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Exploring the Bioactive Potential of Mushroom Aqueous Extracts: Antimicrobial, Antioxidant, and Prebiotic Properties

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Abstract: Mushrooms provide essential nutrients and bioactive compounds, namely glucans, protein, and phenolic compounds. In this study, two aqueous extractions were performed (M1 and M2) using *Pleurotus ostreatus*, *Pleurotus eryngii*, and *Agrocybe cylindracea*. In M1, a hot extraction (extract M1) (90 °C, 700 rpm, 1 h) was performed. In M2, a room-temperature extraction (extract M2A) followed by a hot extraction (extract M2B) (90 °C, 700 rpm, 1 h) of the extract M2A residue was performed. The M2B extracts showed the lowest extraction yields (12.58–21.78%), while the other yields ranged between 30.91% and 46.03%. All extracts had high protein (12.09–32.97 g/100 g of dry extract), glucan (12.69–48.57 g/100 g of dry extract), and phenolic contents (7.90–16.65 mg GAEs/g of dry extract) and high antioxidant (ABTS and ORAC assays), antimicrobial, antibiofilm, and prebiotic activities. So, they have potential to be used as functional ingredients or natural preservatives. Extracts from *A. cylindracea* stood out since they had higher protein content, antioxidant activity, and prebiotic activity (extract M1) and inhibited a higher number of foodborne bacteria (only extract M2A). However, unlike extracts from *P. ostreatus* and *P. eryngii*, at 40 mg/mL, they had cytotoxic effects.

Keywords: mushrooms; aqueous extracts; protein; glucans; phenolic compounds; antioxidant activity; antimicrobial activity; prebiotic activity

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1. Introduction

Mushrooms are an important source of bioactive compounds, namely phenolic compounds, polyketides, terpenes, steroids, and polysaccharides [1]. In recent decades, water-soluble polysaccharides from mushrooms have received increasing attention from the scientific community and pharmaceutical industry. According to previous studies, they have several therapeutic and prophylactic benefits such as immunomodulatory, anti-inflammatory, anti-tumoral, hypoglycemic, hypolipidemic, prebiotic, antioxidant, and antimicrobial properties [2–8].

In recent years, consumer demand for minimally processed food products and clean labels has increased significantly [2]. This new trend in the food market has created new challenges for the food industry, particularly in replacing synthetic food preservatives with natural bioactive compounds. One of the main challenges of the food industry within

food preservation is avoiding the growth of spoilage microflora and pathogens [9]. Some of the more naturally common antimicrobials used as food preservatives are essential oils, plant extracts, organic acids, and chitosan [10]. As mentioned before, water-soluble polysaccharides from mushrooms may have antimicrobial activity and other bioactive properties with potential in food industry applications [2]. However, until now, these applications have been limited.

Water-soluble polysaccharides are obtained from mushrooms through a hot aqueous extraction preceded and followed by purification steps, in which protein and low-molecular components, such as mono- and disaccharides, oligosaccharides, amino acids, lipids, and phenolic compounds, are eliminated [3–5,11–13]. Many of the eliminated compounds have bioactive properties and nutritional value. For instance, phenolic compounds are powerful antioxidants, and the biological value of protein from mushrooms is greater than the biological value of other non-animal source proteins [14]. Retaining these compounds during extraction could significantly enhance the functional and nutritional potential of mushroom-derived ingredients.

To achieve this, the extraction methodologies applied in this study did not include purification steps (e.g., after aqueous extraction, the precipitation of soluble polysaccharides with ethanol was performed), which differentiate them from most procedures used by other research works that extract soluble polysaccharides from mushrooms [11–13,15,16]. Besides enabling the extraction of other essential nutrients and bioactive compounds together with polysaccharides, the absence of purification steps makes these methodologies simpler, faster, cheaper, and more environmentally sustainable, factors that could facilitate their scale-up in the food industry.

In this context, the aim of this study was to develop aqueous extracts from three mushrooms species—*Pleurotus ostreatus*, *Pleurotus eryngii*, and *Agrocybe cylindracea*—using an integrated approach to extract water-soluble polysaccharides and other compounds with potential in the food industry. Furthermore, the extracts' chemical composition (protein, glucans, and total phenolic compounds) and their antimicrobial (minimum inhibitory concentration), antibiofilm, prebiotic, and antioxidant activities, namely regarding ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)) and ORAC (oxygen radical absorbance capacity), as well as their toxicity (using Presto Blue and Ames), were evaluated.

2. Materials and Methods

2.1. Mushroom Samples

The BLC3 Association (Oliveria do Hospital, Portugal) supplied frozen *Pleurotus ostreatus* and *Pleurotus eryngii* from the Pleutrotaceae family and *Agrocybe cylindracea* from the Tubariaceae family. The samples were stored in a freezer at −18 °C until they were analyzed.

2.2. Extraction Methods

Two different aqueous extraction methods (M1 and M2) were developed according to Figure 1. In M1, frozen mushrooms were crushed together with deionized water (1 g of dry mushroom; 20 mL of H_2O) using a food crushing machine. Afterwards, a hot extraction (90 °C, 500 rpm, 1 h) was performed on a heating plate. The extract (M1) was then separated from the insoluble residue through centrifugation (23 °C, 5000 rpm, 20 min).

In M2, a room-temperature extraction (extract M2A) was followed by a hot extraction (extract M2B) of the extract M2A residue. Firstly, frozen mushrooms were crushed together with deionized water (1 g of dry mushroom; 20 mL of H_2O) using a food crushing machine. Then, the crushed bulk was centrifuged to separate the insoluble residue of extract M2A. Finally, deionized water (equal in volume to extract M2A) was added to the insoluble residue, and it was hot-extracted (90 °C, 500 rpm, 1 h) on a heating plate. The extract (M2B)

was separated from the insoluble residue through centrifugation (23 °C, 5000 rpm, 20 min). All extractions were performed in triplicate. The resulting extracts were lyophilized, and the extraction yield was calculated according to the following equation:

$$\frac{(\text{dry weight of the extract})}{(\text{dry weight of the mushroom})} \times 100$$

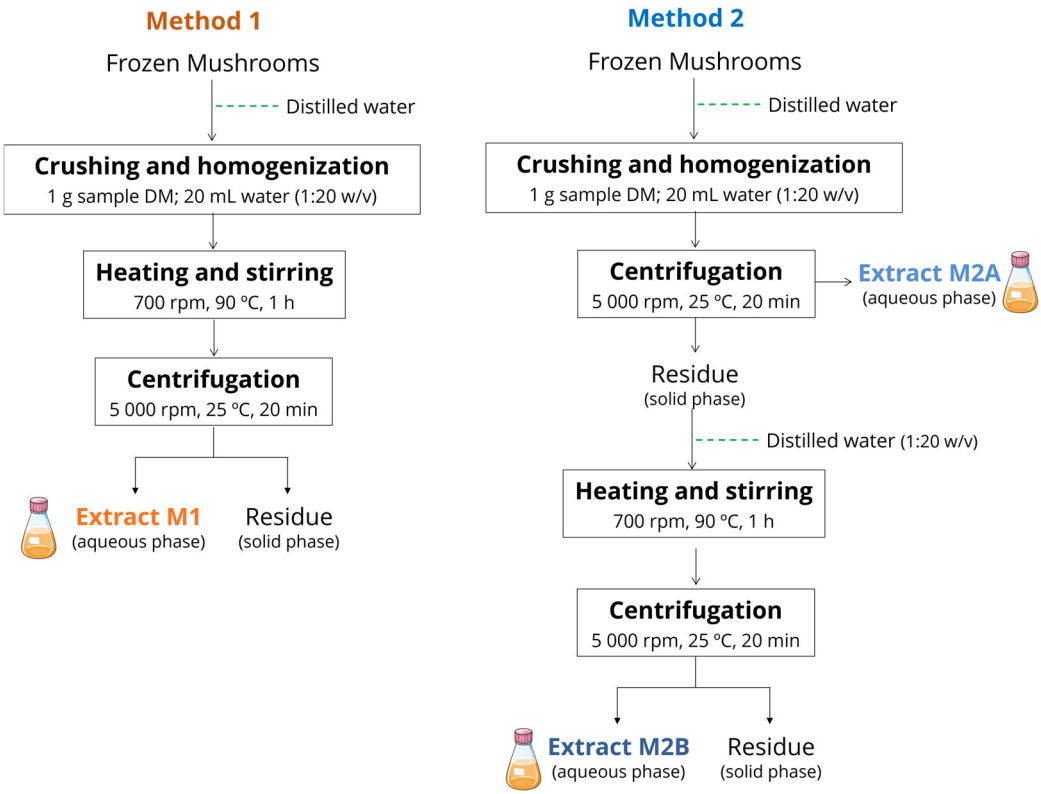

Figure 1. Extraction methods used for the three mushroom species.

2.3. Determination of Protein Dry Weight, Protein, and Total Glucans Content

The dry weight and the protein content of extracts M1, M2A, and M2B were determined according to the AOAC procedures [17]. The protein content was determined by the micro-Kjeldahl method. The nitrogen-to-protein conversion factor used was 4.38 [18]. In turn, the total glucans content was determined through the enzymatic method described by McCleary and Draga [19].

2.4. Quantification of Total Phenolic Content

The determination of the total phenolic content was carried out using the Folin–Ciocalteu method. A total of 50 µL of each extract was added to 50 µL of Folin–Ciocalteu reagent, 1 mL of sodium carbonate (75 g/L), and 1.4 mL of distilled water. After a 1 h reaction, the optical density of each sample was determined using a UVmini 1240 UV—Vis spectrophotometer (Shimadzu, Kyoto, Japan) at 750 nm [20]. The results were expressed in mg of gallic acid equivalents (GAEs)/g of dry extract.

2.5. Determination of Antioxidant Activity

Antioxidant activity was assessed using the ABTS and ORAC methods. Lyophilized extracts were dissolved in deionized water (10 mg/mL) for performing both methods. Regarding the ABTS method, extracts were added to a solution of 2,2′-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid radical cation) (ABTS+). After 6 min of the reaction,

the optical density of each sample was determined using a UVmini 1240 UV—Vis spectrophotometer (Shimadzu) at 734 nm. The results were expressed in mg of ascorbic acid equivalents (AAE)/g of dry extract [20]. The ORAC method was performed exactly as described by Araújo-Rodrigues et al. [21].

2.6. Determination of Toxicity

2.6.1. Cytotoxicity

The cytotoxicity of the extracts was determined by assessing cell viability with the Presto Blue reagent. Caco-2 cells were incubated in culture medium containing the extracts dissolved at a concentration of 4%, or with 4% ultrapure water in the case of the control, at 37 °C, for 24 h. Then, the cell viability reagent was added, and the plate was incubated again at 37 °C for more than 10 min. Finally, the fluorescence of the samples was read at 560/590 nm using a microplate reader (Microplate Reader FLUOstar OPTIMA, Offenburg, Germany). The percentage of metabolic inhibition was calculated according to the following equation:

$$\frac{(\text{sample fluorescence} - \text{control fluorescence})}{\text{control fluorescence}} \times 100$$

The extracts were considered toxic when the percentage of metabolic inhibition was greater than 30%.

2.6.2. Mutagenicity

The mutagenicity of the extracts was evaluated using the Ames test, performed with the strain *Salmonella typhimurium* TA 98 (His-), with and without liver extract (mix S9). The reagents 2-aminoanthracene and daunomycin were used as positive controls in the assays with and without liver extract, respectively. The samples were considered mutagenic when the number of UFCs was greater than 2.5 times the value of the negative control [22].

2.7. Determination of Antimicrobial Activity

The antimicrobial activity of the extracts was evaluated through the determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against 7 Gram-negative bacteria (*Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas agarici* ATCC 25943, *Pseudomonas fluorescens* (internal collection from the Escola Superior de Biotecnologia da Universidade Católica Portuguesa), *Pseudomonas tolaasii* ATCC 51309, *Salmonella enterica* ATCC 13076, and *Yersinia enterocolitica* DSM 11503) and 3 Gram-positive bacteria (*Bacillus cereus* ATCC 14579, *Listeria monocytogenes* ATCC 15313, and *Staphylococcus aureus* ATCC 6538).

The MICs were determined through the microdilution method and colorimetric assays. Firstly, the extracts were decontaminated by exposure to UV radiation for 20 min and dissolved in Mueller Hinton broth (80 mg/mL). Then, 100 µL of each extract was placed in a respective well in a microplate, and serial dilutions were made in the following wells where 50 µL of Mueller Hinton broth had previously been placed. Finally, 50 µL of Mueller Hinton broth with an inoculum of 10^6 CFU/mL of the microorganism under study was added to all wells (except for the negative controls). For each extract and each of the microorganisms studied, two negative controls (extract dissolved in Mueller Hinton broth (40 mg/mL) and only Mueller Hinton broth) and a positive control (100 µL of Mueller Hinton broth with 5×10^5 UFCs) were studied. All microplates were incubated for 24 h at 37 °C, excluding *P. agarici* (25 °C), *P. fluorescens* (25 °C), *P. tolaasii* (25 °C), *Yersinia enterocolitica* (30 °C), and *B. cereus* (30 °C) [23]. After the end of the incubation period, 40 µL of dye p-iodonitrotetrazolium chloride (INT) (0.2 mg/mL) was added to each well, and

the microplate was incubated at 37 °C, 25 °C, or 30 °C for 30 min. Viable microorganisms change the color of INT from yellow/colorless to pink. Thus, it was considered that the MIC of each extract against each microorganism under study corresponded to the lowest concentration at which there was no color change [24].

To determine the MBC, 50 µL of the solutions, where there was no growth of microorganisms, was inoculated in plate count agar and incubated at 37 °C for 24 h. The MBC was considered to correspond to the lowest concentration, where there was no growth of microorganisms. All tests were performed in duplicate [24].

2.8. Determination of Antibiofilm Activity

Antibiofilm activity was determined as described by Costa et al. [25] with some modifications. Briefly, an extract was dissolved in TSB supplemented with 1% (*w*/*v*) glucose (Sigma, St. Louis, MO, USA) at three different concentrations, namely the MIC, one-half of the MIC, and one-fourth of the MIC. These concentrations are commonly employed to explore both direct antimicrobial effects (at MIC) and sub-inhibitory effects (at half and quarter of MIC) that may influence biofilm formation and bacterial behavior without completely inhibiting growth. Additionally, these concentration ranges ensured the solubility and stability of the extracts throughout the assay. In cases where no MIC was observed, the three highest concentrations tested were used (40, 20, and 10 mg/mL). The microorganisms used to screen for antibiofilm activity were *E. coli*, *L. monocytogenes*, *P. aeruginosa*, *P. agarici*, *P. fluorescens*, *P. tolaasii*, and *S. aureus*. Test solutions were inoculated at 2% (*v*/*v*) using an overnight inoculum (ca. 10^8 CFU/mL), transferred into 96-well microtiters (Nunc, Darmstadt, Germany), and incubated for 24 h at 37 °C or 25 °C depending on the microorganism. Afterwards, the contents of the plate were discarded, and each well was carefully washed three times with sterile distilled water to remove any adhered cells. The adhered cells were then fixed with 200 mL/well of methanol for 15 min. Afterward, the methanol was discarded, the plates were left to dry, and then the fixed biofilm was stained with 200 µL/well of crystal violet (CV) for 5 min. Excess staining was rinsed out by placing the plate under slow-running tap water. After this, the plates were air-dried, and the dye that bound to the adhered cells was resolubilized by adding 200 µL/well of 33% (*v*/*v*) glacial acetic acid. The optical density of the solution obtained was measured at 570 nm using a microtiter plate reader. All assays were performed in triplicate, a positive control was prepared using inoculated culture media, and a negative control was prepared using only sterile media. The results were given in biomass formation inhibition percentages and calculated according to the following formula, in which ODassay is the OD measured in the presence of the extract and ODpositive control is the OD measured for the positive control.

$$\%\text{biomass formation inhibition} = 100 - \left(\frac{\text{OD}_{\text{assay}}}{\text{OD}_{\text{positive control}}} \right) \times 100$$

2.9. Determination of Prebiotic Activity

2.9.1. Microorganisms

The strains used to evaluate the potential prebiotic activity of the extracts were *Lactobacillus acidophilus* Ki, *Lactobacillus casei* 01, *Lactobacillus rhamnosus* R11, *Bifidobacterium lactis* BB12, and *Bifidobacterium lactis* BLC1. *L. acidophilus* Ki was obtained from CSK (Leeuwarden, The Netherlands); *L. casei 01* and *B. lactis* BB12 were obtained from Christian Hansen (Hørsholm, Denmark); *L. rhamnosus* R11 was provided by Lallemand (Montréal, QC, Canada); and *B. lactis* BLC was obtained from DSM (Moorebank, Sydney, NSW, Australia). The microorganisms were reactivated, and pre-cultures were prepared in de Man–Rogosa–Sharpe (MRS, Biokar Diagnostics, Allonne, France) broth and incubated overnight at 37 °C. In case

of *B. lactis* BB12 and *B. lactis* BLC, MRS was supplemented with 0.5 g L^{-1} of filter-sterilized L-cysteine-HCl (Fluka, Buchs, Switzerland) to lower the redox potential.

2.9.2. Culture Medium

The culture medium used for the evaluation of prebiotic properties was MRS broth prepared using a mixture of different compounds in order to enable carbon source substitution, i.e., 10 g L^{-1} of peptone (Sigma-Aldrich, EUA), 10 g L^{-1} of meat extract (Merck, Darmstadt, Germany), 5 g L^{-1} of yeast extract (Biokar Diagnostics, France), 1.08 g L^{-1} of tween 80 (Merck), 2 g L^{-1} of ammonium citrate tribasic (Sigma-Aldrich), 2 g L^{-1} of di-potassium hydrogen phosphate (Merck), 5 g L^{-1} of sodium acetate (Merck), 0.2 g L^{-1} of magnesium sulfate (Merck), 0.04 g L^{-1} of manganese sulfate (Sigma-Aldrich), and 20 g L^{-1} of a carbon source. Based on MRS, three different basal media were prepared, i.e., MRS without a carbon source, MRS with 2% (*w*/*v*) glucose (Fluka), and MRS with 1% and 2% (*w*/*v*) of each extract.

2.9.3. Growth Curves via Microplate Assay

Assays were performed by adding a 2% (*v*/*v*) inoculum of each probiotic strain, pre-grown overnight in MRS with 2% (*w*/*v*) glucose, to the different MRS culture media. These inoculated media were transferred to a 96-well microplate (200 µL). In the case of *B. lactis* BB12 and *B. lactis* BLC, the wells were covered with 50 mL of autoclave-sterilized liquid paraffin to maintain anaerobic conditions. The microplate was incubated at 37 °C for 48 h. The optical density of each well at 660 nm (680 Microplate Reader, Bio-Rad, Hercules, CA, USA) was recorded every 30 min using Microplate Manager v5.2.1 software (Bio-Rad). All assays were carried out in triplicate.

2.10. Statistical Analysis

The data were analyzed using the software GraphPad Prism v8.0.2 (GraphPad Software, La Jolla, CA, USA). The mean and standard deviation (SD) within samples were calculated for all cases. The statistical significance of results was determined by one-way ANOVA tests. $p < 0.05$ was considered statistically significant. To summarize and analyze the chemical compositions and cytotoxicity data, principal component analysis (PCA) was performed.

3. Results

3.1. Extraction Yield and Chemical Composition

Three different extraction conditions were used for each mushroom species, resulting in a total of nine different extracts. The results for the extraction yield of each extraction are presented in Table 1. The results show that extraction M1 presented the highest yield in all mushroom species; however, it was not statistically significant in the case of *P. ostreatus* and *P. eryngii* compared to the respective M2A extraction. Extraction M2B showed the lowest yield for all mushroom species, with the *P. eryngii* M2B extract providing the lowest extraction yield of all nine extracts.

The protein and total glucan content results are also shown in Table 1. The results show that all extracts presented a high amount of protein. Among them, extracts M1 and M2A from *P. eryngii* yielded the lowest protein contents with 14.61 and 12.09 g/100 g of dry extract, respectively. In contrast, extract M2B from *A. cylindracea* showed the highest protein content at 32.97 g/100 g of dry extract. The results show that these aqueous extraction approaches were able to extract a high quantity of protein from frozen mushrooms. Hence, these extracts have a high potential for use in the food industry as rich protein ingredients. The results suggest that extract M2B is the most adequate for producing functional

ingredients rich in protein since, for all mushrooms, they had a higher amount of protein than M1 and M2A. Regarding mushroom species, the results indicate that *A. cylindracea* was the best option for protein extraction in the conditions studied since the amount of this nutrient in extracts M1, M2A (excluding *P. ostreatus*), and M2B was higher than in the same extracts from the other two mushrooms. It is known that edible mushrooms have high nutritional value and are a good source of protein [26]. The protein contents obtained from the present aqueous extracts were in line with protein contents reported by other studies for these mushroom species. For instance, different studies have reported protein contents for *P. ostreatus* ranging from 17 to 42 g/100 g of dried fruit bodies [27]. For *P. eryngii*, protein contents also range from 21.33 to 24.08 g/100 g of dried fruit bodies [28]. Regarding *A. cylindracea*, there are studies reporting protein contents ranging from 34.17 to 44.94 g/100 g of dried fruit bodies as well as 14.09 to 27.24 g/g of dried extract, depending on the origin/location of the mushrooms and the substrate used for their growth [26,29].

Table 1. Extraction yield and protein, total glucan, α-glucan, and β-glucan content of extracts M1, M2A, and M2B from *P. ostreatus*, *P. eryngii*, and *A. cylindracea*. The results are presented as the mean ± SD. Different letters in each column represent statistical differences using a p-value < 0.05.

		Extraction Yield (%)	Protein (g/100 g of Dry Extract)	Total Glucans (g/100 g of Dry Extract)	α–Glucans (g/100 g of Dry Extract)	β–Glucans (g/100 g of Dry Extract)
P. ostreatus	M1	37.22 ± 0.84 ^{a}	24.25 ± 1.15 ^{a}	33.75 ± 1.68 ^{a}	21.10 ± 0.57	12.65 ± 1.67
	M2A	34.55 ± 0.09 ^{a}	22.82 ± 0.21 ^{a}	30.17 ± 0.19 ^{a}	15.30 ± 0.75	14.87 ± 2.29
	M2B	21.78 ± 0.78 ^{b}	27.69 ± 1.18 ^{b}	32.04 ± 0.29 ^{a}	22.42 ± 2.97	9.62 ± 3.24
P. eryngii	M1	46.03 ± 2.18 ^{c}	14.61 ± 0.34 ^{c}	37.96 ± 3.62 ^{a}	0.21 ± 0.04	37.76 ±3.64
	M2A	44.46 ± 3.96 ^{c}	12.09 ± 0.72 ^{d}	n.d.	n.d.	n.d.
	M2B	12.58 ± 0.94 ^{d}	21.23 ± 1.05 ^{a}	48.57 ± 9.05 ^{b}	0.38 ±0.01	48.19 ± 9.05
A cylindracea	M1	44.05 ± 0.21 ^{c}	27.44 ± 0.12 ^{b}	23.10 ± 0.98 ^{c}	0.80 ± 0.04	22.30 ± 0.98
	M2A	30.91 ± 0.89 ^{e}	24.67 ± 1.10 ^{a}	12.69 ± 1.78 ^{d}	0.36 ± 0.05	11.66 ± 0.77
	M2B	14.77 ± 1.49 ^{d}	32.97 ± 0.33 ^{e}	14.19 ± 0.78 ^{c}	0.82 ± 0.05	13.37 ± 0.77

n.d.—not determined due to insufficient amount of extract. Extract M1—hot extraction (1 g of mushroom dry matter (DM); 20 mL of water; 90 °C, 700 rpm, 1 h); extract M2A—room-temperature extraction (1 g of mushroom DM; 20 mL of water); extract M2B—hot extraction of residue from M2A (1 g of mushroom DM; 20 mL of water; 90 °C, 700 rpm, 1 h).

In terms of total glucans, extract M2B from *P. eryngii* showed the highest content (48.57 g/100 of dry extract). Due to insufficient extract mass, it was not possible to determine the glucan content for the *P. eryngii* M2A extract. The *A. cylindracea* extract showed the lowest content of total glucans, especially M2A and M2B, with 12.69 and 14.19 g/100 of dry extract, respectively. Hence, when an ingredient rich in glucans is desired for the desired final product, *A. cylindracea* seems less sustainable than *P. eryngii* and *P. ostreatus.* In terms of total glucans, the present *P. ostreatus* extracts yielded a lower amount than what has been reported in other studies for this mushroom. For instance, Synytsya et al. [30] reported 44.2 to 90 g/100 g of dry matter for different *P. ostreatus* water extracts. In the same study, glucans for *P. eryngii* water extracts ranged from 33.6 to 66.4 g/100 g of dry matter, which are in line with the results of the present study. All extracts were a better source of β-glucans than α-glucans, which is corroborated by several previous studies [11,12]. It is important to underline that the total glucan content as well as the α-glucan and β-glucan ratio varies strongly within and between mushroom species depending on extraction methodology, mushrooms' development stage, the composition of the growth subtract, and the parts of mushrooms analyzed (e.g., cap versus stalk), among others [11–13]. The health benefits of β-glucans, namely prebiotic, antiviral, antimicrobial, antioxidant, antilipidemic, antidiabetic,

and anti-inflammatory properties, have been extensively reported in the literature, while α-glucans have been associated with prebiotic and antimicrobial activities [8]. Besides health benefits, glucans from mushrooms also have technological properties important for the food industry, namely thickening, stabilizing, emulsifying, foaming, and gelation properties [31]. So, the extracts produced in the present study have a high potential for use in the food or pharmaceutical industries, due to their high glucan content.

Regarding the total phenolic content, all three mushroom species were found to be good sources of phenolic compounds. Among them, extracts from *P. ostreatus* tended to show the highest number of phenolic compounds, followed by extracts from *A. cylindracea* and finally *P. eryngii* (Table 2), but these differences had no statistical significance. All aqueous extraction methods were able to extract phenolic compounds, with no particular method standing out among them, as the differences among the mean values of extracts from the same mushroom species were not statistically significant ($p > 0.05$). The amounts of total phenolics obtained in each aqueous extract were comparable to what other researchers have previously described, with either aqueous or methanolic extracts. For instance, González-Palma et al. [32] reported 11.36 ± 0.04 mg GAEs/g of polyphenols for an aqueous extract of the dry fruiting body of *P. ostreatus*. Methanolic extracts of dried *P. ostreatus* revealed lower phenolic contents than aqueous extracts, with values varying between 2.39 mg GAEs/g and 9.64 ± 0.33 mg GAEs/g [32,33]. In both cases, the values obtained were lower than the ones reported for the three aqueous extracts obtained in the present study. In the case of *P. eryngii*, Rodríguez-Seoane et al. [34] reported between 8 and 18 mg GAEs/g of total phenolic content in aqueous extracts obtained with different temperatures. The values obtained in this study were also similar or lower compared to methanolic extracts, with studies reporting values between 7.91 ± 1.02 mg GAEs/g and 32.21 ± 0.92 mg GAEs/g of total phenols [33,35]. The results obtained for *A. cylindracea* were lower than those reported in other studies, with values of 30.16 ± 0.07 mg GAEs/g of total phenols from an aqueous extract and 23.47 ± 0.30 mg GAEs/g for a methanolic extract of dried *A. cylindracea* fruiting bodies [36,37].

Table 2. The total phenolic content of extracts M1, M2A, and M2B from *P. ostreatus*, *P. eryngii*, and *A. cylindracea*. The total phenolic content is expressed as gallic acid equivalents. The results are presented as the mean ± SD.

		Total Phenolic Content (mg GAEs/g of Dry Extract)
P. ostreatus	M1	15.80 ± 1.54
	M2A	16.57 ± 0.26
	M2B	16.65 ± 1.01
P. eryngii	M1	9.06 ± 0.63
	M2A	7.90 ± 0.46
	M2B	9.25 ± 0.28
A cylindracea	M1	10.67 ± 0.35
	M2A	13.35 ± 0.68
	M2B	12.79 ± 0.82

Statistically significant differences were not detected among the total phenolic content of all extracts evaluated with $p < 0.05$. Extract M1—hot extraction (1 g of mushroom dry matter (DM); 20 mL of water; 90 °C, 700 rpm, 1 h); extract M2A—room-temperature extraction (1 g of mushroom DM; 20 mL of water); extract M2B—hot extraction of residue from M2A (1 g of mushroom DM; 20 mL of water; 90 °C, 700 rpm, 1 h).

3.2. Antioxidant Activity

The results for antioxidant activity are expressed in Table 3. The results are expressed as ascorbic acid equivalents and trolox equivalents in ABTS and ORAC assays, respectively.

Table 3. The antioxidant activity of extracts M1, M2A, and M2B from *P. ostreatus*, *P. eryngii*, and *A. cylindracea*. The results are presented as the mean ± SD. Different letters in each column and for each antioxidant assay indicate significant differences among the mean values of each extract ($p < 0.05$).

		ABTS (mg AAE/g of Dry Extract)	ORAC (mg TE/g of Dry Extract)
P. ostreatus	M1	5.78 ± 0.31 ^{a}	31.92 ± 0.85 ^{a}
	M2A	6.33 ± 0.83 ^{a}	33.52 ± 4.09 ^{a}
	M2B	4.39 ± 1.12 ^{a}	37.37 ± 1.32 ^{a}
P. eryngii	M1	5.48 ± 0.03 ^{a}	39.29 ± 2.28 ^{a}
	M2A	2.23 ± 0.15 ^{b}	34.31 ± 5.58 ^{a}
	M2B	3.87 ± 0.39 $_{a}$	44.25 ± 0.05 ^{b}
A. cylindracea	M1	5.91 ± 0.45 ^{a}	65.70 ± 2.82 ^{c}
	M2A	12.05 ± 1.26 ^{c}	60.57 ± 3.47 ^{c}
	M2B	6.78 ± 0.44 ^{a}	61.88 ± 4.85 ^{c}

Extract M1—hot extraction (1 g of mushroom dry matter (DM); 20 mL of water; 90 °C, 700 rpm, 1 h); extract M2A—room-temperature extraction (1 g of mushroom DM; 20 mL of water); extract M2B—hot extraction of residue from M2A (1 g of mushroom DM; 20 mL of water; 90 °C, 700 rpm, 1 h).

The results for antioxidant activity showed that all extracts had antioxidant capacity. Extract M2A from *A. cylindracea* showed the highest antioxidant activity through the ABTS method, while the *P. eryngii* M2A extract showed the lowest activity from the same methodology. The other extracts showed no significant differences among them. Regarding the ORAC methodology, the *A. cylindracea* extracts showed the highest antioxidant activity among all mushrooms, with no significant differences among the three extracts. Extract M2B from *P. eryngii* followed the *A. cylindracea* extracts' antioxidant activity, and no significant differences were observed between the remaining extracts. Although statistically significant differences were not detected in the total phenolic compounds (Table 2), *A. cylindracea* showed a higher antioxidant capacity than *P. ostreatus*. Different studies have also reported good and effective antioxidant activity from aqueous extracts from these mushroom species; however, the values reported in these studies were not obtained using the same methodologies nor reported in the same units, making direct comparisons more challenging [37–40]. The results obtained in the present study suggest that antioxidant activity cannot be explained by the total phenolic content alone. According to previous studies, besides phenolic compounds, mushrooms are rich in other molecules with antioxidant properties, namely β-glucans and lectins [1,8].

3.3. Cytotoxicity

The cytotoxicity of each extract was evaluated at a concentration of 40 mg/mL. This concentration was chosen because it was the highest concentration tested among all assays, especially the antibacterial and antibiofilm assays. At this high concentration, it was possible to determine that *P. ostreatus* and *P. eryngii* showed no cytotoxic activity (negative percentages indicate an increase in metabolic activity) while all *A. cylindracea* extracts were cytotoxic (metabolic inhibition percentages superior to 30%), especially M2A and M2B (Table 4).

The results for *P. ostreatus* and *P. eryngii* are in line with other studies. For instance, Boulaka et al. [41] showed that *P. ostreatus* showed no cytotoxicity at 2% (*w*/*v*) against Caco-2 cells. Additionally, *P. ostreatus* and *P. eryngii* extracts also did not show mutagenic activity, demonstrating their safety for food applications. The cytotoxicity observed in extracts from *A. cylindracea* occurred at high concentrations, highlighting the need for further studies to determine the safe concentration limits for potential applications. The goal should be to identify non-toxic concentrations that retain the desired bioactivities.

Table 4. Cytotoxicity of extracts M1, M2A, and M2B from *P. ostreatus*, *P. eryngii*, and *A. cylindracea*.

	Extracts (40 mg/mL)	Metabolic Inhibition (%)
P. ostreatus	M1	−27.67 ± 1.46
	M2A	−47.70 ± 5.55
	M2B	−32.52 ± 11.59
P. eryngii	M1	−3.46 ± 6.38
	M2A	−9.45 ± 3.23
	M2B	27.03 ± 5.19
A. cylindracea	M1	34.00 ± 7.00
	M2A	89.00 ± 1.00
	M2B	74.00 ± 12.00

Extract M1—hot extraction (1 g of mushroom dry matter (DM); 20 mL of water; 90 °C, 700 rpm, 1 h); extract M2A—room-temperature extraction (1 g of mushroom DM; 20 mL of water); extract M2B—hot extraction of residue from M2A (1 g of mushroom DM; 20 mL of water; 90 °C, 700 rpm, 1 h).

3.4. Principal Component Analysis

Principal component analysis allows us to summarize, clarify, and simplify groups of information given by variables. In this case, the different extraction experiments were used as independent variables. The extraction yield, protein, total glucans, total phenolic content, antioxidant activity (ABTS and ORAC), and cytotoxicity were the dependent variables. PCA enabled us to make graphical representations of distance among the different extraction experiments (Figures 2 and 3).

In Figure 3, it is possible to observe that the contribution of the variables and the two components explain a cumulative 66.8% of the total variance in the experimental data (component one is 35.5% and component two is 31.3%). Component one is slightly more correlated with all the variables than component two. The total phenolic compounds, cytotoxicity, antioxidant activity (ABTS and ORAC), and protein are positively correlated with component one and the extraction yield and total glucans are negatively correlated with it. Regarding component two, glucans, cytotoxicity, and the ORAC are positively correlated with it, and the extraction yield, total phenolic compounds, protein, and ABTS negatively correlated with it. An analysis of Figure 1 allows us to observe the differentiated extracts from the three different mushrooms. The different extracts from each mushroom are grouped closer to one another. From Figures 2 and 3, we can observe that extracts from *P. eryngii* showed the highest content of glucans while presenting the lowest amount of protein, total phenolic content, and antioxidant activity (ABTS). *P. ostreatus* extracts showed the highest total phenolic content and lowest cytotoxicity. As for *A. cylindracea* extracts, they presented the highest amount of protein and antioxidant activity (ABTS and ORAC) and a good total phenolic content while also showing high correlation with the cytotoxicity variable. Some of the antioxidant activity might have been related to the presence of peptides, as the protein, ABTS, and ORAC variables are positively correlated within the first component.

PCA allowed us to evaluate and validate the differences among the different mushroom extractions regarding the protein content, total glucans, total phenolic content, antioxidant activity, and cytotoxicity, which were determined analytically. The simple display of the two graphics allows us to observe the behavior of each variable regarding the different mushroom extracts.

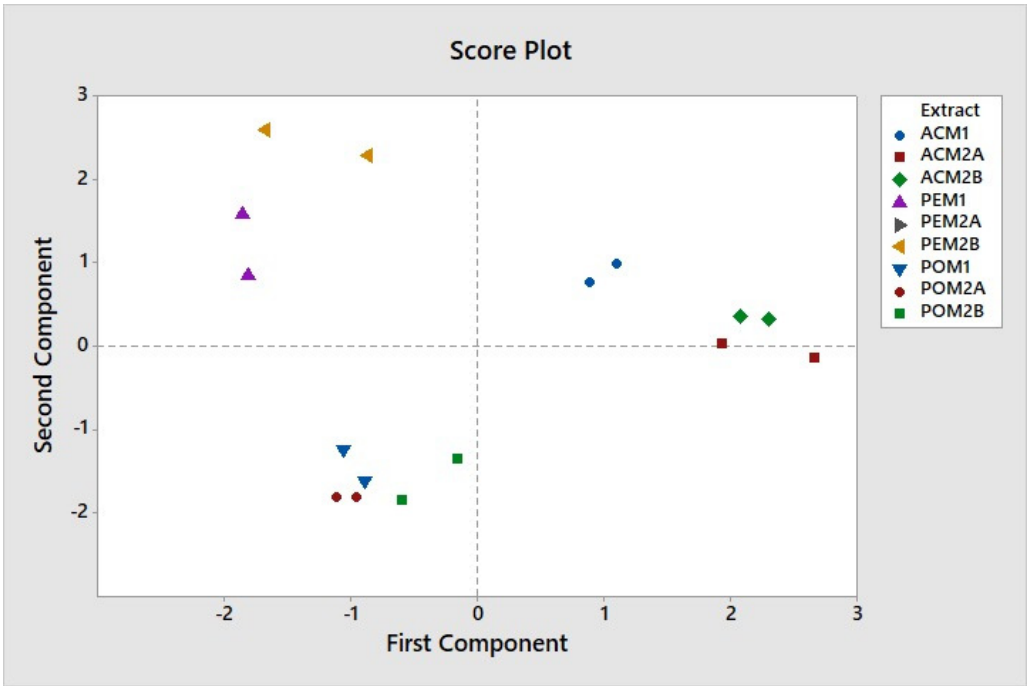

Figure 2. A score plot of the extraction experiments in the sorting space built for the linear regression of the two first components. (AC—*A. cylindracea*; PE—*P. eryngii*; PO—*P. ostreatus*). Extract M1—hot extraction (1 g of mushroom dry matter (DM); 20 mL of water; 90 °C, 700 rpm, 1 h); extract M2A—room-temperature extraction (1 g of mushroom DM; 20 mL of water); extract M2B—hot extraction of residue from M2A (1 g of mushroom DM; 20 mL of water; 90 °C, 700 rpm, 1 h).

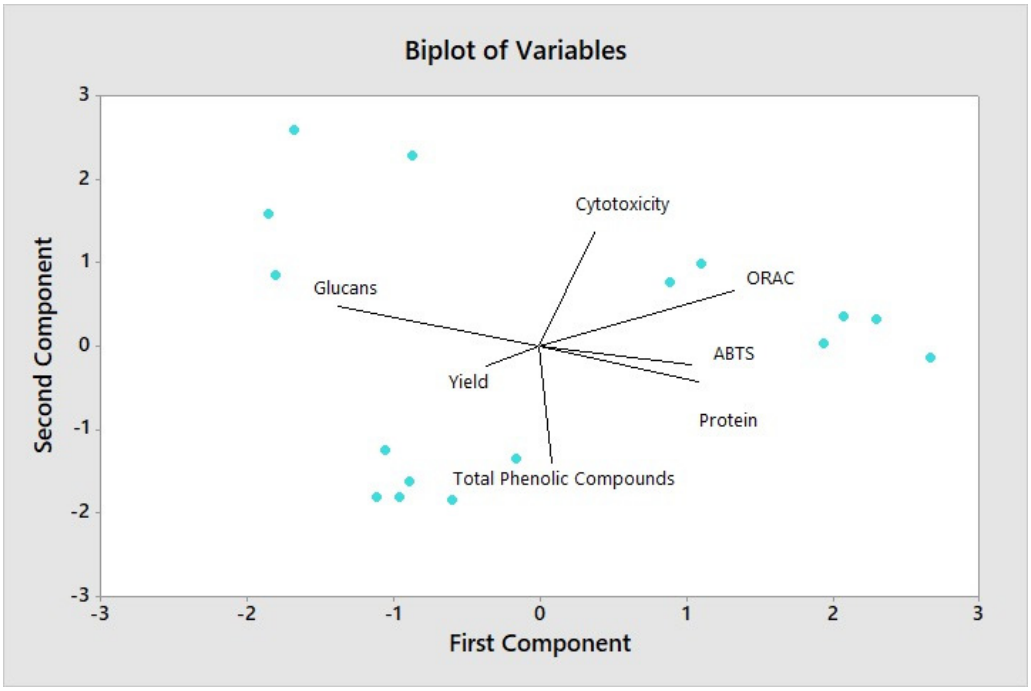

Figure 3. Contribution of variables in first two components.

3.5. Antimicrobial Activity

Regarding the results in antimicrobial activity, Table 5 shows the MICs of the extracts against the tested bacteria. At the highest tested concentration of 40 mg/mL, no extract from either mushroom was able to inhibit *Pseudomonas agarici*, *Pseudomonas fluorescens*, or *Pseudomonas tolaasii*. These bacteria are known mushroom pathogens, which might

be a reason to justify their resistance to these extracts [42–44]. The growth of the other seven bacteria was inhibited by at least one extract. The M2A extract from *A. cylindracea* showed antimicrobial activity against a higher number of bacteria (*Bacillus cereus*, *Listeria monocytogenes, Salmonella enterica, Yersinia enterocolitica*, and *Pseudomonas aeruginosa* at 20 mg/mL) than all other extracts, being the most promising extract to be used as a natural antimicrobial substance. On the other hand, extracts M1 and M2B from the same mushroom (*A. cylindracea*) showed the worst results regarding antimicrobial activity, since they inhibited the growth of none or only two bacteria, respectively. The highest antimicrobial activity was observed for the *P. eryngii* M1 extract against *L. monocytogenes* with an MIC of 2.5 mg/mL. *P. aeruginosa* was the most susceptible bacteria since its growth was inhibited for all extracts, excluding M1 from *A. cylindracea*, with MICs ranging from <5 to 10 mg/mL, <5 to 20 mg/mL, and 20 to 40 mg/mL in extracts from *P. ostreatus*, *P. eryngii*, and *A. cylindracea*, respectively. The second most susceptible bacterium was *S. enterica*, since it was inhibited for all extracts, excluding the M1 and M2B extracts from *A. cylindracea*. In other studies, the antimicrobial screening of extracts from these mushrooms varies greatly in terms of extraction methods and the choice of assay in which the antimicrobial activities are assessed. For these reasons, direct comparisons to the present results are difficult. For instance, aqueous extracts from *P. ostreatus* showed almost no activity (3 out of 29 bacterial pathogens) at a higher concentration of 1000 mg/mL using the disk diffusion method [45]. A *P. ostreatus* extract obtained using equal thirds of glycerol, ethanol, and distilled water showed activity against *B. cereus* and *P. aeruginosa* at 50 mg/mL using the well diffusion method [46]. However, an ultrasound-assisted extraction of the phenol fraction of *P. ostreatus* using hydroalcoholic conditions revealed MICs in the µg/mL range for *E. coli*, *P. aeruginosa*, *B. cereus*, and *S. aureus* [47]. Regarding *P. eryngii*, an extract obtained using a solution of 10% acetic acid in phosphate saline buffer revealed MICs of 25% (*v*/*v*) for *P. aeruginosa*, *E. coli*, and *S. aureus* [48]. In addition, different extracts using acetone and ethanol revealed antibacterial activity against *P. aeruginosa*, *E. coli*, and *S. aureus* at 50 mg/mL using the disk diffusion method [49]. To the best of our knowledge, no antibacterial activity has been reported for *A. cylindracea* in the literature, although there are reports of antifungal activity [40,50]. The antimicrobial activity of mushroom aqueous extracts has been associated with the presence of several bioactive molecules, namely phenolic acids, β-glucans, and lectins [1,8].

Table 5. Minimum inhibitory concentration (mg/mL) of extracts M1, M2A, and M2B from *P. ostreatus*, *P. eryngii*, and *A. cylindracea*.

	P. ostreatus			***P. eryngii***			***A. cylindracea***		
	M1	**M2A**	**M2B**	**M1**	**M2A**	**M2B**	**M1**	**M2A**	**M2B**
B. cereus	20	40	40	n.i.	n.i.	n.i.	n.i.	20	n.i.
E. coli	n.i.	n.i.	n.i.	n.i.	40	40	n.i.	n.i.	n.i.
L. monocytogenes	n.i.	n.i.	n.i.	2.5	n.i.	n.i.	n.i.	20	n.i.
S. aureus	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	40
S. enterica	20	10	20	10	10	20	n.i.	20	n.i.
Y. enterocolitica	40	20	10	n.i.	n.i.	n.i.	n.i.	20	n.i.
P. aeruginosa	10	<5	<5	<5	10	20	n.i.	20	40
P. agarici	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
P. fluorescens	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
P. tolaasii	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.

n.i.—no inhibition observed at the highest tested concentration of 40 mg/mL. Extract M1—hot extraction (1 g of mushroom dry matter (DM); 20 mL of water; 90 °C, 700 rpm, 1 h); extract M2A—room-temperature extraction (1 g of mushroom DM; 20 mL of water); extract M2B—hot extraction of residue from M2A (1 g of mushroom DM; 20 mL of water; 90 °C, 700 rpm, 1 h).

3.6. Antibiofilm Activity

Microorganisms have the ability to grow while irreversibly attached to a surface, forming complex communities known as biofilms. Currently, there is a search for the use of natural products as a source of antimicrobial compounds coupled with antibiofilm activity. Mushrooms are a promising source of bioactive compounds encompassing these bioactivities [51]. Regarding the effect of the present mushroom extracts on the inhibition of biofilm formation by different bacteria, the results are shown in Figure 4. For each microorganism, three concentrations of each extract were tested, namely the corresponding MIC, one-half of the MIC, and one-fourth of the MIC. In cases where no MIC was observed, the concentrations tested were 40, 20, and 10 mg/mL. Due to an insufficient amount of extract, it was not possible to test the antibiofilm activity of the *P. eryngii* M2A extract. The results obtained in Figure 4 reveal that most of the extracts were able to inhibit biofilm formation from the different bacteria, with a high number of extracts being able to inhibit up to 100% of biofilm formation. Even in the cases where no MIC was observed for the antibacterial activity, a high number of extracts was able to inhibit biofilm formation of the bacteria in concentrations ranging from 40 to 10 mg/mL. All extracts, with some exceptions, were able to cause significant biofilm inhibition even at concentrations of one-fourth of the MIC. In some cases, it was observed that inhibition at one-half of the MIC or one-fourth of the MIC was higher than at the MIC or 40 mg/mL. This can be attributed to the extracts not being completely solubilized in the medium at higher concentrations. At lower concentrations, solubilization improved, allowing for the inhibitory compounds to be more homogenously distributed in the wells. The results also show that antibiofilm activity was species-dependent, with extracts being more efficient at inhibiting certain bacteria and not others. For instance, the *P. ostreatus* M2B inhibited 87.2–99.3% of *L. monocytogenes*, *P. fluorescens*, *P. tolaasii*, and *S. aureus* biofilms but only 19.7–24.9% of *E. coli* and *P. aeruginosa* biofilms. Despite being the highest biofilm mass producer, *S. aureus* was the most susceptible bacteria to the extracts, with all extracts causing inhibition ranging from 93.2 to 100%. Overall, the mushroom that showed better antibiofilm activity was *A. cylindracea*, with more extracts being able to inhibit up to 100% of biofilm formation, followed by *P. ostreatus*. *P. eryngii* showed the weakest antibiofilm activity among the three mushrooms. Of the three *A. cylindracea* extracts, extract M2A showed the highest antibiofilm capacity. This extract demonstrated antibacterial activity against five of the tested bacteria, strong antioxidant activity, and a high total phenolic content, which may explain its ability to inhibit biofilm formation.

In Alves et al. [51], mushroom extracts presented high inhibition percentages toward biofilm productions of different Gram-positive and Gram-negative bacteria. In another study by Petrović et al. [52], an *Agrocybe aegerita* methanolic extract was able to reduce *P. aeruginosa* biofilm formation by 84.24% with a concentration of one-half of the MIC. Despite these reports, there are still few studies evaluating the antibiofilm activity of mushroom extracts, especially aqueous extracts, which is why it is important to investigate the mechanism of action by which they inhibit biofilm production. The results obtained in the present study reveal the high capacity of mushroom extracts to inhibit bacterial biofilm formation.

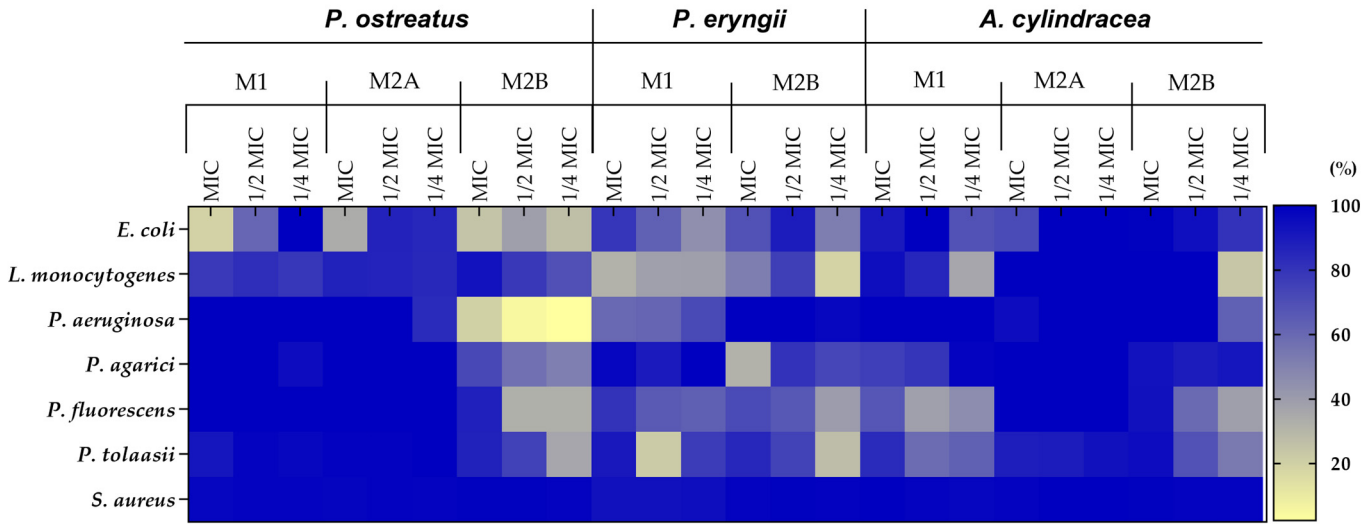

Figure 4. Heat map of biofilm formation inhibition (%) by *P. ostreatus*, *P. eryngii*, and *A. cylindracea* aqueous extracts against Gram-positive and Gram-negative bacteria. Extract M1—hot extraction (1 g of mushroom dry matter (DM); 20 mL of water; 90 °C, 700 rpm, 1 h); extract M2A—room-temperature extraction (1 g of mushroom DM; 20 mL of water); extract M2B—hot extraction of residue from M2A (1 g of mushroom DM; 20 mL of water; 90 °C, 700 rpm, 1 h).

3.7. Prebiotic Activity

The mushroom extracts were also tested regarding their prebiotic activity using different probiotic bacteria. The extracts were tested at 2% and 1% (*m*/*v*) (Figure 5). Unfortunately, due to a lack of sufficient extract, it was not possible to determine the *P. eryngii* M2A prebiotic activity. Observing the results in Figure 5, it is possible to see that the *A. cylindracea* and *P. eryngii* extracts were able to induce growth in most of the probiotic bacteria. Overall, extract M1 at 1% from A. cylindracea stood out since it showed higher prebiotic activity than the other extracts in most of the bacteria studied. This effect on growth was never higher than in the glucose control. In contrast, the *P. ostreatus* M1 and M2A had a negative effect on the growth of all probiotic bacteria while M2B was able to enhance some of the bacteria growth, especially in *Lactobacillus acidophilus* Ki and *Bifidobacterium lactis* BLC. This prebiotic activity aligned with what is reported in other studies. For instance, in a study conducted by Synytsya et al. [30], it was observed that aqueous extracts from *P. eryngii* and *P. ostreatus*, rich in β-glucans and proteins, were able to promote probiotic bacteria growth rates, including *Lactobacillus* and *Bifidobacterium* strains. In the same study, *P. eryngii* extract proved to be a better growth source than *P. ostreatus*. For instance, a *Bifidobacterium* strain grew only with *P. eryngii* extract, while *P. ostreatus* was not able to stimulate its growth [18]. In another work, a hydroethanolic extract from *P. ostreatus* was able to induce the growth of *L. acidophilus* at 1% (*m*/*v*) [53]. In addition, a different study showed that the abundance of families such as Lactobacillaceae and Bifidobacteriaceae was increased in the presence of *P. eryngii* extracts [54]. *A. cylindracea* polysaccharides have also been reported to have prebiotic activity [55].

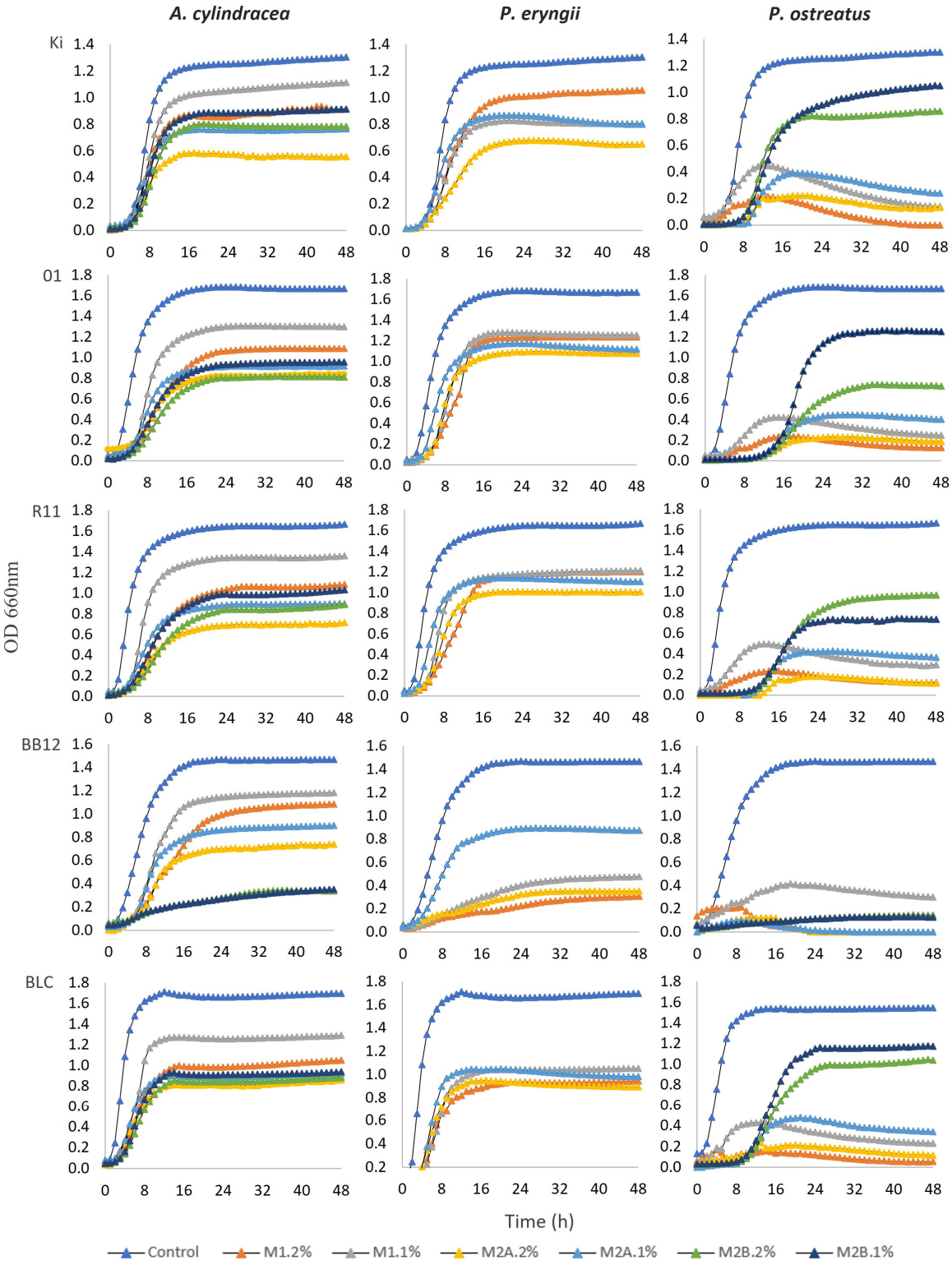

Figure 5. The growth curves of selected strains of prebiotic bacteria in MRS broth containing glucose (control) or each of the extracts from *A. cylindracea*, *P. eryngii*, and *P. ostreatus* at 2% and 1% (*m*/*v*). Extract M1—hot extraction (1 g of mushroom dry matter (DM); 20 mL of water; 90 °C, 700 rpm, 1 h); extract M2A—room-temperature extraction (1 g of mushroom DM; 20 mL of water); extract M2B—hot extraction of residue from M2A (1 g of mushroom DM; 20 mL of water; 90 °C, 700 rpm, 1 h).

In the present study, among all bacteria, *B. lactis* BB12 was the one with the worst prebiotic results, with only the *A. cylindracea* M1 and M2A (2% and 1%) extracts and *P. eryngii* M2B (1%) stimulating its growth. With some exceptions, the highest extract

concentration of 2% was not as efficient at inducing bacterial growth when compared to the lower concentration. One of the reasons for these results might be that at the highest concentration, as well as at the lower concentration, the extract did not solubilize in MRS. Thus, at 2% concentrations, the solutions were not as homogeneous, and the polysaccharides were not as readily available as in the lower concentrations. Concentrations of 1% appeared more homogeneous, thus providing a better source of energy for the probiotic bacteria. In the case of the *P. ostreatus* M1 and M2A extracts, the negative effect on the growth of bacteria was more noticeable at the higher concentration. This might be explained by some compounds that were present in the extracts that inhibited the growth of bacteria. With a lower concentration, bacteria growth was not induced. However, it was still higher compared to the highest concentration. Overall, these results show that polysaccharides obtained under different conditions from these three mushrooms, depending on the bacterial strains and concentration, can be used as prebiotics. The prebiotic activity of β-glucans from mushrooms has been extensively reported in the literature. They are the most studied polysaccharides, considering this bioactivity [8]. Although less studied, in recent years, α-glucans have also been pointed out as prebiotic polysaccharides [8]. Additionally, phenolic acids can also contribute to the prebiotic activity of mushroom aqueous extracts [1,8].

4. Conclusions

The findings of this study reveal notable differences in extraction yields, protein, glucan content, and bioactivity across mushroom species and extraction methods, highlighting their potential as functional ingredients for diverse applications. Among the extracts, M1 showed the highest extraction yield and M2B the lowest, though M2B was the most effective for producing protein-rich ingredients, with *A. cylindracea* identified as the most suitable species for this purpose. Conversely, *A. cylindracea* was less sustainable for glucan-rich applications, whereas the M2B extract from *P. eryngii* excelled. All nine extracts were rich in phenolic compounds, but no mushroom species or extract stood out in this regard. Regarding bioactivities, the M2A extract from *A. cylindracea* demonstrated the strongest antioxidant capacity in both the ABTS and ORAC assays, underscoring its potential as a natural antioxidant. This extract also exhibited the highest antimicrobial activity, inhibiting a broad range of pathogenic bacteria, and high antibiofilm activity, attributed to its phenolic content and robust bioactive properties. In contrast, the M1 and M2B extracts from *A. cylindracea* showed limited antimicrobial efficacy. The M1 extract from *A. cylindracea* exhibited the strongest prebiotic effect at a 1% concentration across most of the studied bacterial strains, indicating its potential for enhancing gut health when incorporated into food products. However, additional studies using simulated gut environments would provide a more accurate understanding of how the extract functions as a prebiotic.

In summary, this study emphasizes the unique functional properties that each mushroom and extraction method offer. *A. cylindracea* is particularly promising for applications where protein enrichment, antioxidant, antimicrobial, antibiofilm, and prebiotic effects are desired. However, *A. cylindracea* extracts showed cytotoxicity at the highest concentration tested (40 mg/mL), which may limit their safe use in certain applications. Nevertheless, antimicrobial and prebiotic activities occurred at lower concentrations, suggesting its potential safe use in these applications. To mitigate this, careful optimization of extraction concentrations and doses is recommended, ensuring that the beneficial bioactive effects are retained while minimizing toxicity. Meanwhile, *P. eryngii* stands out in glucan-rich applications, and *P. ostreatus* presents a balanced profile in antimicrobial and antibiofilm activities. These findings suggest a wide array of applications for these extracts within the food industry.

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