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Abbreviations: AF, Aflatoxin; AFBO, AFB1-exo-8,9-epoxide; AFL, aflatoxicol; C, Curcumin (≥ 94%); CAT, catalase; CDNB, 1- chloro-2, 4-dinitrobenzene; CL, Curcumin from *Curcuma longa* (≥ 65%); CYP, cytochrome P450; DMSO, Dimethylsulfoxide; DTNB, dithio-bisnitrobenzoic acid; EPHX, epoxide hydrolase; GPx, glutathione peroxidase; GST, glutathione S-transferase; Nrf2, NF-E2-related factor 2; NQO1, quinone oxidoreductase; q-PCR, quantitative Real-time PCR; Q, Quercetin hydrate; R, Resveratrol; SOD, superoxide dismutase; UGT, Uridine 5'-diphospho-glucuronosyltransferase.

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ABSTRACT

Aflatoxin (AF) B1, a widespread food and feed contaminant, is bioactivated by drug metabolizing enzymes (DME) to cytotoxic and carcinogenic metabolites like AFB1-epoxide and AFM1, a dairy milk contaminant. A number of natural antioxidants have been reported to afford a certain degree of protection against AFB1 (cyto)toxicity. As the mammary gland potentially participates in the generation of AFB1 metabolites, we evaluated the role of selected natural antioxidants (i.e. curcumin, quercetin and resveratrol) in the modulation of AFB1 toxicity and metabolism using a bovine mammary epithelial cell line (BME-UV1). Quercetin and, to a lesser extent, resveratrol and curcumin from *Curcuma Longa* (all at 5 μM) significantly counteracted the AFB1-mediated impairment of cell viability (concentration range: 96-750 nM). Moreover, quercetin was able to significantly reduce the synthesis of AFM1. The quantitative PCR analysis on genes encoding for DME (phase I and II) and antioxidant enzymes showed that AFB1 caused an overall downregulation of the detoxifying systems, and mainly of GSTA1, which mediates the GSH conjugation of the AFB1-epoxide. The negative modulation of GSTA1 was efficiently reversed in the presence of quercetin, which significantly increased GSH levels as well. It is suggested that quercetin exerts its beneficial effects by depressing the bio-transformation of AFB1 and counterbalancing its pro-oxidant effects.

Keywords: Aflatoxin B1; Aflatoxin M1; Bovine; Mammary epithelial cells; Quercetin

1. Introduction

Aflatoxin (AF) B1 is a natural occurring difuranocoumarin-based mycotoxin, mainly synthesized by *Aspergillus flavus* and *parasiticus* as a secondary metabolite. The distribution of AFB1 is especially abundant in areas with high temperature and humidity, although fungal growth and toxinogenesis may also occur in conditions of drought stress (Gilbert et al., 2016). A wide variety of food and feed commodities, including cereals and oil-rich agricultural crops, can be contaminated both pre- and post-harvest (Abrar et al., 2013; Rodrigues and Naehrer, 2012). AFB1 exposure has been associated with various diseases and health problems in humans, livestock and domestic animals, such as growth impairment and immune suppression (Wild and Gong, 2010). According to sufficient evidence of potent genotoxicity and carcinogenicity, AFs as a group have been classified as a class 1A human carcinogenic agents by the International Agency for Research on Cancer (IARC, 2012).

Bioactivation by hepatic microsomal cytochrome P450 (CYP) enzymes is essential for AFB1 toxicity in all species (Dohnal et al., 2014). Oxidative phase I metabolism, mainly mediated by CYP1A and CYP3A but also CYP2A, generates several water-soluble metabolites including AFB1-exo-8,9-epoxide (AFBO), AFM1, AFB2a, AFQ1 and AFP1. AFBO, a highly reactive intermediate, may bind guanine residues in DNA or RNA to form adducts, inducing DNA mutations, and inhibiting transcription and translation (Kuilman et al., 1998; Wogan et al., 2012). Hydrolysis of AFBO, either spontaneously or enzymatically through epoxide hydrolases (EPHX), leads to the generation of AFB1-dihydrodiol, which can bind lysine residues leading to protein damage and subsequent necrosis (Kuilman et al., 2000; McLean and Dutton, 1995). Toxic outcomes are also associated with intracellular reactive oxygen species (ROS) generation (Marchese et al., 2018; Zhang et al., 2015). AFBO can be readily detoxified by GSH through glutathione S-transferases (GST), mainly GSTA1, that produce polar and less toxic metabolites excreted in the urine as mercapturic acid derivatives. CYP1A mediates also the generation of AFM1, which may be

conjugated to glucuronic acid through uridine 5'-diphospho-glucuronosyltransferases (UGT), and subsequently excreted via the bile. Alternatively, it may enter the systemic circulation being excreted unmodified in the urine or milk of healthy women, dairy cows and other mammals dietary exposed to AFB1 (Diaz and Sánchez, 2015; Fink-Gremmels, 2008; Shuib et al., 2017). The notable hepatic CYP1A constitutive expression in cattle is expected to lead to a relatively higher rate of AFM1 generation compared to other animal species (Nebbia et al., 2003). As a consequence, milk and dairy products may represent a significant source of AFs for humans. Although AFM1 carcinogenic potency is approximately 2–10% with respect to AFB1 in mammalian species, its toxic hazard is generally comparable to that of the parent compound (Marchese et al., 2018). Thus, in the European Union, very low limits have been set for AFM1 in dairy milk (0.050 µg/kg, lowered to 0.025 µg/kg in infant formulae) to protect consumer's health (EC Regulation 1881/2006).

Factors capable of modulating enzymes involved in AFB1 metabolism (e.g. dietary constituents, environmental pollutants, etc.) may dictate the sensitivity to toxic effects by affecting the generation/inactivation of toxic metabolites. A number of natural antioxidant compounds (e.g. curcuminoids and flavonoids) may have the potential to reduce the generation and/or increase the inactivation of AFB1 metabolites through the inhibition of CYP enzyme activity and/or through the induction of phase II (GST and UGT) and antioxidant enzymes (catalase - CAT, glutathione peroxidase - GPx, quinone oxidoreductase - NQO1, and superoxide dismutase - SOD) (Bisht et al., 2010; Limaye et al., 2018; Miron et al., 2017). The *in vivo* protective effects of natural antioxidants against AFB1 toxicity have already been shown in laboratory animals, mainly rats, and in broilers (Eftekhari et al., 2018; Mohajeri et al., 2018; Sridhar et al., 2015) but not in cattle or in dairy cows.

In the last years the possible change in patterns of AF occurrence in food and feed crops due to climate change has become a matter of concern, especially in Southern Europe, where the contamination of maize has reached alarming levels (Battilani et al., 2016). Among the various approaches to reduce the risk of AF ingestion by livestock, one of the most employed is the use of

binders (e.g. clays and zeolites) that complex the mycotoxins in the gastrointestinal tract and reduce their bioavailability (Kabak et al., 2006). However, the prolonged administration of high dosages may reduce the utilization of vitamins and minerals (Chestnut et al., 1992). Hence, there is an increasing interest towards the possible use of natural compounds to both counteract the negative effects of AFs on animal health and reduce the contamination risk of animal products (i.e. milk). Beside liver, extra-hepatic organs may play a role in AFB1 biotransformation. A study performed in a bovine mammary epithelial cell line (BME-UV1) indicates that also the mammary gland may participate in the generation of AFM1, and possibly of other AFB1 metabolites (Caruso et al., 2009). The goal of this study was to evaluate the role of selected natural antioxidants (i.e. curcumin, quercetin and resveratrol) in the modulation of AFB1 toxicity and metabolism in the bovine species. Such an issue has been addressed *in vitro* using the above mentioned BME-UV1 cell line, as a model for the bovine mammary gland.

2. Materials and methods

2.1. Materials and chemicals

AFB1, quercetin hydrate (Q), resveratrol (R), curcumin (≥ 94% purity, C), curcumin from *Curcuma longa* (containing curcumin ≥ 65%, bisdemethoxycurcumin and demethoxycurcumin, CL), Dimethylsulfoxide (DMSO), and all cell culture reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). The Cell Proliferation Reagent WST-1 was from Roche Diagnostics (Mannheim, Germany). HPLC-grade acetonitrile was purchased from Sigma-Aldrich (Taufkirchen, Germany), MS-grade formic acid from Fisher Chemical (Thermo Fisher Scientific Inc., San Jose, CA, USA) and bidistilled water was obtained using Milli-Q System (Millipore, Bedford, MA, USA). All the materials for the quantitative RT-PCR (q-PCR) analysis (including RNA extraction and cDNA synthesis) were supplied by Bio-Rad (Valencia, CA, USA). The BCA Protein Assay Kit

was obtained from Thermo Fisher Scientific. If not specified otherwise, all other chemicals were from Sigma–Aldrich.

2.2. Cell culture and treatments

The BME-UV1 bovine mammary epithelial cell line (kindly provided by Prof. Mario Baratta, Dept. of Veterinary Sciences, University of Torino, Italy) was grown in DMEM medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1000 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg⁄mL amphotericin B. Cells were maintained at 37 °C in an atmosphere of 95% relative humidity and 5% CO2. Cells were trypsinized every 3–4 days for sub-culturing.

For the viability assays, cells were seeded in 96-well culture plates at a density of 5×10^3 cells/well and treated at the conditions described below, after reaching approximately 60% confluency. Six replicates for each experimental condition were included. To determine the concentrations to be used in the co-incubation experiments, cells were treated with increasing concentrations of AFB1 (12-3000 nM), C (0.15-20 μM), CL (0.15-20 μM), Q (0.12-50 μM) or R (0.12-50 μM) for 24 and 48 h. To evaluate the protective effects of the antioxidants against AFB1-cytotoxicity, cells were pre-incubated with or without each antioxidant (5 μM) for 16 h, and subsequently exposed to increasing concentrations of AFB1 (96-750 nM) in the presence or absence of each antioxidant for 24 and 48 h.

For the assessment of gene expression, GSH level and GST activity, cells were seeded in 10-cm dishes at a density of 3×10^6 cells/dish and, after reaching approximately 60% confluency, treated at the following conditions: pre-incubation with or without C or Q (5 μM) for 16 h followed by exposure to AFB1 (375 nM) in presence or absence of C or Q. Then, gene expression and GST activity were assessed after 24 h; GSH level was measured after 2, 6 and 24 h. The effects of the

either antioxidant were evaluated incubating cells with C or Q (5 μM) alone for the corresponding time-points.

For the analytical investigations, cells were seeded in 10-cm dishes at a density of 3×10^6cell/dish and, after incubation at the conditions described below, growth media were collected for the measurement of AFM1. To test the capability of BME-UV1 cells to metabolize AFB1, cells were exposed to increasing concentrations of AFB1 (188-375-750 nM) for 8 and 24 h. To evaluate the effects of the antioxidants on AFM1 synthesis, cells were pre-incubated with each antioxidant (5μM) for 16 h and subsequently co-exposed to AFB1 (375nM) and each antioxidant for 24 h.

For all experiments, chemicals were dissolved in DMSO (used as control), whose final concentration in the growth medium did not exceed 0.1% (v/v). Due to the possibility that a considerable proportion of AFB1 may bind to serum albumin (Caruso et al., 2009), all the incubations with the mycotoxin were performed in cell culture medium containing 1% FBS.

2.3. Cell viability assays

Viability of cells exposed to all the tested substances, except Q, was investigated using the Cell Proliferation Reagent WST-1 (Roche) according to the manufacturer's instructions. Taking into account the ability of Q to reduce tetrazolium salts (Peng et al., 2005), viability of cells exposed to such antioxidant was evaluated by the Neutral Red Uptake (NRU) assay, according to the protocol outlined by Repetto et al. (2008). The absorbance values were measured at 450nm and 540 nm for the WST-1 and NRU tests, respectively, with a microplate reader. Cell viability was expressed as percent relative to control cells (0.1% DMSO).

2.4. Analytical investigations

The presence of AFB1 and AFM1 in growth media was assessed by means of LC/MS-MS analysis using a UHPLC (Dionex Ultimate 3000) system coupled with a triple quadrupole mass spectrometer (TSQ Vantage) (Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with an ESI interface. All the analyses were performed on a reverse-phase C18 Sunshell column (particle size 2.6 µm, inner diameter of 2.1 µm and a length of 100 mm; ChromaNik Technologies Inc., Osaka, Japan) using bi-distilled water (A) and acetonitrile (B) both acidified with 0.2% of formic acid as eluents. The injection volume was 3 µL. The gradient started at 5% B and it was kept for 1 minute, reaching 95% B in 11 min with a flux of 0.35 mL/min (12 min). Then it was kept isocratic for 5 min (17 min), reaching the initial conditions in the next 1 minute (i.e. 95% A and 5% B; 18 min). The initial conditions were kept 8 minutes before running a new analysis, with a total run time for each sample of 26 min.

Compounds under investigation were monitored under positive ionization mode (spray voltage = 3500 V), with the capillary temperature at 270 °C, while the vaporizer temperature was kept at 200 ° C. The sheath gas flow was set at 40 units and the auxiliary gas pressure at 5 units.

Detection was carried out using multiple reactions monitoring (MRM) mode. The following transitions were used for the compounds monitoring: 313.10 / 241.20 (CE 42 eV), 313.10 / 270.10 (CE 28 eV) and 313.10 / 285.10 (CE 25 eV) for AFB1, and 329.07 / 229.00 (CE 42 eV), 329.07 / 259.00 (CE 25 eV) and 329.07 / 273.00 (CE 25 eV) for AFM1.

2.5. RNA extraction and Quantitative RT-PCR (q-PCR)

Total RNA was isolated using the PureZOL™ RNA Isolation Reagent, according to the manufacturer's protocol. RNA purity and quantity was evaluated by absorbance readings using the NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Illkirch Cedex, France). The ratio of the optical densities measured at 260 and 280 nm were >1.9 for all RNA samples. One mg of total RNA was reverse transcribed into cDNA using ISCRIPT cDNA SYNTHESIS KIT

according to the manufacturer's instructions, in a final volume of 20 µL. Sufficient cDNA was prepared in a single run to perform the q-PCR experiments for all the selected genes. Primers for CYP1A1, GSTA1, GSTA2, NQO1, UGT1A1, UGT1A6, and GAPDH were from Girolami et al., (2015), whereas primers for CYP2A13, CYP3A28, CAT, EPXH1, EPXH2, EPXH3, EPXH4, GSTM1, GPx, and SOD were designed on Bos Taurus GenBank and Ensembl mRNA sequences using Primer 3 Software (version 3.0, Applied Biosystems, Foster City, CA). Oligonucleotides were designed to cross the exon/exon boundaries to minimize the amplification of contaminant genomic DNA and were analysed with the NetPrimer tool (available at http://www.premierbiosoft. com/netprimer/index.html) for hairpin structure and dimer formation. Primer specificity was verified with BLAST analysis against the genomic NCBI database. Table 1 summarizes primer information, including sequences, gene accession numbers and amplicon sizes. Each primer set efficiency was comprised between 95% and 100%. GAPDH was selected as the reference gene since its expression was not affected by any of the treatments. q-PCR reactions were performed on 500 ng of cDNA, in a final volume of 20 µl consisting of the 1× iTaq SYBR Green Supermix with ROX and an optimized concentration of each primer set (150–900 nM range). PCR amplification was run on an ABI 7500 Real-time PCR System (Applied Biosystems) using 96-well optical plates under the following conditions: 30 s at 95 °C for polymerase activation, and 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Each reaction was run in triplicate, and a no-template control was included using water instead of cDNA. The modulation of gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method and data were expressed as fold-change compared to control samples (Livak and Schmittgen, 2001); a 2.0 fold-change cut-off was selected.

Table 1

Primers for quantitative RT-PCR analysis.

Gene	Accession no.	Sequence	Amplicon size (bp)
CAT	NM_001035386.2	F: ATTTGAAAGTGCTGAATGAGGAG R: TGGACATCGCTGAAGTTCTT	118
CYP1A1	XM_588298	F: CGAGAATGCCAATATCCAGC R: TGCCAATCACTGTGTCCAG	173
CYP2A13	XM_024979093.1	F: TCGCAGAGTCACCAAGGATA R: AGAACTTGGGGTCTCTCAGC	128
CYP3A28	NM_174531	F: CGTCCCGAAAGGTTCAGTAA R: GCAGGATTCTGACAAGAGCA	139
EPHX1	NM_001034629	F: CCTGGGGCAAGTCAATAAGC R: TCACCCACTTTTCTGGCAAG	101
EPHX2	NM_001075534	F: CGCGGAGAAGGACTTGGT R: TGTCCAGTGTCCACAATCCT	103
EPHX3	NM_001193176.1	F: TCGAAACATCTTCAGGACCTTC R: CCTTGCTCAAAATAGGGGTCC	96
EPHX4	NM_001075855	F: GCCCAGCTGTTCAAATCCAG R: TCCTTTTCTTCCAATGCCAGTG	138
GPx	NM_174076.3	F: GCATCAGGAAAACGCCAAGA R: CTTCTCGCCATTCACCTCG	118
GSTA1	NM_001078149	F: AGAGGGTGTGGCAGATTTGG R: TGGCTCTTCAGCACATTTTCA	141
GSTA2	NM_177515	F: TTACCACTGTGCCCACCTGAT R: CTTGTCCGTGATTCTTCAGCAC	112
GSTM1	NM_175825.3	F: TTTCCTGGTTTATGACGTCCTT R: AAGCGGCTGGACTTCATGTA	138
NQO1	NM_001034535	F: CGGAATAAGAAGGCAGTGCT R: AGCCACAGAAGTGCAGAGTG	130
SOD	NM_174615.2	F: GAGAGGCATGTTGGAGACCT R: TCTGCCCAAGTCATCTGGTT	153
UGT1A1	NM_001105636	F: TGGGTCTGTCTGGATTCTCA R: GGAATCTCCGAGACCATTGA	195
UGT1A6	NM_174762.1	F: CAACACGGTCCTCATCGGA R: GCCCAAAGAGAAAACCACAA	115
GAPDH	NM_001034034	F: GGAGAAACCTGCCAAGTATGAT R: GAGTGTCGCTGTTGAAGTCG	125

2.6. GSH content determination and GST activity assay

Cells were collected using a rubber tipped cell scraper and centrifuged at 200 x g for 5 minutes at room temperature. After pellet suspension in 0.1 M phosphate buffer pH 7.4 (0.1 M Tris acetate, 0.1 M KCl, 1 mM EDTA, and 18 μM butylated hydroxytoluene), cells were lysed by four cycles of freezing–thawing (fresh frozen in liquid nitrogen and thawed at 37 °C), followed by ten cycles of 10 s of sonication on ice. The homogenate was centrifuged at 17000 x g for 15 min at 4 °C and the supernatant stored at −80 °C until analysis. Protein concentrations were measured by BCA Protein Assay Kit.

GSH content was determined with dithio-bis-nitrobenzoic acid (DTNB) on deproteinized samples as described elsewhere (Ugazio et al., 1993). Results were expressed as μg of GSH per mg of protein.

Total GST was assayed using 1-chloro-2,4-dinitrobenzene (CDNB) 1 mM as described by Habig et al. (1974). Results were expressed as nmoles conjugated GSH per min per mg of protein.

2.7. Statistical analysis

All data are shown as mean ± SEM of at least three independent experiments. In case data were normally distributed according to the D'Agostino and Pearson normality omnibus test, significant differences among groups were evaluated by one-way analysis of variance (ANOVA), followed by the Dunnett's or Bonferroni's post-hoc tests. Otherwise, the non-parametric Kruskall-Wallis ANOVA test followed by the Dunn's test was used. Differences were considered statistically significant when the two-sided P value was < 0.05. Analyses were performed with the GraphPad Prism 7.03 software (Graph Pad Software, San Diego, CA, USA).

3. Results

3.1. Effects of natural antioxidants on AFB1-induced cytotoxicity

The natural antioxidants (curcumin, quercetin and resveratrol) were selected based on their ability to ameliorate aflatoxicosis upon dietary supplementation in different species (Eftekhari et al., 2018; Mohajeri et al., 2018; Sridhar et al., 2015). Since the most commonly used, curcumin, is typically administered in the form of turmeric powder, we tested both the high purity compound (C) and the extract from *Curcuma longa* (CL) that contains also the main metabolites (bisdemethoxycurcumin and demethoxycurcumin).

To investigate the capacity of the antioxidants to counteract the impairment of cell viability induced by AFB1 in an *in vitro* model of bovine mammary epithelium, BME-UV1 cells were first exposed to each individual compound at different concentration ranges (AFB1, 12 nM - 3 μM; C and CL, 0.15 - 20 μM; R and Q, 0.125 - 50 μM) for 24 h and 48 h. The concentration response curves showed that AFB1 reduced cell viability in a time- and concentration dependent manner compared to control cells (LC50 at 24 h and 48 h equal to 687 and 180 nM, respectively) (Fig. 1). Conversely, all the antioxidants exhibited significant cytotoxic effects ($P < 0.05$ or less) only at very high concentrations (from 10 μM for CL, from 20 μM for C and R, and from 50 μM Q) (Fig. S1). Based on the dose-response results, the optimal experimental conditions for the co-incubation assays were set. In accordance with the protocol adopted in several studies investigating the protective effects of antioxidants against oxidative stress *in vitro* (Qin et al., 2015; Ramyaa and Padma, 2013), cells were pre-treated for 16 h with each antioxidant individually (5 μM), followed by a co-incubation with AFB1 (concentration range: 96 - 750 nM) and C, CL, R, or Q for 24 and 48 h.

C did not affect the reduced cell viability triggered by AFB1 at any experimental conditions (Fig. S2), while all the other antioxidants were able to afford some protection against AFB1-cytotoxicity albeit to a different extent (Fig. 2). CL and R were significantly ($P < 0.05$ or less)

effective against AFB1 (from 96 to 375 nM) only at 48 h (Fig. 2 B and F), increasing cell viability up to 40% and 50%, respectively. Conversely, Q significantly ($P < 0.05$ or less) protected cells from AFB1 at both 24 h (from 188 to 750 nM) and 48 h (at all the tested concentrations) (Fig. 2 C and D). Moreover, Q enhanced the viability of AFB1-treated cells up to 62% (AFB1 375 nM at 48 h), resulting the most effective compound.

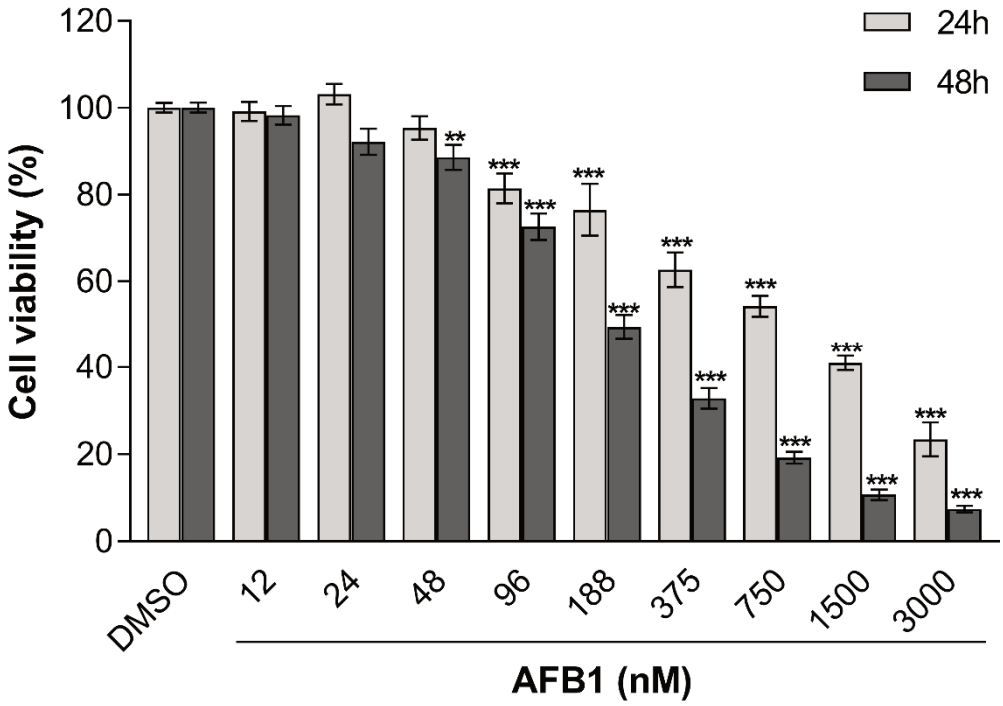

Fig.1. Impairment of cell viability induced by AFB1 (12-3000 nM) in BME-UV1 cells after 24 and 48 h exposure, measured with the WST-1 assay. Results are expressed as percentage of viability compared to the solvent control (0.1% DMSO). Data are represented as mean ± SEM of three independent experiments and analysed by one-way ANOVA followed by Dunnett's test. Statistical differences with respect to the controls are indicated (**$P < 0.01$, ***$P < 0.001$).

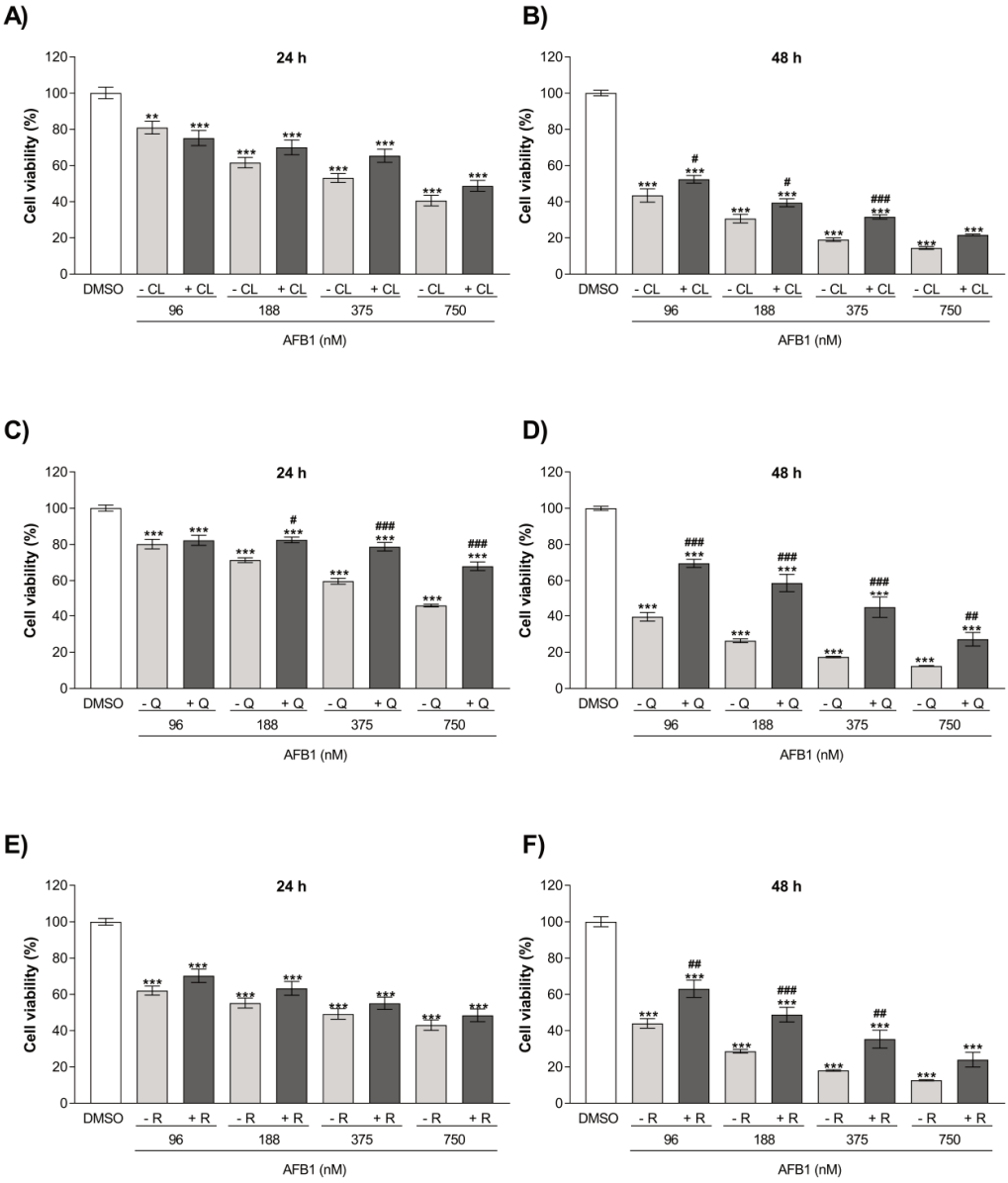

Fig. 2 Protective effects of CL (A-B), Q (C-D) and R (E-F) against AFB1-induced toxicity in BME-UV1 cells, measured with the WST-1 (for CL and R) or NRU (for Q) assays. Cells were pre-incubated for 16 h with or without each antioxidant (5 μM) and then exposed to AFB1 (96-750 nM) in the presence or absence of each antioxidant for 24 and 48 h. Results are expressed as percentage of viability compared to the solvent control (0.1% DMSO). Data are represented as mean ± SEM of three independent experiments and analysed by one-way ANOVA followed by Bonferroni's test. Statistical differences with respect to the controls (**$P < 0.01$, ***$P < 0.001$), and with respect to the cells treated with the corresponding concentration of AFB1 alone ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$) are indicated.

3.2. Effects of natural antioxidants on AFM1 synthesis

The ability of the selected natural antioxidants to modulate the synthesis of AFM1 in BME-UV1 cells was then investigated. First, the metabolic capacity of cells was assessed by the incubation with increasing concentrations of AFB1 (188, 375 and 750 nM) and the measurement of AFM1 in culture media after 8 and 24 h. Analytical results showed that AFM1 was synthetized by BME-UV1 cells proportionally to both AFB1 concentrations and incubation time (Table 2). For the co-incubation assays, cells were treated with a single AFB1 concentration (375 nM) using the same conditions as in the cell viability experiments. Media were collected after 24 h. Q significantly ($P < 0.001$) reduced the rate of AFM1 synthesis by approximately 70% compared to the cells treated with AFB1 alone. All the other antioxidants did not significantly affect AFM1 concentration in culture media, although a trend in declining AFM1 synthesis could be appreciated in cells co-exposed to AFB1 and CL or R (Fig.3).

Table 2

AFM1 synthesis by BME-UV1 cells treated with AFB1 (188 - 750 nM) for 8 h and 24 h, as measured by LC/MS-MS in culture media.

	AFM1 (nM)	
	8 h	**24 h**
AFB1 (188 nM)	14.43 ± 0.07	27.91 ± 0.74
AFB1 (375 nM)	28.18 ± 0.16	44.75 ± 0.91
AFB1 (750 nM)	39.72 ± 5.93	75.43 ± 5.54

Data are expressed as mean ± SEM of three independent experiments.

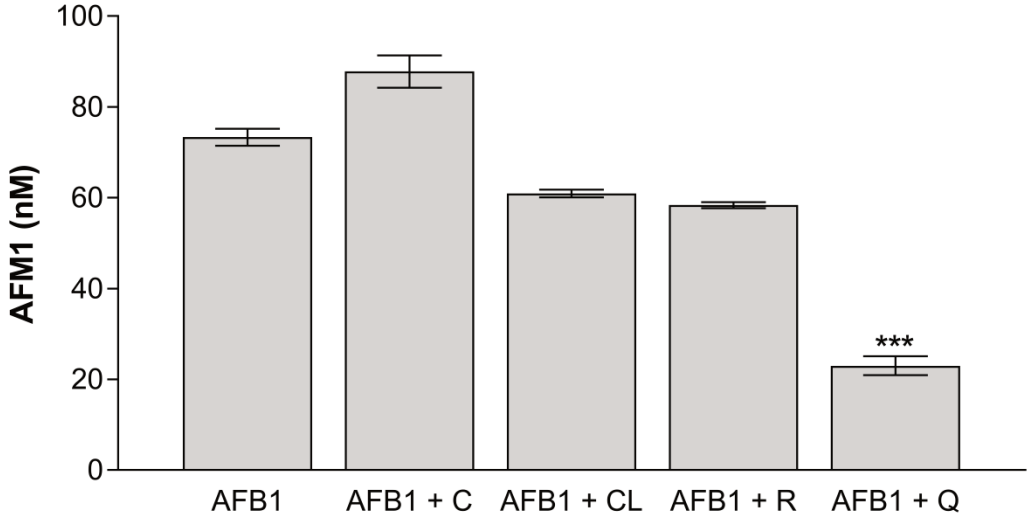

Fig. 3. Effects of C, CL, Q, and R on the synthesis of AFM1 in BME-UV1 cells, measured by LC/MS-MS in culture media. Cells were pre-incubated for 16 h with each antioxidant individually (5 μM) and then co-exposed to AFB1 (375 nM) and each antioxidant for 24 h. Data are represented as mean ± SEM of three independent experiments and analysed by Kruskall-Wallis ANOVA test followed by the Dunn's test. Statistical differences with respect to cells treated with AFB1 alone are indicated (***$P < 0.001$).

3.3. Modulation of gene expression by AFB1 and natural antioxidants

In order to study the possible mechanisms responsible for both the protective effects against AFB1 cytotoxicity and the modulation of AFM1 synthesis exerted by the natural antioxidants in BME-UV1 cells, the modulation of genes encoding for enzymes involved in AFB1 metabolism (i.e. CYP1A1, CYP2A13, CYP3A4, EPHX 1-2-3-4, GSTA1-2, GSTM1, and UGT1A1-6) and in the antioxidant defence (i.e. CAT, NQO1, SOD and GPx) was evaluated. Based on the results of both the viability assays and the analytical investigations, we decided to compare the effects of C and Q that correspond to the least and the most effective antioxidant compound, respectively. BME-UV1 cells were exposed individually to AFB1, C and Q, or co-exposed to AFB1 and either antioxidant at the same conditions used in the viability and biotransformation experiments.

The q-PCR results showed that BME-UV1 cells do not express some of the investigated genes, and namely CYP2A13, CYP3A28, GSTM1, EPHX isoforms 2 and 4, and UGT1A6. In addition, although expressed, CYP1A1 and UGT1A1 were not modulated by any of the treatments, i.e. neither by the single molecules nor under co-incubation conditions (data not shown). The remaining tested genes (i.e. the antioxidant enzymes CAT, GPx, NQO1 and SOD, and biotransformation EPHX1-3 and GSTA1-2) were all significantly ($P < 0.05$ or less) down-regulated by AFB1 to a variable extent, while their mRNA levels were not affected by C or Q alone (Fig. 4). The exposure to AFB1 alone decreased the expression of CAT, GPx and SOD by approximately 2-fold, and those of NQO1 and EPHX3 up to 5-fold. The most down-regulated genes were EPHX1 (10-fold change), GSTA1 (43-fold change) and GSTA2 (16-fold change). The co-incubation of AFB1 with C or Q did not counteract the negative modulation of both the antioxidant and detoxifying enzymes elicited by the mycotoxin, with the exception of GSTA1, whose downregulation was alleviated by approximately 80% in the presence of Q.

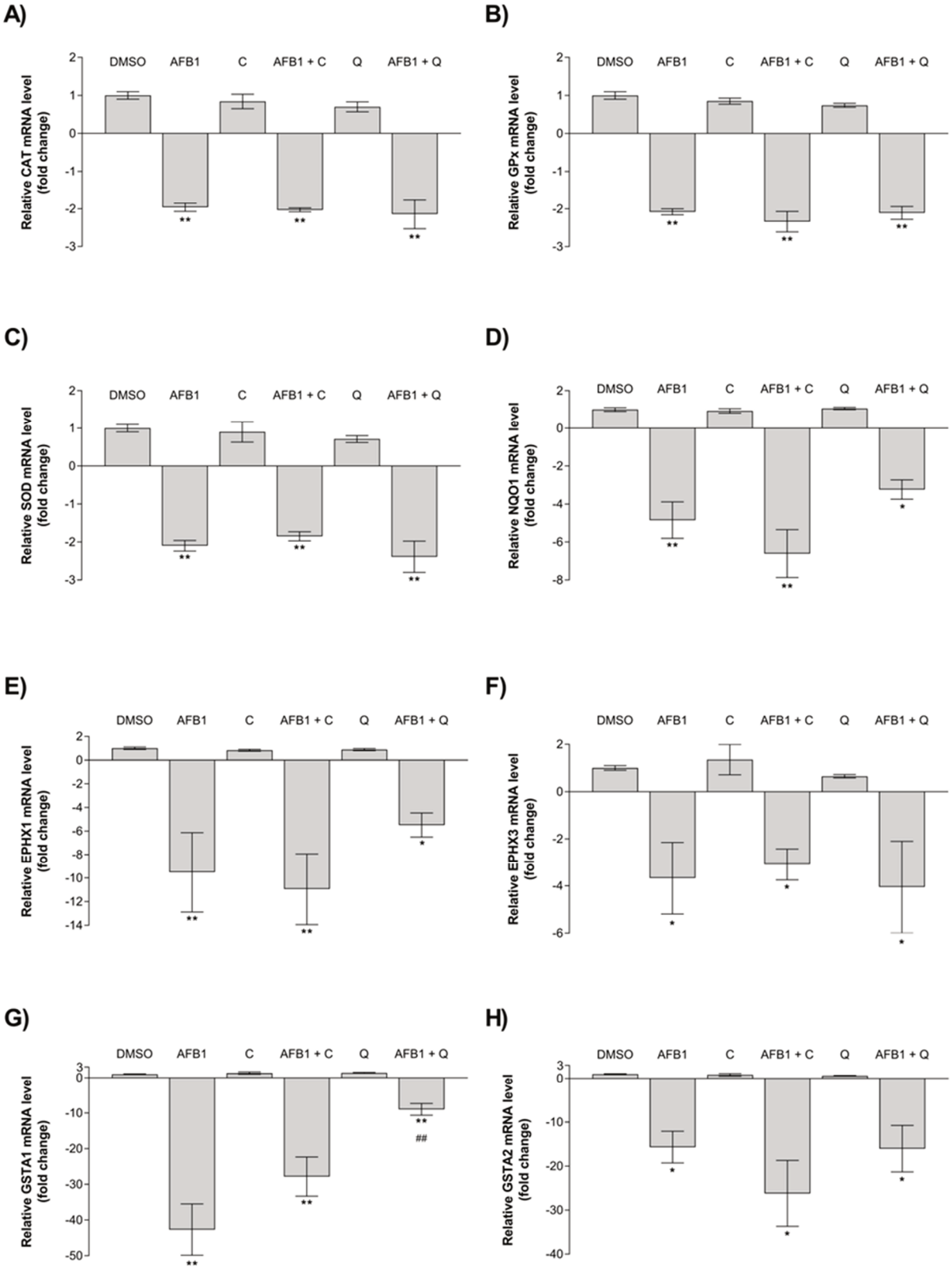
A)
B)
C)
D)
E)
F)
G)
H)
DMSO
AFB1
C
AFB1 + C
Q
AFB1 + Q
Relative CAT mRNA level (fold change)
Relative GPx mRNA level (fold change)
Relative SOD mRNA level (fold change)
Relative NQO1 mRNA level (fold change)
Relative EPHX1 mRNA level (fold change)
Relative EPHX3 mRNA level (fold change)
Relative GSTA1 mRNA level (fold change)
Relative GSTA2 mRNA level (fold change)
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Fig. 4. Gene expression modulation in BME-UV1 cells treated with AFB1 (375 nM), C (5 μM), or Q (5 μM), or co-exposed to AFB1 (375 nM) + C (5 μM) and AFB1 (375 nM) + Q (5 μM). The mRNA expression levels of CAT (A), GPx (B), SOD (C), NQO1 (D), EPHX1 (E), EPHX3 (F), GSTA1 (G) and GSTA2 (H) were determined by quantitative RT-PCR. Results are expressed as fold change compared to the solvent control (0.1% DMSO). Data are represented as mean ± SEM of three independent experiments and analysed by Kruskall-Wallis ANOVA test followed by the Dunn's test. Statistical differences with respect to the controls (* $P < 0.05$, ** $P < 0.01$), and with respect to the cells treated with the corresponding concentration of AFB1 alone ($^{\#\#}P < 0.01$) are indicated.

3.4. Modulation of GST activity and GSH content by AFB1 and natural antioxidants

Based on the results of the gene expression analysis and since the conjugation with GSH catalysed by GST enzymes is the main detoxifying pathway of AFBO, total GST activity and GSH content were assayed in BME-UV1 cells incubated at the same conditions used for the gene expression analysis. The total GST activity assayed with CDNB as the substrate after 24 h did not vary irrespective of the tested compounds and the incubation conditions (data not shown). Table 3 depicts the effects of the different treatments on GSH levels at 2, 6 and 24 h. The GSH content was not affected by the exposure to either antioxidant alone compared to untreated cells at all time-points. AFB1 produced a limited but statistically significant ($P < 0.05$) depletion of GSH compared to control cells at 6 h, which was then compensated at 24 h. The AFB1-mediated decrease in intracellular GSH at 6 h was fully counterbalanced by either C or Q ($P < 0.05$), which also caused the GSH levels to outweigh those of control cells and of AFB1-treated cells at 24 h (up to approximately 50%).

Table 3

GSH content in BME-UV1 cells treated with AFB1 (375 nM), C (5 µM), or Q (5 µM), or co-exposed to AFB1 (375 nM) + C (5 µM) and AFB1 (375 nM) + Q (5 µM) after 2, 6 and 24 h.

	GSH content (µg GSH/mg protein)		
	2h	**6h**	**24h**
DMSO	53.12 ± 0.73	72.43 ± 1.35	81.58 ± 3.23
AFB1	55.03 ± 1.09	50.19 ± 2.87*	95.36 ± 1.50
C	64.69 ± 2.23	82.21 ± 3.61$^{\#}$	97.63 ± 2.65
AFB1 + C	56.89 ± 2.56	78.22 ± 0.98$^{\#}$	122.38 ± 2.19*$^{\#}$
Q	63.25 ± 1.23	83.81 ± 2.15$^{\#}$	94.31 ± 3.25
AFB1 + Q	52.16 ± 0.89	77.03 ± 1.84$^{\#}$	127.22 ± 4.23*$^{\#}$

Data are expressed as mean ± SEM of three independent experiments and analysed by Kruskall-Wallis ANOVA test followed by the Dunn's test; *$P < 0.05$ as compared to DMSO, $^{\#}P < 0.05$ as compared to AFB1.

Discussion

The risk associated with AFB1 exposure has recently increased due to the climate change that is influencing the geographical pattern of both fungal growth and toxinogenesis. In the last decade the unusual contamination of maize in Southern Europe led in some cases to the exceeding of AFM1 maximum limits in dairy milk (Assuncao et al., 2018). Some natural phenolic and flavonoid compounds have already proved to counteract some of the negative effects of AFB1 in laboratory species and broilers (Eftekhari et al., 2018; Mohajeri et al., 2018; Sridhar et al., 2015); however, research in the bovine species is still lacking, and no data are available on the ability of such molecules to modulate AFM1 synthesis. Due to the potential role played by the mammary gland in the generation of AFB1 metabolites (Caruso et al., 2009), resulting in possible damage of the mammary tissue and in health concerns for the milk consumers, our study was performed in the BME-UV1 cell line, as a model for the bovine mammary gland.

The concentration and time-dependent cytotoxicity of AFB1 observed in the present work has been detected in several cell lines with different degree of sensitivity according to the cell type (Clarke et al., 2014; Sun et al., 2015). Caruso et al (2009) described such effect also in BME-UV1 cells, using higher concentrations compared to ours (2-16 μM). However, in a human breast cancer cell line (MCF-7), AFB1 was found to impair cell viability at concentrations of the same order of magnitude employed in our study (Yip et al., 2017). In cultured bovine hepatocytes the LC50 of AFB1 at 24 h was higher compared to our model (4 μM *vs* 0.7 μM) (Kuilman et al., 2000). The lower sensitivity of liver cells might be explained by a more competent metabolic system, resulting in a more efficient detoxification of AFB1 metabolites. Moreover, although no data are available about cattle, it is noteworthy that the serum AFB1 levels measured in naturally exposed populations from different countries are in the range of the concentrations used in the present study (nM) (Hassan et al., 2006; Aydin et al., 2014).

In our study the co-incubation experiments revealed that, with the exception of C, the selected antioxidants (i.e. CL, Q and R) counteracted the AFB1-mediated impairment of cell viability.

Besides, Q resulted the most effective compound, increasing cell viability up to more than 60%. To the best of our knowledge, this is the first report that compares the effects of the above natural antioxidants against AFB1 cytotoxicity. More to the point, no *in vitro* data are available about the modulation of cell viability by C, CL and R in the presence of AFB1. As regards Q, it effectively lowered AFB1-induced cell death in primary cultures of rat hepatocytes by approximately 30%, albeit at a higher concentration (100 μM) compared to that employed in our study (5 μM) (Eftekhari et al., 2018). Although they did not check for cell viability, Barcelos et al. (2011) reported a lower AFB1-mediated DNA damage in human hepatoma cells in the presence of Q (16 μM). Similar positive effects were triggered by Q against ochratoxin A, which is also reported to act as a pro-oxidant like AFB1 (Tao et al., 2018). The pre-treatment with Q (10 μM) significantly was found to restore cell viability to 95% in a monkey kidney cell line (Ramyaa and Padma, 2013) and in human peripheral blood mononuclear cells (Periasamy et al., 2016). On the other hand, R did not protect intestinal Caco-2 cells against both ochratoxin A and deoxynivalenol cytotoxicity (Cano-Sancho et al., 2015), while it slightly reduced the inhibition of cell viability induced by zearalenone (Sang et al., 2016). Taken together, such results point to a higher protective ability of Q against mycotoxin cytotoxicity compared to R, in accordance with what has been recorded in our study.

Taking into consideration the studies reporting on the possible autoxidation of Q under cell culture conditions (Xiao et al. 2015, Xiao et al. 2018), the actual Q concentration in cell culture medium with or without BME-UV1 cells was monitored over 24 hours of observation (data reported as Supplementary Material). Although a decrease in concentration was observed over time both in the presence or absence of cells, it must be noticed that the antioxidative properties of Q could be retained by its metabolites and/or degradation products, as reported in the literature (Xiao et al. 2015, Aragones et al. 2017).

As far as C and CL are concerned, the only *in vitro* study published so far showed that C was not effective in modulating the AFB1-DNA adduct formation in cultured human primary hepatocytes in comparison with other phytochemicals (i.e. diindolylmethane and xanthohumols)

(Gross-Steinmeyer et al., 2009). Conversely, most of the *in vivo* experiments involving the dietary supplementation of turmeric powder (containing CL) demonstrated its efficiency in reducing the liver damage, as well as in improving the serum antioxidant status (Mohajeri et al., 2018). Thus, it would appear that C and CL have different effect on AFB1 toxicity, as observed in BME-UV1 cells. Such discrepancy suggests that C metabolites contained in the CL mixture might play a role in the recorded protective effects.

As reported earlier (Caruso et al., 2009), also in our study BME-UV1 cells were able to biotransform AFB1 to AFM1. It is therefore confirmed the potential additional role of the mammary gland in the generation of this compound, which is a major hepatic AFB1 metabolite in the bovine species (Kuilman et al., 1998). With the exception of C, a trend in the reduction of AFM1 biosynthesis was observed upon the co-incubation with the other examined antioxidants, substantially matching their protective effects against AFB1 cytotoxicity. In this respect it should be noted that not only AFBO but also AFM1 displays cytotoxic properties (Caloni et al., 2006), even if with a less potent effect (Marchese et al., 2018), that could also participate in the tissue damage of the mammary gland.

As outlined above, liver CYP1A, CYP3A, and, to a lesser extent, CYP2A are believed to carry out the generation of AFB1 metabolites, including AFM1 and AFBO (Dohnal et al., 2014; Marchese et al., 2018). Kuilman et al. (2000) reported that the oltipraz-mediated depression of AFM1 synthesis in primary cultures of bovine hepatocytes was mostly attributable to the inhibition of a number of CYP1A and CYP3A-dependent activities. According to the results of the gene-expression analysis, however, neither CYP3A28 nor CYP2A13 transcripts were expressed in the employed BME-UV cell line, pointing to CYP1A(1) as the sole CYP subfamily responsible for AFB1 metabolism in our cell system. Surprisingly, in our study CYP1A1 was not depressed by Q, the most effective antioxidant in limiting AFM1 synthesis. Although the mechanism(s) by which Q affects AFB1 biotransformation remain(s) to be established, it is worth noting that Q was found to strongly inhibit CYP1A-mediated 7-ethoxyresorufin O-deethylase (EROD) activity in liver

microsomes from mice (Pilipenko et al., 2017), rats (Kuo et al., 2004) and pigs (Ekstrand et al., 2015). We found that EROD activity was also strongly inhibited by Q (10, 8, 4, 2 or 1 μM) in bovine liver microsomes (data not shown). In the bovine species this enzyme activity is specifically mediated by CYP1A1 (Sivapathasundaram et al., 2001), which is also well expressed in our test system. This suggests that the Q-related reduction in both AFM1 synthesis and cytotoxicity observed in the present study might involve the inhibition of CYP1A1 catalytic activity.

It is generally accepted that antioxidants can afford protection against AFB1 mainly by positively modulating the natural detoxification mechanisms (El-Bahr, 2015; El-Nekeety et al., 2014). Thus, we investigated the transcriptional response of biotransformation and antioxidant enzymes to AFB1 with or without C and Q, representing the least and the most efficacious compound in our test system, respectively. In our model AFB1 produced an overall downregulation of the detoxifying enzymes (i.e. CAT, GPx, SOD, NQO1, EPHX1-3 and GSTA1-2), while the bioactivating pathway (i.e. CYP1A1) was not affected. Such results are mostly in accordance to what has been already reported in several *in vitro* and *in vivo* studies. Recently, Wang et al. (2018) demonstrated that the dietary exposure to AFB1 (5.0 mg/kg diet for 28 days) decreased both GST and EPHX mRNA levels in broiler liver. Likewise, though no data are available about the modulation of NQO1 by AFB1, the antioxidant enzymes CAT, GPx and SOD were transcriptionally down-regulated by the mycotoxin in rat and broiler liver after the exposure to comparable AFB1 dosage (Abdel-Wahhab et al., 2015; Yarru et al., 2009); the same effects were reported in cultured bovine peripheral blood mononuclear cells exposed to much higher mycotoxin concentrations compared to our study (Bernabucci et al., 2011).

When dealing with CYP1A1, the scenario is more complex. As regards AFB1, opposed outcomes have been described, possibly indicating a species- and even individual-dependent ability to modulate CYP1A transcription. In primary cultures of rabbit hepatocytes AFB1 significantly decreased CYP1A1 mRNA (Guerre et al., 2000), yet in human ones the effect varied between individuals, ranging from gene induction to lack of regulation (Ayed-Boussema et al., 2012).

Moreover, AFB1 exposure produced a significant CYP1A1 increase in a rat hepatoma cell line (Mary et al., 2015), but this was not the case for the human counterpart (Smit et al., 2017). To the best of our knowledge no data are available about the AFB1-mediated regulation of CYP1A1 in tissues other than liver and our results in mammary cells would also suggest a cell type-dependent variability. In our study, either antioxidant (C or Q) alone or under co-incubation conditions also failed to modulate CYP1A1. Even if several reports stated that both C and Q up-regulate CYP1A1 (Jin et al., 2018; Mohammadi-Bardbori et al., 2012), some others demonstrated that such molecules did not influence CYP1A1 at transcriptional level but rather attenuated its induction in response to known activators (e.g. 2,3,7,8-tetrachlorodibenzo-p-dioxin and benzo[a]pyrene) (Choi et al., 2008; Perepechaeva et al., 2017).

As already reported for hepatocytes (Abdel-Wahhab et al., 2015; El-Bahr, 2015; Gross-Steinmeyer et al.), the gene expression profile of detoxifying enzymes in BME-UV1 cells treated with either antioxidant (C or Q) individually did not differ compared to controls; this further supports the hypothesis that such molecules typically exert their protective effects only in the presence of an oxidative damage/stress. In keeping with this, in our study Q decreased the remarkable GSTA1 downregulation induced by AFB1 (40 fold-change). The protective effect triggered by Q in our cell system is in accordance with the gene expression modulation detected in liver from rats orally treated with AFB1 and Q (Abdel-Wahhab et al., 2015).

Surprisingly, the AFB1-mediated mRNA down-regulation of GSTA1-2 was not paralleled by a decrease in GST activity assayed with CDNB, making therefore possibly unnoticeable also the positive modulation of GSTA1 mRNA caused by the co-incubation with Q. In this respect, the use of an unspecific GST substrate like CDNB might partly explain this apparent discrepancy.

Pro-oxidants like AFB1 typically trigger a biphasic response consisting in an acute GSH-depletion followed by a later restoration toward or even over the baseline levels (“rebound effect”), with antioxidants like C and Q being active, in vitro or in vivo, (positively affecting) during both phases (Choi, 2010; Eftekhari et al., 2018; El-Agamy, 2010; Zhang et al., 2016). This was also the

case in our study, thus reinforcing the key role of GSH in the natural antioxidant- mediated protection against AFB1 cytotoxicity and oxidative damage.

In conclusion, data from this investigation suggest that, among the different tested natural antioxidants (C, CL, R, and Q), Q proved the most effective not only in counteracting the cytotoxic effects of AFB1 in a bovine mammary gland cell line, but also in reducing the synthesis of the main milk metabolite AFM1. Remarkably, these positive features were observed at antioxidant concentrations in the low micromolar range. Although additional work is needed to unravel the mechanisms by which Q affects AFB1 biotransformation, and hence the generation of toxic metabolites, the protective effects seem to be at least partly mediated by the enhancement of the antioxidant defense. Further investigations are ongoing to test the effects of the examined antioxidants on cultured bovine liver cells, as well as to verify their in vivo ability in reducing AFM1 excretion in dairy cows.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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