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Persistence of the antagonistic effects of a natural mixture of *Alternaria* mycotoxins on the estrogen-like activity of human feces after anaerobic incubation

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HIGHLIGHTS

- A complex *Alternaria* toxin mixture antagonized the estrogenic properties of feces.
- *Alternaria* mycotoxins might act as endocrine disruptors *in vivo*.
- Tenuazonic acid is unable to act as ER-agonist/antagonist.
- An *Alternaria* toxin mixture reduced the ERα/ERβ nuclear ratio.

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ABSTRACT

Several *Alternaria* mycotoxins are believed to act as endocrine disruptive chemicals (EDCs), since they are reported to bind estrogen receptors in several experimental models. After ingestion of contaminated food commodities, the mycotoxins reach the intestine, where they come into direct contact with food constituents as well as the gut microbiota. Thus, the aim of the present work was to evaluate the modulatory potential of a complex extract of cultured *Alternaria* fungi (CE; containing eleven chemically characterized compounds) on the estrogenic signaling cascade of mammalian cells before and after anaerobic incubation with fecal slurries, in order to simulate an *in vivo*-like condition in the gut. Assessing alkaline phosphatase expression in Ishikawa cells as a measure for estrogenicity, we found the CE to partially quench the intrinsic estrogenic properties of fecal slurries and fecal waters, even after 3 h of fecal incubation. Investigation of the mechanisms underlying the effects observed carried out through an *in vitro*/*in silico* approach revealed the ability of the extract to decrease the ERα/ERβ nuclear ratio, while a possible action of the mycotoxins as ER-antagonists was excluded. Our results suggest that *Alternaria* mycotoxins might act as EDCs *in vivo*, and warrant further investigation in animal models.

Abbreviations: AOH, alternariol; AME, alternariol monomethyl ether; ALT, altenuene; TeA, tenuazonic acid; TEN, tentoxin; ALP, alterperylenol; AST, altersetin; ATX, altertoxin; STTX-III, stemphyltoxin III; AlP, alkaline phospatase; CE, complex *Alternaria* extract; EDC, endocrine disrupting chemical; ER, estrogen receptor; E2, 17-β-estadiol; PBS+CE, extract dissolved in PBS; PBS+DMSO, DMSO dissolved in PBS; PBS+E2, 17-β-estadiol dissolved in PBS; FS+CE, fecal slurry + extract; FS+DMSO, fecal slurry + DMSO; FW +CE, filtered fecal water + extract; FW+DMSO, filtered fecal water + DMSO; PM+CE, fecal particulate matter + extract; LM+CE, living microorganisms + extract; DM+CE, dead microorganisms + extract.

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1. Introduction

The contamination of food commodities by naturally-occurring environmental contaminants is a global food safety issue. Those may be responsible for the onset of acute and chronic toxic effects on different organs or systems, the severity of which depends on several factors such as the type of toxin, the dose, age and health status of the individual (Costa et al., 2019; Thompson and Darwish, 2019). Among the various classes of food contaminants frequently found in food, endocrine disrupting chemicals (EDCs) may affect biosynthesis, metabolism, and bioactivity of endogenous hormones, thus modifying body homeostasis, and developmental and reproductive physiology (Diamanti-Kandarakis et al., 2009). To date, several classes of compounds, either of natural (*e.g.* genistein, coumestrol, 8-prenylnaringenin, zearalenone) or synthetic (*e.g.* polychlorinated biphenyls, phthalates, dioxins, bisphenol A) origin, have been reported to act as EDCs by targeting physiological pathways such as those activated by progesterone, androgens and/or estrogens (Aichinger et al., 2018; Bell, 2014; Diamanti-Kandarakis et al., 2009; Grindler et al., 2018; Patisaul, 2017; Rubin, 2011; White and Birnbaum, 2009). In this context, several food-contaminating fungal species have been reported to produce toxic secondary metabolites, termed "mycotoxins", which have been reported to interfere with estrogenic pathways (Cimbalo et al., 2020; EFSA, 2011a). In particular, contamination of food by mycoestrogens can occur not only in agricultural fields, but also during later stages of the food chain, including storage (Chilaka et al., 2016; Drejer Storm et al., 2014; Sulyok et al., 2007). In addition to the *Fusarium* toxin zearalenone and its derivatives (α- and β-zearalenol), which are widely recognized to exert estrogenic effects (EFSA, 2011a; Tatay et al., 2018), another class of mycoestrogens is produced by molds of the genus *Alternaria*. These fungi can infest a wide range of crops and raw materials and produce a cocktail of potentially toxic compounds with broadly different chemical structures and toxicological properties. Interestingly, among the *Alternaria* mycotoxins known to date, alternariol (AOH) and alternariol monomethyl ether (AME) were reported to exert not only genotoxic effects (EFSA, 2011b), but also to potentially act as EDCs (Frizzell et al., 2013; Stypuła-Trębas et al., 2017). Specifically, AOH was found to increase the expression of the progesterone receptor, the production of estradiol and progesterone in the H295R cell line (Frizzell et al., 2013), as well as to induce androgenic effects (Stypuła-Trębas et al., 2017) *in vitro*. In addition, AOH and AME were described to mediate estrogenic effects by binding to both, α- and β- estrogen receptors (ERs), but with a markedly lower potency as compared to the endogenous hormone estradiol and with a higher affinity for ERβ over ERα (Lehmann et al., 2006). Of note, AME, which differs from AOH by an additional methyl group in its chemical structure, was found to fit better into the binding pocket of the ER and to possess a slightly higher potency compared to AOH (Dellafiora et al., 2018a). The ability of AOH to target casein kinase 2 (Aichinger et al., 2020), as well as the aptitude of AOH and AME to bind to the aryl hydrocarbon receptor (Schreck et al., 2012) were also described as factors potentially able to affect estrogen-related signaling and gene expression (Hohenbichler et al., 2020).

These mycotoxins are often found in food along with other, non-estrogenic, *Alternaria* mycotoxins, like altenuene (ALT), tenuazonic acid (TeA), tentoxin (TEN), altertoxins (ATX) I and II or alterperylenol (ALP) (Arcella et al., 2016; Escrivá et al., 2017). The co-occurrence of multiple mycotoxins in food can be responsible for the onset of effects other than that exerted by the single compounds, *i.e.* additive, synergistic or antagonistic effects (Battilani et al., 2020). After ingestion, the toxic effects mediated by mycotoxins can be altered by several other factors such as the chemical interaction with both food matrix and constituents thereof, and the microorganisms of the gut microbiota. Indeed, we recently reported the ability of human gut bacterial strains to adsorb some *Alternaria* mycotoxins, thus potentially reducing the free-absorbable proportion of mycotoxins (Crudo et al., 2021). In addition, fecal components (*i.e.* gut microorganisms and undigested food constituents) were found to reduce the bioavailability of certain *Alternaria* mycotoxins and, consequently, to suppress their genotoxic effects (Crudo et al., 2020).

Based on this, the main objective of the present work was to assess the influence of gut microbes and fecal materials (*e.g.* fecal water, particulate fecal matter) on the estrogenic properties of a complex extract (CE) of *Alternaria* mycotoxins. In order to better simulate an *in vivo*-like condition and to obtain information about the possible role of different fecal fractions, the estrogenic effects of CE were assessed before and after 3 h of co-incubation with fecal samples (collected from several human donors) and defined fractions thereof. Furthermore, in mechanistic *in vitro*/*in silico* studies we investigated: i) the possible impact of CE on the immunolocalization of α- and β-ERs in nucleus and cytoplasm of Ishikawa cells; ii) the estrogenic/antiestrogenic properties of TeA; iii) the possible action of *Alternaria* mycotoxins as ER-antagonists.

2. Materials and methods

2.1. Materials

For cell culture experiments, Minimum Essential Medium (MEM), Dulbecco's Modifed Eagle Medium/F-12 nutrient mixture (DMEM/ F12), heat-inactivated fetal bovine serum (FBS), l-glutamine and penicillin/streptomycin (P/S) solution were purchased from Invitrogen™ Life Technologies (Karlsruhe, Germany). Charcoal–dextran stripped (CD-) FBS was purchased from Fisher Scientific (Catalog #A3382101). The estrogen-sensitive human endometrial adenocarcinoma cell line Ishikawa was obtained from the European Collection of Authenticated Cell Cultures (Wiltshire, United Kingdom). 17β-Estradiol, and 4-nitrophenyl phosphate were purchased from Sigma-Aldrich (Schnelldorf, Germany).

The complex extract of *Alternaria* mycotoxins used in the present study was obtained by growing the *Alternaria alternata* DSM 62010 strain on long grain rice for 21 days and chemically characterized by LC–MS/MS analysis. Concentrations of the *Alternaria* mycotoxins of the extract at the applied dose (5 μg/mL) and their chemical structures are shown in Table 1 and Fig. S1, respectively. Further information about the extract preparation can be found in Puntscher et al. (2019b). Tenuazonic acid was purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany).

Table 1
Concentration of *Alternaria* mycotoxins the cells were exposed to during treatment with 5 μg/mL of CE.

Mycotoxins	Concentration (nM)
Alternariol (AOH)	15
Alternariol monomethyl ether (AME)	12
Altenuene (ALT)	13
Tenuazonic acid (TeA)	15160
Tentoxin (TEN)	0.2
Altertoxin-I (ATX-I)	141
Altertoxin-II (ATX-II)	201
Alterperylenol (ALP)	180
Stemphyltoxin-III (STTX-III)	301
Altenusin (ALS)	5
Altersetin (AST)	230

2.2. Fecal incubations

Samples tested in the present work were obtained and used in a previous work to investigate the modifications of the genotoxic effects exerted by the *Alternaria* extract after incubation with fecal samples or fractions of them. Detailed information about sample preparation can be found in Crudo et al. (2020). Briefly, fresh fecal samples were collected by four healthy donors (two males and two females) who signed the informed consent for the fecal sample donation. Fecal samples were transferred into an anaerobic tent with 10 % CO_2, 5% H_2 and 85 % N_2 and processed within 3 h of collection. 2% (w/v) fecal slurries were prepared by diluting each fecal sample with a pre-reduced and sterile phosphate buffer saline solution (PBS, 0.1 mol/L, pH 7.4). Fecal slurries (FS) were then spiked with 1% dimethyl sulfoxide (FS + DMSO), with 50 μg/mL of the CE (FS + CE) or subjected to high speed centrifugations (5,200 rcf; 10 min) to obtain samples of fecal water. Samples of fecal water were then sterilized by filtration with 0.2 μm cellulose acetate filters and spiked with the extract (FW + CE; 50 μg/mL) or with DMSO (FW + DMSO; 1%). To obtain samples of particulate matter, aliquots of the FS were individually centrifuged at low speed (175 rcf; 10 min), the corresponding pellets were resuspended in PBS (original volume) and spiked with 50 μg/mL of CE (PM + CE). To assess the ability of the fecal microorganisms to modify the toxic properties of the extract, the FS samples were centrifuged at low speed to remove the particulate matters, followed by centrifugation at high speed (5,200 rcf; 10 min) of the supernatants. Then, the corresponding pellets of living microorganisms were resuspended in PBS (original volume) and spiked with 50 μg/mL CE (LM + CE). Additional samples of dead microorganisms were also prepared as follow: after high-speed centrifugation of the fecal slurry samples stripped of particulate matter, the living microorganism pellets were incubated with a 70 % ethanol solution (room temperature; 5 min). Afterward, the dead microorganisms were pelleted by high-speed centrifugation, resuspended in PBS (original volume) and spiked with the CE (50 μg/mL; DM + CE). For each fecal incubation, samples of PBS containing the CE (50 μg/mL; PBS + CE) were also prepared and used as controls.

The above-mentioned samples were incubated under anaerobic condition for 3 h at 37 °C and 150 revolutions/min. Aliquots of the samples before and after 3 h of anaerobic incubation were collected and immediately stored at −80 °C until the time of analysis. Before using the collected samples for cell treatment purposes, sterilization of samples by filtration with 0.2 μm PTFE filters was carried out.

2.3. Cell culture

The estrogen-sensitive Ishikawa cell line was chosen as a model system because of the constitutive expression of both estrogen (α and β) and aryl hydrocarbon receptors (AhR) (Lehmann et al., 2006; Wormke et al., 2000). Two weeks before starting the experiments, cell stocks kept in liquid nitrogen were taken into culture and grown in MEM supplemented with 1 % L-glutamine, 1% P/S and 5% (v/v) FBS. Cells were grown as a monolayer in humidified incubators (37 °C, 5% CO_2) and passaged twice per week, when confluence reached 80 %. All experiments were carried out by using an "assay medium" composed as follow: DMEM/F-12 supplemented with 5% CD-FBS and 1% P/S solution.

2.4. Alkaline phosphatase (AlP) assay

AlP enzyme activity was measured after 48 h incubation of Ishikawa cells with the samples collected before and after fecal incubation as a measure for ERs activation, as previously described (Aichinger et al., 2018). Before cell seeding, Ishikawa cells were resuspended in assay medium containing CD-FBS with the aim to ensure low hormone levels. Then 10,000 cells/well were seeded into 96-well plates and allowed to grow for 48 h into a humidified incubator (37 °C; 5% CO_2). After 48 h incubation, the collected fecal samples were diluted 1:10 with assay medium, to reach a final CE or DMSO concentration of 5 μg/mL or 0.1 %, respectively. Control media containing 0.1 % DMSO (with 10 % PBS; PBS + DMSO) or 1 nM E2 (with 10 % PBS and 0.1 % DMSO; PBS + E2) were also prepared and used as controls. The final DMSO concentration was 0.1 % in all samples. Thus, the spent medium of each well was replaced with the test and control media, and cells were incubated for a further 48 h. Afterward, cells were washed three times with PBS and lysed by quick freezing at −80 °C for 20 min. After allowing the plates to thaw at room temperature for 5 min, the detection buffer (5 mM 4-nitrophenylphosphate, 1 M diethanolamine, 0.24 mM MgCl2; pH 9.8) was added and the increase of absorption at 405 nm was measured for 1 h every 3 min with a plate reader. The slope of the curves in the linear range was calculated as a measure for the activity of the enzyme. Based on the AlP results obtained by incubating the cells with the feces-containing samples and the relative high content of TeA in the *Alternaria* extract used in the present work, six different concentrations of TeA (1, 10, 15, 20, 50, 100 μM; final DMSO concentration: 0.1 %) were tested in order to assess the ability of the mycotoxin to exert estrogenic/antiestrogenic effects. In particular, to assess its possible antiestrogenic properties, TeA (at the various concentrations) was co-incubated with 1 nM E2 (final DMSO concentration: 0.1 %). The AlP assay protocol used was the same previously described, but no dilution of assay medium with PBS was performed.

2.5. Sulforhodamine B (SRB) assay

To exclude artefacts deriving from cytotoxic effects induced by *Alternaria* mycotoxins or fecal compounds that could have affected the evaluation of AlP assay results, the cellular protein content was determined by SRB assays as a measure for cell viability. Ishikawa cells (in assay medium) were seeded into 96-well plates at a cell density of 10,000 cells/well, allowed to attach for 48 h and, finally, incubated with the samples collected, controls or with TeA for a further 48 h (See Section 2.4). Cells were then fixed by addition of trichloroacetic acid in distilled water (50 % m/v) and incubated for 1 h at 4 °C. Plates were then washed four times with water, allowed to dry overnight and stained for 1 h at room temperature by addition of a 0.4 % (*w/v*) SRB solution. Afterwards, cells were washed twice with water and twice with 1% acetic acid solution to remove the excess of SRB. After allowing the plates to dry overnight, the dye was resolved by addition of TRIS buffer (pH 10). The absorbance at 570 nm was determined with a PerkinElmer Victor3 V plate reader. All conditions for each donor were tested in triplicate and their mean values were related to the positive control (1 nM E2).

2.6. Immunolocalization and quantification of α and β ERs

ERs were immunolocalized and quantified in Ishikawa cells exposed to the controls PBS + DMSO and PBS + E2, as well as in cells treated with the samples FS-DMSO, FS-CE, PBS-CE collected before anaerobic incubation. The choice to analyze only samples collected before the incubation was justified by the results of the AlP assay, which showed no impact of the incubation on the anti-estrogenic properties of the CE-containing samples, and only a decrease, despite slight, in the estrogenic properties of the FS + DMSO controls. Therefore, to maximize the probability to observe differences in terms of nuclear and cytoplasmic receptors between samples of FS + DMSO and FS + CE, samples collected before the anaerobic incubation (for which a greater AlP induction by FS +

DMSO was observed) were chosen for the immunolocalization and quantification of α and β ERs.

For imaging experiments, Ishikawa cells were seeded at a cell density of 5300 cells/well in μ-Slides (poly-l-lysine coated, Ibidi GmbH, Martinsried, Germany) and allowed to attach for 48 h. Then, the spent assay medium of each well was replaced with the test and control media, which were prepared as described in Section 2.4. After 48 h incubation, cells were fixed by addition of formaldehyde (3.7 % in PBS, 15 min) and processed as previously described with minor modifications (Dellafiora et al., 2018b). After the cell membrane permeabilization (0.2 % Triton X, 10 min, room temperature-RT), cells were incubated for 1 h at RT with a solution containing 2% donkey serum (Merck KGaA, Darmstadt, Germany) and 1% BSA (Roth, Graz, Austria) in PBS, in order to block unspecific reactive sites. For the detection of α and β ERs, cells were incubated for 2 h at RT with the following antibodies: ERα D-12 (SC-8005; mouse monoclonal IgG2a; Santa Cruz Biotechnology; dilution of 1:150) and ERβ H-150 (SC-8974; rabbit polyclonal IgG; Santa Cruz Biotechnology; dilution of 1:250). The cytoskeleton architecture was counterstained by using an F-actin probe conjugated to a fluorescent dye (Oregon Green™ 488 Phalloidin; Catalog number O7466; Invitrogen; dilution 1:500). After 2 h, cells were washed tree times with 0.05 % Triton X and twice with PBS, followed by 1:30 h incubation with the secondary antibodies (Alexa Fluor 568 Donkey Anti-Rabbit, A10042; Alexa Fluor 647 Donkey Anti-Mouse, A31571; dilution 1:1000; Life Technologies, Thermo Fisher Scientific). Afterwards, cells were washed tree times with 0.05 % Triton X and twice with PBS, followed by fixation with 3.7 % formaldehyde (in PBS). Finally, cells were washed with PBS, 100 mM glycine in PBS solution was employed to mask reactive sites and cell nuclei were stained by addition of Roti®-Mount FluorCare DAPI (Art. Nr. HP20.1). Slides were stored at 4 °C until the time of analysis. Images were acquired with a Confocal LSM Zeiss 710 equipped with ELYRA PS. 1 system. Images were analyzed with the software Fiji (ImageJ Version 2.0.0-rc-69/1.53c) and for each condition of each donor, n> 16 cells from four different optical fields were randomly chosen for the quantification of ERs. Thus, a total of n>64 cells were analyzed for each condition. Nuclear and cytoplasmatic quantification of α and β ERs was carried out in the central planar section of the cells by measuring the mean signal intensity value (gray value, relative units).

2.7. In silico *analysis*

ALP, AST, TeA and ATX-I were analyzed *in silico* for their capability to interact with ERs in one of the discrete crystallographic conformation reported so far representing the ER in the agonistic conformation (one main conformational state) or in the antagonistic conformation (two main conformational state), where the helix 12 is reorganized to prevent the interaction of co-regulator proteins (Dellafiora et al., 2017, 2015a). In agreement with previous studies (Dellafiora et al., 2015b; Nongonierma et al., 2018), the computational analysis relied on docking simulations and rescoring procedure to estimate the capability of ligands to fit the ER's pocket in the diverse conformational state, followed by pharmacophoric analysis to provide a structural evidence of the results collected. Specifically, the GOLD software (version 2020.1) was used to perform the docking study. Default parameters were used and, in each model, the pocket space to dock ligands was set 10 Å around the centroid of the pocket. For each ligand and in each model, 20 poses were generated. Then, the HINT scoring function was used to re-score docking poses providing a better evaluation of protein-ligand interaction, as previously shown (Dellafiora et al., 2014). In brief, the HINT scoring function calculates the favor of protein-ligand recognition through a sum of hydrophobic atom constants derived from experimental LogPo/w values (the partition coefficient of a molecule in 1-octanol/water). Thus, HINT appears as a "natural" and intuitive force-field providing an

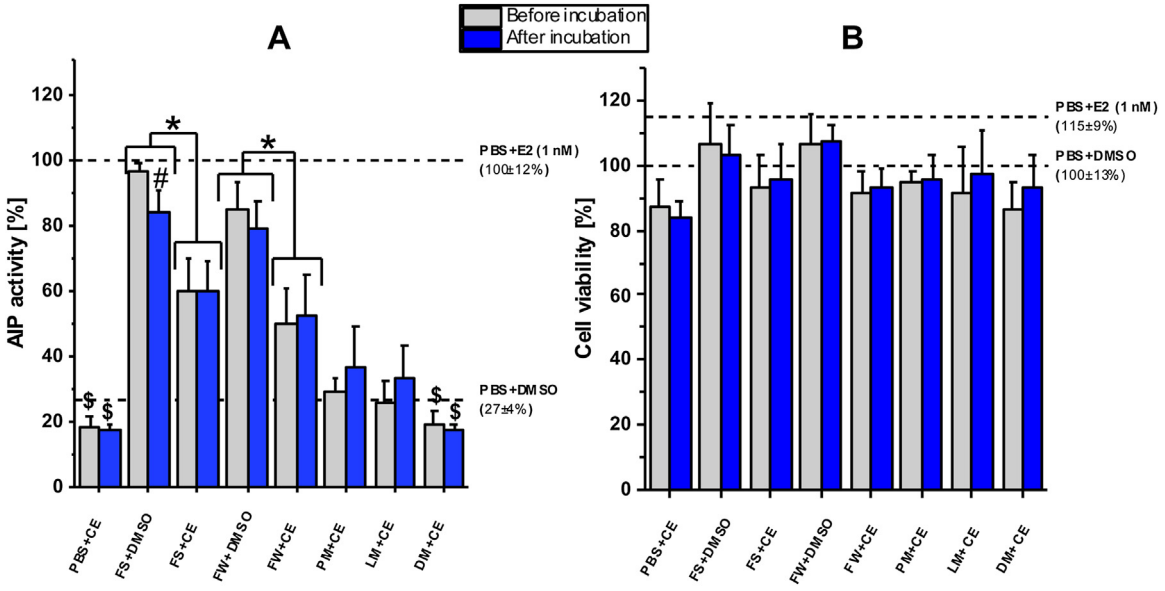

Fig. 1. Effects on AlP activity (A) and cell viability (B) resulting from a 48 h incubation of Ishikawa cells with the collected samples and controls. Feces-containing samples were obtained by incubating the fecal materials (or their fractions) with the *Alternaria* extract for 0 h (before incubation) and 3 h (after incubation). Every condition for each donor was measured in triplicate. Results were expressed as mean + standard deviation of the mean values (n = 4) obtained by the four stool donors and related to the positive (1 nM E2; in the AlP assay) or negative (0.1 % DMSO; in the SRB assay) control. Differences between samples collected before and after the anaerobic incubation were calculated by Student's *t*-test (#; $p < 0.05$). Differences between CE-containing and non-CE-containing samples were calculated by Student's *t*-test (*; $p < 0.05$). $ indicate a reduced AlP activity as compared to the PBS + DMSO control ($p < 0.05$).
PBS + CE: extract dissolved in PBS; FS + CE: fecal slurry + extract; FW + CE: filtered fecal water + extract; PM + CE: fecal particulate matter + extract; LM + CE: living microorganisms + extract; DM + CE: dead microorganisms + extract.

empirical and quantitative evaluation of protein-ligand interaction as a sum of all single atom-atom contributions. In particular, positive and high HINT score correlates with favorable binding free energy and provide the most probable architecture of binding, while negative scores are associated with unfavorable ligand-pocket interaction (Amadasi et al., 2006; Eugene Kellogg and Abraham, 2000). Therefore, in each model, only the best scored pose for each ligand was considered for the pharmacophoric analysis. To do so, the surface pocket of each model was defined using GetCleft algorithm (Gaudreault et al., 2015), while the respective pharmacophoric imagines were derived using the IsoMIF algorithm (Chartier and Najmanovich, 2015). Default parameters were applied with the exception of the maximum distance value between the grid and residues atoms set at 3 with a grid resolution of 1 Å. The 3D model of the three discrete conformational states of ER derived from the crystallogrpahic structures recoded in PDB (Protein DataBank; https://www.rcsb.org/) with PDB code 2YJA (Phillips et al., 2011) for the agonistic conformation, and 1XPC (Blizzard et al., 2005) and 1QKM (Pike et al., 1999) for the two antagonistic conformational states. In agreement with previous studies (Dellafiora et al., 2014), protein structures were processed using the molecular modeling software Sybyl, version 8.1 (www.certara.com). All atoms were checked for atom- and bond-type assignments. Amino- and carboxyl-terminal groups were set as protonated and deprotonated, respectively. Hydrogen atoms were computationally added to the protein and energy-minimized using the Powell algorithm whit a coverage gradient of ≤0.5 kcal (mol Å)−1 and a maximum of 1500 cycles. The structures of ALP, AST, TeA and ATX-I were retrieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) in the 3D structure-data file (.sdf) format.

2.8. Statistical analysis

Independent Student's *t*-test was performed to determine significant differences between samples collected before and after the anaerobic incubations. Differences were considered statistically significant for $p \leq 0.05$ or $p \leq 0.01$. Differences among the amount of ERs found in cells after the various treatments were determined through the Kruskal-Wallis test followed by Dunn's multiple comparison test. All statistical analyses were carried out by using SPSS (v. 23.0, SPSS inc., Chicago, IL, USA) or OriginPro (v. 9.5, OriginLab® Corp., Northampton, MA, USA) software.

3. Results

3.1. Influence of feces-containing samples and TeA on AlP activity

Estrogenic properties exerted by the *Alternaria* extract before and after 3 h of incubation with the fecal materials and corresponding fractions (*i.e.*: fecal water, FW; particulate matter, PM; living and dead microorganisms, LM and DM) were assessed in the endometrial Ishikawa cell line by measuring the AlP activity. As shown in Fig. 1A, incubation of cells with samples of CE dissolved in PBS (PBS + CE; collected before and after the anaerobic incubation) did not lead to increased levels of AlP activity as compared to the solvent control (PBS + DMSO), thus indicating the inability of the CE to exert estrogenic effects. Similar results were obtained following the incubation of PM, LM, and DM with the CE, whose AlP induction did not exceed that of the PBS + DMSO control. Interestingly, incubations of cells with both PBS + CE and DM + CE resulted in a lower ALP activity ($p < 0.05$) as compared to the PBS + DMSO control.

Among the samples tested, fecal slurries (FS + DMSO) collected before and after the anaerobic incubation were found to induce estrogenic effects, activity partially suppressed ($p < 0.05$) by co-incubation of these samples with the CE. Similar results were observed for samples of fecal water (FW), which showed to increase the activity of the AlP enzyme. Also in this case, co-incubation with CE led to a lower AlP activity compared to the respective solvent control (FW + DMSO). With regard to the samples of particulate matter, living and dead microorganisms incubated with the CE, no difference in AlP activity was detected as compared to the solvent control (PBS + DMSO). As for the effect exerted by 3 h of anaerobic incubation, none of the collected samples showed changes in its estrogenic/antiestrogenic properties, except for the samples of FS + DMSO, whose estrogenic properties were found to be slightly reduced. Of note, estrogenic/antiestrogenic properties of the samples collected before and after anaerobic incubation were found not to be affected by the inter-individual variability of the donors, as can be deduced from the rather low standard deviations obtained in the AlP assay (Fig. 1A). To avoid a possible misinterpretation of the estrogenicity data due to cytotoxicity, quantification of the cellular protein content was carried out by the SRB assay. Results of the assay confirmed that the antiestrogenic effects observed in CE-containing samples could not be ascribed to cytotoxicity phenomena (Fig. 1B).

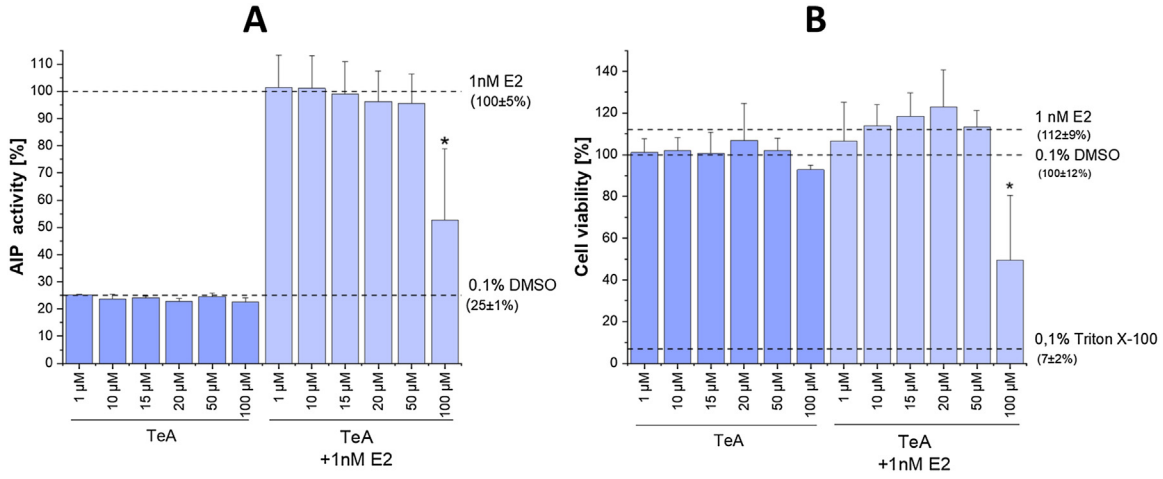

Fig. 2. Effects of different concentrations of TeA on AlP activity (A) and cell viability (B) in presence or absence of 1 nM E2. Results were expressed as mean + standard deviation of tree independent biological replicates and related to the positive (1 nM E2; in the AlP assay) or solvent (DMSO; in the SRB assay) control. 0.1 % Triton X-100 was used as a positive control for cytotoxicity. Significant differences compared to the positive (E2; in the AlP assay) or solvent (DMSO; in the SRB assay) control were calculated by Student's *t*-test (*$p < 0.05$).

Based on the results obtained and on the relative high abundance of TeA in the *Alternaria* extract used, the ability of the mycotoxin to exert estrogenic or antiestrogenic effects was also investigated. As shown in Fig. 2A, incubation of cells with six different concentrations of TeA (ranging from 1 μM to 100 μM) did not result in an increased AlP activity compared to the solvent control. In addition, the co-treatment of cells with TeA and 1 nM E2 was not able to counteract the estrogenic effects induced by E2, except for the highest concentration of TeA tested (100 μM) which mediated a reduction of the AlP activity of about 50 %. However, as shown in Fig. 2B, this strong reduction in the AlP activity was found to be a consequence of a reduced cellular protein content and not a real antiestrogenic effect.

3.2. Localization of α and β ERs into the nucleus and cytoplasm of Ishikawa cells

Quantification of α and β ERs into the nucleus and cytoplasm of Ishikawa cells exposed to FS + DMSO, FS + CE and PBS + CE (collected before the anaerobic incubation), as well as to the controls PBS + DMSO and PBS + E2, was carried out by confocal microscopy.

Fig. 3 shows representative images of the central cross section of an Ishikawa cell island, in which ERα (Fig. 3A) are ERβ (Fig. 3B) are depicted in red and green, respectively. A representative workflow of the image analysis is illustrated in Fig. 3F. Results of the ERs quantification (Fig. 4) clearly showed increased nuclear/cytoplasmic ratios, for both types of ERs, in cells treated with E2, FS + DMSO, FS + CE, and CE, when compared to solvent control (PBS + DMSO). The increased ratios observed were determined by a reduction of the number of ERs into the cell cytoplasm, while no difference in the amount of nuclear receptors was observed (as compared to the solvent control). Thus, all samples tested determined a reduction of the total number (nucleus + cytoplasm) of ERs in cells compared to the solvent control PBS + DMSO. Representative images of Ishikawa cells exposed to PBS + DMSO, PBS + E2, FS + DMSO, FS + CE and PBS + CE are reported in Fig. S2, in which the higher cytoplasmic content of ERα and ERβ in cells treated with PBS + DMSO is visually observable.

Despite no significant difference in the amount of nuclear or cytoplasmic ERs was found between cells exposed to FS + DMSO and FS + CE, which might have partially explained the antiestrogenic effects of the extract, cells exposed to the samples containing the *Alternaria* extract (*i.e.* FS + CE and PBS + CE) showed a slight but significant ($p < 0.05$) decrease of the ERα/ERβ nuclear ratio compared to the cells exposed to the estrogenic samples PBS + E2 and FS + DMSO (Fig. 4C). On the contrary, this difference was not observed at the cytoplasmatic level, where all treatments led to a decreased ERα/ERβ ratio compared to the solvent control (PBS-DMSO; $p < 0.05$) (Fig. 4C).

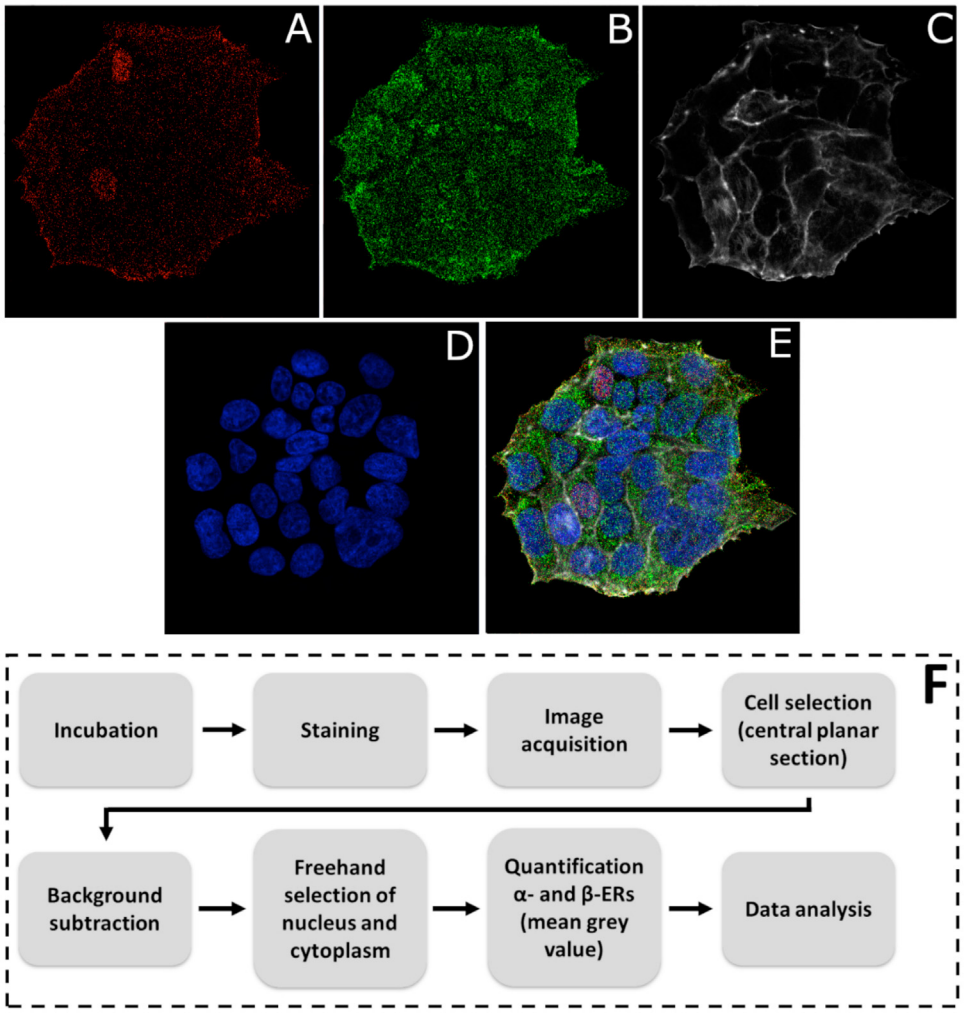

Fig. 3. Representative images of the central cross section of an Ishikawa cell island, showing the localization of the ERα and ERβ. A. ERα (red); B. ERβ (green); C. Cytoskeleton (grey); D. Nucleus (Blue); E. Merged image. F. Schematic description of the ERs quantification workflow.

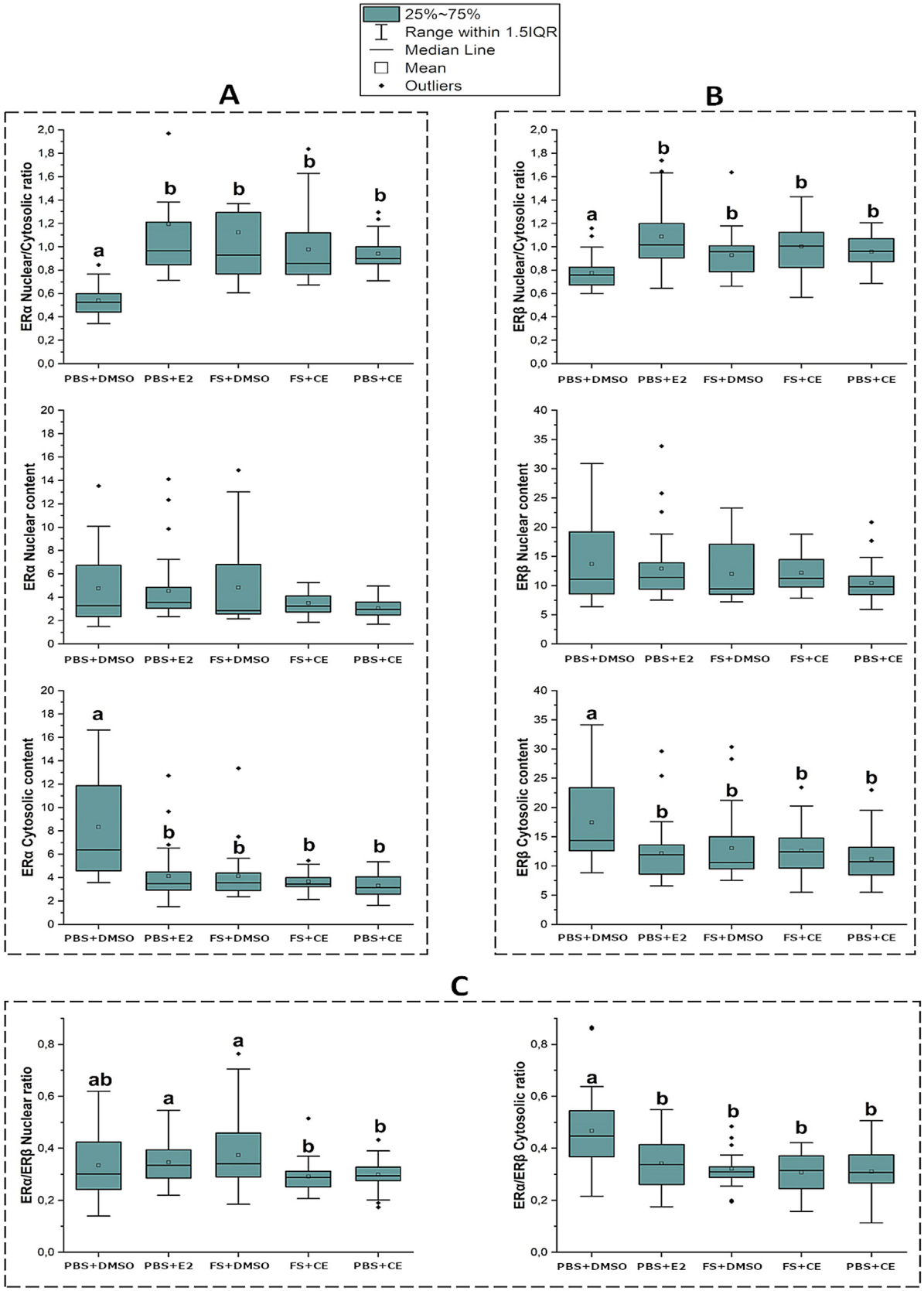

Fig. 4. Effects of CE-containing samples and controls on the number and distribution of ERα and ERβ in Ishikawa cells. Box and whisker plots show the mean and median values, the interquartile range (IQR) and the 1.5 IQR. Outliers were defined as the values above or below the 1.5 IQR. Panels A and B report the nuclear/cytoplasmic ratio, the nuclear content and the cytoplasmic content of ERα and ERβ, respectively. Panel "C" reports the calculated ERα/ERβ nuclear and cytoplasmic ratios. Differences among samples were evaluated by applying the Kruskal-Wallis test followed by the Dunn's multiple comparison test. Samples with different letters (a, b) are significantly different ($p < 0.05$).

3.3. In silico *analysis*

The *in silico* analysis aimed at assessing the capability of ATX-I, ALP, TeA and AST, which were those most resistant during fecal incubations (Crudo et al., 2020), to interact with the discrete conformational states of ER described so far by crystallographic studies. The interaction with the antagonistic conformation of ER or the disruption of its agonistic conformation could provide a mechanistic rationale to explain the antagonistic activity of CE. However, none of the considered compounds were able to satisfy the requirements of ER's pocket in any of its conformational state under analysis. Indeed, as shown in Table 2, ATX-I, ALP, AST and TeA recorded negative scores in all the models considered pointing to their incapability to satisfy the requirements of ER's pocket. Of note, the workflow succeeded to compute the interaction of the respective ligands co-crystallized with the ER in the diverse conformational states under analysis since positive scores were recorded for them. This result eventually pointed to the procedural reliability to compute relevant protein-ligand interactions. Therefore, the negative scores of ATX-I, ALP, AST and TeA were actually due to their incapability to fit the ER pocket.

A closer inspection of binding poses in comparison to the pharmacophoric fingerprint of the diverse conformational states of ER pocket provided a structural explanation of the negative scores recorded. Concerning the agonistic conformation, ATX-I, ALP and AST were found exceeding the space available for ligand resulting in unfavorable interatomic clashes (Fig. 5A). Conversely, TeA showed a volume compatible with the space available for ligand but it was unable to satisfy the pocket requirements and caused hydrophobic/polar mismatches (Fig. 5A). In the two antagonistic states, ATX-I, ALP, TeA and AST showed rather similar occupancy of the binding site, which has additional space compared to that of the agonistic conformation. However, in both models, ATX-I, ALP, TeA and AST did not match the pharmacophoric requirements of pockets since they arranged most of their polar groups in regions markedly hydrophobic (Fig. 5B) causing hydrophobic/polar interferences to the binding event. Therefore, ATX-I, ALP, TeA and AST were not deemed able to satisfactorily interact with ERs due to their incapability to interact neither with the agonistic conformation, nor with the antagonistic states.

4. Discussion

The final toxicological outcome deriving from the ingestion of food contaminated by multiple toxic compounds is strictly dependent by the type and relative concentrations of compounds forming the mixture, which may be modified by several factors such as the interaction with food constituents and metabolization/adsorption phenomena mediated by the gut microbiota. In this context, *Alternaria* mycotoxins, which are often found in food in mixtures, have been shown to exert toxic effects in both *in vivo* and *in vitro* experiments (Crudo et al., 2019). Despite this, the presence of this class of food contaminants in food is not yet regulated due to the shortage of toxicological and occurrence data. In the present work, a naturally occurring mixture of *Alternaria* mycotoxins was tested for its estrogenic properties before and after incubation with fecal slurries and fractions thereof, in order to obtain information about the possible role of microbes and fecal material in modifying the endocrine disruptive properties of the extract.

As reported in Fig. 1, the tested fecal slurries (FS + DMSO) were found to induce estrogenic effects in the Ishikawa cell model, independently from the gender of the donor. The extent of estrogenicity was in the maximum saturation range for the AlP assay and comparable to the effect caused by 1 nM of estradiol. The estrogenic properties of fecal slurries can probably be attributed to the presence of soluble compounds with estrogenic activity, as demonstrated by the absence of significant differences between the estrogenic effects exerted by the fecal slurries and the corresponding fecal water fractions. Potential candidate substances could directly derive from the ingested foods or be of microbial origin. In fact, foods, especially those of plant origin, are an important source of natural and synthetic compounds with estrogenic activity (*e.g.*: phytoestrogens, alkylphenols, mycoestrogens, pesticides), but they can also contain indigestible polyphenols that can be metabolized by intestinal microbes with consequent synthesis of estrogenic compounds (Capriotti et al., 2013; Parida and Sharma, 2019). In addition, feces also contain free and conjugated excreted estrogens, and the deconjugation activity of the microorganisms of the gut microbiota is known to contribute to the increase of the free forms of these endogenous hormones (Parida and Sharma, 2019).

Unlike what was observed for samples of FS + DMSO, the extract alone (PBS + CE) did not lead to any increase of the AlP activity as compared to the solvent control (PBS + DMSO; Fig. 1), and the absence of estrogenic effects was even observed after incubation of the CE with the fecal-water stripped samples of particulate matter, living and dead microorganisms. The inability of the extract to mediate estrogenic effects was probably due to the low concentrations of estrogenic dibenzo-α-pyrones, such as AOH and AME (Table 1). These mycotoxins were previously reported to act as estrogenic compounds starting from higher concentrations (Dellafiora et al., 2018a; Lehmann et al., 2006).

Interestingly, incubation of both, fecal slurries and fecal waters in combination with the extract resulted in antiestrogenic effects even before the anaerobic incubation, and the magnitude of the antiestrogenic effects observed remained unchanged after 3 h of incubation. The absence of differences in the antiestrogenic effects of the extract between the fecal slurry samples collected before

Table 2
Computational scores of AST, ALP, ATX-I and TeA with the diverse conformational state of ER.

ER model	Mycotoxin				Reference compound [a]
	AST	ALP	ATX-I	TeA	
2YJA Agonistic conformation	−1032	−863	−941	−306	1137
1XPC Antagonistic conformation 1	−1155	−980	−1008	−402	1043
1QKM Antagonistic conformation 2	−2767	−853	−2332	−873	126

Note: computational scores are proportional to the capability of compounds to fit ER pocket and negative scores indicate their incapability to satisfy the physico-chemical requirements of pockets (for further detail see Section 2.7).

[a] The ligands co-crystallized with the diverse ER conformations were considered reference compounds to check modeling reliability and served as positive controls to ensure the capability of scoring procedure to well compute a real protein-ligand interaction. E2, a raloxifene-like compound ((2S,3R)-3-(4-hydroxyphenyl)-2-[4-[(2R)-2-pyrrolidin-1-ylpropoxy]phenyl]-2,3-dihydro-1,4-enzoxathiin-6-ol) and genistein were the reference ligands co-crystallized in the PDB structure 2YJA, 1XPC and 1QKM, respectively.

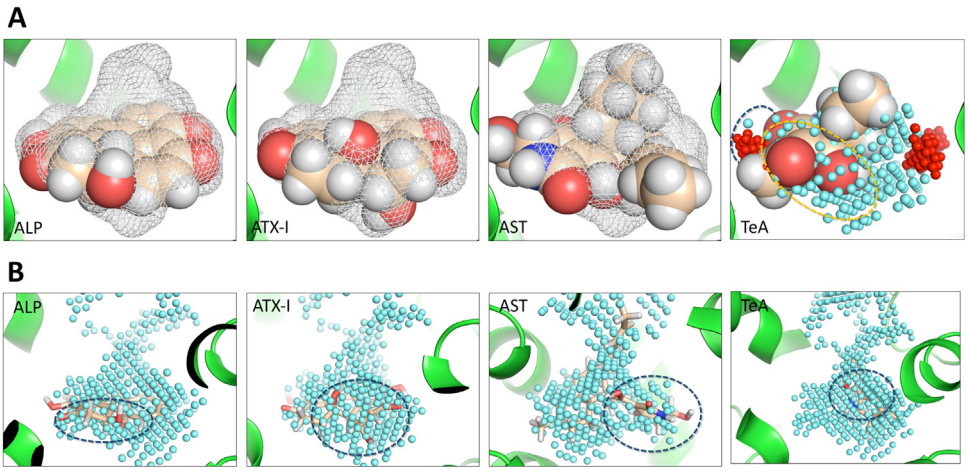

Fig. 5. Computed pose of ATX-I, ALP, AST and TeA within the agonistic or antagonistic conformation of ER. **A.** Binding poses within the agonistic conformation of ER. The protein is represented in cartoon, while ligands are represented in van der Waals spheres. The shape of the binding site is represented by mesh. In all the computed binding architectures (except for TeA), portions of ligand exceeded the boundaries of binding site. Cyan and red spheres indicate regions sterically and energetically able to receive hydrophobic and polar groups, respectively. The dashed blue ring indicates the improper arrangement of TeA's hydrophobic group within a polar region of the pocket, while the orange dashed ring indicates the improper arrangement of TeA's polar group within a hydrophobic region of the pocket. **B.** Binding poses within the antagonistic conformation of ER. The pockets of the two antagonistic conformations are rather similar and ligands adopted very similar pocket occupancy. Therefore, only the binding pose within the model derived from the crystallographic structure having PDB code 1XPC is shown. Protein is represented in cartoon, while ligands are represented in sticks. Cyan spheres indicate regions sterically and energetically able to receive hydrophobic groups. Dashed blue rings indicate the improper arrangement of ligands polar groups within the hydrophobic space of the pocket.

and after anaerobic incubation suggests the inability of the gut microorganisms, as well as of the other fecal fractions, to alter the bioactivity of the mycotoxins responsible for the effects observed.

A slight reduction of AlP activity was also observed in cells exposed to just the extract in PBS, pointing towards a suppressive effect of the latter even in the absence of estrogenic stimuli. The same effect was visible for incubations with a mixture of CE and inactivated microorganisms (DM + CE), a combination that was recently reported to lead to a partial or complete loss of some of the mycotoxins within the extract, while more resistant mycotoxins such as ATX-I, AST, ALP, TeA and TEN were mostly maintained (Crudo et al., 2020). Thus, considering that both PBS + CE (containing the mycotoxins at the original concentrations) and DM + CE (in which losses of AOH, AME, ATX-II, and STTX-III were reported) resulted in similar reduced AlP activity as compared to PBS + DMSO, the antiestrogenic effects observed in FS + CE and FW + CE might be attributable to one or more of the more persistent mycotoxins (*i.e.* ATX-I, AST, ALP, TeA and TEN).

In a previous study (Aichinger et al., 2019), the same *Alternaria* extract was found to suppress estrogenic effects induced by 1 nM E2 in feces-free incubations, without modifying the transcription of the estrogen receptors (*ESR1* and *ESR2*) but enhancing that of *CYP1A1*, whose up-regulation can be stimulated by the activation of AhR (Delescluse et al., 2000). Of note, the ability of the *Alternaria* extract to activate the AhR was demonstrated in a recent work (Hohenbichler et al., 2020), in which the extract activated the AhR signaling pathway in MCF-7 breast cancer cells starting from 20 μg/mL. Although in the present study Ishikawa cells were exposed to a lower CE concentration, an involvement of the AhR in the antiestrogenic effects could not be excluded, considering the employment of a different cell line and the enhanced *CYP1A1* transcription previously reported in Ishikawa cells (Aichinger et al., 2019). Based on these results, we hypothesized a possible degradation (AhR or non-AhR-mediated) of the ERs as a mechanism underlying the antiestrogenic effects of the extract. 2,3,7,8-tetrachlorodibenzo-[p]-dioxin (TCDD), which is one of the most potent AhR agonists, was in fact reported to reduce ERα protein levels by enhancing its proteasome-dependent degradation at a level higher than that of the endogenous hormone estradiol (Matthews and Gustafsson, 2006). In addition, also other food contaminants (such as bisphenol A and phthalates) and drugs have been previously reported to modulate, through different mechanisms, the degradation of both α- and β-ERs (Masuyama and Hiramatsu, 2004; Peekhaus et al., 2004).

As shown in Fig. 4, α- and β-ERs were quantified in nucleus and cytoplasm of Ishikawa cells exposed to the samples FS + DMSO, FS + CE, PBS + CE, PBS + DMSO, and PBS + E2, collected before the 3 h of anaerobic incubation. Although no difference in the absolute nuclear content of α- and β-ERs was observed among the samples, a significant increase of the nuclear/cytoplasmic ratio for both α- and β-ERs was found in all samples compared to the PBS + DMSO solvent control (Fig. 4A–B). Of note, the increased nuclear ratio was a consequence of the strong reduction of the number of ERs found at the cytoplasmic level, which was also responsible for the lower overall cellular content (nucleus + cytoplasm) of receptors detected in samples other than the solvent control (PBS + DMSO).

The reduced number of α- and β-ERs found in the E2-treated cells (compared to the solvent control) was not unexpected, since the E2-induced down-regulation of the ERs is a well-known and essential regulatory mechanism which allows to limit the time of action of the endogenous hormone on cells (Borrás et al., 1994). A similar reduction was also observed in cells exposed to FS-DMSO (Fig. 4A–B), probably as a consequence of the presence of estrogenic compounds able to mimic the endogenous hormone estradiol, as confirmed by the increased AlP activity. Surprisingly, reduction of α- and β-ERs was also observed in the cytoplasm of cells exposed to samples containing the extract, which induced antiestrogenic effects (*i.e.* FS + CE) or did not show any estrogenic properties (*i.e.* PBS + CE). Considering that a lower total number of ERs was detected in all samples tested other than the PBS + DMSO control, this phenomenon could not explain the antiestrogenic effects observed in samples containing the extract, also considering the absolute unmodified number of ERs into the nucleus among the various samples.

As shown in Fig. 4C, the ERα/ERβ cytoplasmic ratio was found to be reduced in all samples other than the PBS + DMSO control. Interestingly, unlike what was observed at the cytoplasmic level, the ERα/ERβ nuclear ratio in cells treated with samples containing

the CE, which led to the appearance of antiestrogenic effects (FS + CE) or did not induce any estrogenic effect (PBS + CE), was found to be similar to that of the solvent control PBS + DMSO, but significantly reduced compared to the estrogenic samples PBS + E2 and FS + DMSO. The modification of the ERα/ERβ nuclear ratio may partially explain the observed antiestrogenic properties of the *Alternaria* extract. As a matter of fact, while the transcription of a certain gene can sometimes be modulated by both types of receptors, α- and β-ERs have also unique target genes, whose transcription can be affected by only one of the two estrogen receptors (Williams et al., 2008). In addition, it has also been reported the ability of ERβ to affect the gene-regulation activity of ERα, sometimes leading to opposite effects (Williams et al., 2008). Thus, it cannot be excluded that the different ERα/ERβ nuclear ratio was responsible for modifications of the transcription of genes encoding proteins involved in the estrogenic pathway (*e.g.* co-repressors or co-activators).

To dispel doubts about a possible ability of mycotoxins contained into the extract to act as direct ER antagonists, a docking simulation was performed. We chose ALP, AST, TeA and ATX-I for this *in silico* analysis because of their resistance to fecal incubations (previously discussed) in opposite to the perylene quinones ATX-II and STTX-III, as well as the dibenzo-α-pyrones AOH and AME, which were shown to be completely or partially lost in the experimental conditions used (Crudo et al., 2020). Moreover, TEN and ALS were described as unable to interact with ER in a previous study (Dellafiora et al., 2018a) and they were therefore not considered in the present study. Although TeA was not likely to act as an ER binder due to the lack of an aromatic system, which has been documented to be a crucial feature for interaction with the ER pocket (Ng et al., 2014), it was included in the analysis in the light of its relative abundance in the *Alternaria* extract. Of note, AST also lacks of an aromatic portion, but was included in the analysis since its overall molecular shape could be compatible with the shape of the ER pocket. However, as reported in Table 2 and Fig. 5, none of the mycotoxins considered in the *in silico* analysis were found able to favorably interact with the ER. Therefore, the computational outcome did not support the existence of ER-dependent mechanisms as the basis of the antiestrogenic effects described in this work.

Considering the high abundance of TeA in the *Alternaria* extract, and to dispel any doubt about its possible involvement in the antiestrogenic effects observed, TeA was also tested *in vitro*. As reported in Fig. 2, results of the AlP assay revealed the inability of TeA to induce estrogenic or antiestrogenic effects, thus confirming the *in silico* results.

The ability of the *Alternaria* extract to induce antiestrogenic effects, which from a toxicological point of view could represent as much danger as the induction of estrogenic effects, raises the question of whether these food contaminants might impair the native functioning of the gastrointestinal tract, which represent the first and most important target of mycotoxins action. In fact, due to their high occurrence in food and low systemic bioavailability (Puntscher et al., 2019a), these mycotoxins could constantly interfere with the estrogenic signaling pathway at the intestinal level. Although the consequences of these interferences are still poorly understood, the key role of this pathway in the progression of various gastrointestinal diseases has already been suggested (Chen et al., 2019). This justifies the need for further studies aimed to better characterize the risk associated to this class of mycotoxins.

5. Conclusions

The present study aimed to investigate the estrogenic effects exerted by a naturally occurring mixture of *Alternaria* mycotoxins after incubation with fecal materials, in order to evaluate possible changes of its properties in a condition more similar to the *in vivo* situation, where mycotoxins are exposed to the action of gut microorganisms and are ingested along with food constituents. We found the estrogenicity of the fecal slurries and corresponding fecal waters to be partially suppressed in mixture with the *Alternaria* extract, even without anaerobic fecal incubation. The antiestrogenic properties of the extract were found to be preserved also in samples subjected to 3 h of fecal incubation, thus describing the *Alternaria* mycotoxins responsible for these effects as potential endocrine disruptors even *in vivo*. Although single mycotoxins responsible of these effects remain to be identified, we propose ATX-I, AST, ALP and TEN as the most likely candidates for subsequent studies. With respect to the underlying mechanisms of action, no antagonistic activity of the mycotoxins ALP, ATX-I, AST and TeA toward the ERs was observed during the *in silico* analysis, and the inability of TeA to exert antiestrogenic effects was also confirmed *in vitro*. Further investigations revealed the ability of the extract to decrease *in vitro* the ERα/ERβ nuclear ratio. Although this result might partially explain the antiestrogenic effects observed in the Ishikawa cell line, the absence of substantial changes in the cytosolic and nuclear contents of ERα and ERß supports the hypothesis of a possible involvement of other mechanisms in the antiestrogenic effects observed. Based on the results obtained, the potential ability of the *Alternaria* mycotoxins of the extract to change the transcription of genes related to the estrogenic pathways (as a consequence of an ERα/ERβ nuclear ratio modification), as well as to activate other intracellular pathways involved in the regulation of the ER-signaling, will be addressed in subsequent in-depth studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2022.01.015.

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