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Comparative antioxidant and antimicrobial properties of *Lentinula edodes* Donko and Koshin varieties against priority multidrug-resistant pathogens

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ABSTRACT

The problematic increase in multidrug-resistant bacteria translates into the urgent need to discover novel and effective antimicrobial substances. Herein, mushrooms could be a promising alternative of natural source of new antimicrobials. The present work aimed to compare the phytochemical composition and antimicrobial activity of methanol and aqueous crude extracts of *Lentinula edodes* var. Koshin and Donko. Disk diffusion method was used to screen the antimicrobial activity and to assess the synergistic effect of the mushroom extracts. Microdilution method was used to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). Phytochemical characterization of mushrooms extracts was achieved by analysis of total phenols, *ortho*-diphenols content and its antioxidant activity. The results noticed a positive relation between phenolic compounds content, antioxidant activity, and antimicrobial capacity of the mushroom's extracts. The *L. edodes* var. Koshin aqueous extracts, which contained the highest amount of total phenolic compounds, exhibited the highest scavenging capacity of ABTS which, in turns, exhibited the highest antimicrobial efficacy in inhibiting the growth of methicillin-resistant *Staphylococcus aureus*. Moreover, the combination between mushrooms extracts and commercial antibiotics showed favorable synergistic effects against tested bacteria. These results suggest that *L. edodes* var. Koshin may represent an important and valuable therapeutic source of compounds to be used against multidrug-resistant bacteria.

1. Introduction

Nowadays, the world is witnessing a dramatic increase related to multidrug-resistant bacteria. The indiscriminate use of antibiotics, among other factors, has been contributed to the development of resistant organisms, leading to easily curable diseases becoming a serious problem (McAdam *et al.*, 2012).

In 2014, the World Health Organization advised that antibiotic resistance reveals serious worldwide threat to Public Health, highlighting the risks associated to the absence of alternative therapies against multidrug-resistant microorganisms (WHO, 2014). In 2015 the European Centre for Disease Prevention and Control estimated 671,689 infections in the EU and European Economic Area caused by multidrug-resistant bacteria, resulting in 33,110 deaths, most of them acquired in healthcare settings (Weist and Högberg, 2016). In 2017, WHO published its first list of antibiotic-resistant "priority pathogens" – a catalogue of 12 families of bacteria which pose the greatest threat to human health. This list was drawn up in a bid to guide and promote research and development of new antibiotics. It is divided into three categories according to the urgency of need for new antibiotics: *critical, high* and *medium priority* (WHO, 2017).

One of the most troubling problems with antibiotic resistance is healthcare associated infections (HCAIs). HCAIs are associated with prolonged hospital stay, which in turns leads, to an increasedcosts, morbidity and mortality (WHO, 2011). Hence, notwithstanding the impossibility to prevent bacteria evolution, it is important to discover novel, natural, and effective antimicrobial substances against pathogenic microorganisms resistant to conventional treatments. Naturally produced antimicrobials have gained an increase of popularity and, among them, mushrooms could be a promising alternative as source of

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new antimicrobials (Gyawali and Ibrahim, 2014; Zdenka *et al.*, 2016). In fact, mushroom species release several bioactive compounds, such as terpenoids, flavonoids, tannins, alkaloids, and polysaccharides which could be used as novel antibiotics. Herein, *Lentinula edodes* (shiitake mushrooms) has been attracted particular attention concerning its antimicrobial activity (Bender *et al.*, 2001). Therefore, in the present work, the *in vitro* antimicrobial activity of *Lentinula edodes* var. Donko and Koshin aqueous and methanolic extracts was screened against several clinical isolates of carbapenem-resistant *Klebsiella pneumoniae* and methicillin-resistant *Staphylococcus aureus* that belong to the WHO *critical* and *high priority* agent groups. Those clinical isolates are from CHTMAD – Hospital Center of Trás-os-Montes and Alto Douro, Portugal. The comparison between the two mushroom varieties was taken into account since there are substantial differences in morphology and growing conditions which may interfere on the content of bioactive compounds and biological activities. In fact, the var. Donko grows slowly at low temperatures, and present a thick cap, short stalk and dark brown color (Figure 1A). On the other hand, var. Koshin develops rapidly at mild temperatures and it has a thin cap and a pale coloration (Figure 1B) (Miles and Chang, 2004).

Considering the results of the antimicrobial activity, the most promising species extracts were combined with commercial antibiotics against different clinical isolates, including multidrug-resistant microorganisms. Moreover, for the first time, the two varieties of *L. edodes* were compared regarding the antimicrobial activity and phytochemical composition.

2. Material and Methods

2.1. Chemicals and drugs

Methanol was of analytical grade purity from Lab-Scan (Lisbon, Portugal). The culture media Brain Heart Infusion agar (BHIA), Mueller Hinton broth (MHB), Mueller Hinton agar (MHA) and all antibiotics were obtained from Oxoid (Humpshire, UK). The saline solution was prepared with NaCl from Merck (Darmstadt, Germany). The dye resazurin was purchased from Sigma–Aldrich (St Louis, MO, USA) to be used as microbial growth indicator.

2.2. Mushrooms material

Lentinula edodes var. Donko and Koshin mushrooms, produced in Floresta Viva company (Amarante, Portugal), were stored at − 20°C, freeze-dried (Dura Dry TM μP, −41°C and 500 mTorr) and grounded to a fine powder.

2.3. Lentinula edodes extracts

Mushrooms extracts were obtained by two different extraction methods: (1) Exhaustive aqueous extracts: 5g of dried mushrooms material was added to 150 mL of distilled water. The mixture was agitated at room temperature (orbital shaker, one hour, 150 rpm), then centrifuged. The supernatant was filtered (Whatman no. 4 filter paper), and again 100 mL of distilled water was added to the pellet. The whole procedure was repeated to a total of 4 times. The total extracted was stored at −20°C before lyophilization to obtain the final extract; (2) Exhaustive methanolic extracts: 5g of dried mushrooms material was added to 150 mL of a 80% methanol solution (methanol/distilled water v/v). The mixture was agitated at room temperature (orbital shaker, one hour, 150 rpm), then centrifuged. The supernatant was filtered and again 100 mL of the previous solution was added to the pellet. The whole procedure was repeated to a total of 4 times. The total extracted volume was concentrated in a vacuum rotary evaporator at 38°C to remove methanol and stored at −20°C before lyophilization to obtain the final extract.

2.4. Microorganisms and culture media

The microorganisms used were clinical isolates from patients hospitalized in various departments of Hospital Centre of Trás-os-Montes and Alto Douro (CHTMAD) - these are located in the cities of Lamego, Peso da Régua, Chaves, and Vila Real, Portuguese north province of Trás-os-Montes and Alto Douro. Ethical approval for this study was granted by the Ethics Committee of Hospital Vila Real (CHTMAD), according to a research collaboration protocol established in 2004. These strains belong to MJH and MJMC collections and are stored at −70°C in aliquots of BHI (Brain Heart Infusion) medium with 15% (v/v) glycerol, in the Microbiology Laboratory of the Veterinarian Science Department at UTAD. Six Gram-negative bacteria, *Klebsiella pneumonia* isolated from biological fluids (MJH 513, MJH 569, MJH 579, MJH 599, MJH 602, MJH 640, MJH 662), and thirteen Gram-positive bacteria, methicillin-sensitive *Staphylococcus aureus* (MSSA) (MJMC 018, MJMC 026, MJMC 109, MJMC 110, MJMC 511) and methicillin-resistant *S. aureus* (MRSA) (MJMC 025, MJMC 027, MJMC 102, MJMC 111, MJMC 507, MJMC 534 B, MJMC 539, MJMC 545, MJMC 552), isolated from wound exudates, were used to screen the antimicrobial activity of the mushroom extracts. All strains were identified by morphological and biochemical tests (morphological identification of colonies, Gram staining, conventional biochemical identification methods and MicroScan WalkAway identification panels), followed by Kirby-Bauer antibiotic sensitivity assays, using different antibiotics (10 μg).

Escherichia coli (CETC 434) and *Staphylococcus aureus* (CETC 976) strains were obtained from *Spanish Type Culture Collection (CETC)*. Ethics approval for this study was granted by the Ethics Committee of Hospital

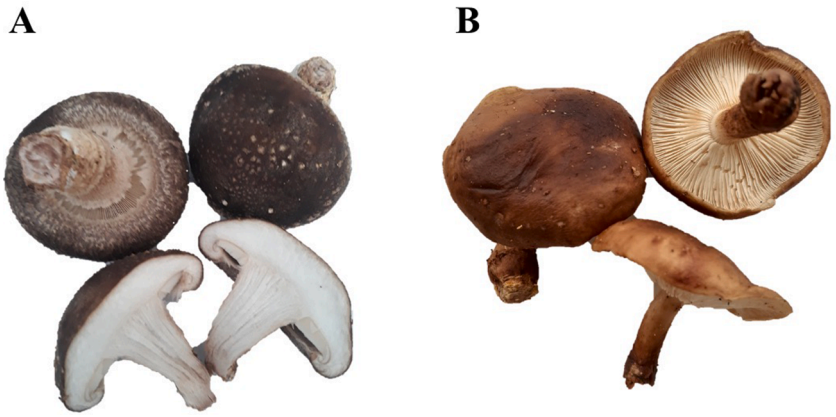

Figure 1. *Lentinula edodes* Donko **(A)** and Koshin **(B)** varieties

Vila Real.

2.5. Antimicrobial activity

Briefly, bacterial suspension with the turbidity adjusted to 0.5 McFarland standard units, were spread with a sterile cotton swab onto Petri dishes (90mm of diameter) containing 4 mm of Mueller-Hinton agar. Six-millimeter diameter sterile paper discs were dispensed on the seeded agar plates and imprinted with 12 μL of 1000 $\mu g.mL^{-1}$ extracts solution (in Dimethyl Sulfoxide (DMSO) 10%). Incubation for 24 h at 37°C, followed by diameter measurement (mm) of the clear inhibitory zones around the discs imprinted with the extracts. In all experiments, a negative control (12 μL DMSO) and a positive control [standard commercial antibiotic gentamicin (10 μg)] were included.

The antimicrobial activity was classified according to the following scheme: noneffective (-): inhibition halo = 0; moderate efficacy (+): 0 < inhibition halo < antibiotic inhibition halo; good efficacy (++): antibiotic inhibition halo < inhibition halo < 2x inhibition halo; strong efficacy (+++): inhibition halo > 2x antibiotic inhibition halo (Aires *et al.*, 2009).

Minimum inhibitory concentration (MIC, lowest concentration of mushroom extract able to inhibit bacterial growth) was evaluated by a resazurin microdilution assay (Sarker *et al.*, 2007). Bacteria tested were picked from overnight cultures in BHIA. A small portion of bacteria was transferred into a bottle with 50 mL of MHB, capped and placed in an incubator overnight at 37°C. After 16 h of incubation, bacterial suspension was adjusted to an optical density of 0.5 measured at OD500 nm. The resazurin solution (3.4 $mg.mL^{-1}$) was prepared in sterile distilled water. A 96-wells sterilized microplate was used and a volume of 100 μL of MHB was used in each well, together with 200 μL of extract solution, or positive control. From the first well (belonging to the first horizontal line) 100 μL was taken and added to the next well and then this step was repeated to each of the following wells in the vertical line, allowing a serial fold dilution of decreasing concentration (range of 1000 $\mu g.mL^{-1}$ to 7.81 $\mu g.mL^{-1}$). In addition, 20 μL of bacterial suspension and 20 μL of resazurin solution was added to each well. Microplates were incubated at 37°C for 18–24 h. All tests were performed in triplicate and MIC was then assessed visually by the color change of resazurin in each well (blue to pink in the presence of bacteria growth). For the determination of minimum bactericidal concentration (MBC, the lowest concentration of mushroom extract at which bacterial growth by at least 99.0%), the content from each well without changes in color was plated on Mueller-Hinton Agar and incubated at 37 °C for 24 h. The lowest concentration that yielded no growth after this subculturing was taken as the MBC.

2.5.1. Synergistic effect

The screening of synergistic effects was performed by the disk diffusion method in solid medium. Taking into account the antimicrobial results, the synergistic effect was only evaluated for *L. edodes* var. Koshin. Two discs were dispensed into the plates by antibiotic and one of them was impregnated with 12 μL of the mushroom extract solution. Seven antibiotics were used: gentamicin (CN), amoxicillin/clavulanic acid (AMC), ciprofloxacin (CIP), vancomycin (VA), imipenem (IPM), ertapenem (ETP) and meropenem (MEM).

2.6. Determination of total phenolic compounds

The total phenolic compounds in the extracts were determined by the Folin–Ciocalteu method as previous described (Gouvinhas *et al.*, 2017), with some modification. Briefly, 10 μL of mushrooms extract at a concentration of 1 $mg.mL^{-1}$ or gallic acid standards (0.01 to 1.0 $mg.mL^{-1}$ in methanol) were mixed with 185 μL of distilled water in a 96-well plate followed by the addition of 25 μL of Folin-Ciocalteu reagent. After an incubation period of 5 min, sodium carbonate (75 μL of 7% Na_2CO_3) was added and further incubated for 2 h in the dark and at room temperature. The absorbance was then measured at 725 nm against a blank on a Biotek Powerwave XS2 plate reader (BioTek Instruments, Inc. USA) at 25 °C. The phenolic content was expressed as mg gallic acid equivalents *per* gram of extract (mg GAE/g dry weight).

2.7. Ortho-diphenols content

For the analysis of the ortho-diphenols content, a colorimetric method, based on a complex reaction with sodium molybdate dehydrate, was applied (Ferreira *et al.*, 2020). Briefly, extract aliquots (60 μL at a concentration of 1 $mg.mL^{-1}$) or gallic acid standards (0.01 to 1.0 $mg.mL^{-1}$ in methanol) were reacted for 25 min with 200 μL of a sodium molybdate dihydrate solution (5% prepared in ethanol/water, 1:1 v/v). The absorbance of the samples and standards was measured at 370 nm against to blank (ethanol/water 1:1, v/v) on a plate reader at 25 °C. The results were expressed as mg of gallic acid equivalents *per* gram of extract (mg GAE/g dry weight).

2.8. Radical Scavenging Activity on ABTS radical

The radical scavenging activity of both mushroom extracts on ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation was measured using a spectrophotometric methodology, according to a previously described procedure (Ferreira *et al.*, 2020). Briefly, an ABTS radical cation stock solution (7.4 mM) was prepared by reacting equal amounts of ABTS and potassium persulfate (2.6 mM) and allowing to stand 16 h in the dark and at room temperature. The radical solution was then diluted with methanol to an absorbance of 0.70 (±0.02) at 734 nm at 25 °C. The samples (10 μL ranging from 0.0625 to 1 $mg.mL^{-1}$) were mixed with 190 μL of the radical solution and after 6 min incubation the absorbance was measured. The results were expressed as Trolox equivalent antioxidant capacity (mM Trolox / g dry weight).

2.9. Statistical Analysis

For the evaluation of antimicrobial activity, all assays were carried out in duplicate and the results are expressed as mean ± standard deviation. Regarding the chemical characterization of the extracts, all assays were performed in triplicate. Data are expressed as mean ± standard deviation and were statistically analyzed by one-way analysis of variance (one-way ANOVA), followed by Holm-Sidak's multiple comparison test. Statistical analyzes were performed using GraphPad Prim for Windows (Version 7) and differences were considered significant when $p < 0.05$ (95% significance).

3. Results

3.1. Antimicrobial susceptibility of clinical isolates

Concerning the assessment of antibiotic susceptibility, the results revealed that some of the clinical isolates used are multidrug-resistant bacteria (Tables 1 and 2) being a problem due to the insufficient alternatives of effective antibiotics. As seen in Table 1, in Gram-positive clinical bacteria isolated from wound infections, the methicillin, ciprofloxacin and levofloxacin had the highest antibiotics resistance. The clinical isolates MJMC 534 B, MJMC 539 and MJMC 552 are resistant to 7, 8 and 9 antibiotics, respectively, being the most multidrug-resistant bacteria. Gentamicin and vancomycin are the most effective antibiotics, as thirteen of the fourteen isolates are sensitive to those antibiotics.

Concerning to Gram-negative clinical bacteria isolated from different types of biological samples (Table 2), all microorganisms were resistant to amoxicillin / clavulanic acid and ertapenem, a carbapenem used as a last resort in the treatment of Extended-Spectrum β-lactamase (ESBL) -producing bacterial infections. The clinical isolates MJH 569 and MJH 599 have the highest number of antibiotic resistances, being evident the

Table 1
Susceptibility of different antibiotics against Gram-positive bacteria.

	MJMC018	MJMC025	MJMC026	MJMC027	MJMC102	MJMC109	MJMC110	MJMC111	MJMC507	MJMC511	MJMC 534 B	MJMC539	MJMC545	MJMC552
Penicillin	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	R	R	R
Methicillin	S	R	S	R	R	S	S	R	R	S	R	R	R	R
Ampicillin	nd	nd	nd	nd	nd	nd	nd	nd	nd	R	nd	nd	nd	R
Amoxicillin/ Cavulanic acid	R	S	R	S	R	S	S	R	R	S	R	R	S	R
Oxacillin	nd	nd	nd	nd	nd	nd	nd	nd	R	S	R	nd	R	R
Clindamycin	nd	nd	nd	nd	nd	nd	nd	nd	I	I	R	R	nd	R
Daptomycin	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	S	S	nd	nd
Erythromycin	nd	nd	nd	nd	nd	nd	nd	nd	nd	S	R	R	nd	R
Gentamicin	S	S	S	S	S	S	S	S	S	S	S	R	S	S
Ciprofloxacin	R	S	R	R	R	S	S	R	R	S	R	R	S	R
Levofloxacin	R	S	R	R	R	S	S	R	R	S	R	R	S	R
Linezolid	nd	nd	nd	nd	nd	nd	nd	nd	S	nd	S	S	S	S
Trimethoprim/ Sulfamethoxazole	nd	nd	nd	nd	nd	nd	nd	nd	S	S	S	S	S	S
Vancomycin	S	S	S	S	S	S	S	S	S	nd	S	S	S	S

S- Susceptible; I- Intermediate; R- Resistant (this classification was made according to the interpretative breakpoints suggested by Clinical and Laboratory Standards Institute-CLSI); nd- not determined.

low sensitivity to the β-lactam antibiotic group.

3.2. Antimicrobial activity evaluation of extracts

The *L. edodes* extracts were tested at concentration of 500 μg.mL^{-1} and it was observed that by increasing the concentration to 1000 μg.mL^{-1}, the size of the inhibition halos also increased. Therefore, only the results obtained with the concentration of 1000 μg.mL^{-1} are presented in this work. Data obtained on screening of antimicrobial activity of the two varieties of *L. edodes* extracts, against Gram-positive and Gram-negative bacteria, are shown in Table 3. Concerning to aqueous extracts, the bacterial inhibition halos varied between 7 – 11 mm and between 7 – 16 mm for var. Donko and var. Koshin, respectively (Figure 2 and Table 3). Methanolic extracts of both varieties were not effective against either isolate tested. Also, Gram-negative isolates were resistant to all extracts tested.

Therefore, and according to the classification scheme proposed by Aires et al. (2009), the aqueous extract was "moderately effective" against all Gram-positive isolates, and "noneffective" against all Gram-negative isolates. The methanolic extract was showed to be "noneffective" for the 23 isolates tested (Table 3).

Since the most promising results were obtained with *L. edodes* var. Koshin mushrooms extracts, the MIC of both extracts (aqueous and methanolic) was only determined for this variety in Gram-positive clinical isolates (Table 4). In aqueous extract, the MIC values ranged from 15.63 to 250 μg.mL^{-1}, and in methanolic extract, the values ranged from 31.25 to 1000 μg.mL^{-1}. The best antimicrobial activity was achieved for MJMC 102 and MJMC 534 B clinical wound infection isolates and, in the MIC assay, they were the most sensitive. Noteworthy, the methanolic extract, that was not effective in the disc diffusion method, was efficient against all strains tested and the MIC values ranged from 500 to 1000 μg.mL^{-1}. Regarding to MBC, the lowest concentration with bactericidal effect was 500 μg.mL^{-1}, to MJMC 111 and MJMC 552 with aqueous extract, and to MJMC 534 B with methanolic, all MRSA isolates.

3.3. Synergistic effect

Data obtained in the assessment of synergistic effect between *L. edodes* var. Koshin aqueous and methanolic extracts and commercial antibiotics are shown in Figure 3 and 3. Regarding to Gram-positive bacteria, both extracts exhibiting favourable synergistic effects with gentamicin (CN), vancomicyn (VA) and ciprofloxacin (CIP). No change in the "Resistant" to "Sensitive" profile was observed in the antibiotics tested, however the disk diffusion method showed an increase in halos size, in some cases approaching the value stipulated by the EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines to change from" Resistant" to "Sensitive", such as the combination of aqueous extract + amoxicillin / clavulanic acid (AMC) in the MJMC 534 B ulcer clinical isolate (increased from 11 mm to 19 mm). Antagonistic effects were also observed with the combination of extracts and amoxicillin/clavulanic acid (AMC) antibiotic.

Gram-negative bacteria (Figure 4) did not showed synergistic effects between extracts and the tested antibiotics. On the other hand, for meropenem (MEM) a decrease of ± 10 mm in halos size was observed, which indicated antagonism effect, and even a change in susceptibility profile (from "Sensitive" to "Resistant").

3.4. Total phenolic compounds and ortho-diphenols components

The total phenolic compounds content in the extracts varied significantly for the two *L. edodes* varieties and extraction solvent used (Figure 5A). Comparing the methanolic and aqueous extracts from var. Koshin (2.07 ± 0.12 and 4.09 ± 0.59 mg GAE/g dry weight, respectively), the results showed that there were significant differences concerning the extraction solvent used, with the aqueous solvent presenting a higher total phenolic compounds content than methanol. Noteworthy,

Table 2
Susceptibility of different antibiotics against Gram-negative bacteria.

	MJH 513	MJH 569	MJH 579	MJH 599	MJH 602	MJH 640	MJH 662
Ampicillin	nd	R	nd	R	nd	R	nd
Amoxicillin/ Clavulanic acid	R	R	R	R	R	R	R
Piperacillin/ Tazobactam	R	R	nd	R	R	R	R
Imipenem	I	I	I	I	I	I	I
Ertapenem	R	R	R	R	R	R	R
Meropenem	R	R	I	S	I	R	I
Cefuroxim	nd	R	nd	R	nd	R	nd
Cefoxitin	R	R	nd	S	R	nd	nd
Cefotaxime	R	ESBL	nd	ESBL	R	R	R
Ceftazidime	nd	ESBL	nd	ESBL	nd	R	R
Ciprofloxacin	R	R	nd	R	R	S	S
Fosfomycin	nd	S	nd	S	nd	S	nd
Nitrofurantoin	nd	R	nd	nd	nd	S	nd
Norfloxacin	nd	R	nd	R	nd	S	nd
Levofloxacin	nd	R	nd	R	nd	S	nd
Gentamicin	S	S	S	S	S	S	I
Trimethoprim/ Sulfamethoxazole	R	R	nd	R	nd	S	R
Tobramycin	R	R	nd	R	nd	S	nd
Amikacin	I	I	nd	I	I	S	I
Colistin	S	nd	nd	nd	S	S	S
Tigecycline	S	nd	nd	nd	S	nd	S

S- Susceptible; I- Intermediate; R- Resistant; ESBL- Extended-spectrum beta-lactamases (this classification was made according to the interpretative breakpoints suggested by Clinical and Laboratory Standards Institute-CLSI); nd- not determined.

Table 3
In vitro antimicrobial activity of positive control and aqueous and methanolic extracts of *L. edodes* var. Donko and Koshin (1000 $\mu g.mL^{-1}$), determined by the diameter of inhibition zones (mm).

		AQUEOUS EXTRAC		METHANOLIC EXTRAC		CONTROL	
	Isolate	Donko	Koshin	Donko	Koshin	CN	DMSO
GRAM +	MJMC 018	7 ± 0.0 (+)	10 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{24 \pm 0.2}$	6 ± 0.0
	MJMC 025	8 ± 0.0 (+)	8 ± 0.0 (+)	6 ± 0,0 (-)	6 ± 0,0 (-)	S $^{22 \pm 0.2}$	
	MJMC 026	8 ± 0.0 (+)	7 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{22 \pm 0.0}$	
	MJMC 027	8 ± 0.0 (+)	7 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{22 \pm 0.05}$	
	MJMC102	8 ± 0.0 (+)	12 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{25 \pm 0.5}$	
	MJMC109	9 ± 0.0 (+)	11 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{25 \pm 0.5}$	
	MJMC110	9 ± 0.0 (+)	11 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{25 \pm 0.5}$	
	MJMC111	9 ± 0.0 (+)	10 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{24 \pm 1.0}$	
	MJMC507	10 ± 0.0 (+)	10 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{25 \pm 0.0}$	
	MJMC511	10 ± 0.0 (+)	10 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{26 \pm 0.5}$	
	MJMC534 B	11 ± 0.0 (+)	16 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{27 \pm 0.0}$	
	MJMC 539	9 ± 0.0 (+)	9 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	R $^{15 \pm 0.15}$	
	MJMC 545	7 ± 0.0 (+)	8 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{23 \pm 0.25}$	
	MJMC 552	9 ± 0.0 (+)	9 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{25 \pm 0.2}$	
	CETC976	7 ± 0.0 (+)	7 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{22 \pm 0.0}$	
GRAM -	MJH513	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	S $^{17 \pm 0.0}$	6 ± 0.0
	MJH569	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	S $^{21 \pm 0.0}$	
	MJH579	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	S $^{19 \pm 0.5}$	
	MJH599	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	S $^{18 \pm 0.0}$	
	MJH602	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	R $^{22 \pm 0.5}$	
	MJH640	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	S $^{20 \pm 0.0}$	
	MJH662	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	R $^{13 \pm 0.0}$	
	CETC434	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	S $^{20 \pm 0.0}$	

Results are expressed as mean ± SD (standard deviation) of 2 replicates. Note: 6 mm value corresponds to disc diameter.

the amount of total phenolic compounds content in aqueous var. Koshin extract (4.09 ± 0.59 mg GAE/g dry weight) was significantly higher than in the aqueous extract from var. Donko extract (2.12 ± 0.49 mg GAE/g dry weight), suggesting that *L. edodes* varieties may differ on the content of phenolics compounds.

The overall trend observed for the total phenolic compounds (Figure 5A) content was also observed for the *ortho*-diphenols content (Figure 5B), i.e. there were significant differences between varieties of *L. edodes* and between the solvents used for extraction. As observed for the total phenolic compounds content, the content of *ortho*-diphenols in aqueous extracts from var. Koshin (0.11 ± 0.02 mg GAE/g dry weight) was significantly higher than in methanolic extract (0.04 ± 0.01 mg GAE/g dry weight), indicating that there were significant differences between the solvents used for extraction. Likewise, the content of *ortho*-diphenols in aqueous of var. Koshin extract (0.11 ± 0.02 mg GAE/g dry weight) was significantly higher than *ortho*-diphenols in aqueous var. Donko extract (0.08 ± 0.01mg GAE/g dry weight) in line with previously described for the total phenolic compounds content of the extracts.

3.5. *In vitro* Antioxidant activity

As shown in Figure 6, a higher ABTS•+ radical scavenging activity was observed for the aqueous extract of var. Koshin (1.53 ± 0.13 mmol Trolox / g dry weight) when compared with the methanolic extract of var. Koshin (0.77 ± 0.10 mmol Trolox/ g dry weight). Noteworthy, there was observed a significant difference between the aqueous extract of var. Koshin (1.53 ± 0.13 mmol Trolox / g dry weight) and aqueous extract of var. Donko (1.17 ± 0.10 mmol Trolox / g dry weight).

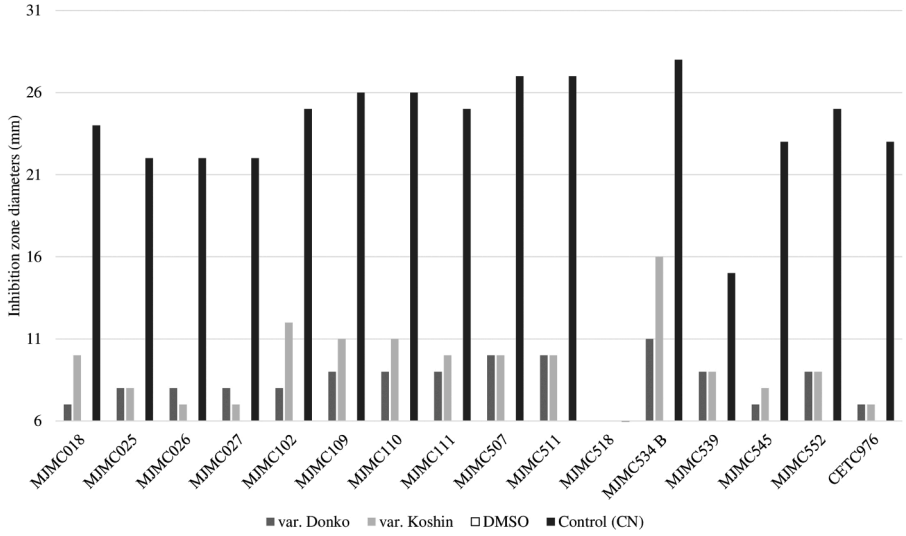

Figure 2. *In vitro* antimicrobial activity of positive control (Gentamicin) and aqueous extracts of *L. edodes* var. Donko and Koshin (1000 μg.mL^{-1}) against Gram-positive isolates, determined by the diameter of inhibition zones (mm).

Table 4
Minimum Inhibitory Concentration (μg.mL^{-1}) and Minimum Bactericidal Concentration for aqueous and methanolic extracts of *L. edodes* var Koshin.

		AQUEOUS EXTRACT		METHANOLIC EXTRACT		CONTROL
	Isolate	CMI	CMB	CMI	CMB	CMI
MRSA	MJMC 025	250	>1000	1000	>1000	125
	MJMC 027	125	>1000	500	>1000	125
	MJMC102	62.5	>1000	250	1000	7.81
	MJMC111	62.5	500	500	>1000	7.81
	MJMC507	125	>1000	250	1000	7.81
	MJMC534 B	62.5	1000	500	500	7.81
	MJMC 539	125	>1000	500	>1000	62.5
	MJMC 545	125	>1000	500	>1000	125
	MJMC 552	250	500	500	1000	125
MSSA	MJMC 018	62.5	>1000	500	>1000	7.81
	MJMC 026	125	>1000	1000	1000	125
	MJMC109	125	1000	1000	>1000	125
	MJMC110	62.5	1000	500	1000	62.5
	MJMC511	62.5	1000	500	1000	7.81
	CETC976	15.63	n.d.	31.25	n.d.	7.81

Moreover, the ABTS•+ radical scavenging activity of the aqueous extract of var. Donko (1.17 ± 0.10 mmol Trolox / g dry weight) was significantly higher than the methanolic extract of var. Koshin (0.77 ± 0.10 mmol Trolox/ g dry weight) (Figure 6). These results are in accordance with the results obtained for the total phenolic compounds and *ortho*-diphenols contents of both methanolic and aqueous extracts. On the other hand, no differences in the ABTS+. scavenging activity were observed between the methanolic extracts of var. Koshin and Donko.

4. Discussion

The rapid emergence of resistant bacteria is universal compromising the efficacy of the existing available antibiotics. Currently, the most notorious multidrug-resistant bacteria have been identified as the so-called "ESKAPE" which includes *S. aureus*, and *K. pneumoniae* being responsible for significant high morbidity and mortality (Li and Webster, 2018).

According to the OECD's projections, by 2050 Italy, Greece and Portugal "will have the highest mortality due to antimicrobial resistance" among EU members (OECD, 2018). Hence, there is an urgent need for the development of new and effective drugs against current multidrug-resistant pathogens (Zaman *et al.*, 2017). Natural resources have been widely exploited, being fungal species an important potential source of bioactive compounds with exceptional therapeutic value (Poucheret *et al.*, 2006). Herein, the two varieties of *Lentinula edodes* were assessed as an alternative to fight antibiotics resistance. Accordingly, the present study aimed to evaluate the antimicrobial properties of *L. edodes* var. Koshin and Donko aqueous and methanolic extracts and its synergistic effect with current clinical antimicrobials in multidrug resistant bacteria isolates obtained from a Portuguese hospital. Noteworthy, for the first time, the two varieties of *L. edodes* were compared concerning to phytochemical composition and antimicrobial activity.

The evaluation of antimicrobial activity, by disc diffusion method, revealed that both aqueous extracts were effective against Gram-positive bacteria (MSSA and MRSA), and both methanolic extracts were not effective against either isolates. Aqueous extracts from the *L. edodes* var. Koshin showed the best results, since the highest antimicrobial activity was obtained against to MRSA MJMC 534B. This is a result of great importance since the incidence of MRSA, especially in Portuguese hospitals, is one of the most important in Europe (45%) (ECDC, 2017). Noteworthy, it should be pointed out that MRSA MJMC 534B is clinical wound infection isolate causing high morbidity and mortality; longer hospital stays, delay in wound healing, increase economic burden and discomfort. Therefore, these results obtained with aqueous extracts on Gram-positive isolates are an important step towards to search for new effective agents against *S. aureus* in infected wounds. These results are in accordance with previous reports, in which different extracts from 48 mushroom species including *L. edodes*, were evaluated and it was observed that Gram-positive bacteria were more sensitive than Gram-negative bacteria. Among all extracts, the aqueous extracts of *Clitocybe geotropa* and *Lentinula edodes* showed the highest antimicrobial activity against all strains tested (Venturini *et al.*, 2008). In agreement, in another study, the aqueous *L. edodes* demonstrated notable antimicrobial activity against MRSA (Hearst *et al.*, 2009). The antimicrobial differences between the two *L. edodes* varieties and two solvents (aqueous and methanol) may be due to the total phenols and *ortho*-diphenols contents present, which was higher for the aqueous extract of the Koshin variety. In fact, several authors have previously associated the antimicrobial activity of different natural sources to phenolic compounds (Barros *et al.*, 2008; Alves *et al.*, 2012). Usually, the differences observed between varieties can be explained by several factors, namely, genetic, physiological and morphologic characteristics, agroclimatic conditions and ripening stage and in this case, the differences in phenols content, as well as antioxidant activity are probably,

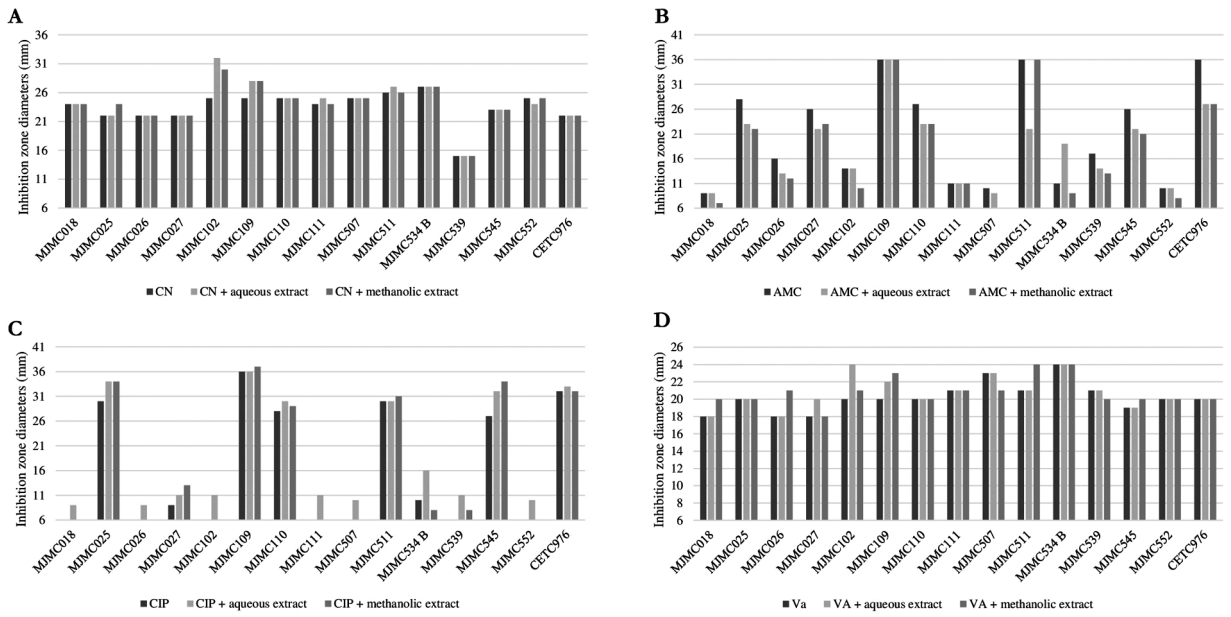

Figure 3. Inhibition zone diameter (mm) in the synergistic effect assessment with combination of *L. edodes* var Koshin aqueous/methanolic extracts and (A) Gentamicin, (B) Amoxicilin /clavulanic acid, (C) Ciprofloxacin, (D) Vancomycin on Gram-negative isolates.
Guidelines EUCAST/CLSI for *Staphylococcus aureus*: CN S ≥18 mm; AMC) ≥20; CIP S ≥21 mm; VA S ≥12 mm.

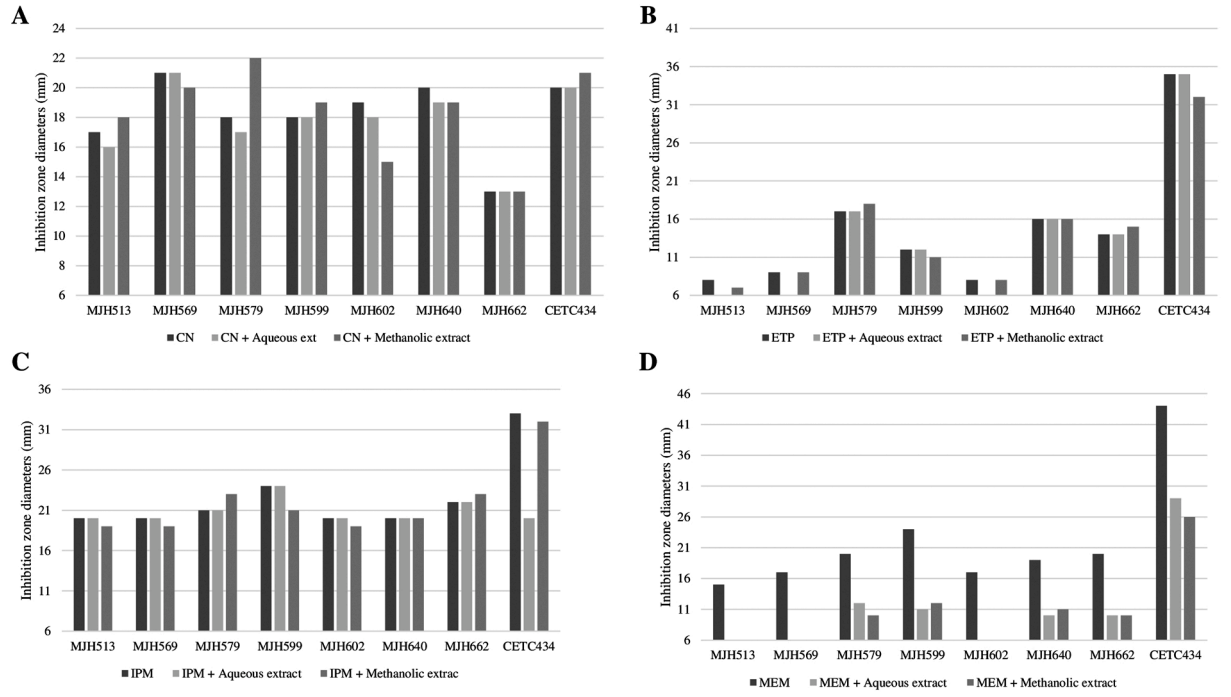

Figure 4. Inhibition zone diameter (mm) in the synergistic effect assessment with combination of *L. edodes* var Koshin aqueous/methanolic extracts and (A) Gentamicin, (B) Ertapenem, (C) Imipenem, (D) Meropenem on Gram-negative isolates.
Guidelines EUCAST/CLSI for *Klebsiella pneumoniae*: CN S ≥14 mm; ETP S ≥25 mm; IPM S ≥17 mm; AMC S ≥19 mm; MEM S ≥16 mm.

mainly due to genetic, morphologic characteristics and growing conditions.

It should be highlighted that more studies should be performed in order to elucidate the mechanism of bacteriostatic or bactericide effect and the specific phenolic/ *ortho*-diphenols compounds found in these extracts should be tested against selected bacteria in order to identify molecules responsible for the mushrooms bioactivity.

In the MIC determination, methanolic extracts also had antimicrobial activity, however with higher MIC values. These results do not match those obtained in disc diffusion method. However, the absence of an inhibition zone does not necessarily mean that the extract is not effective against that microorganisms, although rather that the diffusion was not complete, especially for the less polar compounds that diffuse more slowly into the culture medium (Moreno *et al.*, 2006).

Regarding the evaluation of synergistic effect, this is a pioneer study between *L. edodes* different extracts and commercial antibiotics and constitutes an important step, since the current available data are mainly correlated with plant extracts and not with mushroom extracts

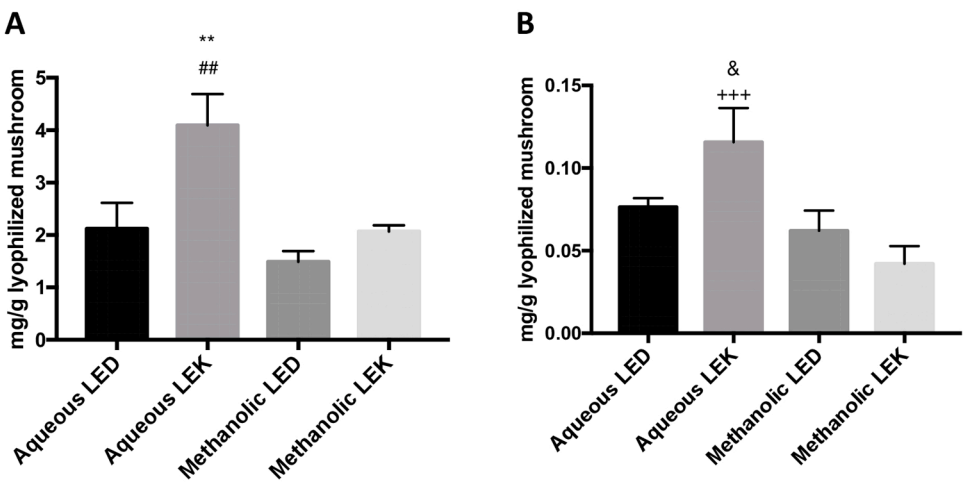

Figure 5. Total phenols **(A)** and ortho-diphenols **(B)** composition in aqueous and methanolic extracts from *L. edodes* var. Donko and Koshin.
Results are presented as mean ± standard deviation (n =3). Statistical comparisons were made using the one-way ANOVA, followed by the Holm-Sidak's multiple comparisons test, ($^{**}p< 0.01$ *vs.* aqueous var. Donko extract; $^{\#\#}p < 0.01$ *vs.* methanolic var. Koshin extract; $^{\&}p< 0.05$ *vs.* aqueous var. Donko extract; $^{+++}p < 0.001$ *vs.* methanolic var. Koshin extract).

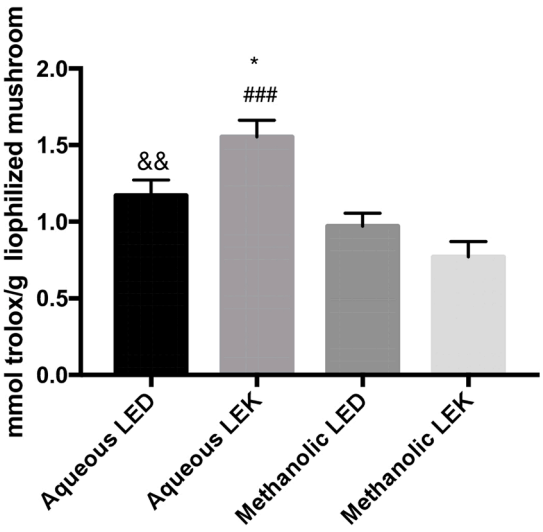

Figure 6. Antioxidant properties of aqueous and methanolic extracts from *L. edodes* var. Donko and Koshin expressed as Trolox equivalent antioxidant capacity.
Results are presented as mean ± standard deviation (n =3). Statistical comparisons were made using the one-way ANOVA, followed by the Holm-Sidak's multiple comparisons test, ($^{*}p< 0.05$ *vs.* aqueous var. Donko extract; $^{\#\#\#}p < 0.001$ *vs.* methanolic var. Koshin extract; $^{\&\&}p< 0.01$ *vs.* methanolic var. Koshin extract).

(Alves *et al.*, 2014). The synergistic effect between mushrooms extracts and antibiotic may constitute a strategy employed for protection against increasing microorganism's resistance. The results obtained showed that there was a synergistic effect between both mushrooms extracts and some standard antibiotics for Gram-positives bacteria namely gentamicin, vancomicyn, and ciprofloxacin. No change in the "Resistant" to "Sensitive" profile were observed, however, in some cases, the halo value size approached the value determined by EUCAST to change from "Resistant" to "Sensitive". Similar to this result, Alves *et al.* (2014) reported synergistic effect against MRSA using methanol and aqueous extracts from different wild mushroom species, and synergistic effects were observed for two quinolones (ciprofloxacin and levofloxacin) and for some β-lactams (penicillin, ampicillin and cefoxitin). Other authors have also reported synergistic effects of plant extracts and quinolones (ciprofloxacin and levofloxacin) against MRSA (An *et al.*, 2011).

Antagonistic effects were also observed with extracts + amoxicillin/clavulanic acid combination, which may be explained by the amount of metabolites present in the extracts that may lead to decrease of the active substances action, through competitiveness by the same action target and / or through inhibition of antibiotic active principle. Gram-negative isolates showed antagonism with the addition of extracts to meropenem. The carbapenem group represents a last line in the fight against multidrug resistant bacterial infections, due to the broad spectrum of action against Gram-negative and Gram-positive bacteria, including ESBL-producing microorganisms. Carbapenems act by inhibiting bacterial cell wall synthesis by binding and inactivating PBPs (Penicillin-binding proteins). The intensity of binding to PBPs depends the type of carbapenem. Meropenem has a high affinity for PBP 3 relative to imipenem and ertapenem (Meroueh *et al.*, 2006). Here, the possibility that some compounds, present in the mushroom extract, will first bind to PBP 3 may justify this antagonistic effect.

These extracts, as well as specific compounds, should be evaluated for further conclusions. Moreover, more studies are required in order to clarify the mechanisms of action that support the observed effects upon each antibiotic and microorganism.

Given the problem associated with antibiotic resistance worldwide, *Lentinula edodes* is a potential source of antibacterial compounds that can be used prophylactically to prevent the risk of infection and may be used in combination with antibiotics, to reduce the time of infection and the possible occurrence of resistance phenomena.

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Author Contributions

J.G. and A.A. carried out the chemical and the antimicrobial experiments and have equally contributed to the realization of the research. J. G., A.A., C.F., F.M.N., G.M. and M.J.S. wrote the manuscript. C.F., G.M. and M.J.S. proposed the subject, designed and supervised the antimicrobial study. F.M.N. designed and supervised the chemical study. All authors reviewed and contributed to the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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