

Article

# Valorization of Olive By-Products as Substrates for the Cultivation of *Ganoderma lucidum* and *Pleurotus ostreatus* Mushrooms with Enhanced Functional and Prebiotic Properties

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**Abstract:** The successful management of olive by-products constitutes a major challenge due to their huge volume, high organic content, and toxicity. Olive-mill wastes (TPOMW) and olive pruning residues (OLPR) were evaluated as substrates for the cultivation of *Ganoderma lucidum* and *Pleurotus ostreatus*. Chemical composition, glucans, total phenolic content, and antioxidant activity were measured in mushrooms, and their prebiotic potential was assessed by examining their effect on the growth of four intestinal bacteria. Several substrates based on olive by-products had a positive impact on *P. ostreatus* mushroom production, whereas only one performed adequately for *G. lucidum*. Increased ratios of OLPR to wheat-straw resulted in an increase of crude protein content in *P. ostreatus* fruit-bodies by up to 42%, while *G. lucidum* mushrooms from OLPR-based substrates exhibited an up to three-fold increase in  $\alpha$ -glucan, or a significant enhancement of  $\beta$ -glucan content, when compared to beech sawdust (control). The mushrooms' FTIR spectra confirmed the qualitative/quantitative differentiation detected by standard assays. In regard to prebiotic properties, mushrooms powder supported or even enhanced growth of both *Lactobacillus acidophilus* and *L. gasseri* after 24/48 h of incubation. In contrast, a strain-specific pattern was observed in bifidobacteria; mushrooms hindered *Bifidobacterium bifidum* growth, whereas they supported a similar-to-glucose growth for *B. longum*.

**Keywords:** olive mill waste; lignocellulosic residues; *Ganoderma lucidum*; *Pleurotus ostreatus*; medicinal mushrooms; glucan; prebiotic; *Lactobacillus*; *Bifidobacterium*; waste valorization

## 1. Introduction

The particular organoleptic and nutritional properties of olive oil, in conjunction with the need to improve human diet, have resulted at a continuous increase in olives production during the last few decades. Nowadays, over 750 million olive trees are cultivated worldwide, 95% of which grow in the Mediterranean region, while the global production of olive oil is projected to reach 3.1 million metric tons in 2018/2019 (International Olive Council, <http://www.internationaloliveoil.org>). However,

the cultivation of olive trees and operation of olive mills generate huge amounts of plant residues and effluents, respectively. The annual production of pruning exceeds 15 million tons, all of which are usually burnt on farms and result in air pollution due to aerosols rich in organic compounds [1]. Furthermore, large quantities of a highly toxic and recalcitrant sludge-like waste (known as “alperujo”) are produced by two-phase olive oil mills [2]. Moreover, centralized alperujo treatment is not feasible in most countries due to the olive mills’ small capacity, seasonal operation, and scattered distribution [3].

Olive by-products—especially olive-oil mill waste—have attracted scientific interest, and various physicochemical or biological processes have been proposed for reducing their pollution load [4–7]. However, their wide-scale implementation is often technically complicated and/or not financially viable. Hence, the adoption of innovative processes for the generation of value-added products presents an alternative worth investigating in order to successfully exploit such wastes by reducing their environmental impact.

Mushroom cultivation constitutes a noteworthy and sustainable practice through which lignocellulosic residues are enzymatically biotransformed into fungal biomass with particular nutritional and/or medicinal properties. Though cereal straw and hardwood sawdust are commonly used as substrates for the production of most mushrooms species [8], many of them are also cultivated on various residues, e.g., cottonseed hulls, corn cobs, sugarcane bagasse, cotton gin trash, coffee husks, grape marc, vineyard pruning, banana straw, palm leaves, soybean stalk, waste paper, and nut shells [9–17], as well as on olive mill wastes [18–21]. Among the most widely appreciated mushrooms are those produced by *Pleurotus ostreatus* and *Ganoderma lucidum*. The former (commonly known as the ‘oyster mushroom’) is the third most cultivated species worldwide [22]. Its culinary/nutritional value and the relatively easy cultivation process has resulted in the large spread of its cultivation over the last 20 years. Mushrooms, as well as the mycelia of *P. ostreatus*, contain bioactive compounds such as polysaccharides, lectins, lipopolysaccharides, peptides, and triterpenoids to which a plethora of medicinal properties are attributed, including anticancer, antitumor, anti-inflammatory, immunostimulatory, and immunomodulatory activities [23]. On the other hand, *G. lucidum*, commonly known as the reishi mushroom, has been extensively used as a pharmaceutical product. Over 400 chemical compounds present in *G. lucidum* biomass have been classified as bioactive, including polysaccharides, proteins/peptides, steroids, sterols, and fatty acids, which are associated to antioxidative, antiaging, antifatigue, hypoglycemic, immunomodulating, anti-inflammatory, antitumor, antibacterial, antiviral, hypolipidemic, sleep regulating, and analgesic properties [23,24]. Furthermore, both *P. ostreatus* and *G. lucidum* have demonstrated promising prebiotic properties, possibly due to their indigestible polysaccharides and, particularly,  $\beta$ -glucan content [25,26].

Fungal (mushroom) polysaccharides comprise chitin,  $\alpha$ - and  $\beta$ -glucans, mannans, xylans, and galactans; they are mainly found as linear and branched glucans with various glycosidic linkages, e.g., (1→3), (1→6)- $\beta$ -glucans, and (1→3)- $\alpha$ -glucans, while some contain arabinose, glucuronic acid, galactose, mannose, xylose, or ribose [27]. Glucans, in particular, have deservedly earned much attention thanks to their exceptional but not yet fully understood immunobiological activity [28]. Their presence in several mushroom species has been associated with various functional and medicinal properties [29–33].

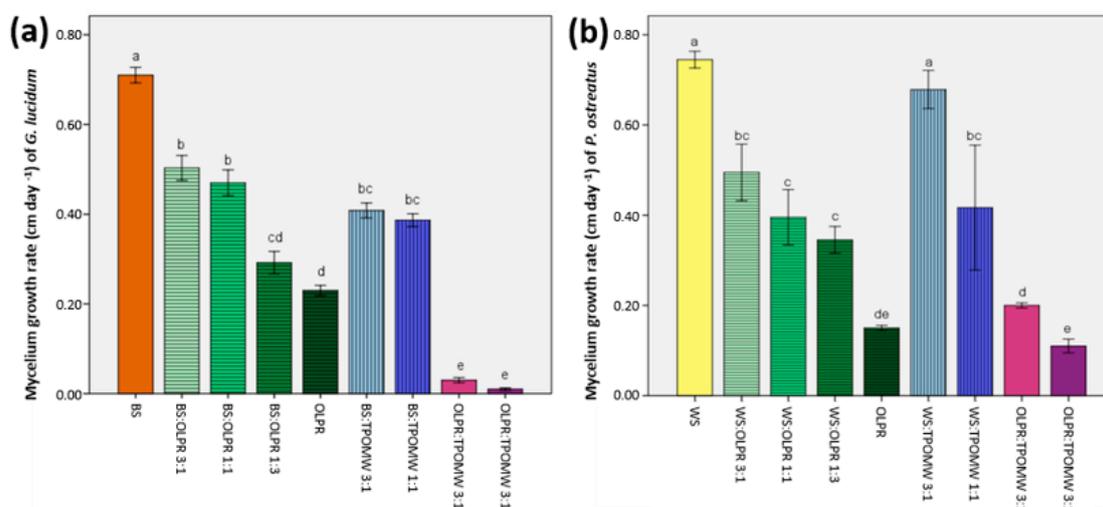
The nutritional composition of mushrooms, as well as their content in various bioactive ingredients, are differentiated quantitatively and qualitatively depending on the strain, cultivation conditions, and substrate used, as previously demonstrated with *Cyclocybe cylindracea*, *Hericium erinaceus*, and *Pleurotus* spp. [10,18,19,34,35]. In general, it has been shown that the addition of materials rich in phenolic and antioxidant compounds in cultivation substrates of the aforementioned species lead to a significant increase in the respective components in fruit-bodies. However, no data exist about the cultivation of *G. lucidum* on olive by-products/wastes or in regard to the effect of production substrates on mushroom composition and functional properties. In addition, very limited information is available on the prebiotic properties of lyophilized mushroom powder deriving from different species, cultivation processes, and media [25].

In the frame of the present study, the suitability of olive mill wastes and olive cultivation residues (in comparison to commonly/widely used substrates) for the production of *G. lucidum* and *P. ostreatus* mushrooms was examined. In addition to cultivation parameters, both the crude composition and the content of fruit-bodies in selected bioactive compounds were evaluated, and the effect of various substrates was assessed. Finally, the prebiotic potential of mushrooms was investigated in vitro based on selected intestinal microbial strains cultivation.

## 2. Results and Discussion

### 2.1. Initial Assessment of Substrates for Fungal Growth

The first part of this study aimed at determining the growth of *G. lucidum* and *P. ostreatus* in substrates consisting of olive pruning residues (OLPR) and two-phase olive mill wastes (TPOMW) in various mixtures with each other or with beech sawdust (BS) and wheat straw (WS), respectively. The growth rate of *G. lucidum* was significantly higher on BS as compared to all other substrates (Figure 1). In general, values in ‘race tubes’ containing up to 50% OLPR were comparable to those measured in 50% TPOMW. Increasing the OLPR content led to significant decrease in growth rates, while mixtures of OLPR and TPOMW were the worst performing media and were excluded from further experiments. In the case of *P. ostreatus*, the control (WS) and the WS:TPOMW 3:1 substrate equally supported mycelium growth; however, growth rates were significantly reduced when TPOMW or OLPR were added at a ratio of 50% or higher (Figure 1). It is worth mentioning that the three ratios of OLPR to WS did not differ significantly, whereas the OLPR substrate alone, as well as the OLPR and TPOMW mixtures, showed relatively low growth rates.



**Figure 1.** Mycelium growth rates of (a) *Ganoderma lucidum* and (b) *Pleurotus ostreatus* during the colonization of nine substrates prepared on the basis of three main ingredients (BS: Beech sawdust, WS: Wheat straw, TPOMW: Two-phase olive mill waste, and OLPR: Olive pruning residues) and their various mixtures as estimated in “race” tube experiments. Values (cm day<sup>-1</sup>) are expressed as means  $\pm$  standard errors of means,  $n = 4$ . A lack of letters in common indicates statistically significant differences (Gabriel’s  $t$ -test,  $p < 0.05$ ) for comparisons of treatment means between different substrates.

Hence, the outcome of this initial assessment revealed that increasing amounts of TPOMW in substrates retarded mycelium growth due to the elevated toxicity exerted mainly by the higher polyphenolic content. This effect was more pronounced for *G. lucidum*, while similar behavior was noted in OLPR-based media for both species examined. Especially in regard to *P. ostreatus*, a reduction of growth was only observed when olive by-products were used at high ratios. Such results are in accordance to earlier findings, where the addition of composted (or not) TPOMW at a ratio of up to 20% in substrates of several *Pleurotus* species (including *P. ostreatus*) contributed to a growth increase,

which was adversely affected when TPOMW exceeded 40% [21]. However, the fungal growth response to the nature of substrates also depends on the species examined. Thus, and in contrast to what was observed in the present study for *G. lucidum*, *H. erinaceus* strains showed satisfactory growth in substrates containing OLPR or TPOMW, exceeding, in most cases, the values obtained by the BS control [18].

## 2.2. Evaluation of TPOMW- and OLPR-Based Substrates for Mushroom Cultivation

Previously obtained results led to the elimination of the worst performing substrates. Thus, OLPR 25%, 50%, and 75%, and TPOMW 25% and 50%, in mixtures with the conventional substrates (i.e., BS for *G. lucidum* and WS for *P. ostreatus*; BS or WS alone were also used as controls for each species) were further examined in regard to their suitability to support mushroom production in comparison to the control substrates.

*G. lucidum* completed incubation within 26–33 days in most of the substrates examined, but it performed significantly slower on BS and BS:OLPR 3:1 (up to 43 days; Table 1). In contrast, these two particular substrates (BS and BS:OLPR 3:1) provided the best earliness (time required for the appearance of mushroom primordia) values (45 to 46 days), while the rest were notably slower, in particular those containing TPOMW (77–84 days). Total yield ranged from 19 to 275 g, and the corresponding biological efficiency (BE; i.e., fresh weight of mushrooms produced over the dry weight of the substrate) values varied from 5 to 61% (Table 1). The control substrate (BS) provided significant higher values in respect to the other treatments, while only BS:OLPR 3:1 exhibited a satisfactory performance among all substrates containing olive by-products.

**Table 1.** Mushroom cultivation parameters for *Ganoderma lucidum* and *Pleurotus ostreatus* in six substrates consisting of beech sawdust (BS), wheat straw (WS), two-phase olive mill waste (TPOMW), and olive pruning residues (OLPR) in various mixtures (*w/w*). Values are expressed as means  $\pm$  standard deviation of means,  $n = 4$ . Lack of superscript letters in common indicates statistically significant differences (Gabriel's *t*-test,  $p < 0.05$ ) for comparisons between substrates.

<i>Ganoderma lucidum</i>						
	BS	BS:OLPR 3:1	BS:OLPR 1:1	BS:OLPR 1:3	BS:TPOMW 3:1	BS:TPOMW 1:1
Incubation period (days)	43.00 $\pm$ 0.00 <sup>a</sup>	43.00 $\pm$ 0.00 <sup>a</sup>	26.33 $\pm$ 0.67 <sup>c</sup>	31.25 $\pm$ 0.63 <sup>b</sup>	33.00 $\pm$ 1.16 <sup>b</sup>	26.33 $\pm$ 0.67 <sup>c</sup>
Earliness (days)	45.75 $\pm$ 0.25 <sup>b</sup>	45.00 $\pm$ 3.68 <sup>b</sup>	52.75 $\pm$ 4.50 <sup>ab</sup>	63.67 $\pm$ 15.50 <sup>ab</sup>	77.00 $\pm$ 18.54 <sup>a</sup>	83.50 $\pm$ 19.50 <sup>a</sup>
Total yield (g)	275.22 $\pm$ 14.48 <sup>a</sup>	193.42 $\pm$ 13.52 <sup>b</sup>	61.46 $\pm$ 10.8 <sup>c</sup>	19.10 $\pm$ 8.3 <sup>d</sup>	36.10 $\pm$ 14.47 <sup>cd</sup>	61.46 $\pm$ 10.84 <sup>c</sup>
Biological efficiency (%)	61.24 $\pm$ 3.22 <sup>a</sup>	40.24 $\pm$ 2.81 <sup>b</sup>	20.52 $\pm$ 3.62 <sup>c</sup>	4.54 $\pm$ 1.97 <sup>d</sup>	12.05 $\pm$ 4.83 <sup>c</sup>	20.52 $\pm$ 3.62 <sup>c</sup>
<i>Pleurotus ostreatus</i>						
	WS	WS:OLPR 3:1	WS:OLPR 1:1	WS:OLPR 1:3	WS:TPOMW 3:1	WS:TPOMW 1:1
Incubation period (days)	26.00 $\pm$ 0.00 <sup>c</sup>	28.00 $\pm$ 0.33 <sup>b</sup>	30.67 $\pm$ 2.33 <sup>ab</sup>	36.47 $\pm$ 2.19 <sup>a</sup>	25.00 $\pm$ 0.00 <sup>d</sup>	27.00 $\pm$ 0.00 <sup>b</sup>
Earliness (days)	40.67 $\pm$ 4.81 <sup>bc</sup>	37.25 $\pm$ 1.53 <sup>c</sup>	41.27 $\pm$ 1.16 <sup>bc</sup>	46.00 $\pm$ 2.00 <sup>b</sup>	40.50 $\pm$ 1.44 <sup>bc</sup>	58.67 $\pm$ 5.55 <sup>a</sup>
Total yield (g)	215.87 $\pm$ 15.43 <sup>a</sup>	263.84 $\pm$ 46.40 <sup>a</sup>	255.72 $\pm$ 28.04 <sup>a</sup>	134.87 $\pm$ 4.82 <sup>b</sup>	220.75 $\pm$ 3.17 <sup>a</sup>	256.87 $\pm$ 44.34 <sup>a</sup>
Biological efficiency (%)	77.26 $\pm$ 5.52 <sup>a</sup>	82.60 $\pm$ 14.53 <sup>a</sup>	56.79 $\pm$ 6.23 <sup>b</sup>	39.73 $\pm$ 1.42 <sup>c</sup>	73.68 $\pm$ 1.06 <sup>a</sup>	71.33 $\pm$ 12.31 <sup>ab</sup>

The use of hardwood sawdust or cereal straw, cotton seed husk, and corn cobs is common in the commercial production of *G. lucidum* [36–38]. However, the highly increased market demand during the last twenty years led to the exploitation of several other substrates, which demonstrated an increase in BE of up to 40% by replacing part of the sawdust by residues such as stillage grain from rice-spirit distilleries or tea wastes [39,40]. An even higher increase (up to 75%) in BE was reported on substrates consisting of maize straw supplemented with wheat and maize bran [41]. Olive by-products were evaluated for the first time as substrates in *G. lucidum* cultivation. Though the BS (control) performed notably better than the other media tested, BS:OLPR 3:1 showed that it is a promising alternative substrate (worth examining in future experiments with additional strains to assess their potential

suitability) in *G. lucidum* mushroom production since it supported relatively high yields with very satisfactory earliness values.

On the other hand, *P. ostreatus* colonized most substrates within 26–28 days, but ratios of OLPR exceeding 50% (*w/w*, in respect to WS) delayed substrate colonization by up to 10 days (Table 1). Significant differences were observed in regard to earliness in *P. ostreatus*; mushroom appearance required the most time in WS:OLPR 3:1 (46 days) and WS:TPOMW 1:1 (59 days), whereas primordia formation was noted within 37–41 days in the other treatments examined (Table 1). The evaluation of yield and BE for *P. ostreatus* evidenced that the supplementation of the conventional substrate with OLPR or TPOMW up to 50% had a positive impact on mushroom performance, i.e., an increase in yield ranging from 5 to 49 g kg<sup>-1</sup> and BE enhancement by up to 5.5% (Table 1). It is noteworthy that only WS:OLPR 1:3 presented a markedly negative effect on *P. ostreatus* mushroom production.

Hence, most of the substrates based on olive by-products supported similar or even better performance in *P. ostreatus* when compared to the control (WS). Both WS:OLPR and WS:TPOMW mixtures performed well, since BE reached 83% and 74%, respectively. This is in agreement with the outcome of previous pertinent studies reporting that addition of TPOMW in rather low ratios (20% *w/w*) resulted in improved *P. ostreatus* mushroom production (by up to 30%), while a further increase could be achieved only after supplementation with composted TPOMW due to the reduced toxicity and higher nutrient availability [21]. In general, the use of different fungal strains in combination with the high variation in the physicochemical properties of olive by-products, which are much affected by soil and climatic conditions, variety, and olive oil extraction process [42,43], results in a considerable variability in the values of mushroom cultivation parameters when such materials are used as substrates, e.g., BE: 50–137% for *P. ostreatus* cultivated on—supplemented or not—olive by-products [10,15,19,21,44].

### 2.3. Assessment of Nutritional Composition of Mushrooms Produced on Olive By-Products

In the frame of the present study, the outcome of proximate analysis on *G. lucidum* mushrooms revealed that the main ingredients (with the exception of crude fat) differed—albeit not always significantly—among fruit-bodies cultivated on various substrates (Table 2). In general, it was observed that mushrooms grown on the control (BS) contained less ash and protein compared to most of those produced on other substrates, while mushrooms from BS:OLPR 1:3 showed the highest content (Table 2). Of additional interest was the significantly higher content in crude fibers detected in fruit-bodies formed on BS:OLPR 3:1 (62 g kg<sup>-1</sup> d.w.). In addition, no significant differences were noted when crude fat values were compared among treatments. In the case of *P. ostreatus*, the addition of OLPR to WS (i.e., from plain WS to WS:OLPR 1:3) resulted in a progressive reduction in ash, crude fat, and fiber content, as well as in an increase of crude protein content by up to 42% (WS versus WS:OLPR 1:3). Furthermore, small variations were detected in total carbohydrates and gross energy content of both mushroom species.

In general, variations noted in mushroom composition were linked to the use of different substrates, e.g., the increases in ash and protein content of mushrooms were associated with high ratios of TPOMW, which could be attributed to its higher concentration in metals and nitrogen, respectively [21,43]. This outcome is in accordance to previous results obtained in *P. ostreatus* produced on paper scraps [45] or after the supplementation of wood-based substrates of *G. lucidum* with tea waste (which is richer than sawdust in nitrogen and minerals [39]). Moreover, a significantly higher protein content was detected in cultivated mushrooms growing on substrates rich in nitrogen, e.g., spent beer grains supplemented with bran, wheat straw mixed with sugar beet, olive leaves mixed with TPOMW for *P. ostreatus* [10,46,47], beech sawdust supplemented with wheat bran for *Hericium americanum* [48], or wheat straw amended with poultry manure or rolled oats and soybean flour for *C. cylindracea* [49,50]. Therefore, nitrogen supplementation, apart of improving mushroom yield, also contributes at obtaining a final product with an elevated protein content.

**Table 2.** Crude composition and bioactive compounds content in *Ganoderma lucidum* and *Pleurotus ostreatus* mushrooms cultivated in six substrates consisting of beech sawdust (BS); wheat straw (WS); two-phase olive mill waste (TPOMW), and olive pruning residues (OLPR) in various mixtures. Values are in g kg<sup>-1</sup> d.w., except of gross energy (kcal 100 g<sup>-1</sup> d.w.), total phenolics (mg gallic acid equivalents per g d.w.), antiradical activity (mmol Trolox equivalents per g d.w.), and reducing power (mmol Trolox equivalents per g d.w.), and are expressed as means ± standard deviation of means, *n* = 4. Lack of superscript letters in common indicates statistically significant differences (Gabriel's *t*-test, *p* < 0.05) for comparisons of treatment means between substrates.

<i>Ganoderma lucidum</i>						
	BS	BS:OLPR 3:1	BS:OLPR 1:1	BS:OLPR 1:3	BS:TPOMW 3:1	BS:TPOMW 1:1
Ash	3.10 ± 0.08 <sup>c</sup>	3.08 ± 0.12 <sup>c</sup>	3.41 ± 0.33 <sup>c</sup>	5.20 ± 0.32 <sup>a</sup>	3.68 ± 0.07 <sup>bc</sup>	4.26 ± 0.18 <sup>b</sup>
Crude fiber	47.93 ± 1.86 <sup>b</sup>	62.48 ± 1.18 <sup>a</sup>	43.80 ± 2.02 <sup>b</sup>	21.57 ± 9.66 <sup>c</sup>	52.89 ± 3.77 <sup>ab</sup>	49.34 ± 1.56 <sup>b</sup>
Crude fat	2.21 ± 0.08 <sup>a</sup>	2.45 ± 0.05 <sup>a</sup>	2.03 ± 0.49 <sup>a</sup>	2.04 ± 0.50 <sup>a</sup>	1.56 ± 0.52 <sup>a</sup>	1.10 ± 0.12 <sup>a</sup>
Crude protein	16.84 ± 0.62 <sup>bc</sup>	17.06 ± 0.15 <sup>bc</sup>	15.28 ± 0.55 <sup>c</sup>	23.25 ± 4.08 <sup>abc</sup>	18.85 ± 0.55 <sup>ab</sup>	22.21 ± 0.79 <sup>a</sup>
T. carbohydrates	77.86 ± 1.05 <sup>a</sup>	77.41 ± 2.07 <sup>a</sup>	79.28 ± 3.47 <sup>a</sup>	69.52 ± 3.29 <sup>b</sup>	75.92 ± 1.85 <sup>a</sup>	72.44 ± 1.38 <sup>ab</sup>
Gross energy	399 ± 13 <sup>a</sup>	400 ± 17 <sup>a</sup>	397 ± 4 <sup>a</sup>	389 ± 11 <sup>a</sup>	393 ± 2 <sup>a</sup>	388 ± 3 <sup>a</sup>
α-glucan	2.09 ± 0.48 <sup>b</sup>	1.68 ± 0.68 <sup>b</sup>	6.84 ± 1.99 <sup>a</sup>	6.22 ± 2.27 <sup>a</sup>	3.08 ± 1.38 <sup>ab</sup>	3.87 ± 0.30 <sup>ab</sup>
β-glucan	35.83 ± 2.05 <sup>a</sup>	43.10 ± 6.38 <sup>a</sup>	35.06 ± 5.14 <sup>a</sup>	31.27 ± 6.19 <sup>a</sup>	34.72 ± 1.03 <sup>a</sup>	32.87 ± 2.00 <sup>a</sup>
Total phenolics	3.25 ± 0.09 <sup>b</sup>	4.23 ± 0.27 <sup>a</sup>	3.89 ± 1.15 <sup>ab</sup>	4.02 ± 0.51 <sup>a</sup>	2.98 ± 0.31 <sup>b</sup>	2.99 ± 0.32 <sup>b</sup>
Antiradical activity	8.49 ± 1.32 <sup>a</sup>	9.56 ± 0.41 <sup>a</sup>	8.46 ± 1.05 <sup>a</sup>	10.41 ± 1.37 <sup>a</sup>	7.18 ± 2.78 <sup>a</sup>	6.37 ± 0.72 <sup>a</sup>
Reducing power	12.99 ± 1.95 <sup>b</sup>	13.96 ± 0.41 <sup>b</sup>	18.90 ± 0.89 <sup>a</sup>	13.76 ± 4.88 <sup>ab</sup>	12.93 ± 3.82 <sup>b</sup>	13.26 ± 3.93 <sup>b</sup>
<i>Pleurotus ostreatus</i>						
	WS	WS:OLPR 3:1	WS:OLPR 1:1	WS:OLPR 1:3	WS:TPOMW 3:1	WS:TPOMW 1:1
Ash	8.49 ± 0.82 <sup>a</sup>	7.95 ± 0.10 <sup>a</sup>	6.49 ± 0.14 <sup>b</sup>	6.32 ± 0.45 <sup>b</sup>	8.99 ± 0.03 <sup>a</sup>	9.42 ± 1.90 <sup>a</sup>
Crude fiber	18.99 ± 1.97 <sup>a</sup>	17.16 ± 1.24 <sup>a</sup>	16.54 ± 1.94 <sup>a</sup>	14.01 ± 1.34 <sup>ab</sup>	15.47 ± 1.13 <sup>ab</sup>	12.97 ± 2.44 <sup>b</sup>
Crude fat	2.54 ± 0.17 <sup>a</sup>	2.47 ± 0.14 <sup>a</sup>	1.87 ± 0.04 <sup>b</sup>	1.62 ± 0.21 <sup>b</sup>	2.74 ± 0.17 <sup>a</sup>	2.70 ± 0.21 <sup>a</sup>
Crude protein	15.22 ± 1.29 <sup>c</sup>	16.00 ± 0.37 <sup>c</sup>	19.88 ± 2.34 <sup>a</sup>	21.54 ± 0.24 <sup>a</sup>	17.08 ± 0.58 <sup>b</sup>	19.32 ± 0.24 <sup>ab</sup>
T. carbohydrates	73.75 ± 2.81 <sup>a</sup>	73.58 ± 4.27 <sup>a</sup>	71.76 ± 0.87 <sup>a</sup>	70.52 ± 1.46 <sup>a</sup>	71.19 ± 0.98 <sup>a</sup>	68.56 ± 3.64 <sup>a</sup>
Gross energy	379 ± 5 <sup>a</sup>	381 ± 14 <sup>a</sup>	383 ± 2 <sup>a</sup>	383 ± 9 <sup>a</sup>	378 ± 4 <sup>a</sup>	376 ± 9 <sup>a</sup>
α-glucan	8.75 ± 0.37 <sup>a</sup>	7.25 ± 1.98 <sup>a</sup>	6.32 ± 2.07 <sup>a</sup>	5.69 ± 2.40 <sup>a</sup>	6.00 ± 1.14 <sup>a</sup>	2.17 ± 0.58 <sup>b</sup>
β-glucan	30.64 ± 2.45 <sup>a</sup>	28.02 ± 1.61 <sup>a</sup>	28.87 ± 3.45 <sup>a</sup>	25.58 ± 0.21 <sup>a</sup>	31.49 ± 0.43 <sup>a</sup>	29.56 ± 2.42 <sup>a</sup>
Total phenolics	2.01 ± 0.11 <sup>c</sup>	3.09 ± 0.34 <sup>a</sup>	2.73 ± 0.17 <sup>ab</sup>	2.57 ± 0.27 <sup>b</sup>	2.81 ± 0.24 <sup>a</sup>	2.99 ± 0.34 <sup>a</sup>
Antiradical activity	2.49 ± 0.14 <sup>b</sup>	3.79 ± 0.47 <sup>ab</sup>	2.81 ± 0.57 <sup>b</sup>	5.59 ± 1.21 <sup>a</sup>	3.27 ± 0.43 <sup>ab</sup>	4.68 ± 0.67 <sup>a</sup>
Reducing power	5.78 ± 0.88 <sup>a</sup>	6.69 ± 1.27 <sup>a</sup>	6.85 ± 1.39 <sup>a</sup>	5.55 ± 0.22 <sup>a</sup>	3.47 ± 0.27 <sup>b</sup>	3.94 ± 0.04 <sup>b</sup>

In regard to glucans, *G. lucidum* generally presented lower content in α-glucan (1.7–6.8%) and higher content in β-glucan (31.3–43.1%) than *P. ostreatus* (2.2–8.8% and 25.6–31.5%, respectively) (Table 2). In terms of α-glucan content, the use of olive by-products exhibited opposite effects on the two species studied. Hence, the addition of OLPR to BS at ratios exceeding 50% resulted in a three-fold increase in α-glucan content in *G. lucidum* (i.e., BS versus BS:OLPR 1:3), whereas the opposite was noted in *P. ostreatus* mushrooms, where increased ratios of the control substrate (WS) favored higher content in α-glucan (8.75% in WS versus 6.32% and 2.17% in WS:OLPR 1:1 and WS:TPOMW 1:1, respectively). In regard to β-glucans, high content was found in fruit-bodies, while a notable increase (by 20% reaching up to a total of 43%) was recorded in *G. lucidum* mushrooms when cultivated in a BS:OLPR 3:1 substrate (versus the control, BS). However, no significant differences were recorded, which can be attributed to the high variability observed among replicates in BS:OLPR treatments. On the other hand, a similar content in β-glucans (26–31%) was found in *P. ostreatus* fruit-bodies produced in different substrates.

To the best of our knowledge, no data are available concerning qualitative and quantitative variation in mushroom glucans production when different cultivation substrates are used. This is the first time that glucans have been comparatively evaluated in *G. lucidum* fruit-bodies produced on various substrates. Results revealed that the nature of substrate significantly affects relative content in glucans structural types (α- and β- glucan). In addition, *G. lucidum* fruit-bodies were shown to be among the richest in β-glucan content (i.e., 31–43%) when compared to a wide range of mushrooms from various species previously examined [51]. A similar enhancement in β-glucans content in mushrooms cultivated on olive by-products was reported in *H. erinaceus* and *Pleurotus* spp. [18,19] and

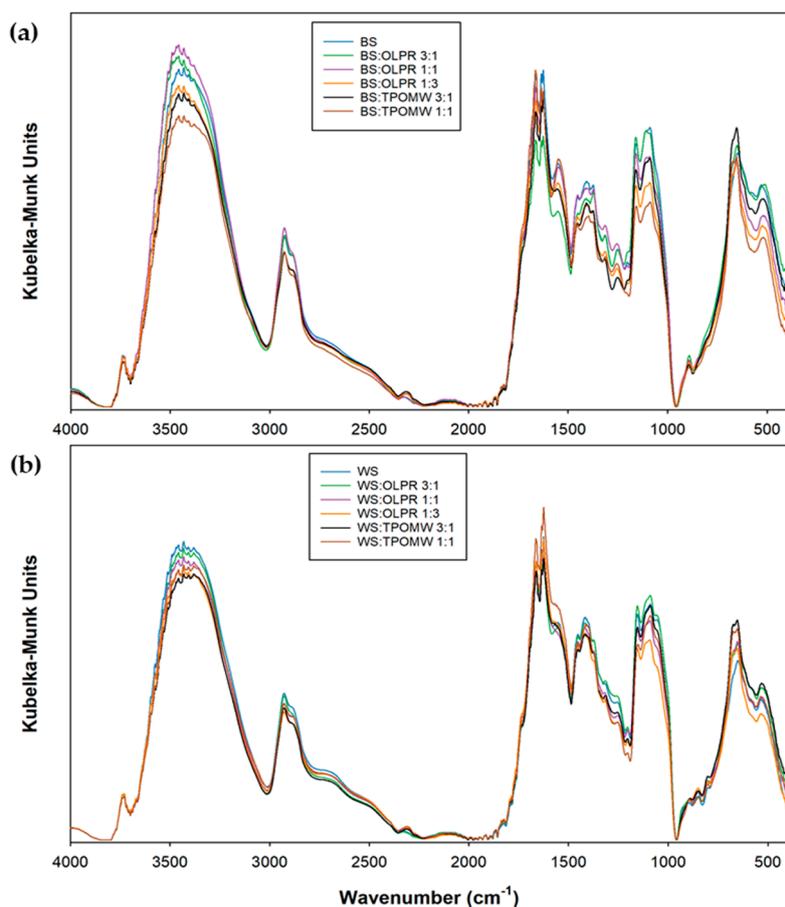
was attributed to the activation of  $\beta$ -glucan synthase due to the toxicity of olive mill by-products [52]. Studies using soy residues [53,54] or wheat straw [55] also showed that the glucan content in *G. lucidum* is affected by the substrate composition, which, in turn, has an impact on the antimicrobial, antioxidant, and cytotoxic properties of mushroom extracts.

In general, higher concentrations of total phenolics (TPC) and antioxidant activity were recorded in *G. lucidum* mushrooms in respect to *P. ostreatus* (Table 2). Fruit-bodies of both species demonstrated significant increase in TPC when produced on substrates containing OLPR, i.e., by up to 30% and 54% for *G. lucidum* and *P. ostreatus*, respectively (as compared to TPC content in fruit-bodies cultivated in BS and WS, respectively). In contrast, the addition of TPOMW in substrates resulted in TPC increase in *P. ostreatus* mushrooms only, indicating that such interactions also depend on the fungal species used.

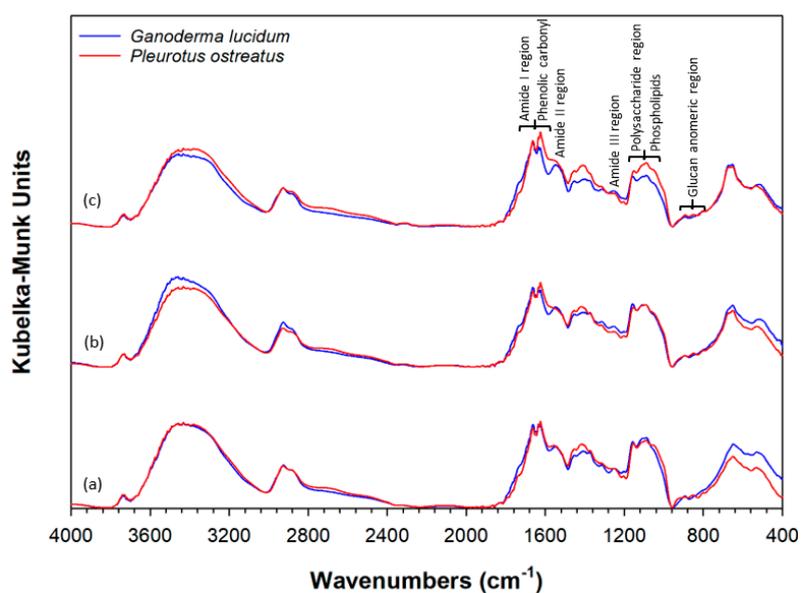
Similar trends were observed for the antioxidant activity of fruit-bodies (Table 2). *G. lucidum* mushrooms exhibited increased antiradical activity and reducing power values in BS:OLPR substrates only (significantly different in the case of reducing power, i.e., up to 45% higher than the control). On the other hand, *P. ostreatus* mushrooms showed higher values for antiradical activity only; the respective values in fruit-bodies from olive-based by-products were more than double in respect to the those obtained from the control substrate. Finally, a statistical analysis of the results showed that a significant correlation between TPC and antioxidant activity in *P. ostreatus* only ( $r = 0.87$  for TPC versus antiradical activity, and  $r = 0.45$  for TPC versus reducing power,  $p < 0.05$ ), demonstrating that phenolics are among the main antioxidant compounds in *Pleurotus* mushrooms. An increase in total phenolics concentration, which was also assessed for *Pleurotus eryngii*, *P. nebrodensis*, and *H. erinaceus* [18,19], seems to be associated with the selective absorption of substrates' organic components—including phenolic and terpenic compounds—by several cultivated mushrooms, and it is closely associated with elevated antioxidant activities [19].

#### 2.4. Fourier Transform Infrared (FTIR) Analysis

The analysis of the recorded FTIR spectra revealed a notable/extended variation in *G. lucidum* mushrooms produced in different substrates as opposed to *P. ostreatus* mushrooms, where differences in spectra were mostly observed in the region  $1800\text{--}400\text{ cm}^{-1}$  (Figure 2). In both species, differences were mainly related to the quantitative changes of compounds present in mushrooms which were consequently associated with the respective region vibrations (Figure 3). The spectral regions at  $1670\text{--}1610$ ,  $1550$ , and  $1240\text{ cm}^{-1}$  are related to the C=O stretching (Amide I band), N-H in-plane/C-N stretching (Amide II band), and C-N stretching (Amide III band) of proteins, respectively, and demonstrate that *G. lucidum* fruit-bodies contains higher amounts of protein than those of *P. ostreatus*, which is in general agreement with measurements of crude protein content (Table 2). In addition, since the region  $1180\text{--}1000\text{ cm}^{-1}$  could provide information regarding the C-O stretching vibration in polysaccharides and the  $\text{PO}_2$  stretching ( $1080\text{ cm}^{-1}$ ) of phospholipids [56], our results demonstrated that *G. lucidum* mushrooms show a higher carbohydrates content (at  $1160\text{ cm}^{-1}$ ) than *P. ostreatus*, which is also in accordance to the crude carbohydrates content measured (Table 2).

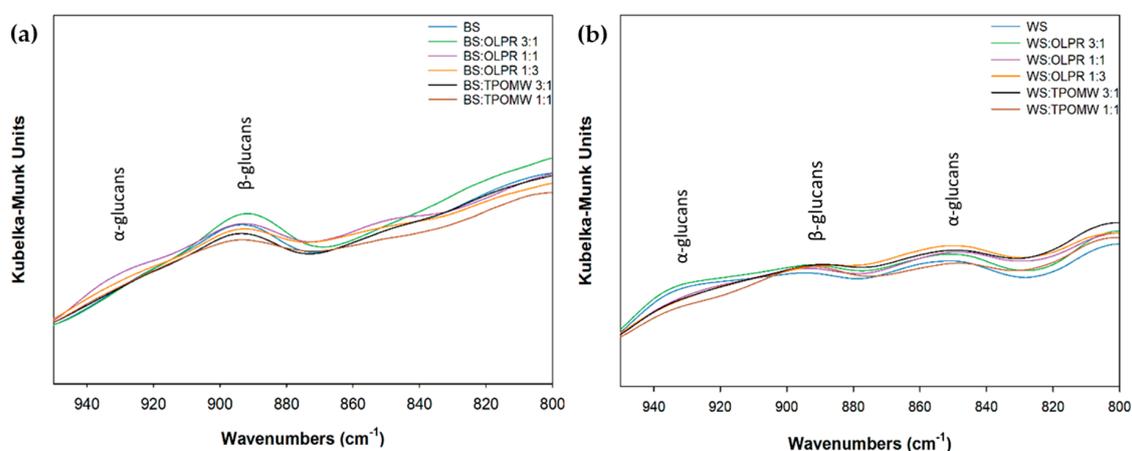


**Figure 2.** FTIR spectra of (a) *Ganoderma lucidum* and (b) *Pleurotus ostreatus* mushrooms produced on different substrates (and their mixtures), i.e., BS: Beech sawdust, WS: Wheat straw, TPOMW: Two-phase olive mill waste, and OLPR: Olive pruning residues.



**Figure 3.** Spectroscopic comparison of samples from *Ganoderma lucidum* and *Pleurotus ostreatus* mushrooms produced on (a) control substrate (BS and WS, respectively), (b) mixture of control substrate and olive pruning (BS:OLPR 1:1 and WS:OLPR 1:1), and (c) mixture of control substrate and two-phase olive-mill waste (BS:TPOMW 1:1 and WS:TPOMW 1:1).

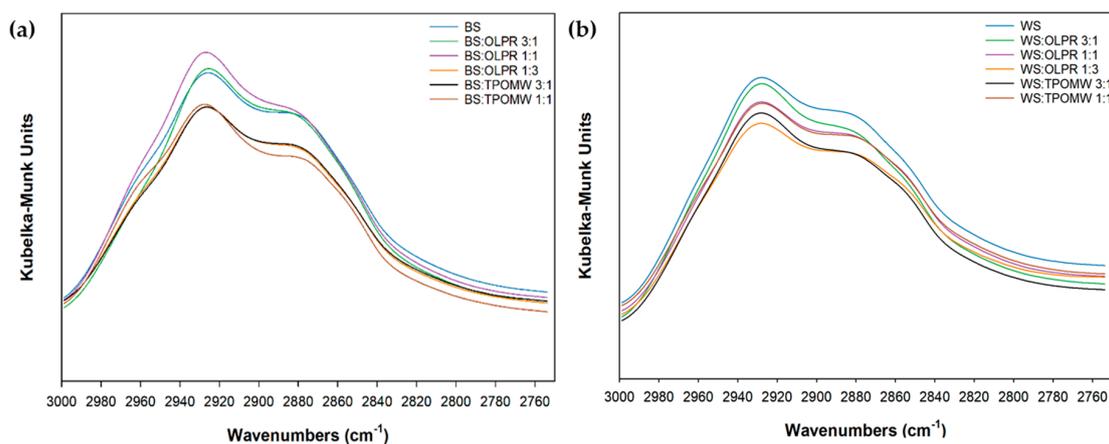
A comparison between the different substrates of each species was also performed in order to examine the effect of each substrate on mushroom content in compounds of interest. The region  $950\text{--}750\text{ cm}^{-1}$  provides information regarding the anomeric region of glucans (Figure 4); more specifically, the region at  $890\text{ cm}^{-1}$  for the C–H deformation in  $\beta$ -glucans and the regions at  $930$  and  $850\text{ cm}^{-1}$  for the asymmetric ring vibration and the C–H deformation in  $\alpha$ -glucans, respectively [56,57]. In regard to glucans, *G. lucidum* mushroom spectra corresponding to different substrates were in close agreement with the respective content measured by the commercial kit (Table 2). Moreover, due the very low content in  $\alpha$ -glucans in most of *G. lucidum* treatments, the peaks related to the  $\alpha$ -anomeric structure of glucans were almost absent. However, the ability of FTIR to detect the two substrates (i.e., BS:OLPR 1:1 and BS:OLPR 1:3) that produced mushrooms with relatively higher content in  $\alpha$ -glucan was noteworthy. On the other hand,  $\beta$ -glucan in *P. ostreatus* mushrooms was marginally detected, mostly due to the very similar content in  $\beta$ -glucans among treatments and the high—in some cases—standard deviation between replicates in the same treatment. Nevertheless,  $\alpha$ -glucan region absorption intensities in *P. ostreatus* followed the previously measured  $\alpha$ -glucan content (Table 2).



**Figure 4.** Glucans and recorded spectra by diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy in the region  $950\text{--}800\text{ cm}^{-1}$  of (a) *Ganoderma lucidum* and (b) *Pleurotus ostreatus* mushrooms produced on different substrates (BS: Beech sawdust, WS: Wheat straw, TPOMW: Two-phase olive mill waste, and OLPR: Olive pruning residues).

Information regarding lipids in cell membranes of mushrooms can be obtained through the FTIR spectrum in three regions and originates from various types of molecular vibrations: (i) The acyl chain vibrations (i.e.,  $\text{CH}_3$  and  $\text{CH}_2$  asymmetric and symmetric stretching vibrations, as well as  $\text{CH}_2$  bending and rocking vibrations), (ii) the headgroup vibrations (i.e.,  $\text{PO}_2^-$  stretching vibration), and (iii) the interface regions (i.e.,  $\text{C}=\text{O}$  stretching vibration) [56]. However, due to the high content in a wide range of organic compounds, which results in an extended peak overlapping, information regarding the lipid/fat content of the produced mushrooms could be acquired from the region  $3000\text{--}2750\text{ cm}^{-1}$ , and, more specifically, by the peaks at  $2930$  and  $2880\text{ cm}^{-1}$  corresponding to the  $\text{CH}_2$  asymmetric and  $\text{CH}_3$  symmetric stretching of acyl chain respectively (Figure 5). For both mushroom species, peak intensity seems to follow the measured crude fat of mushrooms from different substrates (Table 2). However, some differences can be observed, probably due to the very similar fat content of the mushrooms from different treatments and the relatively high standard deviation observed among replicates within individual treatment (Table 2).

Finally, little information could be acquired regarding the phenolic content (and consequently the antioxidant activity) of mushrooms from the recorded spectra. The characteristic regions for phenolic compounds, e.g.,  $1670\text{--}1600\text{ cm}^{-1}$  for the carbonyl vibration [56], are related to proteinic, phenolic, and flavonoid carbonyl, and, therefore, the recorded FTIR intensities in this region cannot be associated with an individual group of compounds.



**Figure 5.** Acyl chain vibration of lipids and recorded DRIFT spectra in the region 3000–2750  $\text{cm}^{-1}$  of (a) *Ganoderma lucidum* and (b) *Pleurotus ostreatus* mushrooms produced on different substrates (BS: Beech sawdust, WS: Wheat straw, TPOMW: Two-phase olive mill waste, and OLPR: Olive pruning residues).

### 2.5. Evaluation of Prebiotic Potential of *G. lucidum* and *P. ostreatus* Mushrooms

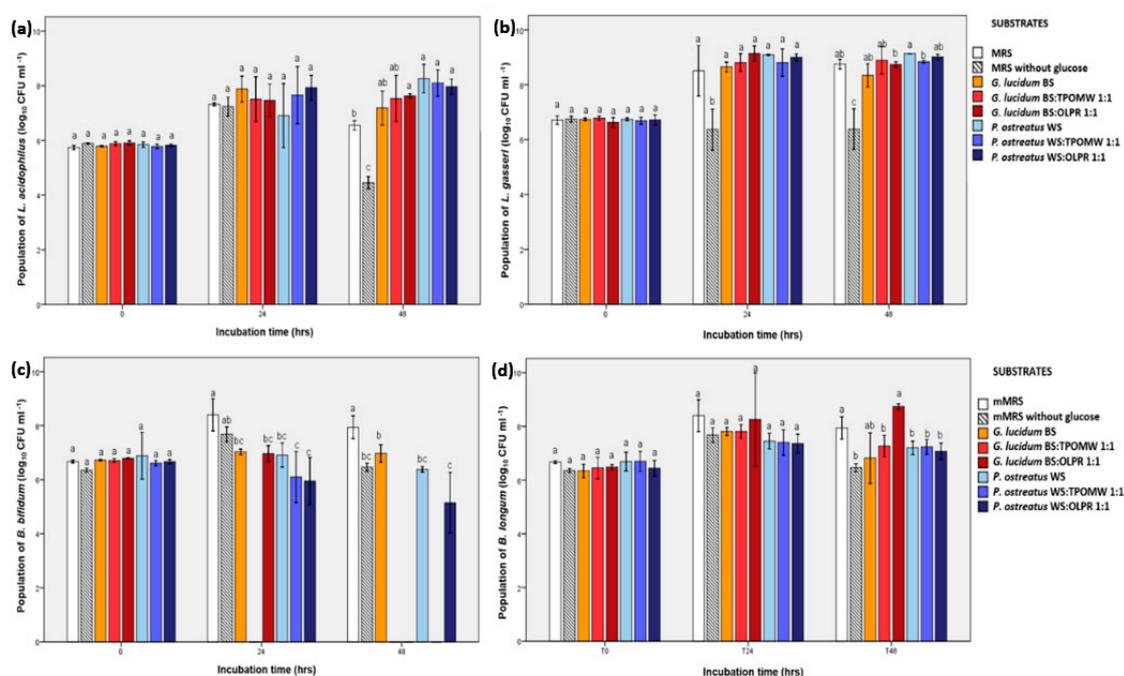
In the present work, the growth of four intestinal bacterial isolates (*Lactobacillus acidophilus*, *L. gasseri*, *Bifidobacterium bifidum*, and *B. longum*) was studied by using lyophilized powder from two edible/medicinal mushrooms (*P. ostreatus* and *G. lucidum*) deriving from different substrates as the sole carbon source. No significant differences were observed in initial inocula ( $t = 0$ ) of each of the four bacterial strains among all tested substrates (Figure 6a–d). In the case of lactobacilli, mushrooms supported a comparable-to-glucose bacterial growth of both strains after 24 h of incubation (Figure 6a,b), and of *L. gasseri* strain after 48 h of incubation (Figure 6b). Interestingly, in the case of *L. acidophilus*, mushrooms of *P. ostreatus* (from all tested substrates) and *G. lucidum* (from BS:OLPR 1:1) induced a significant increase in bacterial levels compared to glucose after 48 h of incubation, while the rest of *G. lucidum* treatments exhibited similar growth levels compared to standard medium (Figure 6a).

The potential lactogenic effect of polysaccharide extracts from several *Pleurotus* spp. (e.g., *P. ostreatus*, *P. eryngii*, *P. citrinopileatus*, and *P. salmoneo-stramineus*) has been previously reported [30,58]. Furthermore, the growth of lactobacilli strains was supported by extracts of *G. lucidum* based on in vitro fermentation models [26,59,60] and animal studies [31,61]. The previously reported induction of *Lactobacillus* spp. growth by *P. ostreatus* and *G. lucidum* polysaccharides was also evident in the present work based on lyophilized samples of entire fruit-bodies. Our results were also in line with the fact that, in some cases, the growth rates of lactobacilli in mushroom extracts were higher than those of bifidobacteria, which is probably related to the more efficient fermentation profile of *Lactobacillus* spp. [26,58].

A more differentiated, strain-specific pattern of bacterial growth was observed in the case of bifidobacteria after 24 h and 48 h of incubation (Figure 6c,d). In detail, all mushroom treatments used as the sole carbon source-induced growth of *B. longum* within 24 h, similar to that of glucose control; moreover, this effect was also evident only in the case of *G. lucidum* cultured in BS:OLPR 1:1 or BS after a 48 h cultivation of *B. longum* (Figure 6d). In contrast, the growth of *B. bifidum* was significantly hindered in all mushroom substrates after 24 h and 48 h of incubation, with a more drastic effect (i.e., undetectable bacterial levels at both time points) observed in the case of *G. lucidum* in BS:TPOMW 1:1. Furthermore, no detectable growth of *B. bifidum* was recorded in *G. lucidum* BS:OLPR 1:1 and *P. ostreatus* WS:TPOMW 1:1 after 48 h of incubation (Figure 6c).

In the past, probiotic strains of *Bifidobacterium* spp. (e.g., *B. longum*, *B. pseudocatenulatum*) were used in order to determine the prebiotic capacity of extracts from *G. lucidum* [26,59,60] and *Pleurotus* spp. [30,58]. *B. bifidum* was tested for the first time as an indicator of mushroom prebiotic activity in the present study. In vitro and animal-based data have suggested the bifidogenic potential of *G. lucidum*

and *Pleurotus* spp. extracts, with an emphasis in the marked variability of the effect exerted by the bacterial strain and by differences in the chemical structure of polysaccharides [58–60]. The strain and substrate specific effects on bifidobacterial growth were also evident in our study, implying that the biological potency of the tested mushrooms could be modified by regulating the formulation of their cultivation substrate to meet the nutritional requirements of the probiotic strains examined. In line with previous data [26], our results suggested a short period of enhanced *B. longum* growth for both mushrooms, with a more prolonged bacterial growth, especially in the case of *G. lucidum* BS:OLRP. This result could be attributed to the presence of simple sugars (e.g., glucose) that are rapidly consumed by bifidobacteria and the substrate-dependent variation in the polysaccharides content of mushrooms [26,62]. In contrast, the *B. bifidum* growth on de Man Rogosa and Sharpe (MRS) culture medium was significantly higher compared to mushroom-based substrates in all cases, indicating that the latter were not suitable for this particular probiotic microorganism.



**Figure 6.** Populations of (a) *L. acidophilus*, (b) *L. gasseri*, (c) *B. bifidum*, and (d) *B. longum* at inoculation (T0) and after 24 and 48 h of incubation, which were grown on lyophilized *G. lucidum* and *P. ostreatus* mushroom powder as the sole carbon source. Mushrooms were cultivated on conventional substrates (BS and WS for *G. lucidum* and *P. ostreatus*, respectively) and on mixtures with two phase olive mill wastes (TPOMW) and olive tree pruning (OLPR). Columns represent means of bacterial populations ( $\log_{10}$  CFU  $\text{mL}^{-1}$ )  $\pm$  standard errors of means, ( $n = 4$ ). A lack of letters in common indicates statistically significant differences (Duncan's *t*-test,  $p < 0.05$ ) for comparisons of treatment means between different substrates.

### 3. Materials and Methods

#### 3.1. Biological Material

In the frame of this study, *G. lucidum* LGAM 9720 and *P. ostreatus* LGAM 1123 strains were examined. Both strains were previously isolated from the wild (Greece), were routinely maintained on potato dextrose agar (PDA; Conda, Spain), and were preserved in agar slants and as submerged cultures at 4 °C in the fungal Culture Collection of Laboratory of General and Agricultural Microbiology (Agricultural University of Athens, Athens, Greece).

### 3.2. Substrates for Fungal Growth—Determination of Mycelium Growth Rates

The suitability of the following media (mainly composed of olive-based by-products) to serve as substrates for fungal growth was evaluated: (i) Olive pruning residues (OLPR) were used alone or in mixtures with beech sawdust (BS) for *G. lucidum* or with wheat straw (WS) for *P. ostreatus* in ratios of 25, 50, and 75% (*w/w*, f.w.); (ii) two phase olive mill waste (TPOMW) and BS or WS were mixed as in case (i); and (iii) TPOMW was mixed with OLPR in ratios of 25 and 50% (*w/w*, f.w.); BS and WS alone were used as controls, while the moisture content of the substrates was 52–65%. Olive based by-products were obtained from an olive mill in Kalamata (Peloponnese, southwest Greece), while WS and BS were derived from a wood processing industry in Athens and a mushroom cultivation farm in Evvoia (central Greece), respectively. Glass ‘race tubes’ (200 × 30 mm) were filled with the aforementioned substrates, sterilized (121 °C, 1.1 atm, 1 h) and then inoculated with a 6 mm diameter agar plug taken from the actively growing periphery of *G. lucidum* or *P. ostreatus* developing on a Petri dish with PDA. Fungal growth took place in an incubation chamber at 25 °C in the dark. Mycelium growth was recorded daily until the substrate was completely colonized, and linear growth rates were calculated as previously described [63].

### 3.3. Mushroom Cultivation Substrates—Assessment of Production Parameters

On the basis of the results obtained through the comparative assessment of mycelium growth rates in ‘race tubes’ containing the substrates initially tested, five mixtures were selected for further examination in respect to mushroom production (i.e., BS or WS with OLPR in ratios of 3:1, 1:1, and 1:3, and BS or WS in mixtures with TPOMW in ratios of 3:1 and 1:1); BS and WS substrates were used as controls for *G. lucidum* and *P. ostreatus*, respectively.

For the inoculation of mushroom cultivation substrates, cereal grain spawn was prepared according to method previously described [15]. Then, polypropylene autoclavable bags were filled with 1 kg of substrate (moisture content: 50–68%), sterilized (121 °C, 1.1 atm, 1 h), and inoculated with spawn at a 5% *w/w* rate. Four replicates per substrate and strain were used. The incubation of substrates was performed at 25 °C in the dark; for primordia formation, the temperature and relative air humidity were set at 16 °C and 95%, respectively, and illumination was provided (700 lux m<sup>-2</sup>, 12h day<sup>-1</sup> with fluorescent lamps). As soon as primordia were formed, the CO<sub>2</sub> level was maintained at 800–1000 ppm, the temperature and relative humidity were set at 18 °C and 85%, respectively, and illumination was increased to 1000 lux m<sup>-2</sup>.

To evaluate the suitability of different substrates for supporting mushroom production, the following parameters were studied: (i) Incubation time, defined as the time between inoculation and complete colonization of the substrate by fungal hyphae; (ii) earliness, defined as the time elapsed between the day of inoculation and the day of primordia appearance, (iii) total yield, expressed as fresh weight of mushrooms harvested; and (iv) biological efficiency, calculated as the percentage ratio of fresh mushroom weight over the dry weight of the substrate. The entire cropping period lasted 90 and 120 days for *P. ostreatus* and *G. lucidum*, respectively.

Following harvest, *P. ostreatus* and *G. lucidum* mushrooms were freeze-dried in a Telstar Cryodos apparatus and milled to fine powder. Pertinent samples were stored at –20 °C until subjected to analyses.

### 3.4. Chemical Analysis of Mushrooms

Ash, crude fiber and crude fat were determined according to methods described by the Association of Official Agricultural Chemists, AOAC International [64]. Nitrogen content was assessed by a CHN elemental analyzer (Carlo Erba, EA1108, Isomass Scientific Inc., Calgary, Canada), and crude protein was calculated by employing the converting factor 4.38 [10]. Nitrogen-free extracts (total carbohydrates) were estimated by the formula: 100 – (moisture + protein + fat + ash contents), and gross energy (kcal

100 g<sup>-1</sup> f.w.) was calculated according to the equation: Energy = 4 × (g protein + g carbohydrate) + 9 × (g fat) [65].

The Mushroom and Yeast Beta-Glucan assay kit (Megazyme Int., Bray, Ireland) was used for the determination of total and α-glucans content in mushroom samples according to the manufacturer's instructions. The content of β-glucans was calculated by subtracting α-glucans from total glucans.

### 3.5. Measurement of Total Phenolic Content and Antioxidant Activity of Mushrooms Methanolic Extracts

Methanolic extracts were prepared as previously described [66]. In detail, 0.5 g of freeze-dried mushroom samples were extracted with 10 mL of methanol (48 h, 100 rpm at room temperature). Separation was performed by centrifugation in 2500 rpm (10 min), and the precipitate was re-extracted with 2.5 mL of the same solvent for 2 h. Then, the two supernatants were combined, concentrated to 2 mL, and maintained in GS vials tubes in deep freeze. The determination of total phenolic content (TPC) was carried out by the Folin–Ciocalteu method [67]. The antioxidant activity of methanolic extracts were determined as previously described [68] by measuring (i) the radical scavenging activity through the use of the stable free radical molecule DPPH and (ii) the reducing antioxidant potential through the ferric ion reduction activity power (FRAP). The antioxidant activity of mushroom methanolic extracts was evaluated in terms of radical scavenging activity and reducing antioxidant potential using the DPPH and the ferric ion reducing power (FRAP) assays, respectively, as previously described [68]. In the first assay, 0.025 mL of methanolic extract was added in 0.975 mL DPPH (0.1 mM in MeOH) in eppendorf tubes and vortexed. After the mixture was left to stand for 30 min in the dark, the reduction of DPPH was determined by measuring the absorption at 515 nm (U-2001 Spectrophotometer, Hitachi, Tokyo, Japan). DPPH alone served as the blank. In regard to the FRAP assay, 0.05 mL of each extract was added in 0.05 mL FeCl<sub>3</sub> solution (3 mM in 5 mM HCl) in an eppendorf tube, and then it was vortexed and incubated at 37 °C for 30 min. Then, 0.9 mL of 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) solution (1 mM in 0.05 M HCl) was added, and the absorbance of the product of the reaction between Fe<sup>2+</sup> and TPTZ was measured at 620 nm against a blank. For the blank, the FeCl<sub>3</sub> solution was replaced by distilled water. All assays were performed in triplicate, and the quantifications were based on calibration curves using syringic acid for TPC and Trolox for radical scavenging activity and FRAP.

### 3.6. FTIR Analysis

The FTIR spectra were recorded by a Nicolet 6700 spectrometer (ThermoScientific, Waltham, MA, USA) equipped with a deuterated triglycine sulfate (DTGS) detector (Nichrome source with a potassium bromide beam-splitter) and Omnic 7.3 software. For each freeze-dried sample, 64 scans of the infrared region between 4000 and 400 cm<sup>-1</sup> at a resolution of 4 cm<sup>-1</sup> were recorded in triplicates and averaged. Afterwards, the recorded spectra were transformed using the Kubelka–Munk algorithm, which corrects the lack of linearity between the spectral intensity and sample concentration and is applied in diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy where quantitative analysis or relevant comparisons are required. The Kubelka–Munk-transformed spectra were then smoothed by the Savitzky–Golay algorithm [5 points each side (total window of 11 smoothing points) and a zero-order polynomial], adaptively baseline corrected, and normalized by mean. All spectral transformations were performed by the SpectraGryph 1.2.7 software (<https://www.ffmpeg2.de/spectragryph/>).

### 3.7. Determination of Mushrooms Prebiotic Potential

The mushrooms' prebiotic properties were assessed for each species in three out of the six fungus/substrate combinations examined (and previously presented) by using lyophilized samples from fruit-bodies deriving from: (i) OLPR in mixture (1:1, w/w) with BS or WS for *G. lucidum* and *P. ostreatus*, respectively; (ii) TPOMW and BS or WS in the same ratio as in (i); and (iii) BS and WS alone (control substrates) for *G. lucidum* and *P. ostreatus*, respectively.

Four bacterial strains originally isolated from human feces, i.e., two *Lactobacillus* strains (*L. acidophilus* DSM20079 and *L. gasseri* from the Culture Collection of Harokopio University) and

two *Bifidobacterium* strains (*B. bifidum* DSM20456 and *B. longum* from the Culture Collection of Harokopio University), were cultivated in Man, de Rogosa, Sharpe (MRS) or modified MRS (mMRS), respectively, under anaerobic conditions at 37 °C. Modified MRS is commonly used for the cultivation of bifidobacteria; it differs from MRS by containing 0.05% HCl-cysteine, a reducing agent which lowers the oxido/reduction potential in culture media to ensure anaerobic conditions [69]. Activated cultures were subsequently inoculated (1% *v/v*) in culture media (MRS, mMRS, with and without glucose: controls) and in culture media with lyophilized mushroom samples as the sole carbon source (2% *w/v*). In order to avoid bacterial contamination from the non-sterile mushrooms, gentamicin was added in the mMRS medium only (4 mg L<sup>-1</sup>). Cultures were incubated under anaerobic conditions at 37 °C for 48 h (Bactron 1.5, SHELLAB, Cornelius, OR, USA). Samples were taken at 0, 24, and 48 h in order to test the viability of the bacterial strains based on plate-count techniques.

### 3.8. Statistical Analysis

Four replicates for each treatment were used in “race tubes” and in mushroom cultivation experiments. Results are presented as mean ± standard deviation. An analysis of variance followed by a Duncan’s *t*-test at 5% level of probability, which was performed for assessing differences between the means of the various substrates examined, while relationships between variables (at significance levels of 0.05 and 0.01) were determined by Pearson’s correlation coefficient through the use of SPSS (version 22, IBM, Armonk, NY, USA) software.

## 4. Conclusions

The supplementation *P. ostreatus* substrates by olive by-products increased total mushroom yields and reduced the time required for mushroom formation. In contrast, all alternative substrates had a negative effect on *G. lucidum* cultivation parameters. As concerns the crude composition of mushrooms, high ratios of OLPR to wheat-straw resulted in an increase of crude protein and a reduction of ash, crude fiber, and fat content in *P. ostreatus* fruit-bodies. On the other hand, *G. lucidum* mushrooms exhibited up to a three-fold increase in  $\alpha$ -glucan or a significant enhancement of  $\beta$ -glucan content when cultivated on OLPR-based substrates (in comparison to beech sawdust). In addition, several substrates based on olive by-products led to the production of fruit-bodies with increased total phenolic content and antioxidant activity. FTIR spectra confirmed the qualitative/quantitative differentiation of mushrooms composition and demonstrated their suitability as an inexpensive and fast method for determining relevant changes in fruit-bodies content. Moreover, mushroom powder supported/enhanced the growth of *L. acidophilus* and *L. gasseri* after 24 and/or 48 h incubation, while a strain-specific pattern was observed in bifidobacteria; both mushrooms hindered *B. bifidum* growth, and they supported a similar-to-glucose growth for *B. longum*.

**Author Contributions:** Carried out the mushroom cultivation experiments, carried out the analytical and statistical analyses, and wrote the original draft, G.K.; performed the experiments related to the assessment of the prebiotic potential of mushrooms, M.P., E.K.M., and M.K.; wrote the respective part of the manuscript, E.K.M. and A.K.; conducted determination of mushrooms glucan content, carried out the FTIR analysis, and wrote the respective part of the manuscript, G.B.; supervised different aspects of the study, G.I.Z., A.K., and P.A.T.; conceived and designed the experiments, G.I.Z., A.K., V.P., and G.K.; compiled the final version of the manuscript including revision and editing, G.I.Z.; contributed at reviewing and editing, A.K., E.K.M., G.B., P.A.T., V.P., and G.K.; responsible for project administration and funding acquisition, A.K., V.P., and G.I.Z. All authors have read and approved the final submitted manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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