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Protective Effects of New Medicinal Mushroom, *Grifola gargal* Singer (Higher Basidiomycetes), on Induced DNA Damage in Somatic Cells of *Drosophila melanogaster*

Pablo Daniel Postemsky,[1] Ana María Palermo,[2] & Néstor Raúl Curvetto[1]

[1]Laboratorio de Biotecnología de Hongos Comestibles y Medicinales (LBHCM), CERZOS-CONICET-UNS; [2]Instituto Investigaciones Científicas y Técnicas para la Defensa (CITEDEF)

*Address all correspondence to: Néstor R. Curvetto. Laboratorio de Biotecnología de Hongos Comestibles y Medicinales (LBHCM), CERZOS-CONICET-UNS, Camino La Carrindanga Km 7, (8000) Bahía Blanca, Argentina; Tel.: +54 291 4861666; Fax: +54 291 4861527; micouns@criba.edu.ar.

ABSTRACT: *Grifola gargal* is an edible mushroom with attributed antioxidant properties. Different sources of *G. gargal* materials, i.e., fruit bodies and mycelia grown in liquid or solid media, were used to study its potential protective capacity when somatic mutation and recombination is induced in *Drosophila melanogaster* using DMBA (7-12-dimethyl-benz(α)anthracene) as promutagen. Heterozygote larvae (*white/white*$^+$) were grown in media with different concentrations of DMBA. *Grifola gargal* fruit bodies (*Gg*FB) or mycelia from liquid culture (*Gg*LC) or from solid culture (*Gg*WG), i.e., biotransformed wheat kernel flour, were added to the culture media in combined treatments with DMBA. Water, DMBA solvent, or wheat flour (WF) plus DMBA solvent were used as negative controls. Larval mortality increased from 9% to 11% in negative controls to 31% to 36% in DMBA treatments. The addition of *Gg*FB, *Gg*LC, or *Gg*WG materials produced a protective effect on 25 μmol/vial DMBA-induced mortality. Mutations observed in SMART, as light spots per 100 eyes (LS/100 eyes), increased with increasing doses of DMBA; this was also true when considering the mutation incidence expressed as percentage of eyes exhibiting light spots (% eyes with LS). Interestingly, mycelia from *Gg*FB, *Gg*LC, or *Gg*WG, in the presence of 25 μmol/vial DMBA, showed lower values in SMART of both the total LS/100 eyes and the percentage of eyes with LS. Thus, *Grifola gargal* materials were not only nontoxic, but in combination with 25 μmol/vial DMBA lowered the mortality induced by the promutagen and showed antimutagenic effects. Protective effects of *G. gargal* against DMBA are discussed in terms of the onset of desmutagenic and/or bioantimutagenic mechanisms of detoxification in the host organism, probably due to some bioactive compounds known to occur in higher mushrooms.

KEY WORDS: 12-dimethylbenz[α]anthracene; somatic mutation and recombination test; *Gifola gargal*; wild-forest edible mushrooms

ABBREVIATIONS: DMBA: 7,12-dimethylbenz[α]anthracene; **SMART:** somatic mutation and recombination test; ***Gg*FB:** *G. gargal* fruit bodies; ***Gg*LC:** *G. gargal* mycelia obtained from liquid culture; ***Gg*WG:** *G. gargal* mycelia cultivated on wheat grains; **WF:** wheat flour made of whole kernels; **LS:** light spots

I. INTRODUCTION

Scientific research carried out during the last two decades in Asian countries like China and Japan has gathered a body of information on the use of mushrooms as food and medicinal agents. Mushrooms such as lingzhi (*Ganoderma lucidum*), shiitake (*Lentinus edodes*), and yiner (*Tremella fuciformis*) that have been collected, cultivated and used for hundreds of years, are now being evaluated as edible and medicinal resources,[1] and numerous species of wild-growing mushrooms are widely consumed as a delicacy in central and eastern Europe.[2]

Mushrooms are also being investigated as dietary supplements for their putative antigenotoxic, antioxidant, and anticarcinogenic activities.[1,3,4]

Taira et al.[5] found that *Agrocybe cylindracea*, *Pleurotus ostreatus*, and *Lentinus edodes* inhibit or suppress the DNA damage induced by several mutagens to *Drosophila melanogaster*. Similar findings were reported for *Agaricus brasiliensis* (=*A. blazei*

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sensu Heinem.) in different human cell–based bioassays,[6,7,8,9] and for other edible mushrooms using the *Salmonella* spp. bioassay.[10]

The *Grifola* genus (basidiomycete of the Polyporaceae, Aphyllophorales) has shown to possess important nutraceutical properties. In fact, *Grifola frondosa* (Maitake) has been used in eastern Asian medicine for millennia.[11]

The mycelia of this mushroom inhibited mutagenesis, carcinogenesis, metastasis, and tumor growth in mice and in cancer cell lines in *in vitro* studies,[12,13,14,15] and exhibited immunomodulating activities,[16,17,18,19] as well as antihypertensive, cholesterol-lowering, and antidiabetic effects.[20,21,22] One of the most promising studied components is a mixture of a fraction of complex branched polysaccharides called D-fraction.[23]

The native species, *Grifola gargal,* which grows on living or fallen trunks or stumps of *Nothofagus obliqua* (roble beech), a deciduous tree from southern Argentina and south and central Chile,[24] has been used as food by native people.[25]

To evaluate this mushroom's potential antigenotoxicity, the Somatic Mutation and Recombination Test (SMART) in the eye of *Drosophila melanogaster* was chosen. In brief, in this test larvae are exposed to the substance to be evaluated and then the eyes of the emerging flies are analyzed; if a mutation or recombination event has occurred, a white spot (LS, light spot) appears. The frequency of LS estimates the frequency of the above-mentioned events. It is an efficient tool for the detection of a wide spectrum of genotoxic activities exhibited by pure compounds or complex mixtures as well as for studies on antimutagenic properties of substances, foods, or food components, including mushrooms.[5,26,27]

Drosophila melanogaster is a valuable experimental model because, despite the differences among species, similarities in the activation metabolism of xenobiotics in the fly and vertebrate systems do exist and allow the extrapolation of results.[28,29] It is also worthwhile to mention that it presents an orthology of 68% with regards to the human genes that control cancer.[30]

Promutagenic agents, which require metabolic activation, are useful chemical tools to be used as positive controls in the evaluation of the reduction of DNA damage by hypothetically antigenotoxic agents. DMBA (7,12-dimethylbenz[α]anthracene) is a polycyclic aromatic hydrocarbon compound that becomes a very reactive epoxide after oxidation, and is thus able to react with and covalently bind to DNA, causing mutations and chromosomal aberrations. This chemical can even act as a transplacental mutagen and cause increased cell division and fixation of DNA lesions as mutations.[31] It is a ubiquitous anthropogenic contaminant produced by incomplete combustion of fossil fuels or coal, during processing of food by frying or roasting, and it is also present in cigarette smoke. Once DMBA enters the food chain, it can be consumed as contaminated food.[32] This compound has been used as a promutagen in *Drosophila* assays.[5,28,33]

Antioxidant activity of *G. gargal* mushrooms are described elsewhere,[34,35] but to the best of our knowledge there are no previous reports about the genotoxic or antigenotoxic properties of this edible mushroom.

The aim of this study was to assess whether *Grifola gargal*—as a whole mushroom or its mycelia—is free of either toxic or genotoxic effects and hence safe to be consumed as food; and also to search for its potential ability to prevent the damage elicited by the promutagen DMBA on eukaryotic cells.

II. MATERIALS AND METHODS

A. Promutagen

The chemical 7,12-dimethylbenz[α]anthracene (DMBA) was obtained from Sigma Chemical Co., St Louis, MO, USA (Sigma-Aldrich 2004-2005 Catalog #D-3254). DMBA solutions were prepared using a mix of ethanol (CAS N° 64-17-5), polysorbate 20 (Tween 20), dimethyl sulfoxide (DMSO, CAS N° 67-68-5), and dimethyl formamide (DMF) (12:3:1:1) as solvent. Distilled water or DMBA solvent were used as controls. The DMBA dose used in the eye SMART (25 µmol/vial) was selected after evaluation of both a toxicity curve using 12.5, 25.0, 50.0, 75.0, and 100.0 µmol/vial and a genotoxicity curve with 12.5, 25.0, and 50.0 µmol/vial. The purpose of this approach was to avoid false-negative results caused by high toxicity doses of the promutagen.

It should also be mentioned here that in order to predict carcinogenic effects across species, it is necessary that the metabolism and mechanism of action of the chemical at the highest level tested be the same as at the low levels where human ex-

posure would occur. Then, selection of a dose that is too high, where toxicokinetics result in different metabolism, may impair the use of the results for risk assessment.[36]

B. *Grifola gargal*

Grifola gargal fruit bodies were collected at the Lanín National Park, Neuquén, Argentina. Appropriate identification criteria, which included habit of growth, morphology, and cultivation characters, were followed.[24] Selected specimens were immediately frozen, liophylized, milled using a Willey-type mill, and kept at room temperature until use. A pooled mix with equal amounts of milled fruit bodies from these specimens was used in all the experiments (*Gg*FB). *Grifola gargal* mycelium from liquid culture (*Gg*LC) was obtained using strain CIEFAP #191 from the CIEFAP (Centro de Investigación y Extensión Forestal Andino Patagónico, Argentina). The inoculum was done according to Postemsky et al.[37] The liquid culture system was prepared using 3-L flasks with 500 mL of basal medium (30 g/L glucose, 6 g/L yeast extract, 2 g/L peptone, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L $MnSO_4 \cdot 5H_2O$, 0.5 g/L K_2HPO_4, pH 4.5) and inoculated at a 10% v/v ratio. The incubation was done under low-speed agitation (30 rpm) at 21°C and darkness. After 20 d, the mycelium was recovered and rid of excess liquid by centrifugation at 200 × g. Then it was freeze dried and ground with a mortar and pestle. The dehydrated mycelium was kept at room temperature.

Grifola gargal mycelium from solid-state fermentation growth was obtained as described elsewhere.[38] Briefly, a mixture of 250 g whole wheat kernels, 0.6 g $CaCO_3$, and 3.53 g $CaSO_4$ was embedded overnight with 190 mL water and then sterilized at 1 atm for 90 min. Inoculation was performed using one-quarter of a fully colonized Petri dish from a 20-d culture grown on MYPA with addition of sunflower seed hull powder.[37] After 20 d at 24 ± 1°C in darkness, the mycelium completely covered the wheat grains, which were then dried at 60°C in an oven with forced air circulation. The flour from these biotransformed wheat grains (*Gg*WG) was obtained using an Udy Cyclone Mill (USA). Wheat flour (WF) to be used in control treatments was obtained following the same procedure but skipping the inoculation step.

C. Nutritional Analysis

Carbohydrates, crude fats, proteins, ash, and mineral nutrient element contents of different *G. gargal* materials are presented in Table 1. Carbon content was analyzed with an Elemental Carbon Analyzer (LECO, USA), and the nitrogen content by Kjeldhal's method. The protein content of *Gg*FB and *Gg*LC was estimated by multiplying total N content by a factor of 4.38,[39] while for the protein content in *Gg*WG samples, the factor 5.7 was used.[40] The lipid fraction was gravimetrically determined after an exhaustive solvent extraction in hexane. Ashes were gravimetrically determined after sample incineration in a muffle furnace at 525°C for 3 h.

Main mineral nutrient elements content in those materials was quantified by using an induced plasma emission spectrophotometer (Shimadzu ICPS 1000 III, Japan).

D. Somatic Mutation and Recombination Test in *Drosophila melanogaster*

To study the effect of *G. gargal* mycelium sources on the genetic damage induced by DMBA, the *white/white*$^+$ (*w/w*$^+$) spot test in *Drosophila melanogaster* was used.[28,33] This test essentially measures the occurrence of clones of white ommatidia known as light spots (LS) in the eyes of *w/w*$^+$ heterozygous females. Virgin females of the genotype *w/w* were mated to *y/Y* males for 72 ± 4 h in regular culture medium. Inseminated females were transferred to bottles with medium containing the test solutions and allowed to oviposit for 4–8 h; series of untreated larvae were also run at the same time. The eyes of the emerging heterozygous *w/w*$^+$ females were examined in a solution of 90% ethanol, 9% Tween 20, and 1% water for the presence of LS at a 40–60× magnification[28] and the number of ommatidia in each LS clone was recorded.

Drosophila melanogaster strains *yellow* and *white* were kindly provided by the National Commission of Atomic Energy (CNEA) of Argentina.

E. Larval Feeding

The culture media were prepared with water (815.4 mL) and 3.08 mL lactic acid. This solution (pH 3.0) was warmed to 70°C in a microwave and then, according to treatments, 36.9 mL of DMBA solution, its solvent, or water were added. Finally, a mix of 136.7 g of commercial mashed potato

TABLE 1. Nutritional Composition of *Grifola gargal* Fruit Bodies (*Gg*FB), Mycelia from Liquid Culture (*Gg*LC), and Wheat Flour Obtained by Milling Biotransformed Wheat Grains (*Gg*WG) (values are given on a dry weight basis)

	*Gg*FB	*Gg*LC	*Gg*WG
Nutritional composition (g/100g dry material)			
Carbohydrates	86.5	78.1	79.6
Protein	6.2	14.0	15.4
Fat	5.2	7.0	3.0
Ash	2.1	1.0	2.0
Elemental analysis (mg/kg dry material)			
C	40.7×10^4	40.5×10^4	43.9×10^4
N	1.4×10^4	3.2×10^4	2.7×10^4
P	220	500	300
S	90	410	370
K	900	1480	390
Mg	179	230	229
Ca	53	38	347
Na	40	173	34
Fe	11.7	3.8	25.2
Zn	5.9	4.6	4.5
Cu	3.4	1.2	1.1
Cr	<0.05	<0.05	<0.05
Se	<0.02	<0.02	<0.02

flakes and 17.1 g agar was added to the warm solution and thoroughly homogenized. For *Gg*LC or *Gg*FB treatments, 15.4 g of solid medium was replaced by the mycelium; in the case of *Gg*WG treatments and WF control, 76.9 g of solid medium was replaced by the corresponding flour. The final pH was 3.5 and the density was 1 g/mL. The medium was poured into 30-mL flat-bottom glass tubes (21 × 100 mm) at a rate of 6.5 mL per tube; once cooled, 60 mg dry yeast powder was spread on the surface. The treated and control series were maintained in a growing chamber at 60% RH and 25°C until fly hatching. Fifty µL of lactic acid (pH 3.0) were added weekly to each tube in order to prevent contamination and also to help in maintaining adequate moisture.

F. Toxicity Evaluation

Toxicity of different doses of DMBA, and wheat flour or *G. gargal* either in the absence or in the presence of DMBA, was determined by counting the number of pupae that emerged from a certain number of larvae. Larvae grown for 64–72 h in regular media were transferred to vials with the treatment substances and allowed to develop until pupation, when pupae were counted. Toxicity was measured by the proportion of larvae that survived to pupation. When assaying chemicals for geno/antigenotoxicity using a bioassay, such as SMART in *Drosophila melanogaster*, doses above LD50 should be discarded, because they could give false-negative results.[41] No DMBA dose used in the present study exceeded this mortality limit.

G. Statistical Analysis

Statistical comparisons of mortality rates to evaluate toxicity were made by Chi-square test and the Fisher exact probability test according to Lowry[42] ($\alpha = 0.05$). The standard error of mortality percent-

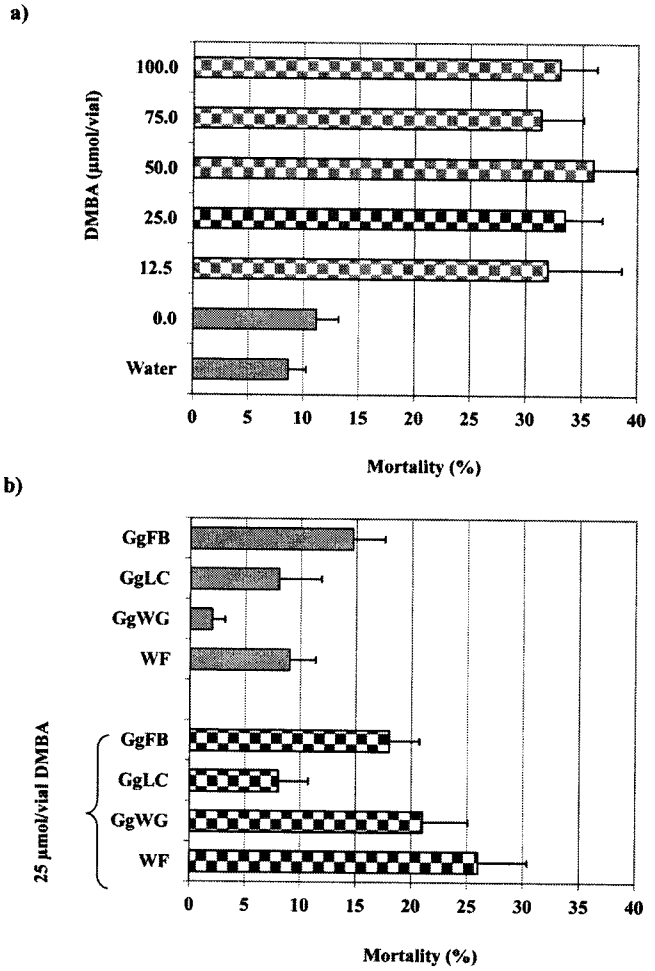

FIGURE 1. Mortality in *Drosophila melanogaster larvae* exposed to DMBA solvent or water (**a**, gray bars); DMBA doses (**a**, square filled bars); DMBA solvent plus *Grifola gargal* mycelia from different sources (**b**, gray bars); or 25 µmol /vial of DMBA plus *Grifola gargal* mycelia from different sources (**b**, square filled bars). Error bars represent standard error: SE (%) = $(P_{Survivors} \times P_{Died}/n)^{½} \times 100$.

ages was determined with the equation SE (%) = $(P_{Survivors} \times P_{Died}/n)^{½} \times 100$.

The frequency of spots was evaluated using the Chi-square test for proportions ($\alpha = \beta = 0.05$).[43,44] The somatic reversion frequencies detected were presented as the number of LS per 100 eyes (LS/100 eyes).

The percentage of eyes exhibiting LS was obtained (% eyes with LS) and the statistical comparisons of % eyes with LS were made by Chi-square test according to Lowry[42] ($\alpha = 0.01$; 0.05).

It is possible to obtain an indication of the genotoxic degree when the ratio between the LS/100 eyes and % eyes with LS become more separated from the unit.

The clone size of each LS (number of ommatidia affected) was also counted. Light spots separated for more than four ommatidia were considered as individual events, and the corresponding data were scored into three categories corresponding to each larval instar according to Vogel and Nivard.[28] Analysis of the frequency of LS per size class was also carried out with the Chi-square test.[43,44]

III. RESULTS

A. Toxicity Evaluation

Toxicity, expressed as mortality (%) of larvae, is shown in Fig. 1. The DMBA solvent did not significantly affect the larvae survival ($p > 0.05$) (Fig. 1a). None of the DMBA concentrations produced a mortality higher than 50% ($p > 0.05$). Irrespective of the DMBA concentration (Fig. 1a), the mortality significantly increased 2.3–2.8 times

TABLE 2. Frequency of Light Spots (LS) and Percentage of Eyes with LS, in *Drosophila melanogaster* after Chronic Exposure to Different Concentrations of DMBA Plus *Grifola gargal* Materials

Treatments[a]	N° of eyes scored	N° of LS	N° LS /100 eyes[c]				% eyes with LS[e]	Diagnosis[f]		
			3rd[d]	2nd	1st	Total		Pair Comp.	I	II
1. Water	383	82	16	4	1	21	19			
2. DMBA 0[b]	326	122	34	2	1	37	28	vs. 1	+	**
3. DMBA 12.5[b]	480	260	47	6	1	54	38	vs. 2	w+	**
4. DMBA 25[b]	545	482	77	10	1	88	57	vs. 2	+	**
5. DMBA 50[b]	550	603	99	10	1	110	65	vs. 2	+	**
6. *Gg*LC	450	105	19	3	1	23	18	vs. 1	–	NS
7.*Gg*FB	304	52	14	3	0	17	15	vs. 1	–	NS
8. WF	450	90	16	2	2	20	19	vs. 1	–	NS
9. *Gg*WG	450	94	17	4	0	21	17	vs. 1	–	NS
10. *Gg*LC + DMBA 25	422	295	61	8	1	70	50	vs. 6	+	**
								vs. 4	w+	*
11. *Gg*FB + DMBA 25	513	221	31	11	1	43	34	vs. 7	+	**
								vs. 4	+	**
12. WF+ DMBA 25	375	298	67	11	1	79	60	vs. 8	+	**
								vs. 4	–	NS
13. *Gg*WG + DMBA 25	450	255	49	7	1	57	42	vs. 9	+	**
								vs. 4	+	**
								vs. 12	w+	**

[a]Treatments: *Gg*FB, *G. gargal* fruit bodies; *Gg*LC, *G. gargal* mycelia obtained from liquid culture; *Gg*WG, *G. gargal* mycelia cultivated on wheat grains; WF, wheat flour. In order to facilitate pair comparison interpretation, treatments were numbered.

[b]DMBA dose expressed in µmol/vial.

[c]The number of LS/100 eyes is (LS/Eyes scored) ×100.

[d]Number of LS/100 eyes per larval instar: 1st instar includes LS of 33–>64 ommatidea, 2nd instar includes LS of 5–32 ommatidea, and 3rd instar includes LS of 2–4 ommatidea, according to Vogel and Nirvard.[28]

[e]Eyes with LS (%) is (Eyes with LS/Eyes scored) × 100.

[f]Diagnosis: pair comparison was done between the treatment in the correspondent file vs. the one in the pair comparison column.

Mutation analysis was done through two statistical approaches: (I) statistical diagnoses of mutation frequencies according to Frei and Würgler:[43] +, positive; w+, weakly positive; –, negative; i, inconclusive; ($\alpha = 0.05$); and (II) statistical analysis of % eyes frequencies using the Chi-square test according to Lowry[42] (*, $\alpha = 0.05$; **, $\alpha = 0.01$; NS, $\alpha = 0.05$).

over the one produced by the solvent ($p < 0.05$). The pair comparison of the DMBA assayed doses showed no differences among them ($p > 0.05$). These results emphasize the point that by extending the DMBA range concentration by a factor of 8, mortality and hence the toxic response did not increase.

When mushroom materials *Gg*FB, *Gg*LC, *Gg*WG, and WF, in the presence of DMBA solvent, were compared with the DMBA solvent alone, no increment in toxicity ($p > 0.05$) was observed (Fig. 1b). Interestingly, when pair comparison of mortality ratios produced by 25 µmol/vial DMBA with *G. gargal* mycelia (*Gg*FB, *Gg*LC, *Gg*WG) and WF treatments (Fig. 1b) was done against the one produced by 25 µmol/vial DMBA alone (Fig. 1a), it was found that only the treatments including *G. gargal* did have a protective effect on mortality induced by DMBA ($p < 0.05$).

B. DMBA Genotoxicity

Genotoxicity results after evaluation with *D. melanogaster* eye-spot test are shown in Table 2. The parameters used were the number of light spots per 100 eyes (LS/100 eyes) and the % of eyes with light spots [eyes with LS (%)]. Also, the following relationship (LS/100 eyes)/(% eyes with LS) = average in number of LS per damaged eye (with LS) was considered.

Treatments 1 and 2 represent water and DMBA

solvent controls, showing a *positive* difference between them, with an increase from 21 to 37 LS/100 eyes and a significant increase in the % eyes with LS ($\alpha = 0.01$) (Table 2). Additionally, the average number of LS per damaged eye was increased from 1.11 and 1.32, respectively.

Treatments containing DMBA (3 to 5, Table 2) showed that the number of LS/100 eyes and % eyes with LS increased in a sigmoidal way, showing the genotoxic effect of DMBA in the present experimental conditions.

Regarding DMBA genotoxicity, *weak positive* results were found between 12.5 and DMBA solvent control, while 25 and 50 μmol/vial gave *positive* increases in the LS/100 eyes. No differences in LS/100 eyes were found by pair comparisons between doses ($\alpha = 0.05$), but significant differences were detected in the percentage of eyes with LS ($\alpha = 0.01$). The increasing doses of DMBA raised the average of LS per damaged eye from 1.42 (in 12.5 μmol/vial) to 1.69 (in 50 μmol/vial).

The LS frequencies in response to the above-mentioned DMBA doses were 22.5, 23.2, and 14.5 LS/mmol DMBA, respectively. Thus, the highest number of LS/mmol of DMBA was for 25 μmol/vial of DMBA.

C. *Grifola gargal* Genotoxicity and Antigenotoxicity

The effect of DMBA solvent on the frequency of induction of total LS/100 eyes was significantly attenuated by *Grifola gargal* materials and by the WF from 37 to 17–23 LS/100 and from 28 to 15–19% eyes with LS, respectively (see treatments 6 to 9), reaching the values corresponding to those in water control (treatment 1, Table 2). It should be noted that water instead of DMBA solvent was used for genotoxicity diagnosis. The average in the number of LS per damaged eye in treatments containing *Gg*FB, *Gg*LC, and *Gg*WG were 1.13, 1.28, and 1.24, respectively, and 1.05 in the case of WF treatment. These values were lower than the ones produced by DMBA solvent.

As previously mentioned, for testing the antigenotoxic activity of *G. gargal* materials, the 25 μmol/vial dose of DMBA was chosen. A *positive* and clear antigenotoxic effect against 25 μmol/vial of DMBA was found in the presence of *Gg*FB and *Gg*WG materials (treatments 11 or 13 vs. 4, Table 2), while when *Gg*LC was present in the growing media a *weakly positive* reduction in the total LS/100 eyes was observed (treatments 10 vs. 4, Table 2). Additionally, *Gg*FB and *Gg*WG were more effective in reducing the % of eyes with LS, i.e., from 57% in 25 μmol/vial of DMBA to 34%–42% ($\alpha = 0.01$), respectively; the *Gg*LC reduction in this evaluation parameter was to 50% ($\alpha = 0.05$); finally, no difference was found in WF comparison against 25 μmol/vial of DMBA.

Otherwise, the average in the number of LS per damaged eye in *Gg*FB, *Gg*LC, and *Gg*WG treatments was low in comparison with the ones produced by DMBA: 1.27, 1.40, and 1.35, respectively, vs. 1.55. The same was also true for WF treatment: 1.32. Thus, even when all assayed materials decreased the average number of LS in damaged eyes, only *G. gargal* materials could reduced the LS/100 eyes and the % of eyes with LS.

Interestingly, the results showed that DMBA genotoxicity could be diminished only by the mushroom-containing treatments, while the wheat flour *per se* did not allow such protection. Additionally, the *Gg*WG compared to WF had a *weakly positive* reduction of 30% in the total LS/100 eyes (treatments 13 vs. 12, Table 2). When the different mushroom materials are analyzed for the above-mentioned antigenotoxic properties, the results presented in Table 2 indicate that *Gg*FB is the most efficient one, though as previously indicated all three materials exert the protective effect when compared to the promutagen alone.

D. Genotoxicity in Each Larval Instar

The size of LS and their distribution among size classes provide information on the time point of induction of the loss of heterozygosity event.[33] Figure 2 and Table 2 show the LS/100 eyes distribution for each larval instar.

All materials assayed did not show genotoxicity values at the 1st instar (maximum value 2 LS/100 eyes). The incidence of LS among treatments represented 1%–8% at this larval stage, and, because of this low value, either *negative* or *inconclusive* results were found in all comparisons.

At the 2nd larval instar the number of LS/100 eyes, being 4 and 2 for water and DMBA solvent, respectively, was lightly increased to 6–10 LS/100 eyes in the case of the different DMBA doses. No differences at this instar regarding the number of LS/100 eyes were found between 25 and 50 μmol/

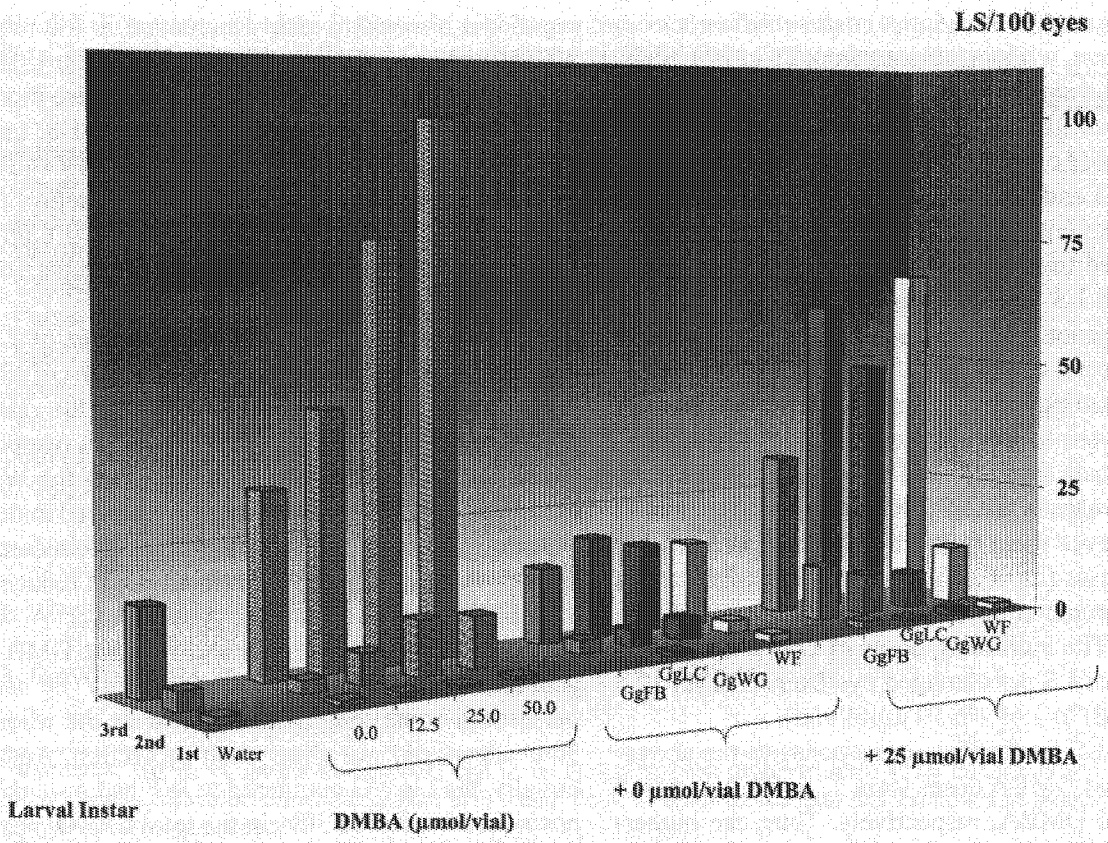

FIGURE 2. Genotoxicity (LS/100 eyes) analysis through SMART in *Drosophila melanogaster*. Data are shown for the three larval instars. Data were obtained after chronic DMBA exposition (12.5, 25.0, 50.0 µmol/vial) from egg to pupae, with water and DMBA diluent mixture as controls. Different treatments containing either *Grifola gargal* mycelia from different sources, wheat flour, and their combination with 25 µmol/vial DMBA are also shown. *Gg*FB, *G. gargal* fruit bodies; *Gg*LC, *G. gargal* mycelia obtained from liquid culture; *Gg*WG, *G. gargal* mycelia cultivated on wheat grains; WF, wheat flour.

vial DMBA (Fig. 2). In particular, at this larval instar, reduction frequencies against 25 µmol/vial DMBA were *inconclusive* with regard to the *Gg*WG treatments (13 vs. 4, Table 2), and *negative* in the remaining ones. However, an interesting *weak positive* result was found between *Gg*WG and WF (7 vs. 11 LS/100 eyes, Table 2).

At the 3rd larval instar the major *positive* results were found by considering LS frequency comparisons. Figure 2 clearly shows the markedly increased effect of the different DMBA doses on LS/100 eyes, which can be related to the progressive DMBA metabolic activation and an increase in the number of target cells.

Positive antigenotoxic effects in this larval instar, in the presence of 25 µmol DMBA, was exhibited by both *Gg*FB and *Gg*WG treatments, while *weakly positive* effects were detected in the case of *Gg*LC treatment and also WF treatment, which previously had a *negative* result when the total reduction of frequency was considered (see total LS/100 eyes in Table 2). It is important to note that only the *G. gargal* biotransformed wheat flour treatment could produce two antigenotoxic reductions: at the 2nd and 3rd larval instars.

IV. DISCUSSION

Grifola gargal is an appreciated edible mushroom of the Patagonian woods. The fruit bodies are big, fleshy, and have an almond fragrance, which is also present in the mycelium from submerged liquid cultivation and from biotransformed whole wheat grains.

It is of utmost importance to ascertain the toxic and genotoxic status before the use of this wild mushroom in new safe food products. Also, its putative antigenotoxic value will eventually increase the interest in this mushroom in the future. It has

been previously stated that the toxicity of different substances and mushrooms can be assessed through the mortality of *D. melanogaster* larvae, as a prerequisite to the determination of the genotoxicity and/or antigenotoxicity.[41]

In the present study it was shown that larvae grown on water and DMBA solvent exhibited a low mortality ratio, while the mortality elicited by the whole DMBA dose range studied tripled control values, with no significant differences among concentrations.

When analyzing for the genotoxic response to DMBA, the number of LS/100 eyes fit a sigmoid curve, as was also true for % of eyes with LS (Table 2). Considering that the working dose of DMBA for the eye spot test in *D. melanogaster* should be the one producing a significant genotoxic response with a nonsignificant increase in mortality, it was found that 25 µmol/vial DMBA, i.e., 3.8 mM, was adequate to perform the genotoxic/antigenotoxic evaluation. A strong genotoxic activity of DMBA at the whole dose range assayed can be appreciated by considering the % of eyes exhibiting LS, the high values and the close parallelism with the LS/100 eyes, and the increasing values in the average number of LS per damaged eye.

Vogel et al.[45] found up to 20-fold variation in induced LS frequencies between different *D. melanogaster* strains. This genotype-dependent variability in response to promutagens is a known phenomenon, and such a difference has been related to an increased cytochrome P450–dependent metabolism.[46,47] The susceptible strain *Leiden standard* tolerated a 2 mM DMBA dose, resulting in a genotoxic response of 41 LS/mmol.[33] The *D. melanogaster* strain used throughout the present study (*CNEA yellow* and *white* strains) is more resistant since it tolerated up to 2–3 times more DMBA concentration and produced 23 LS/mmol, that is, 43% lower.

As previously mentioned, *Grifola gargal* is considered an edible mushroom, and it may be found in Chilean food shops;[48] hence, it was important to test for its toxicity.

After evaluation of the toxicity through treatments containing *G. gargal*, and in comparison with wheat flour, larvae mortality ratios were in the range of 2%–16%, which did not significantly differ from those results produced either by the DMBA solvent or water; hence, the absence of *G. gargal* mushroom toxicity was demonstrated. In addition, the three biotypes of mycelia showed a protective effect against 25 µmol/vial DMBA, while wheat flour showed no protective effect.

It is important to emphasize that no genotoxicity was found when evaluating the different biotypes of *G. gargal* and wheat flour, particularly because, as mentioned above, this mushroom is collected for human consumption. Moreover, those *G. gargal* biotypes lowered the genotoxicity showed by DMBA solvent.

The mutation frequency values (total LS/100 eyes, Table 2) for all treatments, including *G. gargal* biotypes or wheat flour together with 25 µmol/vial DMBA, were significantly decreased in the following order: *Gg*FB, *Gg*WG, *Gg*LC, and WF treatments. However, WF treatment showed a *negative* result in frequency comparisons and could not decrease the % eyes. Thus, the SMART in *D. melanogaster* clearly demonstrated that *G. gargal* mycelia from those different biotypes markedly attenuate DMBA genotoxicity, while wheat flour is able to exert protective effects only if previously biotransformed by *G. gargal*.

In *Drosophila* larvae under chronic chemical exposure, small spots can originate by segmental aneuploidy, which results from non-disjunction of chromosomes during mitosis, which leads to a reduced proliferation capacity. An alternative and nonexclusive origin for the appearance of those small spots arises from the occurrence of late damage caused by the activation of DMBA promutagen. Large spots, on the other hand, are produced by early damage in the imaginal disk cells of larvae and continuous mitosis of cells expressing the marker.[49,50]

In the present SMART protocol in *D. melonogaster*, DMBA genotoxicity greatly increased with increasing doses up to the 3rd larval instar; slight increments were detected at the 2nd larval instar, while the same genotoxic incidence was kept in the 1st larval instar.

Genotoxic responses obtained at the 3rd larval instar, a stage with a higher number of target cells, were more evident for both 25 µmol/vial of DMBA and its solvent, with a significant increase in the mutation frequencies observed.

At the 2nd larval instar there was no difference between the count of LS/100 eyes corresponding to 25 and 50 µmol/vial of DMBA. A plateau start-

ing from 25 µmol/vial of DMBA was observed. The latter suggests that *D. melanogaster* cannot metabolize a greater amount of promutagen.

With regard to *G. gargal* treatments, a marked antigenotoxicity was found in *Gg*FB, an effect which was pronounced at the 3rd instar, but not so evident at the 2nd instar. By taking all treatments as a whole, a lower effectiveness in reducing the antigenotoxic response was observed in the case of *Gg*WG; nevertheless, it showed antigenotoxicity at the 2nd and 3rd instars. This fact emphasizes the point that mycelium coming from different sources elicits different antigenotoxic responses.

It is interesting to note that despite the low ratio of mycelium contained in the DMBA treatment in the presence of *Gg*WG (ca. 0.4% on a dry-weight basis), a reduction in the mutation frequencies could be observed through a *positive* effect at the 3rd instar and a *weakly positive* effect at the 2nd instar. Metabolites coming from the biotransformed wheat kernel and from the *G. gargal* mycelia could be acting together to prevent DMBA damage on DNA.

Interestingly, Huang et al.,[51] looking at *G. frondosa* fruitbodies, mycelium from submerged culture, and biotransformed wheat flour for antioxidant activity, reducing power, DPPH radical scavenging ability, and ability to chelate ferrous ions, found that fermented wheat kernels do possess a high reducing power, free-radical scavenging ability, and ferrous ions chelating ability, even when they carry a low proportion of mycelia.

An increase in the phenolic content was found in *G. frondosa* biotransformed wheat, but no close correlation was found between phenolic content and antioxidant attributes,[51] and it could be concluded that these compounds are not the only substances that could be responsible for such properties in their mushrooms extracts.

It should also be mentioned that *G. gargal* antioxidant properties have been already attributed to its phenolics content, especially flavonoids.[34,35]

It is also quite probable that biotransformed wheat kernel, with the strain of *G. gargal* used in the present work, also increases its phenolic content, as has been demonstrated to be the case with *G. frondosa* and other fungi.[51,52]

With regard to these molecules and DNA metabolism, Ferguson,[53] in her review on the roles of polyphenol substances on DNA stability, shows some of these molecules to possess antimutagenic properties.

Hitherto no evidence was found about mushroom phenolics against PAHs genotoxicity, despite the abundant research done on the activity of plant phenolic compounds against PAHs.[53]

The antimutagenic effect found in mushrooms against PAHs, like DMBA, could be by desmutagenic action, i.e., inactivation of derived mutagens by direct interaction,[10] or by bioantimutagenic action, i.e., those that interfere in cellular endogenous processes that otherwise would lead to mutagenic effects.[5,54–56]

Some mushroom materials, such as mushrooms powders,[5] aqueous, organic, or alcoholic mushroom extracts[54–57] and some molecules isolated from mushrooms like polysaccharides,[58] linoleic acid,[59] polipeptide/proteins,[5] and triterpenoids,[60] have shown to possess antimutagenic activity against the genotoxicity elicited by PAHs.

Results suggest that *G. gargal* is not a toxic or genotoxic mushroom and is hence safe to be consumed as functional food. However, more research is needed to investigate the metabolic bases of its protective antigenotoxic activity.

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