sporocarp part.

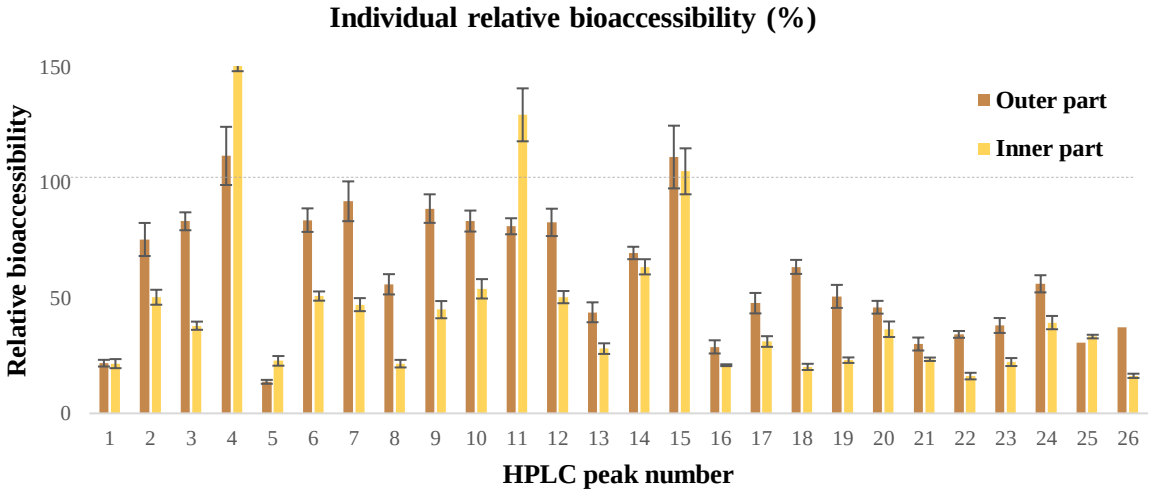


Figure 25. Individual bioaccessibility percentages of triterpenes after *in vitro* digestion of *G. lucidum*'s sporocarp parts. Peaks are numbered according to Table 8.

Regardless the sample, the most bioaccessible compounds in relative terms included the lucidenic acid I (peak 4), and ganoderic acid AM1 (peak 15) (ranging from 111% to 163%, and 111% to 105%, for GO and GI, respectively), whereas the 20-hydroxylucidenic acid F (peak 11) was also among the most bioaccessible compounds in GI (129%, **Figure 25**). These compounds exhibited bioaccessibility values exceeding 100%, which suggests that they were likely products of transformation from related or conjugated compounds during digestion. This transformation could occur through processes such as oxidation, isomerisation or hydrolysis. Moreover, these high values can also be a result of the improved release, or extraction of these compounds from the matrix during digestion, due to the mechanical, chemical and/or enzymatic processes, and do not reflect *de novo* synthesis.

In the external sporocarp part, it is also noteworthy the high bioaccessibility of dehydrolucidenic acid N (89%, **Figure 25**), which became the most intense peak in the chromatogram (**Figure 24**), i.e, and the most concentrated compound in the bioaccessible fraction of GO (0.34±0.02mg/g sample fw, **Table 9**). This compound was already the predominant triterpene in the starting material of the inner sporocarp part (0.19±0.009 mg/g fw), position that was maintained in this sample's bioaccessible fraction (0.08±0.007 mg/g fw).

Indeed, in addition to relative bioaccessibility (expressed as a percentage of the initial compound concentration available after digestion), absolute bioaccessibility—the actual concentration of a bioaccessible compound in the sample (mg/g) - is a critical metric. This distinction also highlights how compounds with apparently low to intermediate relative

bioaccessibility may still contribute significantly to the bioaccessible fraction due to their initial high concentrations. For example, peak 23 (with coelution of lucidenic acid A, ganoderlactone B, ganolucidic acid D) presented a relative bioaccessibility of only 38% for the outer part (**Figure 25**), which might seem low. However, due to its high initial concentration in the sample (0.79 ± 0.01 mg/g in the starting material) (**Table 9**), the absolute amount of almost 52% in the bioaccessible fraction remained significant at 0.30±0.02mg/g sample (fw) (**Table 9**). Conversely, compounds with higher relative bioaccessibility may have low absolute bioaccessibility due to their lower initial concentrations. A clear example of this is peak 11 (20-hydroxylucidenic acid F), which, despite having a bioaccessibility of 81% from the outer part (**Figure 25**), reached an absolute concentration of just 0.05±0.005 mg/g in the BF (**Table 9**).

Table 9. Initial concentration of triterpenes in sporocarp parts and their absolute bioaccessibility

Peak	Triterpenes (mg/g sample fw)			
	Outer part		Inner part	
	SM	BF	SM	BF
1	0.150±0.004	0.030±0.002	0.050±0.003	0.010±0.001
2	0.070±0.002	0.050±0.002	0.040±0.002	0.020±0.001
3	0.070±0.004	0.050±0.003	0.050±0.003	0.020±0.001
4	0.050±0.002	0.050±0.004	0.030±0.002	0.050±0.005
5	0.070±0.003	0.010±0.001	0.020±0.002	0.004±0.000
6	0.130±0.003	0.110±0.006	0.080±0.004	0.040±0.002
7	0.110±0.006	0.100±0.005	0.100±0.004	0.050±0.003
8	0.070±0.003	0.038±0.003	0.020±0.001	0.004±0.000
9	0.380±0.005	0.340±0.023	0.190±0.010	0.080±0.007
10	0.240±0.013	0.200±0.018	0.080±0.005	0.050±0.004
11	0.060±0.001	0.050±0.005	0.010±0.001	0.020±0.002
12	0.150±0.006	0.120±0.010	0.070±0.003	0.030±0.002
13	0.070±0.003	0.030±0.003	0.014±0.001	0.004±0.000
14	0.090±0.005	0.060±0.006	0.020±0.001	0.010±0.001
15	0.050±0.003	0.050±0.002	0.020±0.002	0.020±0.002
16	0.180±0.014	0.050±0.005	0.040±0.001	0.010±0.000
17	0.700±0.020	0.330±0.029	0.160±0.005	0.050±0.004
18	0.510±0.013	0.330±0.011	0.110±0.004	0.020±0.002
19	0.160±0.016	0.080±0.008	0.030±0.002	0.010±0.000
20	0.400±0.015	0.180±0.004	0.070±0.003	0.020±0.002
21	0.040±0.003	0.013±0.001	0.010±0.001	0.002±0.000
22	0.600±0.014	0.210±0.011	0.100±0.004	0.020±0.001
23	0.790±0.015	0.300±0.025	0.140±0.006	0.030±0.003
24	0.120±0.009	0.070±0.003	0.030±0.002	0.010±0.001
25	0.310±0.018	0.090±0.005	0.060±0.003	0.020±0.000

26	0.320±0.014	0.120±0.005	0.060±0.003	0.010±0.001
Total triterpenes	5.900±0.130^{A}	3.090±0.130^{a}	1.600±0.098^{B}	0.620±0.030^{b}

Data are mean ± SD (n=3), expressed as mg/g of sample fw. SM: Starting Material. BF: Bioaccessible Fraction. Different uppercase letters indicate difference (Student's *t* test, $p<0.05$) of the triterpene content between the starting materials of inner and outer sporocarp parts, whereas lowercase letters indicate difference between the bioaccessible fraction's triterpenes of inner and outer parts.

Very limited studies can be found in the literature regarding the *in vitro* bioaccessibility of triterpenes from *G. lucidum* or related products, all published within the past three years. Cai et al. (2021) subjected *G. lucidum* spore powders in different forms (with unbroken, broken or removed sporoderm) to *in vitro* digestion, and reported only the absolute bioaccessibilities of total triterpenes, with values of 4.6±0.3, 5.8±0.3, and 8.3±0.4 mg/g for the respective samples. Nonetheless, the paper provides an approximated triterpene concentration in the initial solid residue in a graphic, and although it is not clear whether this represents the starting material concentration, by calculation it could represent relative triterpene bioaccessibilities of about 90% from unbroken spores and about 35% from the samples with no or disrupted sporoderm. This study concluded that the disruption or removal of spore walls can facilitate the liberation of bioactive compounds, but whereas the absolute bioaccessibility values of triterpenes in these samples are higher, the percentages in relation to the starting material can be relatively low. Moreover, authors reported triterpene concentrations in the solid digestive residues (colonic material) ranging from 9.2±0.8, to 32.5±1.1 mg/g, being higher than these compound's concentrations in the aqueous intestinal phase (bioaccessible fraction) after digestion, and suggested that triterpenes may remain mostly attached to the solids during digestion with only a small fraction being dissolved in the supernatant. To the best of our knowledge, this is the only study of IVD that employed HPLC-DAD to analyse triterpenes. Chromatograms of showing the triterpene profile of the starting material and bioaccessible fraction of one of the samples are presented, but only two compounds are identified, and no information on individual compound quantitation can be found, being the total triterpene content measured spectrophotometrically.

Qi et al. (2024) also studied the triterpene bioaccessibility from *G. lucidum*'s spore powder subjected to different processes of wall disruption. The average triterpene bioaccessibility from unbroken spore powder was 5.37%, while 24.97% and 32.90% of compounds were bioaccessible from spores subjected to mechanical milling and ultra-fine grinding at low temperature. These authors have also suggested that triterpenes can remain attached to solids during digestion, with only a part of them being transferred to the aqueous gastrointestinal fluid. In addition, they associated the relatively low bioaccessibility of these compounds to their poor solubility and

chemical stability (Ho et al., 2016). Triterpenes present intermediate polarity, and in the present work, as in many reports from the literature, we have shown the high recovery of these compounds using hydroethanolic solvent. Because of that intermediate polarity of triterpenic compounds from *G. lucidum*'s, we have assessed the bioaccessibility as the fraction of compounds solubilized in the aqueous phase, and have not evaluated the micellar fraction, i.e., the fraction of bioaccessible lipophilic compounds dispersed within bile-salt mixed micelles.

In the same year, Li et al. (2023) studied not the mushroom itself, but *G. lucidum* fermented whole wheat, assessing the effect of different drying methods on the content and bioaccessibility of bioactive components of this product. These authors reported high values of total triterpene bioaccessibility, ranging from 184.02% to 228.92% for the different dried fermented wheat samples. No information regarding the behaviour of individual compounds during digestion could be found to compare with the data obtained in the present study as the total triterpene content was measured using a spectrophotometric assay.

As far as we are concerned, no studies have been directed to the estimation of triterpene bioaccessibility from *G. lucidum*'s sporocarps, even as a whole or in separate parts. The bioaccessibility and bioavailability of dietary compounds is influenced by numerous factors related to the compound itself, to the edible matrix where it is contained, and to the individuals. *G. lucidum* spores have not only a different amount of triterpenes, which is one of the factors affecting the compound bioaccessibility, but also a distinct matrix composition and chemical and physical barriers to the compound liberation than the sporocarps. The same analogy is valid for the *G. lucidum*'s sporocarp parts, which were shown in the present work to present different content of triterpenes. Despite the similar content of macronutrients and minerals, the inner and outer portions of *G. lucidum* sporocarps can present different components within these classes, for instance, distinct ratio of fibre to digestible polysaccharides and so on, and different presence of non-nutritional compounds, as also evidenced in this study. Additionally, they have different morphological aspect, suggesting different chemical and physical interactions within the matrix. These factors are related to the differential average bioaccessibility of triterpenes from the outer part of the sporocarp in relation to its inner counterpart.

Moreover, this is the first study to present the individual bioaccessibility of the compounds, separated, identified and quantified by HPLC-DAD-(ESI-)HRMS/MS. Whereas the total content and total relative bioaccessibility of triterpenes were different among these two samples ($p<0.05$), it can be noticed that, overall, specific compounds for either sample presented the highest

bioaccessibility values, indicating that they can be differentially absorbed and associated to benefits observed after the consumption of *G. lucidum* sporocarps or their products.

5.5 PREBIOTIC ACTIVITY

Figure 26 shows the performance of non-bioaccessible residues in the duodenal phase, thus representing the colonic material obtained after *in vitro* digestion of the external (CMGO) and internal (CMGI) parts of *G. lucidum* sporocarp. The optical density at 620 nm was monitored over a 48-hour period at one-hour intervals. In all graphs, CMGO and CMGI proved more effective in promoting the growth of colonic bacteria used in this experiment compared to fructooligosaccharides (FOS), while demonstrating similar efficacy to inulin, which served as the primary positive controls. In **Figures 26A**, **26B**, and **26C**, CMGO and CMGI displayed a lower performance than the positive control consisting of MRS medium with glucose; however, in Figure **26D**, CMGO and CMGI matched the performance of the medium supplemented with glucose. The CMGO and CMGI made the bacteria have exponential growth and reach the peak in about 15 hours, and then the growth stabilized until the 48 hours.

These findings suggest that CMGO and CMGI possess significant prebiotic activity for the *Lactobacillus* genus and excellent prebiotic activity for the *Bifidobacterium* genus. The results align with studies reporting the prebiotic activity not only of the fruiting body of *G. lucidum* but also of its spores and mycelia, likely associated with the substantial carbohydrate content in these structures. Research on broken and unbroken spores has shown that their oligosaccharides, composed of arabinose, mannose, glucose, and galactose, exert prebiotic effects on the Lactobacillaceae and Bifidobacteriaceae families, and can withstand acidic environments (Yang et al., 2022).

Polysaccharides from the *G. lucidum* fruiting body have been shown to play a crucial role in modulating human faecal microbiota composition and function as prebiotics, particularly targeting species such as *Bacteroides ovatus* and *Bacteroides uniformis* (Yang et al., 2022). Xia et al. (2022) also reported that oligosaccharides from *G. lucidum*'s fruiting body had a beneficial effect on gut microbiota regulation, as observed in simulated fermentation tests through changes in gas production, short-chain fatty acid levels, pH, and bacterial structure. They found an increased relative abundance of beneficial bacteria, including *Lactobacillus*, *Bifidobacterium*, *Faecalibacterium*, and *Prevotella*, along with a significant decrease in pathogenic genera such as *Escherichia*, *Shigella*, and *Dorea* (Xia et al., 2022).

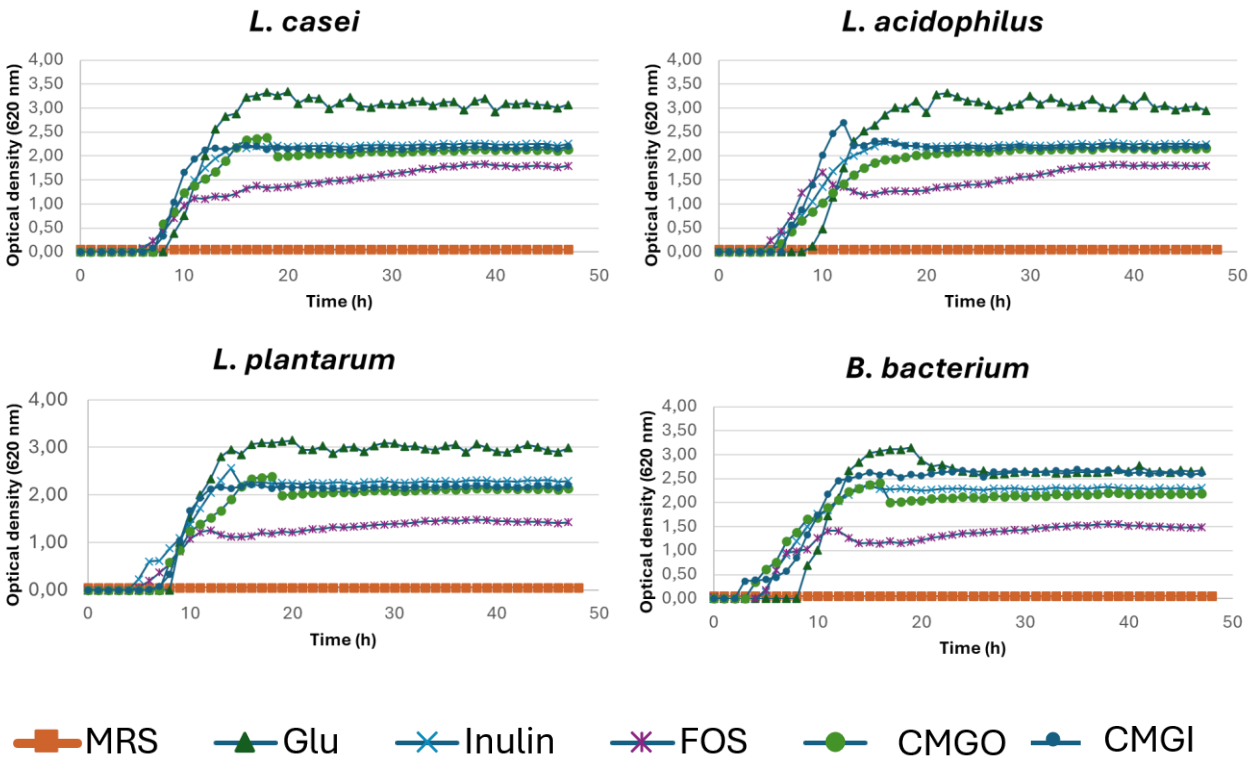


Figure 26. Prebiotic activity of colonic material obtained after IVD of outer and inner parts of *G. lucidum* sporocarps for *Lactobacillus casei* (A), *Lactobacillus acidophilus* (B), *Lactobacillus plantarum* (C) and *Bifidobacterium bacterium* (D). MRS (Man-Rogosa-Sharpe), Glu (Man-Rogosa-Sharpe broth with glucose 2%), FOS (fructooligosaccharides), CMGO (colonic material of outer part) and CMGI (colonic material of inner part).

Additionally, polysaccharides from the fruiting bodies and mycelia of *G. lucidum* and *Poria cocos*, administered at a dose of 750 mg/kg/day for 15 days in six-week-old C57BL/6J mice, were assessed for intestinal microbiota modulation using Enterobacterial Repetitive Intergenic Consensus (EPIC-PCR) and 16S amplicon sequencing. The results indicated a prebiotic effect via modulation of the intestinal microbiota composition. Treated mice showed reduced levels of potentially pathogenic bacteria, increased populations of beneficial bacteria associated with obesity control, enhanced short-chain fatty acid production, xylan and other polysaccharide degradation, and lactic acid production (Khan et al., 2018).

5.6 Determination of Beta-Glucan

The inner sporocarp part showed higher β-glucan content (15.43% w/w) in contrast to the outer skin (9.77% w/w), but both have considerable β-glucan content for cultivated mushroom. These finds and the content of carbohydrates could explain the superior prebiotic activity, evidenced by rapid bacterial growth and higher optical density across all tested strains, suggesting

an enhanced fermentable substrate availability despite the fact of outer skin of *G. lucidum* sporocarp exhibited lower prebiotic potential, particularly for *B. animalis*, comparing with the inner sporocarp.

These findings align with prior research that reported about the relatively lower β-glucan content for cultivated mushroom comparing with wild mushroom. 13.5g/100g dry mass of β-glucan was reported to *Agaricus bisporus* (portobello variety) comparing with 40.9g/100g dry mass of β-glucan in *Tricholomopsis rutilans* (Mirończuk-Chodakowska et al., 2017).

Table 10. Glucans in the colonic solid residue from digested *G. lucidum*'s sporocarp parts

Sample	Analyte	Mushroom β-Glucan (% w/w) "as is"
CMGI	**Total Glucan**	15.5094
	α-Glucan	0.0765
	β-Glucan	15.4330
CMGO	**Total Glucan**	9.8626
	α-Glucan	0.0904
	β-Glucan	9.7722

Data expressed as weight percentage (%, w/w). CMGO: colonic material obtained after IVD of the outer part of *G. lucidum* sporocarp. CMGI: colonic material obtained after IVD of the inner part of *G. lucidum* sporocarp.

6. CONCLUSION

In conclusion, the findings indicated that there is little variation between the inner and outer parts of *G. lucidum* in terms of total macronutrient and mineral composition, and that both are rich in carbohydrates (about 80% of total nutrients). The results align well with previously reported data for whole fruiting bodies, and at a first moment may suggest that further separation of sporocarp parts may not provide substantial advantages depending on the application. On the other hand, specific fractions of compounds within a macronutrient class may vary, as showed for the higher content of β-glucans in the inner part of the sporocarp (15% *vs.* 9%). However, both the external and internal parts presented a considerable amount of β-glucans for cultivated mushrooms.

UAE had the best performance on the triterpene's extraction comparing with MAC and SOX, and was the method selected for the characterization of triterpenes from the inner and outer parts also needed for the study of bioaccessibility. Through a comprehensive analysis of the chemical composition of the internal and external amounts of triterpenoids, although the triterpene content of the external skin is about 2 or 3 times that found in the inner flesh. The outer part also provided a higher bioaccessibility of these compounds when compared with the inner part. On the other hand, the colonic residue obtained after the digestion of inner sporocarp part provided the most efficient prebiotic activity, whereas both samples supported bacterial growth across all tested strains, demonstrating prebiotic activity equivalent or superior to inulin and fructooligosaccharides used as positive controls. The inner flesh residue supported faster bacterial growth, particularly in *Lactobacillus* species. The higher β-glucan content in the inner flesh (15.43% w/w) compared to the outer skin (9.77% w/w) likely contributed to this enhanced prebiotic effect.

This is the first time a study has examined the variations of components and their digestive behavior between different parts of *G. lucidum* sporocarps. These results demonstrate that both the external and internal parts of the fruiting body of *G. lucidum* have beneficial properties, opening the way for studies that can be directed towards increasing productivity and efficiency in the production of supplements with the fruiting body of the mushroom. Understanding the differential composition and bioaccessibility of compounds from various parts of *G. lucidum* sporocarp can inform better utilisation strategies in functional food and nutraceutical applications, enhancing their potential health benefits.

7. FUTURE PERSPERCTIVES

- To evaluate the dietary fibre content (soluble and insoluble fibre, and β-glucans) in both *G. lucidum*'s sporocarp parts;
- To assess the antitumoral, anti-inflammatory and antioxidant activities of the compounds in the bioaccessible fraction of sporocarp parts;
- To estimate the intestinal absorption of bioaccessible compounds from *G. lucidum*'s sporocarp using a cellular model.

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PUBLICATIONS

Works presented in Conferences

1. Regina Soares, Daniele Bobrowski Rodrigues, Lavínia Veríssimo, Tiane Finimundy, **Neivaldo J. Murrube**, Isabel P. Fernandes, Miguel D. Gonçalves, Lillian Barros, Sandrina A. Heleno. Effect of chestnut flower extract on the flavour stability of craft beers. 2023. *International Seminar "ArtiSaneFood: Bio-preservation and Risk Modelling Approaches"*, Portugal.
2. Daniele Bobrowski Rodrigues, **Neivaldo J. Murrube**, Rossana Cardoso, Peter Petros, Eric Puro, Lillian Barros. The influence of tea preparation on *Ganoderma lucidum'*s triterpene bioaccessibility. 2024. *8th International Conference in Food Digestion*, Portugal.
3. Daniele Bobrowski Rodrigues, **Neivaldo J. Murrube**, Rossana Cardoso, Peter Petros, Eric Puro, Lillian Barros. Triterpenic and phenolic composition of *Ganoderma lucidum* extracts as affected by the sporocarp part and extraction technique. 2024. *12th International Medicinal Mushrooms Conference (IMMC12)*, Italy.
4. Daniele Bobrowski Rodrigues, **Neivaldo J. Murrube**, Rossana Cardoso, Peter Petros, Eric Puro, Lillian Barros. Higher bioaccessibility of triterpenes in *G. lucidum* sporocarp external skin in contrast to the inner flesh highlight it as a valuable source for the intake of bioactive compounds. 2024. *12th International Medicinal Mushrooms Conference (IMMC12)*, Italy.