How gamma-rays and electron-beam irradiation would affect the antimicrobial activity of differently processed wild mushroom extracts?

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Running Head: Antimicrobial activity of irradiated mushrooms

Abstract

Aims: The effects of irradiation (gamma-rays and electron-beams), up to 10 kGy, in the antimicrobial activity of mushroom species (*Boletus edulis*, *Hydnum repandum*, *Macrolepiota procera* and *Russula delica*) differently processed (fresh, dried, freeze) were evaluated.

Methods and Results: Clinical isolates with different resistance profiles from hospitalized patients in Local Health Unit of Mirandela, Northeast of Portugal, were used as target microorganisms. The mushrooms antimicrobial activity did not suffer significant changes that might compromise applying irradiation as a possible mushroom conservation technology.

Conclusions: 2 kGy dose (independently of using gamma-rays or electron-beams) seemed to be the most suitable choice to irradiate mushrooms.

Significance and Impact of Study: This study provides important results in antimicrobial activity of extracts prepared from irradiated mushroom species.

Keywords: Wild mushroom extracts; Irradiation technology; Gamma-irradiation; Electron-beam irradiation; Multi-resistant bacteria

Introduction

The interest of the scientific community for mushrooms (extracts and derived compounds) has been increasing due to their potential in prevention or treatment of different diseases of the modern world. Different mushroom species were previously reported for their anticancer, anti-inflammatory, immunosuppressive, and antibacterial properties (Asfors and Ley 1993; Wieczorek *et al*. 1993; Ferreira *et al*. 2010; Alves *et al*. 2014).

In particular, several authors reported the antimicrobial activity of extracts prepared from different mushroom species including *Boletus edulis* Bull. (Kosanić *et al*. 2012), *Hydnum repandum* L. Fr., (Ozen *et al*. 2011), *Macrolepiota procera* (Scop.) Singer, and *Russula delica* Fr. (Türkoğlu *et al*. 2007; Yaltirak *et al*. 2009; Alves *et al*. 2012b). In this sense, mushrooms have been recognized as functional foods and as a source for the development of drugs and nutraceuticals (Lindequist *et al*. 2005; Poucheret *et al*. 2006).

Nevertheless, and in spite of these undeniable qualities, mushrooms are one of the most perishable products and tend to loose quality immediately after harvest. A short shelf life (1-3 days at room temperature) is one of the disadvantages for their distribution and marketing as a fresh product. Their shelf life is limited due to postharvest changes, such as browning, cap opening, stipe elongation, cap diameter increase, weight loss and texture changes; but also due to a high respiration rate and lack of physical protection to avoid water loss or microbial attack (Akram and Kwon 2010; Fernandes *et al*. 2012).

Irradiation is recognized as a safe and effective preservation method, being used worldwide to increase the shelf life of foods (*e.g*, fruits and legumes, spices, grains, meat or seafood) (Andrews *et al*. 1998; Fernandes *et al*. 2012). Gamma-irradiation (Beaulieu *et al*. 2002) and electron-beam (Koorapati *et al*. 2004) are considered potential tools in extending the shelf life of fresh mushrooms. Furthermore, different regulatory agencies

ensure that food irradiation is a safe process in relation to the processing of food for humans (US-FDA 1991; WHO 1994).

Our research group has been investigating the effects of mushrooms (including the above mentioned species *B. edulis*, *H. repandum*, *M. procera* and *R. delica*) irradiation by evaluating their nutritional, physical and chemical parameters, concluding that these parameters are not affected in high extension with this preservation technology (Fernandes *et al*. 2013a-c, 2014a-c).

Nevertheless, to the authors' knowledge, there are no available reports about the effects of irradiation on mushrooms antimicrobial activity. Therefore, in the present study, the antibacterial properties of extracts prepared from irradiated (gamma radiation and electron-beam) wild mushrooms were assessed against clinical isolates with different resistance profiles from hospitalized patients in Local Health Unit of Mirandela, Northeast of Portugal.

Materials and methods

Standards and Reagents

To estimate the dose and dose rate of gamma-irradiation it was used a chemical solution sensitive to ionizing radiation, Fricke dosimeter, prepared in the lab following the standards (ASTM 1992) and Amber Perspex dosimeters (batch V, from Harwell Co., UK). To prepare the acid aqueous Fricke dosimeter solution, the following reagents were used: ferrous ammonium sulfate(II) hexahydrate, sodium chloride and sulfuric acid, all purchased from Panreac S.A. (Barcelona, Spain) with purity PA (proanalysis), and water treated in a Milli-Q water purification system (Millipore, model A10, USA).

Methanol was of analytical grade purity from Lab-Scan (Lisbon, Portugal). The culture media Muller Hinton broth (MHB), Wilkins-Chalgren Broth (WCB) and Columbia agar

(CA) with 5% horse blood were obtained from Biomerieux (Marcy l´Etoile, France), respectively. The dye *p*-iodonitrotetrazolium chloride (INT) was purchased from Sigma–Aldrich (Spruce Street; St. Louis, USA) to be used as microbial growth indicator. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA) before use.

Samples and samples irradiation

Macrolepiota procera, Boletus edulis, Hydnum repandum and *Russula delica* (**Figure 1**) were collected in Trás-os-Montes, in the Northeast of Portugal; the first mushroom species were collected in November 2011 and the three other species were collected in November 2012, and further irradiated.

B. edulis and *H. repandum* fresh fruiting bodies were divided in three groups (each species) with three mushrooms per group and submitted to gamma-irradiation according to the procedure described by Fernandes *et al*. (2013a): control (non-irradiated, 0 kGy), sample 1 (irradiated with 1 kGy) and sample 2 (irradiated with 2 kGy). *M. procera* fruiting bodies were divided in two groups with nine mushrooms per group, and further submitted to different processing technologies according to the procedure described by Fernandes *et al*. (2014a): freezing (at -20º C in a freezer) and drying (at 30 ºC in an oven). Each group was further subdivided in three subgroups and submitted to gamma-irradiation: control (non-irradiated, 0 kGy); sample 1 (irradiated with 0.5 kGy) and sample 2 (irradiated with 1 kGy). *R. delica* fruiting bodies were divided in four groups with six mushrooms per group, dried at 30 ºC in an oven and then submitted to electron-beam irradiation according to the procedure described by Fernandes *et al*. (2014b): control (non-irradiated, 0 kGy), sample 1 (irradiated with 2 kGy), sample 2 (irradiated with 6 kGy), and sample 3 (irradiated with 10 kGy).

Extracts preparation

Each mushroom lyophilized sample (~ 1.5 g) was extracted using a methanol:water (80:20; 30 mL) mixture at -20 °C for 1.5 h. After 15 min in an ultrasonic bath, filtered through Whatman nº 4 paper. The residue was then re-extracted with additional 30 mL of methanol:water mixture at -20 °C for 1.5 h and the previous steps were repeated. The combined extracts were evaporated at 40 ºC under reduced pressure to remove methanol (rotary evaporator Büchi R-210, Flawil, Switzerland), lyophilized, redissolved in water, at a concentration of 200 mg/mL, and stored at -20 ºC for further use.

Microorganisms and culture media

The microorganisms used were clinical isolates from patients hospitalized in various departments of the Local Health Unit of Mirandela, Northeast of Portugal.

Two Gram negative bacteria (*Escherichia coli* and *Proteus mirabilis*, isolated from urine) and two Gram positive bacteria (MSSA- Methicillin-sensitive *Staphylococcus aureus*, isolated from Wound exudate and MRSA- methicillin-resistant *Staphylococcus aureus*, isolated from expectoration) were used to screen the antimicrobial activity of the mushroom extracts.

Characterization of antibiotic susceptibility of target strains

The characterization of antibiotic susceptibility and the identification of the target strains were performed using VITEK® 2 Compact card (Biomérieux, Lyon, France). These cards allow the simultaneous determination of susceptibility to antimicrobial agents and the strain identification, including aerobic and facultative anaerobic Gram negative bacilli (VITEK® 2 Compact AST-N192), and Gram positive cocci such as some fastidious

aerobic Gram positive (VITEK® 2 Compact AST-P619). The sensibility of the microorganisms to each antibiotic is identified by the MIC.

Determination of the antimicrobial activity of the extracts

MIC determinations were performed by the microdilution method and the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay following the methodology suggested by Kuete *et al*. (2011a, b) with some modifications.

Initially, 50 µL of each mushroom extract (200 mg/mL) was diluted in 450 µL of MHB (final concentration of 20 mg/L) and then, 200 µL of this extract solution was added in each well (96-well microplate). Dilutions were carried out over the wells containing 100 µL of MHB and afterwards, 10 µL of inoculum (1×10^8 cfu/mL) were added to all the wells. Two negative (one with MHB and the other with the mushroom extract) and one positive (with MHB and the inoculum) controls were prepared. The plates were incubated at 37 °C, for 24 h, in an oven (Jouan, Berlin, Germany).

The MIC of the samples were detected after adding INT (0.2 mg/mL, 40 µL) and incubating at 37 °C for 30 min. Viable microorganisms reduced the yellow dye to a pink color. MIC was defined as the lowest mushroom extract concentration that prevented this change and exhibited complete inhibition of bacterial growth. All the assays were carried out in duplicate.

Results

Before analyzing the potential antimicrobial activity of the studied mushrooms, the resistance profiles of the bacterial isolates are described in **Table 1**. From its interpretation, it is possible to conclude that *E. coli* and MSSA constitute wild strains with small resistance to the tested antibiotic groups, excluding the result obtained with

MSSA against benzylpenicillin. On the other hand, *P. mirabilis* and MRSA showed resistance against the quinolone group (ciprofloxacin and levofloxacin) and β-lactamic antibiotics (amoxicillin/clavulanic acid). In addition, *P. mirabilis* was also resistant to trimethoprim/sulfasoxazole (antimethabolic) and nitrofurantoin. Overall, the obtained MIC values are in the same range as those obtained in a previous study (Alves *et al*. 2012b). However, the magnitude of the MIC values obtained using antibiotics is nearly 1000-fold lower when compared with the same values obtained using mushroom extracts (**Table 2**). However, the values depicted in each table should not be directly compared, since results in **Table 1** were achieved using pure compounds, while those in **Table 2** derive from using crude extracts with several possible interferences.

Centering the discussion in the antimicrobial activity of mushroom extracts, a primary observation allows concluding that the Gram positive bacteria presented more susceptibility, in agreement with other reports (Barros *et al*. 2007; Barros *et al*. 2008a, b; Venturini *et al.* 2008; Alves *et al*. 2012b), despite the results obtained by Ozen *et al*. (2011), which indicated that *H. repandum* extracts had a maximal antibacterial activity against a Gram negative bacteria (*Pseudomonas aeruginosa*).

Among all tested extracts, those obtained from fresh *B. edulis* presented the highest activity against all tested bacterial strains, followed by dried samples of *M. procera*. Similar resistance levels were previously reported for *B. edulis* samples assayed against *E. coli* and MSSA (Kosanić *et al*. 2012). The results reported herein for dried *R. delica* extracts indicate slightly higher antibacterial activity, namely against *P. mirabilis* and MRSA, than those obtained in our laboratory with the same species (Alves *et al*. 2012b). Nevertheless, the results from both studies might be considered as belonging to the same range. Since the studied species have no phenolic compounds (Vaz *et al*. 2011), the antimicrobial activity could be due to the presence of steroids, sesquiterpenes, organic

acids, or peptides, which represent antimicrobial compounds commonly found in mushrooms (Alves *et al*. 2012a).

The mushroom species studied herein, among several others, might benefit from an increased shelf life when submitted to irradiation, either using gamma-rays (Beaulieu *et al*. 2002; Fernandes *et al*. 2013a-c, 2014a) or electron-beams (Koorapati *et al*. 2004; Fernandes *et al*. 2014b, c) as energy sources. In all studied cases, the chemical composition and antioxidant activity of each mushroom species were not negatively affected by irradiation treatment. Considering the ability to maintain the antioxidant activity, we hypothesized that irradiated mushrooms might likewise present the same antimicrobial activity. The results obtained for differentially processed mushrooms (fresh, freeze or dried) treated with gamma or electron-beam irradiations with varying doses are presented in **Table 2**. As a first remark, it should be clarified that the used heterogeneous samples were defined to prove the feasibility of irradiation treatment in the maintenance of antimicrobial activity, independently of mushroom species, mushroom processing, irradiation source and irradiation dose. As it can be concluded from **Table 2**, the antimicrobial activity was not adversely affected in most of the cases, except gamma-irradiated *B. edulis* against *P. mirabilis*. In fact, this activity was sometimes potentiated, as it was verified for the frozen samples of *M. procera* against *E. coli* and MSSA, or the dried sample of *R. delica* against *E. coli*.

Some matrix effects must indeed be considered. For instance, the 1 kGy dose does not seem to be the preferable choice to treat *B. edulis* and *H. repandum* (which have a better response to the 2 kGy dose), but the same dose had advantageous effects when used to irradiate the *M. procera* samples. This indicates that the chemical composition of a determined mushroom might impart different outcomes as a result of the same irradiation dose. Nevertheless, the results obtained for irradiated samples and controls are very

similar, indicating that there are no significant changes in the antimicrobial activity, which might compromise the feasibility of this technology (under the limitations of the used sampling). As a general conclusion, the results indicate that the 2 kGy should be considered as the best choice in terms of antimicrobial activity maintenance.

Discussion

The development of antibacterial agents' resistance and the decrease of effective antifungal agents are leading the researchers towards investigating novel chemical structures and considering new natural sources of compounds. Usually, compounds found in natural matrices exert their antimicrobial activity by interacting with the microorganism's cell membrane or cell wall, altering its permeability and causing cell destruction. The antimicrobial activity might also be explained by infiltration into bacterial cells, followed by promoting the coagulation of the cell content (Taguri *et al*. 2006; Tian *et al*. 2009).

The antimicrobial activity of different mushroom species was previously reported, particularly in what regards their effectiveness against Gram positive and Gram negative bacteria (Türkoğlu *et al.* 2007; Ozen *et al*. 2008; Venturini *et al.* 2008; Yaltirak *et al.* 2009; Alves *et al*. 2012a,b; Kosanić *et al*. 2012; Alves *et al.* 2014; Smolskaitė *et al*. 2014). In this study, instead of using commercial bacterial cultures, the antibacterial activity was evaluated using clinical isolates from different body fluids. The obtained bacteria had the common feature of showing some resistance against the typically used antibiotics.

After several studies have been conducted proving that neither the chemical composition, nor the antioxidant activity of different mushroom extracts were adversely changed by irradiation treatment, the feasibility of this technology was tested herein by verifying its

effects in the antimicrobial activity of different mushroom species. Besides using different mushrooms, samples were processed differently (fresh, dried, freeze) according to the most common practices applied to mushrooms. Likewise, different doses (0.0, 0.5, 1, 2, 6 and 10 kGy) and two irradiation sources (gamma-rays and electron-beams) were evaluated. Under all the assayed conditions, the antimicrobial activity did not suffer significant changes that might compromise applying irradiation as a possible mushroom conservation technology. As a final remark, the 2 kGy dose (independently of using gamma-rays or electron-beams) seemed to be the most suitable choice. Nevertheless, future assays with additional mushrooms and/or bacterial cultures will certainly be needed before guaranteeing the complete adequacy of this treatment.

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Conflict of Interest

The authors have no conflicts of interest.

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Figure Legends

Figure 1. Representative samples of the control population of *M. procera*, *B. edulis*, *H. repandum* and *R. delica* (from the left to the right). Mushrooms of the other groups (irradiated) are similar to the control.

Table 1. MIC values (μg/mL) of different antibiotics against Gram-negative bacteria and Gram-positive bacteria.

	Escherichia coli		*Proteus mirabilis*		*MRSA*		*MSSA*	
Benzylpenicillin					≥ 0.5	R	≥ 0.5	R
Ampicillin	≤ 2	S	≤ 2	S				
Oxacillin					≥ 4	R	≤ 0.25	S
Claritromycin					na	S	na	R
Clindamycin					≤ 0.25	S	≤ 0.25	S
Daptomycin					0.25	S	0.25	S
Erythromycin					≤ 0.25	S	≥8	R
Fosfomycin					≤ 8	S	≤ 8	S
Moxifloxacin					1	I	≤ 0.25	S
Gentamicin	≤ 1	S	≤ 1	S	≤ 0.5	S	≤ 0.5	S
Ciprofloxacin	≤ 0.25	S	≥ 4	R	na	R	na	S
Levofloxacin	≤ 0.12	S	≥ 8	R	4	R	0.25	S
Linezolid					2	S	2	S
Teicoplanin					≤ 0.5	S	≤ 0.5	S
Tigecycline					≤ 0.12	S	≤ 0.12	S
Tetracycline					≤ 1	S	≤ 1	S
Trimethoprim/Sulfasoxazole	≤ 20	S	≥ 320	R	≤ 10	S	≤ 10	S
Vancomycin					≤ 0.5	S	1	S
Amoxicillin/Clavulanic acid	≤ 2	S	16	R	na	R	na	S
Cephalothin	4	S	8	S				
Cefuroxime	4	S	≤ 1	S				
Cefuroxime Axetil	4	S	≤ 1	S				
Cefotaxime	≤ 1	S	≤ 1	S	na	R	na	S
Ceftazidime	≤ 1	S	≤ 1	S				
Ceftriaxona	na	S			na	R	na	S
Ertapenem	≤ 0.5	S	1	I				
Meropenem	≤ 0.25	S	0.8	S				
Amikacin	≤ 2	S	≤ 2	S				
Tobramycin	≤ 1	S	≤ 1	S				
Fusidic acid					≤ 0.5	S	≤ 0.5	S
Mupirocin					≤ 2	na	≤ 2	na
Rifampicin					≤ 0.5	na	≤ 0.5	na
Nitrofurantoin	≤ 16	S	≥ 512	R	≤ 16	S	≤ 16	S
Piperacillin/Tazobactam	≤ 4	S	≤ 4	S				

S- susceptible; I- intermediate; R- resistant (this classification was made according to the interpretative breakpoints suggested by Clinical and Laboratory Standards Institute - CLSI); na- not applicable.

Table 2. MIC values (mg/mL) of irradiated wild mushrooms against clinical isolates of Gram-negative and Gram-positive bacteria.

		Escherichia coli	*Proteus mirabilis*	*MRSA*	*MSSA*
gamma-irradiated *Boletus edulis*					
0 kGy	fresh	10	5	2.5	5
1 kGy	fresh	10	20	5	5
2 kGy	fresh	10	10	2.5	5
gamma-irradiated *Hydnum repandum*					
0 kGy	fresh	10	>20	10	5
1 kGy	fresh	20	>20	20	10
2 kGy	fresh	10	>20	10	5
gamma-irradiated *Macrolepiota procera*					
Control	dried	20	20	5	10
	frozen	20	>20	5	10
0.5 kGy	dried	20	20	5	10
	frozen	10	>20	5	5
1 kGy	dried	20	20	5	10
	frozen	10	>20	5	5
electron-beam irradiated *Russula delica*					
Control	dried	>20	10	5	10
2 kGy	dried	>20	10	5	5
6 kGy	dried	20	10	5	10
10 kGy	dried	20	10	5	5

MIC- minimum inhibitory concentration; MRSA- methicillin-resistant *Staphylococcus aureus*; MSSA- Methicillin-sensitive *Staphylococcus aureus*.

Figure 1.