UNIVERSITÀ DI PARMA

UNIVERSITA' DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN
SCIENZE DEGLI ALIMENTI

CICLO XXXIII

A Toxicological Evaluation of the Interactions between *Alternaria* Mycotoxins, Food Constituents and Gut Microbiota: Implications for Human Health

Coordinatore:
Chiar.ma Prof. **Chiara Dall'Asta**

Tutori:
Chiar.ma Prof. **Chiara Dall'Asta**
Chiar.ma Prof. **Doris Marko**

Dottorando: **Francesco Crudo**

Anni Accademici 2017/2018 – 2019/2020

PHD THESIS

FRANCESCO CRUDO

A Toxicological Evaluation of the Interactions between *Alternaria* Mycotoxins, Food Constituents and Gut Microbiota: Implications for Human Health

PHD THESIS SUPERVISORS:

Prof. Chiara Dall'Asta

Department of Food and Drug, University of Parma

(Italy)

Prof. Doris Marko

Department of Food Chemistry and Toxicology, University of Vienna

(Austria)

"The role of the infinitely small in nature is infinitely great."

— Louis Pasteur—

ACKNOWLEDGMENTS

This research project would not have been possible without the invaluable support of many people, to whom I am deeply grateful.

First and foremost, I would like to express my eternal gratitude to my supervisors, Prof. Chiara Dall'Asta and Prof. Doris Marko, who guided me with great professionalism and competence during these three years of PhD course in Food Science.

I would also like to extend thanks to Prof. David Berry and his collaborators, who contributed significantly to my professional growth in the microbiology field and, thanks to whom, I was able to carry out an important part of this doctoral thesis.

Special thanks are reserved for Dr. Luca Dellafiora and Dr. Georg Aichinger for their valuable scientific advice and the moral support they have provided me over these years.

I would also like to thank Prof. Benedikt Warth for his valuable advice, as well as the staff of the Mass Spectrometry Center (Faculty of Chemistry; UNIVIE) for skillful and reliable technical support.

I would like to recognize the invaluable research support provided by Dr. Giorgia Del Favero (Core Facility of Multimodal Imaging, Faculty of Chemistry; UNIVIE) and Dr. Elisabeth Varga, as well as to thank all PhD students, research fellows, researchers, professors, technicians and administrative staff of UNIPR and UNIVIE that I had the pleasure of meeting during these three years of PhD spent between Parma and Vienna.

Finally, I wish to express my gratitude to Fondazione Cariparma for the financial support provided under the TeachInParma Project

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PREFACE

Food contamination by natural or anthropogenic toxic compounds has become a problem affecting both developed and developing countries. Among the chemical contaminants of natural origin, *Alternaria* mycotoxins, which are secondary toxic fungal metabolites produced by species belonging to the genus *Alternaria*, are still not regulated due to the shortage of toxicological and occurrence data.

Following the latest call for data inquired by EFSA in 2016, several studies have been carried out on this class of emerging contaminants, which have provided useful data for a future risk assessment. However, only a few works focused on the toxic effects exerted by mixtures of *Alternaria* mycotoxins (which is a situation closer to a real-world scenario compared to testing of a single substance), as well as the role of the gut microbiota, microbial metabolites and food constituents in modifying the toxicokinetics and/or toxicodynamics of the *Alternaria* mycotoxins.

Based on this, the scientific work included in this PhD thesis has been designed with the aim of evaluating the possible impact of the factors reported above on the toxicology of *Alternaria* mycotoxins.

The present PhD thesis is structured as follows:

- A "*General Introduction"* part, in which topics useful to introduce the aims of the thesis are briefly discussed, such as those related to the chemical risk assessment of food-related compounds, the co-occurrence of multiple xenobiotics in food, and the role of the gut microbiota in human health;
- The *"Aims of the thesis"* part, which summarizes the general aims and the specific objectives of each scientific work included in this doctoral thesis;
- The body of the thesis, which is organized into five chapters. Each chapter represents a scientific work (published, submitted or in preparation) carried out during the doctoral program:
    - "*Chapter 1"* is a systematic review of the scientific works dealing with the evaluation of the co-occurrence and combinatory effects of: (i) different *Alternaria* toxins; (ii)

Alternaria toxins in combination with other mycotoxins; (iii) *Alternaria* toxins in combination with bioactive compounds of food origin;

- "*Chapter 2*" and "*Chapter 3*" are original research articles concerning the study of the effects of fecal incubations on the genotoxic and estrogenic properties, respectively, of a complex extract of *Alternaria* mycotoxins, in which the role of the different fecal fractions (e.g. gut microorganisms and particulate matter) in the potential modification of the effects has been evaluated;
- "*Chapter 4*" is a research article in which the potential bidirectional relationship between gut microbiota and *Alternaria* mycotoxins was investigated;
- "*Chapter 5*" is an original research article dealing with the evaluation of the ability of the gut microbial metabolite urolithin C to interfere with the toxicokinetic of the *Alternaria* mycotoxin alternariol in a Caco-2 transwell model.

- A "*General Conclusions and Future Perspectives*" part, which summarizes the results obtained during the PhD project and suggests directions for potential future works in this research context.

LIST OF ABBREVIATIONS

Chemicals

ALP	Alterperylenol
ALS	Altenusin
ALT	Altenuene
AME	Alternariol monomethyl ether
AOH	Alternariol
AST	Altersetin
ATX-I	Altertoxin I
ATX-II	Altertoxin II
CE	Complex extract of *Alternaria* mycotoxins
E2	17β-estradiol
PBS	Phosphate-Buffered Saline
STTX-III	Stemphyltoxin III
TeA	Tenuazonic acid
TEN	Tentoxin
UroC	Urolithin C

Bacteria

AF	*Alistipes finegoldii*
AM	*Akkermansia muciniphila*
AT	*Alistipes timonensis*
B.sp	*Bifidobacterium sp.*
BC	*Bacteroides caccae*
BE	*Bacteroides eggerthii*
BL	*Bifidobacterium longum*
BT	*Bacteroides thetaiotaomicron*
BV	*Bacteroides vulgatus*
CI	*Clostridium innocuum*
EC	*Escherichia coli*
LH	*Lactobacillus hominis*
PD	*Parabacteroides distasonis*
RB	*Ruminococcus bicirculans*

Miscellaneous

AP	Apical compartment
AlP	Alkaline phospatase
BL	Basolateral compartment
DM	Dead microorganisms
ER	Estrogen receptor
FPG	Formamidopyrimidin-DNA-glycosylase enzyme
FS	Fecal slurry
FW	Fecal water
LM	Living microorganisms
PM	Particulate matter
SULT	Sulfotransferase
UGT	Uridine 5'-diphospho-glucuronosyltransferase

GENERAL INTRODUCTION

Food represents a very complex matrix, as it contains not only nutrients such as carbohydrates, lipids, proteins, minerals and vitamins, but also a multitude of other molecules of plant or animal origin, which are constitutively present in raw materials. However, food might also represent a potential reservoir of natural or anthropic toxic chemicals, the so-called "food contaminants", which are not intentionally added to food but may contaminate food during the various stages of the food chain (from farm to fork) as a consequence of environmental contamination, wrong cultivation practices or unsuitable processes of production, packaging, transport or holding (Council of the European Communities, 1993).

Contamination of food by chemical contaminants, such as polycyclic aromatic hydrocarbons, heavy metals or naturally occurring toxins, as well as by pesticides and veterinary drugs residues, represents an issue for both developed and developing countries due to the derived economic losses and the implications on human health (Hussain and Dawson, 2013; Marasas et al., 2008).

The complete elimination of all contaminated food from the market is something that cannot be achieved, given the high number of contaminants known to date and their frequent occurrence. Therefore, in order to protect the health of consumers, maximum levels are generally set for substances that could represent a real risk for humans. The definition of these maximum levels is based on a process, known as "risk assessment", which involves the integration and analysis of several data related to the exposure to a given substance and its potential to cause harmful effects (Benford, 2017).

On a European scale, maximum levels for certain food contaminants were established by the Commission Regulation (EC) No 1881/2006, which includes also some secondary toxic metabolites produced by common and widespread species of molds, named "mycotoxins".

In particular, mycotoxins are secondary metabolites of low molecular weight produced by species belonging mainly to the genera *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* (Berthiller et al., 2013). Many of these metabolites are thermoresistant, being not completely destroyed even by food cooking processes that involve the use of high temperatures (Kabak, 2009). For this

reason, the control of factors involved in the promotion of both, the fungal colonization and the capability of fungi to produce mycotoxins within the raw materials, represents a key point in the fight against mycotoxin contamination. Examples of factors influencing mycotoxin production are the temperature, the use of fungicides and/or fertilizers and, especially, the relative humidity (Tola and Kebede, 2016). Despite the application of good practices during the production, storage and delivery of food, the difficulty in completely controlling the production of mycotoxins makes this class of toxic compounds still relevant for the food safety area.

The biological roles of mycotoxins for the fungal species that produce them are still not fully clarified. However, numerous hypotheses have been formulated over the years and, among these, their probable involvement in the mechanisms of oxidative stress control and protection against other organisms are among the most accredited ones (Reverberi et al., 2010). Among the mycotoxins known to date, only aflatoxins (B1, B2, G1, G2, and M1), ochratoxin A, patulin, deoxynivalenol, zearalenone, fumonisins (B1 and B2), T-2 and HT-2 toxins are currently regulated (European commission, 2006), while others, such as those produced by *Alternaria* species, are still under evaluation due to limited toxicological and occurrence data.

Alternaria is a ubiquitous fungal genus which includes saprophytic, endophytic and pathogenic species able to grow on several different substrates. The species *A. alternata*, *A. arborescens*, *A. brassicae*, *A. brassicicola, A. infectoria, A. radicina* and *A. tenuissima,* are the most frequently reported in food (Lee et al., 2015). This fungal genus can infest both fresh and processed food and can produce more than 70 different toxins, some of which might be discussed to pose a threat to human health due to the adverse effects observed *in vitro* and *in vivo* (genotoxic, mutagenic, androgenic, estrogenic, fetotoxic and teratogenic effects) and the frequent occurrence in food (Crudo et al., 2019). Of note, several authors reported the frequent occurrence of multiple *Alternaria* mycotoxins in food, as well as the co-occurrence of *Alternaria* mycotoxins along with mycotoxins produced by other types of molds.

In this regard, the risk assessment on chemical contaminants, which is the basis for the development of regulatory measures, is currently based on the

integration of the available data on the occurrence and toxicity of a given compound, without considering the risk associated with a possible co-exposure to multiple toxic chemicals (Benford, 2017). The exposure to chemical mixtures can result in effects different from those exerted by the mixture components taken individually (i.e. additive, synergistic or antagonistic effects). As an example, mixtures of type B trichothecenes, which are mycotoxins mainly produced by *Fusarium* species, showed synergistic, additive or antagonistic cytotoxic effects in the human intestinal Caco-2 cell line, depending on the combinations and relative concentrations tested (Alassane-Kpembi et al., 2013). Modifications of the toxic effects of mycotoxins were also reported following co-incubation with bioactive compounds naturally occurring in food. In recent studies, the estrogenic effects of the *Fusarium* mycotoxins zearalenone and α-zearalenol were quenched by the co-incubation with the hop polyphenols xanthohumol and 8-prenylnaringenin (Aichinger et al., 2018), while the isoflavone genistein, which is known to act pro-estrogenic, interacted synergistically or antagonistically with both the mycoestrogens zearalenone and alternariol, depending on the combination ratios and the concentration range (Vejdovszky et al., 2017). All these findings suggest that mycotoxin-mycotoxin and mycotoxin-bioactive interactions might occur upon consumption of contaminated food, sometimes resulting in the appearance of more pronounced effects than those exerted by the single compounds taken individually.

The suppression of the toxic effects mediated by xenobiotics may also occur as a consequence of the interaction of the latter with food constituents other than bioactive compounds. In particular, many agricultural products and by-products, especially those rich in non-degradable dietary fibers, were shown to adsorb xenobiotics introduced with the diet, thus reducing the free-absorbable portion of xenobiotic. As an example, dietary fibers extracted from wheat bran were reported to efficiently adsorb the polycyclic aromatic hydrocarbons naphthalene, acenaphthene, phenanthrene and pyrene (Zhang et al., 2016), while grape pomace has been reported to sequester several mycotoxins, including aflatoxin B1, zearalenone, ochratoxin A, and fumonisin B1 (Avantaggiato et al., 2014).

Apart from food constituents, the toxicological impact of xenobiotics can be also influenced by the interaction with the gut microbiota, which is known to represent, together with intestinal epithelial cells, the host's first line of defense against pathogens and toxins (Iacob et al., 2019; Lazar et al., 2018).

In particular, the gut microbiota consists of dynamic multispecies populations of microorganisms that inhabit the intestinal tract. Although bacteria dominate microbial communities, the gut environment also arbors a wide array of other species of microorganisms such as fungi, archaea and protozoans (Lozupone et al., 2012; Stefanaki et al., 2017). These microorganisms, together with several viruses (mainly bacteriophages) normally housed in the intestine, contribute to the maintenance of the gut homeostasis (Vemuri et al., 2020). Because of the several functions and interactions with the host, the gut microbiome has been reported to act as an additional "organ" of the human body. In particular, its members are involved in the metabolization of indigestible food components, in the synthesis of vitamins (e.g. K, B1, B6), in nutrients and minerals absorption, in the stimulation of the development of the immune system, in the modulation of human metabolism, in the fight against pathogens, as well as in several other functions such as in xenobiotic metabolism (Clarke et al., 2019; Putignani et al., 2014; Ramakrishna, 2013; Rowland et al., 2018; Wilson and Nicholson, 2017). In this context, thanks to the huge array of microbial enzymes and the complex interactions with the host, gut microbiota can affect the metabolism of drugs, food contaminants and food bioactive compounds through direct or indirect mechanisms, thus leading to modifications of the beneficial/toxic effects of the compounds (Clarke et al., 2019). As an example, microbes were reported to bioactivate drugs, as in the case of the prodrug sulfasalazine, which is converted by bacterial azoreductase enzymes into two active compounds named "sulfapyridine" (used for the treatment of rheumatoid arthritis) and "5-aminosalicylic acid" (used in inflammatory bowel disease patients) (Sun et al., 2019). However, gut microorganisms were also found to inactivate drugs (e.g. digoxin; used to treat various heart conditions) (Sun et al., 2019), as well as to reactivate drugs after liver-mediated inactivation, as in the case of the chemotherapeutic prodrug irinotecan. Specifically, the pharmacologically

active metabolite of irinotecan, produced at the liver level, is subsequently inactivated by human glucuronosyltransferases and excreted into the intestine through the bile. At this level, β-glucuronidase enzymes of microbial origin can deconjugate the inactivated compound (Guthrie et al., 2017). In addition, the ability of gut microorganisms to bind to xenobiotics (thus reducing the free-absorbable proportion), as well as to modulate the expression of human enzymes involved in xenobiotic metabolism, were also reported (Clarke et al., 2019). On this basis, the role of the gut microbiota in modifying the final toxicological outcome should always be considered when evaluating the potential risks related to food contaminants introduced with the diet.

Although xenobiotics may exert direct harmful effects on human health, their potential ability to cause indirect toxic effects by altering the gut microbial composition should also be considered. In fact, considering the key role of the gut microbiota in the maintenance of human health, any factor able to modify its composition and activity might influence both normal physiology and disease susceptibility of its host (Hasan and Yang, 2019). Among the various factors influencing the gut microbial composition, xenobiotics such as mycotoxins are also included (Liew and Mohd-Redzwan, 2018). As an example, a significant decrease of the levels of *Escherichia coli* and an increase of the *Bacteroides*/*Prevotella* group was previously reported in rats treated with the *Fusarium* mycotoxin deoxynivalenol (Saint-Cyr et al., 2013), while T2-toxin increased the aerobic bacteria in the intestine of rats and swine (Tenk et al., 1982). Modifications of the gut microbiota composition by ochratoxin A, aflatoxin B1 (Baines et al., 2013; Guo et al., 2014; Ouethrani et al., 2013; Wang et al., 2016), as well as by mixtures of fumonisins B1+B2 (Burel et al., 2013) and zearalenone+ deoxynivalenol (Piotrowska et al., 2014) were also reported.

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AIMS OF THE THESIS

The general purpose of the present PhD thesis was to verify whether interactions of *Alternaria* mycotoxins with food constituents and the gut microbiota may interfere with the toxicological properties of the members of this class of food contaminants. Additionally, considering that factors capable to modify the composition and activity of the gut microbiota might influence both normal physiology and disease susceptibility of its host, the present PhD thesis was also aimed at assessing the ability of *Alternaria* mycotoxins to affect the growth or activity of human gut bacterial strains.

The specific aims of the scientific works included in this thesis are reported below.

- *Chapter 1:*
    - To summarize the most relevant literature concerning the occurrence and toxicity of *Alternaria* mycotoxins, studied either individually or in combination with other mycotoxins or bioactive compounds of food origin, in order to highlight the need to assess their combined toxicity to better support the risk assessment of this class of mycotoxins.
- *Chapter 2:*
    - To investigate the ability of microorganisms, undigested food constituents and soluble substances of human feces to modify the composition and the genotoxic properties of a complex extract of *Alternaria* mycotoxins.
- *Chapter 3:*
    - To assess the impact of *in vitro* short-term anaerobic fecal incubations on the ability of an *Alternaria* mycotoxins extract to interfere with the estrogenic pathways;
    - To investigate the mechanisms underlying the effects exerted by the extract.
- *Chapter 4:*
    - To evaluate the capability of a complex extract of *Alternaria* mycotoxins to affect the growth or activity (biofilm production) of human gut bacterial strains;

  - To verify whether the same gut bacterial strains are able to metabolize or adsorb the *Alternaria* mycotoxins of the extract, thus contributing to the reduction of the free-absorbable portion of mycotoxins.

- *Chapter 5:*
  - To assess the ability of the gut microbial metabolite urolithin C, which is structurally related to the *Alternaria* mycotoxin alternariol, to interfere with the *in vitro* absorption and phase II metabolism of the mycotoxin.

1

Chapter

Review Article

Co-Occurrence and Combinatory Effects of *Alternaria* Mycotoxins and other Xenobiotics of Food Origin: Current Scenario and Future Perspectives

Francesco Crudo [1], Elisabeth Varga [2], Georg Aichinger [2], Gianni Galaverna [1], Doris Marko [1,2], Chiara Dall'Asta [1] and Luca Dellafiora[1,*]

[1] Department of Food and Drug, University of Parma, Parco Area delle Scienze 27/A, 43124 Parma, Italy; francesco.crudo1@studenti.unipr.it (F.C.); gianni.galaverna@unipr.it (G.G.); doris.marko@univie.ac.at (D.M.); chiara.dallasta@unipr.it (C.D.)

[2] Department of Food Chemistry and Toxicology, Faculty of Chemistry, University of Vienna, Währinger Str. 38, 1090 Vienna, Austria; elisabeth.varga@univie.ac.at (E.V.); georg.aichinger@univie.ac.at (G.A.)

* Correspondence: luca.dellafiora@unipr.it

Adapted from

Crudo, F., Varga, E., Aichinger, G., Galaverna, G., Marko, D., Dall'Asta, C., Dellafiora, L., 2019. Co-Occurrence and Combinatory Effects of Alternaria *Mycotoxins and Other Xenobiotics of Food Origin: Current scenario and Future Perspectives. Toxins (Basel). 11, 1–29. https://doi.org/doi:10.3390/toxins11110640*

Abstract

Mycotoxins are low-molecular weight compounds produced by diverse genera of molds that may contaminate food and feed threatening the health of humans and animals. Recent findings underline the importance of studying the combined occurrence of multiple mycotoxins and the relevance of assessing the toxicity their simultaneous exposure may cause in living organisms. In this context, for the first time, this work has critically reviewed the most relevant data concerning the occurrence and toxicity of mycotoxins produced by *Alternaria* spp., which are among the most important emerging risks to be assessed in food safety, alone or in combination with other mycotoxins and bioactive food constituents. According to the literature covered, multiple *Alternaria* mycotoxins may often occur simultaneously in contaminated food, along with several other mycotoxins and food bioactives inherently present in the studied matrices. Although the toxicity of combinations naturally found in food has been rarely assessed experimentally, the data collected so far, clearly point out that chemical mixtures may differ in their toxicity compared to the effect of toxins tested individually. The data presented here may provide a solid foothold to better support the risk assessment of *Alternaria* mycotoxins highlighting the actual role of chemical mixtures on influencing their toxicity.

1. Introduction

Mycotoxins are low-molecular-weight toxic compounds synthetized by different types of molds belonging mainly to the genera *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* (Berthiller et al., 2013). They may enter the food chain worldwide as a consequence of the ability of mycotoxin-producing molds to infect a wide number of crops and food commodities (EFSA, 2011). It has been reported that up to 25 % of world crops may be contaminated with mycotoxins and over 4.5–5.0 billion people are thought to be chronically exposed to these food contaminants (Enyiukwu et al., 2014). However, a much higher prevalence of detected mycotoxins can be found depending either on the considered mycotoxin or crop (up to 80% in certain circumstances), as recently reported (Eskola et al., 2019). Although the highest levels of food contamination are more frequently found in low-income countries, mycotoxins actually represent a growing threat also on account of climate changes (Van Der Fels-Klerx et al., 2016). The contamination of food and feed by mycotoxins results in significant economic losses worldwide, not only in terms of food and feed spoilage, but also in terms of a burden on human health, animal productivity and international trade (Dellafiora and Dall'Asta, 2017). In particular, mycotoxins may pose a toxicological concern for humans and animals since they may exert a wide number of effects including acute toxic, mutagenic, carcinogenic, teratogenic, estrogenic and immunotoxic actions. Among the various categories of mycotoxins, those produced by the genus *Alternaria* are gaining increasing interest due to their frequent occurrence in food, the recent insights on their genotoxic potential and mechanisms of action, and their consequent possible effects on human health (EFSA, 2011; Van Egmond et al., 2007). The *Alternaria* toxins belong to the group of the so called "emerging" mycotoxins. They are compounds of possible concern due to their abundance, occurrence or toxicity, but the limited available data do not allow a comprehensive risk assessment with an acceptable degree of certainty.

Alternaria species are ubiquitous plant pathogens and saprophytes that may contaminate a wide variety of crops and raw materials due to their

environmental adaptability, particularly to their tolerance to low temperature and water stress conditions. They produce a cocktail of secondary metabolites and more than 70 *Alternaria* toxins have been characterized so far (EFSA, 2011). Based on their chemical structures, *Alternaria* toxins may be divided into five groups (Figure 1): (i) dibenzo-α-pyrones, including alternariol (AOH), alternariol monomethyl ether (AME), and altenuene (ALT); (ii) perylene quinones, including the altertoxins I, II, III (ATX-I, ATX-II and ATX-III, respectively), stemphyltoxin I and III (STTX-I and STTX-III, respectively), and alterperylenol/alteichin (ALP); (iii) tetramic acid derivatives, including tenuazonic acid (TeA) and iso-tenuazonic acid (iso-TeA); (iv) *A. alternata* f. sp. lycopersici toxins, which includes several phytotoxins such as AAL-TA and ALL-TB sub-groups (v) miscellaneous structures, as tentoxin (TEN), which has a cyclic tetrapeptidic structure (EFSA, 2011; Ostry, 2008). However, many other mycotoxins might be produced by *Alternaria* spp. such as dihydrotentoxin, isotentoxin, altenuisol (ALTSOH), altenusin, infectopyrone, altersetin, macrosporin A, altersolanol A, monocerin, altenuic acids I, II, and III (Escrivá et al., 2017).

Due to the broad spectrum of adverse effects observed in vitro (e.g., genotoxic, mutagenic, clastogenic, androgenic, and estrogenic effects) and in vivo (e.g., fetotoxic and teratogenic effects), some of the *Alternaria* mycotoxins most frequently found in food may pose a severe threat to human health, especially for the most exposed categories such as infants, toddler and vegetarians (Arcella et al., 2016). Nevertheless, for most *Alternaria* mycotoxins, neither the toxicity nor the occurrence in food is adequately described. The current limitation of data hinders the proper assessment of risks to human health and, consequently, it prevents the establishment of specific regulations (European commission, 2006). Therefore, the need of additional representative data to support the proper risk assessment of *Alternaria* toxins, especially for AOH, AME, TeA, TEN and ALT, was claimed by the expert Committee "Agricultural Contaminants" of the EU commission in 2012 (Sanco, 2012). In 2016, a call to collect data for the human exposure assessment to *Alternaria* toxins (AOH, AME, TeA and TEN) was published by the European Food Safety Authority (EFSA) (EFSA, 2016).

In this respect, the chemical risk assessment of food-related compounds is currently based on the integration of knowledge about the single exposure to a given substance and its potential to individually cause harmful effects (Alexander et al., 2012). However, food is typically contaminated simultaneously by more than one mycotoxin. It is noteworthy that the simultaneous occurrence of compounds (either toxicants or bioactive food constituents) may lead to combinatory interactions (namely, additive, synergistic or antagonistic effects) that may significantly change the final toxicological outcome depending on the overall composition of chemical mixtures (see Section 3.2.). In addition, mycotoxins may be present in food along with a high number of bioactive compounds, showing a huge variety of chemical structures and mechanisms of action, which may further modify their toxic impact. On this basis, risk assessment studies should take into account this complexity rather than relying on individual evidences, to better evaluate the overall risk associated with the consumption of mycotoxins-contaminated food.

Therefore, in the framework of supporting a better risk assessment of *Alternaria* mycotoxins, this work aims at consolidating the current knowledge on occurrence and combined actions of *Alternaria* mycotoxins. The relevance of investigating the effects and occurrence of chemical mixtures to support the thorough assessment of the actual risk this class of mycotoxins may pose to humans is pointed out. In more detail, this work presents the current state-of-the art in terms of co-occurrence and combinatory effects of: (i) different *Alternaria* toxins; (ii) *Alternaria* toxins in combination with other mycotoxins; (iii) *Alternaria* toxins in combination with bioactive compounds of food origin.

2. Natural Occurrence and Co-Occurrence of *Alternaria* Mycotoxins in Food

The occurrence of *Alternaria* mycotoxins in food and feed has been reviewed over the years (Bottalico and Logrieco, 1998; Fernández-Cruz et al., 2010; Fraeyman et al., 2017; Ostry, 2008; Scott, 2001). However, in most cases, the occurrence and the relative concentrations of single or a small group of

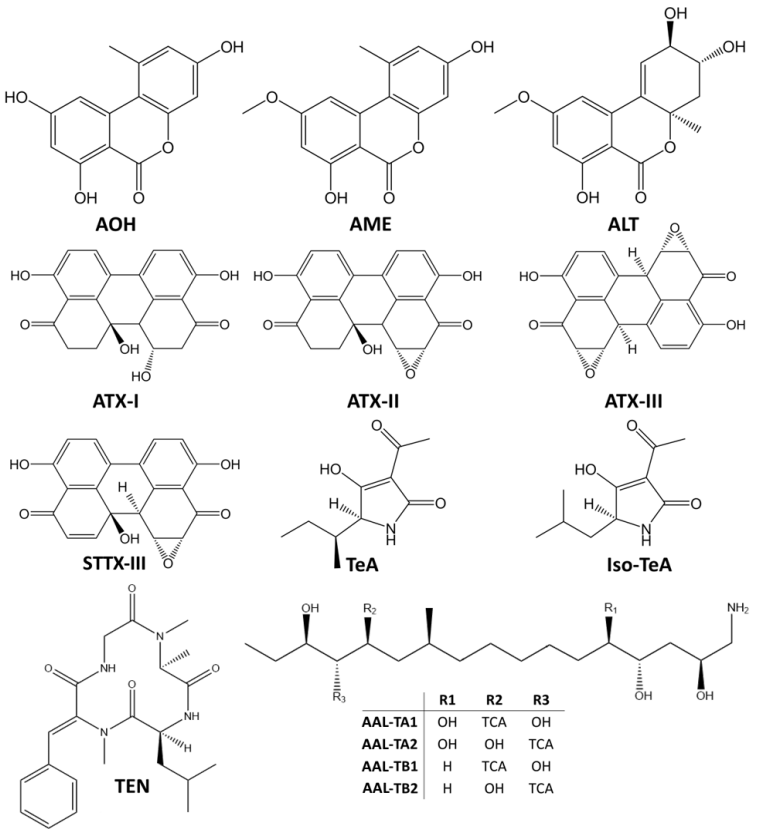

Figure 1. Chemical structures of the main *Alternaria* mycotoxins. AOH – alternariol; AME – alternariol monomethyl ether; ALT – altenuene; ATX-I, ATX-II, ATX-III – altertoxin I, II and III; STTX-III – stemphyltoxin III; TeA – tenuazonic acid; Iso-TeA – iso-tenuazonic acid; TEN – tentoxin; AAL-TA1-2 *Alternaria alternata* f. sp. *lycopersici* toxins sub-group A 1 and 2; AAL-TB1-2 *Alternaria alternata* f. sp. *lycopersici* toxins sub-group B 1 and 2; TCA - tricarballylic acid.

toxins has been reported, whilst the simultaneous co-occurrence of a high number of mycotoxins likely co-occurring together was not systematically assessed.

This section presents a collection of the co-occurrence of multiple *Alternaria* toxins in food commodities. In addition, data on the co-occurrence of *Alternaria* mycotoxins along with other mycotoxins and food constituents are reviewed. The key references covered in this review addressing the natural co-occurrence of different *Alternaria* mycotoxins are summarized in Table 1, while a schematic overview of the literature concerning the study of the co-occurrence of *Alternaria* and other mycotoxins is provided in Table 2.

Detailed information concerning the number of samples analyzed, mycotoxin concentrations, as well as the methods and instruments used are available online at http://www.mdpi.com/2072-6651/11/11/640/s1 (Supplementary material; Table S1).

2.1. Co-Occurrence of Different Alternaria *Toxins in Food*

With regard to the co-contamination of food by different *Alternaria* toxins, AOH, AME, ALT, TeA, TEN, and ATX-I are the most frequently investigated compounds, while broader sets of compounds, including for instance ATX-II, IsoALT, AAL-TA1, AAL-TA2, ALP, macrosporin, ALTSOH, and Val-TeA, are rarely reported.

As shown in Table 1, the presence of *Alternaria* mycotoxins has been well-documented both in fresh and processed food, including fruits and vegetables, nuts, seeds, cereals, and fermented beverages. Among the food commodities investigated so far, apples, tomato, and their derivative products have been more frequently explored than other types of fruits and vegetables. Notably, most of them were found simultaneously contaminated by both AOH and AME, and, in some cases, also by up to five different mycotoxins. One of the first investigations were performed by Stinson and co-workers (Stinson et al., 1981) who reported the contamination of apples and tomatoes with several *Alternaria* toxins already back in 1981. The observed contamination determined by HPLC-UV was in the low mg/kg range for AOH and AME, and in the μg/kg range for ALT and TeA in the case of apple samples. In tomatoes, TeA showed the highest contamination levels with up to 139 mg/kg. Furthermore, the presence or absence of ATX-I was assessed by thin layer chromatography. In the last ten years, multi-analyte measurements using liquid chromatography coupled to mass spectrometry became more and more important. The contamination with seven different *Alternaria* mycotoxins (AOH, AME, ALT, TeA, TEN, ATX-I, and ALP) and two phase-II metabolites (AOH-3-sulfate and AME-3-sulfate) was reported in tomato sauce, sunflower seed oil and wheat flour samples by Puntscher et al. (Puntscher et al., 2019). In this study, the simultaneous contamination in the μg/kg range was reported in sample(s) from Austria, Croatia and Italy.

Infant foods were also found to be contaminated by multiple *Alternaria* mycotoxins. As an example, Gotthard and co-workers reported that tomato sauce and apple-pear-cherry puree were simultaneously contaminated by AOH, AME, TeA, and TEN (Gotthardt et al., 2019). In addition, those mycotoxins were also found in cereal-based infant formulas and they were reported along with ATX-I in wheat- and spelt-based food. These results are particularly relevant considering that the young population (infants and toddlers) show a higher exposure to *Alternaria* toxins in comparison to the other population categories due to their high food consumption in relation to body weight (Arcella et al., 2016).The most important dietary contributors to these mycotoxins were fruits and fruit products, vegetable oil, cereal-based foods and fruiting vegetables (tomatoes) wherein multiple mycotoxins were often found simultaneously, as shown in Table 1. This scenario is further complicated by the possible presence of so called "masked mycotoxins". This term refers to modified forms of mycotoxins as a result of their metabolic transformations in plants. Masked mycotoxins have been reported to abundantly co-occur in contaminated food and raw materials along with their respective parent counterparts (Gratz, 2017). The most common masked mycotoxins are covalently linked sulfate or glucoside groups as a result of plant phase-II metabolism (Nathanail et al., 2015). After ingestion, these phase II plant metabolites can be hydrolyzed during the digestion releasing the respective toxic parental compounds (Berthiller et al., 2013). The transformation of masked mycotoxins to metabolites with higher toxicity than the parent compounds was also described in vitro (Dellafiora et al., 2016, 2017b), further highlighting the toxicological potential of the masked forms of mycotoxins (referred to as "maskedome"). Nevertheless, masked mycotoxins are not routinely screened, and this may result in an underestimation of the actual amounts of mycotoxins in foods. In this respect, Puntscher et al. (Puntscher et al., 2018) reported the presence of some modified forms of AOH and AME (i.e., AOH-3-glucoside, AOH-9-glucoside, AOH-3-sulfate and AME-3-sulfate) in tomato sauce samples from Italy. In particular, one sample was found contaminated not only with AOH, AME, TeA and TEN, but also with AOH-3-glucoside, AOH-3-sulfate and AME-3-sulfate. Similarly, Walravens and co-workers found tomato products

(juices, sauces and concentrates) contaminated with AOH-3-sulfate and AME-3-sulfate, with a prevalence ranging from 11% to 26% and from 32% to 78%, respectively (Walravens et al., 2016). The authors reported the highest prevalence of AOH and AME in tomato sauces (86% and 78%, respectively), while ALT was most frequently detected in tomato concentrates (56%). In addition, a prevalence of TEN-contaminated products, ranging from 21% to 64% (in sauces and juices, respectively), was also reported and, interestingly, all the tested samples showed a high contamination with TeA. More recently, another study highlighted the contamination of both fresh and dried tomato samples by different *Alternaria* toxins, among which TeA was found the most frequent and abundant compound (Sanzani et al., 2019).

The frequent co-occurrence of multiple *Alternaria* mycotoxins was also described in many other foods, including peppers. As an example, Gambacorta and co-workers (Gambacorta et al., 2018) analyzed samples of fresh, dried, grounded, and fried sweet pepper, wherein AOH, AME, TeA, and TEN were found together (limit of quantifications in the low μg/kg range). In particular, TeA was detected in all samples, while AOH was detected in 86%, 43%, 100% and 14% of fresh, dried, grounded and fried products, respectively. Fresh pepper samples were mostly contaminated by AME (57% of fresh pepper samples), while fried peppers were the least AME-contaminated samples (14% of fried peppers samples). ALT was detected only in 43% and 13% of fresh and grounded samples, respectively.

Beside fruits and vegetables, cereals and derived products play an important role in the exposure to *Alternaria* toxins, representing the main source of exposure for infants and toddlers (Arcella et al., 2016). According to EFSA (EFSA, 2011), the highest mean concentrations of AOH, AME, TeA and TEN in grains were observed as follows: AOH (spelt, oats, rice); AME (oats, rice); TeA (wheat, barley, rye, spelt, oats and rice); TEN (rye). Nevertheless, in addition to the above-mentioned mycotoxins, some authors reported also the presence of other compounds in grains, although the actual co-occurrence was not clearly specified. Specifically, ragi, sorghum and spelt were found contaminated by ALT (Ansari and Shrivastava, 1990; Müller and Korn, 2013), while ATX-I was detected in spelt and wheat (Gotthardt et al., 2019; Puntscher et al., 2019). Among the least investigated mycotoxins,

macrosporin, which is produced primarily by the *Stemphylium* genus but it can be produce by *Alternaria* spp. too (Lou et al., 2013), was found in corn and wheat silage (Shimshoni et al., 2013), while ALP was detected in wheat flour samples (Puntscher et al., 2019). The presence of macrosporin was also detected in dried fruits and nuts, such as almonds, dried grape berries, hazelnuts, peanuts, and pistachios (Varga et al., 2013), often in combination with other *Alternaria* mycotoxins. In a study performed by Mikušová et al. (Mikušová et al., 2014), dried grape berries from three Slovak winemaking regions were simultaneously contaminated by up to eight *Alternaria* mycotoxins, i.e., AOH, AME, ALT, TeA, TEN, ATX-I, ATX-II, and macrosporin, whose highest concentrations were 1308 μg/kg, 776 μg/kg, 4120 μg/kg, 159.6 μg/kg, 43.1 μg/kg, 31175 μg/kg, 624 μg/kg, and 762 μg/kg, respectively. Notably, TEN was detected in all the analyzed samples.

Alternaria toxins can be found also in beverages such as fruit juices, beers and wines (Asam et al., 2009; Juan et al., 2017; López et al., 2016; Prelle et al., 2013; Scott et al., 2006; Zhao et al., 2015a), as well as in food supplements used for various purposes (Veprikova et al., 2015). Milk thistle-based supplements for liver diseases were simultaneously contaminated by AOH, AME, TEN, and TeA with maximum concentrations of 4560 μg/kg, 3200 μg/kg, 1280 μg/kg, and 2140 μg/kg, respectively. The same mycotoxins were detected, even though at a lower concentration, in supplements used to treat menopause symptoms (containing red clover, flax seeds and soy) or for general health support (containing among others green barley, nettle, goji berries and yucca). The maximum concentration of TeA was found in supplements for general health support (6780 μg/kg), while milk thistle-based supplements showed the highest average concentrations of all mycotoxins. Notably, the beneficial effects of health-promoting compounds of food supplements might be impaired to various extents by the presence of mycotoxins. In addition, taking into account that food supplements are thought to supply specific deficiencies, the presence of mycotoxins might have a higher impact on specific categories of consumers. These aspects require urgent investigations to timely support the enforcement of specific regulations.

Table 1. Co-occurrence of *Alternaria* toxins in food.

Food/Foodstuff	*Alternaria* Mycotoxins							Reference
	AOH	AME	ALT	TeA	TEN	ATX-I	Other	
Fruits, Vegetables and Derivatives								
Apple	X	X	X	X		X		(Stinson et al., 1981)
Apple juice	X	X						(Asam et al., 2009; Lau et al., 2003)
Apple juice (concentrated)	X	X						(Delgado and Gómez-Cordovés, 1998)
Apple-pear-cherry (puree infant formula)	X	X		X	X	–	ALP (–)	(Gotthardt et al., 2019)
Berry juice	X	X			–			(Juan et al., 2017)
Cherry-banana (puree infant formula)	X	X		X	–	–	ALP (–)	(Gotthardt et al., 2019)
Cranberry juice	X	X						(Scott et al., 2006)
Cranberry nectar	X	X						(Lau et al., 2003)
Citrus juice	–	X [a]		X [a]	–			(Zhao et al., 2015a)
Grape juice	X	X						(Scott et al., 2006)
Ketchup	X[a]	–	X [a]					(Pavón et al., 2012)
Ketchup	X	X		X	X			(Zhao et al., 2015a)
Mixed juice (fruits and vegetables)	X	X [a]	X [a]	X		–	Iso-ALT (–), AAL TA1 (–), AAL TA2(–)	(Hickert et al., 2016)
Orange juice	X	X						(Asam et al., 2009)
Pepper	X	X		X				(da Cruz Cabral et al., 2016)
Prune nectar	X	X						(Lau et al., 2003)
Soya beans	X	X						(Oviedo et al., 2012)
Strawberry	X[a]	X[a]						(Juan et al., 2016b)

Table 1. *(Continued)*

Food/Foodstuff	*Alternaria* Mycotoxins							Reference
	AOH	AME	ALT	TeA	TEN	ATX-I	Other	
Sweet pepper	X	X	X	X				(Gambacorta et al., 2018)
Tangerine (flavedo)	X	X						(Magnani et al., 2007)
Tomato	X	X	X	X		–		(Stinson et al., 1981)
Tomato	X[a]	X[a]		X [a]		–	ATX-II (–)	(Hasan, 1995)
Tomato	X	X	X					(Pavón et al., 2012)
Tomato (dried)	X[a]	X[a]	X[a]		X [a]			(Noser et al., 2011)
Tomato (puree and ketchup)	X	X	–	X	X [a]	–	Iso-ALT (–), AAL TA1 (–), AAL TA2(–)	(Hickert et al., 2016)
Tomato (sun-dried)	X	X	X					(Pavón et al., 2012)
Tomato juice	–	X		X	X			(Zhao et al., 2015a)
Tomato sauce	X	X	X [a]	X	X [a]	X	ALP (X), AOH-3-S (X[a]), AME-3-S (X)	(Puntscher et al., 2019)
Tomato sauce (puree infant formula)	X	X		X	X	–	ALP (–)	(Gotthardt et al., 2019)
Tomato soup (puree infant formula)	–	X		X	–	–	ALP (–)	(Gotthardt et al., 2019)
Vegetable juice	X	X						(Asam et al., 2009)
Cereals and Derivatives								
Bakery products (wheat- and rye- based)	X	X	–	X	X	–	Iso-ALT (–), AAL TA1 (–), AAL TA2(–)	(Hickert et al., 2016)
Bread	X[a]	X		X	X			(Zhao et al., 2015b)
Cereal grains	X[a]	X[a]	–		X [a]			(López et al., 2016)
Corn silage	X[a]	X[a]			X [a]		MACRO (X[a])	(Shimshoni et al., 2013)
Dried noodles	X[a]	X		X	X			(Zhao et al., 2015b)

Table 1. *(Continued)*

Food/Foodstuff	*Alternaria* Mycotoxins							Reference
	AOH	AME	ALT	TeA	TEN	ATX-I	Other	
Maize-based snacks	X	X						(Kayode et al., 2013)
Millet (infant formula)	X	X		X	X	–	ALP (–)	(Gotthardt et al., 2019)
Oat (infant formula)	–	X		X	X	–	ALP (–)	(Gotthardt et al., 2019)
Ragi	–	X	X	X		–		(Ansari and Shrivastava, 1990)
Rice (infant formula)	X	X		X	X	–	ALP (–)	(Gotthardt et al., 2019)
Sorghum	–	X	X	X		–		(Ansari and Shrivastava, 1990)
Spelt (infant formula)	X	X		X	X	X	ALP (–)	(Gotthardt et al., 2019)
Wheat	X [a]	X [a]	X [a]	X [a]				(Müller and Korn, 2013)
Wheat	X [a]	X [a]		X [a]				(Azcarate et al., 2008)
Wheat (infant formula)	X	X		X	X	X	ALP (–)	(Gotthardt et al., 2019)
Wheat flour	X [a]	X		X	X			(Zhao et al., 2015b)
Wheat flour	X [a]	X	–	X	X [a]	X	ALP (X), AOH-3-S (-), AME-3-S (-)	(Puntscher et al., 2019)
Wheat silage	X [a]	X [a]			X [a]		MACRO (X^a)	(Shimshoni et al., 2013)
Weathered wheat	X	X		X				(Li and Yoshizawa, 2000)
Dried Fruits and Nuts								
Almonds	X [a]	X [a]			X [a]			(Wang et al., 2018)
Almonds	X [a]	X [a]			X [a]	–	MACRO (X^a)	(Varga et al., 2013)
Chestnuts	X [a]	X [a]			X [a]			(Wang et al., 2018)
Dried figs	X [a]	X [a]			–			(Wang et al., 2018)

Table 1. *(Continued)*

Food/Foodstuff	*Alternaria* Mycotoxins							Reference
	AOH	AME	ALT	TeA	TEN	ATX-I	Other	
Dried grape berries	X	X	X	X	X	X	ATX-II (X), MACRO (X)	(Mikušová et al., 2014)
Dried jujubes	X [a]	X [a]			X [a]			(Wang et al., 2018)
Dried persimmons	X [a]	X [a]			–			(Wang et al., 2018)
Dried raisins	X [a]	X [a]			–			(Wang et al., 2018)
Dried raisins	X	X	–	X	–			(Wei et al., 2017)
Dried wolfberries	X [a]	X [a]	–	X	X			(Wei et al., 2017)
Hazelnuts	X [a]	X [a]			X [a]			(Wang et al., 2018)
Hazelnuts	X	X			X	X [a]	MACRO (X)	(Varga et al., 2013)
Peanuts	X [a]	X [a]			X [a]	–	MACRO (X [a])	(Varga et al., 2013)
Pine nuts	X [a]	X [a]			X [a]			(Wang et al., 2018)
Pistachios	–	–			–	–	MACRO (X [a])	(Varga et al., 2013)
Walnuts	X [a]	X [a]			X [a]			(Wang et al., 2018)
Other Food and Foodstuff								
Beer	X [a]	–	X [a]	–	X [a]			(Prelle et al., 2013)
Food supplement (antioxidants)	X [a]	X [a]		X [a]	X [a]			(Veprikova et al., 2015)
Food supplement (milk thistle)	X	X		X	X			(Veprikova et al., 2015)
Food supplement (phytoestrogens)	X	X		X [a]	X			(Veprikova et al., 2015)
Red wines	X	X						(Scott et al., 2006)
Sesame seeds	X	X		X				(Ezekiel et al., 2012)
Sunflower seed oil	X	X	–	X	X	X [a]	ALP (X [a]), AOH-3-S (-), AME-3-S (-)	(Puntscher et al., 2019)

Table 1. *(Continued)*

Food/Foodstuff	*Alternaria* Mycotoxins							Reference
	AOH	AME	ALT	TeA	TEN	ATX-I	Other	
Sunflower seeds	–	–	–	X [a]	X [a]			(López et al., 2016)
Sunflower seeds	X	X		X				(Chulze et al., 1995)
Sunflower seeds	X	X	X [a]	X	X	X [a]	Iso-ALT (X [a]), AAL TA1 (–), AAL TA2(–)	(Hickert et al., 2016)
Sunflower seeds	X [a]	X [a]		X	X		ALTSOH (X [a]), Val-TeA (X [a])	(Hickert et al., 2017)
Vegetable oils (rapeseed and sunflower seeds)	X	X	–	X [a]	X	–	Iso-ALT (–), AAL TA1 (–), AAL TA2(–)	(Hickert et al., 2016)
White wines	X	X						(Scott et al., 2006)
Wines	X [a]	–	–	X [a]	–			(López et al., 2016)

X: certain co-occurrence; X [a]: uncertain co-occurrence; – : checked but not detected.

2.2. Co-Occurrence of Alternaria *Toxins with other Mycotoxins*

As discussed above, many food categories may be contaminated by more than one *Alternaria* mycotoxin. However, food commodities can be simultaneously contaminated by a high number of different mycotoxins produced by molds other than *Alternaria*. In particular, mycotoxins produced by *Aspergillus*, *Fusarium*, and *Penicillium* genera frequently co-occur with *Alternaria* mycotoxins (Table 2). Among them, the most investigated and frequently detected were those produced by the genera *Fusarium* and *Aspergillus* [e.g., aflatoxins, enniatins (ENNs) and beauvericin], while the least frequently examined or detected were ochratoxins (ochratoxin A, OTA; ochratoxin B, OTB).

A study conducted by Gambacorta et al. (Gambacorta et al., 2018) investigated the co-occurrence of 17 different mycotoxins in fresh, fried, dried or grounded sweet pepper products. Notably, all of them were contaminated by more than one mycotoxin simultaneously. In more detail, 6 out of 39 samples contained 2, 3 or 4 different mycotoxins, while the remaining samples were positive for a number of mycotoxins ranging from 5 to 16. The fried peppers showed the lowest average level of contamination (with an average mycotoxin contamination of 231 μg/kg), while the fresh pepper samples were the most contaminated (27,280 μg/kg). TeA was the most frequently detected mycotoxin (100% of samples) with an average concentration of 4817.9 μg/kg. With regard to the other *Alternaria* toxins, 93%, 56%, 33%, and 9% of pepper samples were found to be contaminated by TEN, AOH, AME and ALT, respectively. These compounds (except for ALT) were found to co-occur along with 7 other *Fusarium* mycotoxins (nivalenol, HT-2 toxin, T-2 toxin, fumonisin B_1, fumonisin B_2, deoxynivalenol (DON) and zearalenone (ZEN)), 4 other *Aspergillus* mycotoxins (the aflatoxins B_1, B_2, G_1, and G_2), and OTA in the most contaminated sample. It is worth mentioning the average low level of contamination of fried samples. In this respect, the frying process might have a role in lowering the content of *Alternaria* mycotoxins, though it was not directly assessed by the authors. It would be in agreement with other studies pointing to a significant reduction of mycotoxin content upon fry cooking (Bhat et al., 2010). In addition, high-temperature treatments already proved to be

effective in mitigating the content of certain *Alternaria* mycotoxins (Janić Hajnal et al., 2016), supporting the possible role of fry cooking in reducing the content of *Alternaria* mycotoxins. The effects of three extrusion processing parameters (moisture content, feeding rate and screw speed) on the degradation of TeA, AOH and AME in whole wheat flour have been investigated. With the optimal parameters, a reduction of 65.6, 87.9 and 94.5% was achieved for TeA, AOH and AME, respectively (Janić Hajnal et al., 2016). As a general remark, the thermal stability of *Alternaria* mycotoxins needs to be further investigated, along with the possible formation of toxic by-products, to identify effective food processing for reducing their content in food.

The co-occurrence of AOH with the *Fusarium* mycotoxins ZEN and DON and, with the ergot alkaloid ergometrine was described in beer (Bauer et al., 2016). In particular, ergometrine, a toxin produced by Claviceps spp. used in pharmaceutical applications (Fanning et al., 2017), was detected at low concentrations in 93% of the beer samples (0.07–0.47 μg/L, median 0.15 μg/L). AOH (0.23–1.6 μg/L, median 0.45 μg/L) and ZEN (0.35–2.0 μg/L, median 0.88 μg/L) were detected in all the beer samples, while DON was found in 75% of samples (2.2–20 μg/L, median 3.7 μg/L). In the light of the low concentrations reported above, the authors concluded that beer should not be considered among the most important source of dietary intake of AOH, ZEN and DON.

In another study, 253 samples of dried fruits and nuts were analyzed for the presence of 16 mycotoxins (aflatoxins, ochratoxins, *Alternaria* toxins and trichothecenes) (Wang et al., 2018). The authors reported that 124 samples were contaminated with at least one mycotoxin, while more than half (66 out of 124 samples) were contaminated by at least two mycotoxins. AME was the most frequently detected mycotoxin (44/124), followed by AOH (found in 31 out of 124 samples) and enniatin B_1 (found in 30 out of 124 samples). The most contaminated sample contained eight different mycotoxins (i.e., the aflatoxins B_1 and B_2, the enniatins B and B_1, beauvericin (BEA), TEN, AOH, and AME). Among the number of combinations found, the most common were binary (such as BEA + AME) and tertiary (such as

BEA + AME + AOH) combinations. Ochratoxin B was found occurring along with the *Alternaria* toxins AOH, AME and TEN only in two samples.

The co-occurrence of ochratoxin A with AOH and aflatoxin B_2 was described with a low frequency in berry juice (only 1 out of 32 samples was found positive) (Juan et al., 2017). Additionally, although 47% of berry juices were negative for all the investigated mycotoxins, at least one mycotoxin was present in 53% of the samples, with percentage distributions of 9%, 9%, 22%, and 13% for 1, 2, 3 and 4 co-occurring mycotoxins, respectively. Moreover, TEN and aflatoxin B_1 were not detected in any of the analyzed samples, while aflatoxin B_2 + aflatoxin G_2 + AME + AOH and aflatoxin G_2 + AME + AOH were the most frequently found combinations. Importantly, in 87% of the contaminated samples at least one *Alternaria* mycotoxin was detected: AOH was most frequently found (73%; concentrations from 2.5 to 85 ng/mL) followed by AME (67%; concentrations from 267 to 308 ng/mL). Similarly, the co-occurrence of *Alternaria* toxins with other mycotoxins was also reported in dried fruit samples from China (apricots, raisins, dates, and wolfberries) (Wei et al., 2017). In particular, 64.6% of the samples were contaminated by at least one mycotoxin, while 31.4% of the samples were contaminated with two to four compounds. TeA was the most abundant (from 6.9 to 5665.3 μg/kg) and frequently detected compound, followed by TEN (20.5% of samples) and mycophenolic acid (MPA; 19.5% of samples). MPA is produced by various *Penicillium* species and it is used as an immunosuppressant drug to prevent organ rejection after transplantation. In terms of safety, its occurrence in food may rise concern on account of its potential to predispose susceptible individuals to infectious diseases (Wei et al., 2017). The combinations TeA + TEN and TeA + MPA were found with a prevalence of 13.2% and 11.4%, respectively (Wei et al., 2017). In addition, TeA was simultaneously detected along with OTA in 7% of samples, with an apparently inverse relationship: the higher the concentration of TeA, the lower the concentration of OTA. This might be due to competition phenomena between mycotoxin-producing fungi or due to degrading processes, as reported by Müller et al. (Müller et al., 2015). They described an inverse correlation between the increase of AOH, AME and TeA production and the decrease of *Fusarium* toxins (DON and ZEN) possibly

due to the degradation of the latter by *Alternaria* strains. In this context, in vitro studies on the synthesis of mycotoxins during the co-incubation of *Alternaria* strains with other fungi may be useful to investigate the existence of a possible mutual influence, which seems likely to exist on the basis of low level of co-occurring mycotoxins reported so far in the literature.

As already reported in Section 2.1, food supplements might be highly contaminated by *Alternaria* toxins. However, *Alternaria* toxins can be found in food supplements also along with other mycotoxins. As an example, Veprikova and co-workers found 66 out of 69 samples contaminated by more than one mycotoxin. Specifically, 58% of milk thistle-based supplements contained more than 12 different mycotoxins simultaneously, while one of the most contaminated samples contained 14 different mycotoxins, i.e., AOH, AME, TEN, 3-acetyl-DON, beauvericin, fusarenon-X, ZEN, HT-2 toxin, T-2 toxin and enniatins B, B_1, A and A_1 (Veprikova et al., 2015). The most common combinations described were ENNs +HT-2/T-2 + AOH + AME + TEN and ENNs + AOH + AME + TEN + MPA. As a general remark, the state-of the-art of food supplements contamination warns about a potentially dangerous scenario. Indeed, although to date no maximum limits of *Alternaria* mycotoxins have been defined for food, the relatively high concentrations of mycotoxins occasionally detected in food supplements might suggest the need to perform dedicated risk assessment studies. Therefore, further occurrence and exposure studies have to be done urgently paving the ground to timely enact specific regulations for food supplements.

Table 2. Co-occurrence of *Alternaria* toxins with other mycotoxins.

Food/Foodstuff	Co-Occurring Mycotoxins				Reference
	2 Mycotoxins	3 Mycotoxins	4 Mycotoxins	>4 Mycotoxins	
Berry juices	AFB2, AME AFB2, AOH AOH, AME	AFB2, AFG2, AOH AFB2, AOH, OTA AFB2, AFG2, AME AFB2, AFG2, AOH AFG2, AME, AOH AFB2, AME, AOH	AFB2, AFG2, AME, AOH AFG1, AFG2, AME, AOH AFB2, AFG1, AFG2, AME		(Juan et al., 2017)
Sweet pepper (*Capsicum annuum*)	AFB1, TeA	ZEN, TEN, TeA	FB2, TEN, TeA, ZEN	*n* 16 (NIV, AOH, TeA, HT-2, FB2, OTA, T-2, FB1, TEN, AME, AFB1, DON, AFG1, AFB2, AFG2 and ZEN)	(Gambacorta et al., 2018)
Durum wheat	n.a.	n.a.	n.a.	*n* 7 (EN B, EN B1, EN A1, AME, DON, HT2 and T2)	(Juan et al., 2016a)
Dried fruits (raisins, dried apricots, dates and wolfberries)	TeA, MPA TeA, TEN	n.a.	n.a.	n.a.	(Wei et al., 2017)
Maize-based snacks	n.a.	n.a.	n.a.	*n* 6 (FB1, FB2, FB3, BEAU, AME, EMOD)	(Kayode et al., 2013)
Nuts and dried fruits	AFB2, TEN AFG1, AME ZEN, AOH BEA, AME T-2, AME ENB1, TEN	AFB2, TEN, AME AFB2, AOH, AME ENA1, ENB1, AME BEA, AOH, AME T-2, BEA, AME AFB2, ENB, AOH	ZEN, TEN, AOH, AME ENA1, ENB, TEN, AME AFB2, TEN, AOH, AME AFB1, BEA, AOH, AME AFG1, AFG2, ENB1, TEN AFG1, ENB1, AOH, AME	*n* 8 (AFB1, AFB2, ENB, ENB1, OTB, TEN, AOH, AME)	(Wang et al., 2018)
Beer	AOH, ZEN	n.a.	Ergometrine, AOH, ZEN, DON	n.a.	(Bauer et al., 2016)
Food supplements (milk thistle - based)	n.a.	n.a.	AOH, AME, TEN, MPA Other	*n* 14 (AOH, AME, TEN, 3-ADON, FUS-X, ENN-B, ENN-B1, ENN-A, ENN-A1, BEA, DON, HT-2, T-2, ZEN) etc.	(Veprikova et al., 2015)

n.a.: information not available

3. Individual Toxicity of Main *Alternaria* Toxins and Combined Toxicity with other Mycotoxins and Bioactive Compounds of Food Origin

Alternaria species may produce a huge variety of different mycotoxins showing a great variability in terms of chemical structures (Zwickel et al., 2018). AOH, AME, TeA, ALT, and altertoxins (I, II, III) are considered the most relevant for food toxicology, taking into account their occurrence and/or toxicity. Nevertheless, in vivo toxicological data currently available are not adequate for a proper risk assessment and, therefore, they are not sufficient to define toxicological standard values for the establishment of maximum limits in food and feed. At present, the only LD_{50} values are available, even if they refer to a limited number of compounds (Table 3).

As a general remark, except for these few mycotoxins, very few data are available for the other members of the *Alternaria* mycotoxin family, which still remain largely uncharacterized in terms of toxicity and mechanisms of action.

As already discussed, the simultaneous occurrence of more than one *Alternaria* mycotoxin, also in combination with other mycotoxins produced by different fungi, is common in food. In this respect, it is important to remark that the risk assessment of mycotoxins currently relies on single substance effects (Benford, 2017; EFSA, 2011), neglecting any possible mutual combined actions due to simultaneous exposure. These mycotoxin-mycotoxin interactions might modify the individual toxicity of compounds, likely resulting in a final toxic outcome different from the single compound tested alone. In addition, it must be considered that many extra-nutritional constituents (such as bioactive food constituents) are widely present in food, and their biological activity may also interfere with mycotoxin activity. The combined actions can be referred to as: (i) additive effects, when the final toxicity is the sum of the individual toxic effects of compounds; (ii) synergistic effects, when the resulting total toxicity is greater than the sum of individual effects or iii) antagonistic effects, when the opposite is the case and the combinatory effect is less than additive (Chou, 2006). Several mathematical models and methods are commonly used to evaluate the nature of the combined effects of toxic compounds. Among them, the most common are the independent joint action model and the combination index-

isobologram method. The first one allows to calculate an expected additive value from the effects of the single compounds (Webb, 1963) that can in turn be compared to a measured combinatory effect. The combination index-isobologram method allows to take into account the shape of dose-response curves when determining the type of interaction (synergism, additive effect and antagonism) (Chou, 2006; Chou and Talalay, 1984). This is considered the state-of-the-art model; however, it can be challenging to meet the requirements to apply it.

As shown below, the evidence collected so far clearly states that synergistic effects of mycotoxins in mixtures with other compounds (either mycotoxins or other food components) may have important consequences on the single-compound activity. This might have an impact on the assessment of risk related to the presence of *Alternaria* mycotoxins in food, which should consider the mixtures, rather than focusing on single-compound evidences. The individual toxicity of the main *Alternaria* mycotoxins and the effects of their combination with other mycotoxins or food constituents are reported in the following sections.

Table 3. LD_{50} values of *Alternaria* mycotoxins currently available.

Mycotoxin	Animal Species	Route of Exposure	LD_{50} (mg/kg b.w.)	Reference
AOH	Mouse (DBA/2)	intraperitoneal	>400 [1]	(Pero et al., 1973)
AME	Mouse (DBA/2)	intraperitoneal	>400 [1]	
TeA	Mouse	intravenous	115 (female) 162 (male)	(Smith et al., 1968)
		oral	81 (female) 186 (male)	
	Mouse (ICR)	intravenous	125 (male)	(Woodey and Chu, 1992)
		intraperitoneal	150 (male)	
		subcutaneous	145 (male)	
		oral	225 (male)	
	Rat	intravenous	157 (female) 146 (male)	(Smith et al., 1968)
		oral	168 (female) 180 (male)	
	Chicken embryo	injection	548 [2]	(Griffin and Chu, 1983)
	White leghorn chicken	oral	37.5 [3]	(Giambrone et al., 1978)

[1] LD_{50} values of AOH and AME were not reached at the maximum dose tested, corresponding to 400 mg/kg, [2] Unit of measurement: μg/egg, [3] Information about sex not available.

3.1. Individual Toxicity of Alternaria Mycotoxins

3.1.1. Genotoxic Effects

Among the best characterized *Alternaria* toxins, those with genotoxic properties are considered of most concern for human health by regulatory authorities. This particularly applies to AOH and AME,
for which the EFSA concluded that "the estimated mean chronic dietary exposures at the upper bound and 95th percentile dietary exposures exceeded the TTC value" in their latest exposure assessment (Arcella et al., 2016), and thus called for more data regarding exposure and toxicity of those metabolites (EFSA, 2016).

In human cells, both AOH and AME have been reported to induce DNA strand breaks in the comet assay at concentrations ≥1 μM (Fehr et al., 2009), to act clastogenic at ≥2.5 μM (Lehmann et al., 2006) and to possess mutagenic potential at ≥10 μM, as measured by HPRT and TK gene mutation assays (Brugger et al., 2006). An in vivo study on mice did not find AOH to cause systemic DNA damages in liver tissue and bone marrow (Schuchardt et al., 2017). However, the authors argue that any toxicity of the substance would probably be limited to the gastrointestinal tract due to poor bioavailability, but did not include corresponding organs in their survey.

Concerning the mechanisms of action, both AOH and AME were found to act as a topoisomerase (TOP) poison at micromolar concentrations, affecting the activity of both TOP I and TOP II, with a certain preference for the α isoform of TOP II (Fehr et al., 2009). Those enzymes are needed to untangle the DNA for replication or transcription, a process which involves the induction of a transient DNA strand break that is re-ligated at the end of the catalytic cycle. Poisoning of these enzymes by small molecules results in a toxin-dependent stabilization of the covalent DNA–topoisomerase complex (i.e., the so-called "cleavable complex"). Stabilization of the cleavable complex by TOP "poisons" hinders release of TOP in the catalytic cycle and re-ligation of the DNA, thus resulting in a persistence of the initially induced strand break. Thus, TOP poisons are commonly described to act genotoxic (Pommier, 2013).

An additional mechanism contributing to the toxicity of *Alternaria* toxins is the induction of intracellular reactive oxygen species (ROS), which indicate oxidative stress. ROS production induced by AOH and AME might play an important role in the inhibitory effects on cell proliferation observed in different cellular models (Fernández-Blanco et al., 2016a; Tiessen et al., 2017).

Of note, ALT and iso-ALT were not found to affect topoisomerase activity (Fehr et al., 2009), probably due to their less planar structure not allowing for DNA intercalation in comparison to AOH/AME (Dellafiora et al., 2015). However, it was observed that extracts from cultured *Alternaria* strains by far exceeded the genotoxicity of their dibenzo-α-pyrone contents (Schwarz et al., 2012a). This has led to the discovery of the epoxide-carrying perylene quinone ATX-II as a major contributing factor to the genotoxicity of naturally occurring mixtures of *Alternaria* toxins (Fleck et al., 2012; Schwarz et al., 2012b). Later on, not only ATX II, but also the structurally related STTX-III was found to be more mutagenic then AOH. Regarding their mode of action, these mycotoxins were also found to act as inhibitors of TOPs at high concentrations. However, their main genotoxic mode of action is thought to be the formation of DNA adducts, a hypothesis which still awaits experimental confirmation (Fleck et al., 2016; Schwarz et al., 2012b).

Of note, there is speculation that yet not characterized secondary metabolites might also possess genotoxic properties, as an *Alternaria* extract very low on dibenzo-α-pyrones, which was additionally stripped off ATX-II and STTX-III, still maintained substantial DNA-damaging properties (Aichinger et al., 2019).

3.1.2. Endocrine-Modulating and other Toxic Effects

AOH and AME, as well as other related metabolites, were reported to elicit estrogenic effects in cellular systems. In particular, AOH was described to be able to activate both ER-α and β but with a greater affinity (approximately ten-fold higher) for ER-β (Lehmann et al., 2006; Vejdovszky et al., 2017a), although the binding strength is 10,000-fold weaker than the endogenous hormone estradiol. AME was found to be slightly more potent than AOH at 10 μM, and the methylation at the 9-OH group was thought to improve the

molecular fitting within the estrogen receptor pocket (Dellafiora et al., 2018). AOH was additionally found to induce androgenic effects in the yeast androgen bioassay (Stypuła-Trębas et al., 2017). Recently, computational studies reported that mutations of the androgen receptors might affect the capability of AOH to bind and possibly stimulate the activation of receptors (Dellafiora et al., 2019b). Moreover, increases in progesterone and estradiol levels, as well as in progesterone receptor expression, were reported in human adrenocarcinoma H295R cells treated with AOH, supporting its actual role as endocrine disruptor (Frizzell et al., 2013). However, in naturally occurring mixtures of *Alternaria* toxins, endocrine-disrupting effects of AOH and related metabolites might be "quenched" by cytotoxic and anti-estrogenic properties of co-occuring compounds, as recently demonstrated in Ishikawa cells (Aichinger et al., 2019).

In addition to the above listed toxic effects, AOH and AME were found to modulate innate immunity in both human bronchial epithelial BEAS-2B cells and mouse macrophages RAW264.7, through the suppression of the lipopolysaccharide-induced innate immune responses (Grover and Lawrence, 2017). This activity was also confirmed in THP-1 derived macrophages by Kollarova et al. (Kollarova et al., 2018): AOH, in fact, suppressed lipopolysaccharide (LPS)-induced NF-κB pathway activation, induced transcription of the anti-inflammatory cytokine IL-10, and reduced the transcription of the pro-inflammatory cytokines IL-8, IL-6 and TNF-α.

TeA deserves a particular mention as, unlike the other *Alternaria* mycotoxins, it exerts toxic effects mainly by inhibiting the release of proteins from the ribosome. Although a low toxicity of this mycotoxin has been reported in vitro (Schwarz et al., 2012a; Zhou and Qiang, 2008), in vivo studies carried out on several animal models highlighted more severe effects such as emesis, tachycardia and haemorrhages (Fraeyman et al., 2017).

3.2. Combinatory Effects of Alternaria Mycotoxins

There are only a few studies investigating the combinatory effects of *Alternaria* mycotoxins, though food may be quite often simultaneously contaminated by more than one single compound as shown, for instance, for AOH and AME (Section 2.1). Notably, these two mycotoxins are not of

particular concern in terms of cytotoxic effects, also on account of the high concentrations required to cause harmful effects when tested individually. However, the simultaneous exposure to AOH and AME may have significant effects on the overall toxicity in respect to their individual testing. In more detail, their combined effects (1:1 concentration ratio) were invested by Bensassi et al. on the human intestinal cell line HCT-116 (Bensassi et al., 2015). No significant difference in cell viability was detected at 25 μM up to 24 h of exposure when mycotoxins were tested either individually or in combination. Conversely, both mycotoxins reduced cell viability about 30% after 24 h of exposure when tested individually, while they reduced viability about 50% when tested in combination. In this study, the nature of interactive effects was described to be additive, while Fernández-Blanco and co-workers reported synergistic effects in Caco-2 cells after 24 h of exposure to AOH and AME in a 1:1 binary combination and in a concentration range from 3.125 to 30 μM (Fernández-Blanco et al., 2016a). Moreover, the AOH-AME binary combination reduced cell proliferation to a greater extent than AOH alone at all tested concentrations, while it had greater effects than AME alone at 15 and 30 μM. The binary mixture also caused a greater dose-dependent reduction of cell proliferation after 48 h of incubation (in the concentration range 7.5–30 μM) than AOH or AME tested alone. In this case, the nature of the interactive effects was described as synergistic or additive at small or higher fraction affected, respectively.

The effects exerted by the simultaneous exposure to AOH and the genotoxic *Alternaria* mycotoxin ATX-II were investigated by Vejdovsky et al. (Vejdovszky et al., 2017b) on two intestinal (HT-29, HCEC-1CT) and one hepatic (HepG2) cell line. Seven different concentrations, ranging from 500 nM to 10 μM for ATX-II and from 5 μM to 100 μM for AOH were tested for binary combinations (constant ratio of 1:10, ATX-II:AOH). As a result, HT-29 cell line was found to be the least sensitive to cytotoxic effects mediated by the two tested mycotoxins and significant differences in cell viability were found starting from the combination 5 μM:50 μM (ATX-II:AOH). Among the different concentrations tested, the highest decrease in cell viability observed was of nearly 40%. Notably, the cell treatment at low mycotoxin concentrations led to an increase in mitochondrial activity in the co-treated

samples. HepG2 cells were found the most sensitive to the cytotoxic effects exerted by AOH, while HCEC-1CT cells proved to be the most sensitive to the effects of ATX-II. Combining these two mycotoxins, an increased sensitivity to cytotoxic effects was also found in the HepG2 cell line, leading to a reduction in cell viability starting from the combination 1 μM:10 μM (ATX-II:AOH). Although most of the tested 1:10 combinations showed additive effects, antagonistic effects were reported in HCEC and HepG2 cell lines, while only one of the combinations analyzed showed synergistic effects on HepG2 cell line (750 nM ATX-II:750 nM AOH, 1:1 ratio). Modifications of microRNAs expression profile after incubation of HepG2 cells with the mixture 10 μM AOH:1 μM ATX-II may partially explains such effects. The combined exposure caused a significant increase of miR-224 expression after 12 h of exposure, which was no longer over-expressed after 24 h, while miR-192 and miR-29a were respectively down-regulated and up-regulated after 24 h. In addition, miR-29a was up-regulated also in samples treated with AOH alone, suggesting a possible role in the up-regulation of this miRNA by the binary mixture. Interestingly, these three microRNAs are involved in the regulation of apoptotic processes and the observed modifications led the authors to conclude that such miRNAs may be in part involved in the antagonistic effects observed for some of the combinations tested.

As previously described, *Alternaria* mycotoxins are often found in food commodities along with *Fusarium* mycotoxins. In a recent study (Fernández-Blanco et al., 2016b), the cytotoxic effects and the type of interactions of AOH combined with *Fusarium* mycotoxins enniatin B and DON were evaluated after 24, 48, and 72 h of exposure in Caco-2 cells. For binary and tertiary combinations, five different concentrations, ranging from 0.3125 to 5 μM for enniatin B and DON, and from 1.875 to 30 μM for AOH, were tested. The binary combinations enniatin B + AOH (1:6 ratio) led to higher cytotoxic effects compared to AOH tested alone at all the timepoints and concentrations tested. However, no difference between enniatin B tested alone and in mixture was observed, suggesting that the cytotoxic effects were mainly mediated by enniatin B. With regard to the binary combinations DON + AOH (1:6 ratio), the resulting cytotoxicity after 24 h of

exposure was lower than that exerted by DON tested alone. On the contrary, an opposite trend was observed after 48 and 72 h of exposure. As expected, the tertiary mixtures enniatin B + DON + AOH (ratio 1:1:6) led to a greater decrease, albeit of slight intensity, of cell viability compared to the binary combinations. Although the pattern was not uniform along the fraction affected, the application of the isobologram analysis described the interactions in the binary mixtures as additive and synergistic, depending on the concentrations and timepoints tested. Interestingly, the ternary combinations showed antagonistic effects, which were described as due to competition mechanisms at the same receptor site. In this respect, it is worth mentioning the marked diversity of these mycotoxins in terms of chemical structures. Taking into account that the competition to the same protein site usually requires strict conservation of key structural motifs (McKinney, 2000), the inherent structural heterogeneity among enniatin B, DON and AOH is not fully compatible with their capability to physically compete with the same site. Therefore, both the molecular mechanisms and the network of biological targets involved in such antagonistic behaviour need to be precisely described to better understand the effects of the enniatin B/DON/AOH ternary combination.

The effects of binary and ternary combinations of AOH with the DON's acetylated derivatives 3-ADON and 15-ADON were also investigated on HepG2 cells up to 72 h of incubation (Juan-García et al., 2016). Constant ratios of 16:1 (AOH: 3-acetyl-ADON and AOH:15-acetyl-DON) and 16:1:1 (AOH:3-acetyl-DON:15-acetyl-DON) were chosen to test these mixtures, with concentrations ranging from 3.2μM to 24μM for AOH, and from 0.2μM to 1.5 μM for DON's derivatives. Cytotoxicity ranking was found to be the same for all tested time points (AOH+3-acetyl-DON + 15-acetyl-DON > AOH + 3-acetyl-DON > AOH + 15-acetyl-DON) and a concentration-dependent decrease in HepG2 cell viability was found in all tested mixtures. The effects caused by binary and ternary mixtures were described to be mainly synergistic, but some exceptions were found for AOH + 3-acetyl-DON at 72 h (where additive effects were observed at higher fraction affected), and for AOH + 15-acetyl-DON (where additive or antagonistic effects were observed depending on the concentration and timepoint tested).

Binary effects of TeA with the *Fusarium* mycotoxins enniatin B, ZEN, DON, nivalenol and aurofusarin (AURO) were also evaluated on Caco-2 cells with two different concentration sets, named "low concentrations" (none or slight cytotoxic effect) and "high concentrations" (pronounced cytotoxic effect) (Vejdovszky et al., 2016). TeA combinations at "low concentrations" of mycotoxins did not show significant differences between the measured and expected effects (calculated on the basis of the Independent Joint Action model). This indicates that the combinations of TeA at "low concentrations of mycotoxins" only determined additive effects. On the contrary, binary combinations at "high concentrations" led to lower cytotoxic effects then the calculated additive effects. Additional investigations allowed getting more details about the type of interactions between TeA and *Fusarium* mycotoxins. No difference in cytotoxicity was found in samples co-treated with enniatin B and ZEN keeping the concentration of *Fusarium* mycotoxins constant (from 5 to 50 μM depending on the mycotoxin) and varying that of TeA (from 1μM to 250 μM). Indeed, the cytotoxicity of binary mixtures with TeA was found to be equivalent to the toxicity of toxins tested individually. Notably, the toxic effect induced by 10μM DON was reduced in a concentration-independent manner by the combination with TeA at concentrations between 10 μM and 200 μM. A similar trend was found for the combination with 10 μM nivalenol, although differences were not statistically significant. Keeping in mind that TeA and the *Fusarium* mycotoxins DON and nivalenol are known to inhibit protein synthesis in vitro (Vejdovszky et al., 2016), the lower cytotoxic effects of binary mixtures might be due to a molecular interplay at the level of protein synthesis inhibition. Nevertheless, considering that nivalenol and DON inhibit protein synthesis by different mechanisms (i.e., by inhibiting the initiation or elongation-termination steps, respectively) (Rocha et al., 2005), the observed effects cannot be straightforwardly explained in terms of mechanisms of action pointing out the need of investigating further the molecular basis of such interaction. In this respect, the inhibition of protein synthesis by TeA may modify the expression of specific factors, including metabolizing enzymes, and consequences on the pattern of metabolites produced by cells are thought likely. This is of particular relevance as some trichothecenes

metabolites might be involved in mediating ribotoxic effects of parent mycotoxins, as supported recently by in silico studies (Dellafiora et al., 2017a). On this basis, TeA might have indirect effects on trichothecenes toxicity acting on their metabolism and changing the relative abundance of ribotoxic metabolites produced.

Recently, an interesting study was performed by Solhaug et al. that investigated the ability of AOH, DON and ZEN in binary and tertiary mixtures to affect immune response checking the differentiation of monocytes to macrophages (Solhaug et al., 2016). The differentiation process leads to several changes, including modifications of the expression of some cell surface markers such as CD14, CD11b and CD71. AOH, DON and ZEN were able to modify the expression of these markers in THP-1 monocytes, but with some differences: while AOH affected the expression of the all set of markers, DON did not modify the expression of CD71 and ZEN altered only the expression of CD-14. Since CD-14 was the only marker modified by all the three mycotoxins, its expression was used to evaluate the type of interactions in binary and ternary mycotoxins mixtures by applying the "Concentration Addition" (CA) and the "Independent Joint Action" (IA) models. Since authors did not find significant differences between the experimental data and the predicted models, the type of interaction was described to be additive. Remarkably, at the lowest concentrations of the AOH + ZEN combination, the confidence interval of the predicted CA model did not overlap with the experimental values, suggesting a possible synergistic effect. The same results were obtained for the binary combinations through the application of the isobologram analysis. To verify if the observed inhibitory effects of AOH, DON, and ZEN on the up-regulation of CD14 led to a real reduction in macrophage activation, the pro-inflammatory cytokine TNFα and its gene expression were quantified after incubation with single mycotoxins. Contrary to what was observed for AOH and ZEN, DON induced an increased secretion of TNFα following the increase of TNFα gene expression, in spite of its inhibitory action on the up-regulation of CD14. The expression of NF-kB, a protein complex involved in TNFα expression, might provide a plausible explanation to these differences. Indeed, ZEN was reported to reduce the expression of NF-kB

(Pistol et al., 2014), and, recently, also AOH showed the ability to suppress the lipopolysaccharide-induced NF-kB pathway activation, resulting in the reduction of TNFα (Kollarova et al., 2018). In contrast, DON was found to induce both NF-kB activation and TNF-α expression, but the signaling pathway was different from those activated by ZEN and AOH (Pestka, 2010).

3.3. Combined Effects with Bioactive Food Constituents

Beside the combined action of the different members of *Alternaria* mycotoxins group, also in combination with mycotoxins produced by fungi other than *Alternaria*, it is important to take into consideration even the complex interactions that these mycotoxins may have with the other bioactive compounds of food origin.

In this contest, Vejdovszky et al. recently investigated the combinatory estrogenic effects of the isoflavone genistein (GEN) in combination with ZEN and AOH (Vejdovszky et al., 2017c). To elucidate the combinatory effects, the human endometrial adenocarcinoma Ishikawa cell line was chosen as a model system and the phosphatase alkaline (ALP) activity assay was used to measure estrogen receptor activation. The xenoestrogens under investigation were tested at different concentrations (ranging from pM to μM) after 48 h of incubation. All of them increased the ALP activation when tested individually, with the following order of potency in terms of EC50: E2 (17β-estradiol; used as positive control) > ZEN > GEN > AOH. Moreover, these xenoestrogens did not only differ in terms of potency, but also in terms of efficacy as none of them (at any concentration) was able to determine the same effects induced by 1 nM E2. A possible explanation for this finding is that AOH, ZEN and GEN might act as partial agonists. The lower capability to satisfy the pharmacophoric requirements of estrogen receptors pockets in comparison to E2 (Dellafiora et al., 2019a, 2017c) might provide a structural rational to explain such evidence. With regards to binary mixtures of GEN with ZEN or AOH, some of them resulted in significantly higher effects than the respective compounds tested individually, clearly pointing out the existence of synergistic effects. However, combinations of GEN-AOH activated ALP to a lower extent than ZEN-AOH mixtures. It must be

highlighted that in many studies ZEN was found to be more estrogenic than AOH, and this could partly justify the lowering of estrogenic effect observed in combinations (Vejdovszky et al., 2017c). In addition, while the authors noted the preference of AOH and GEN to ERβ, ZEN was previously described with a higher affinity for ERα (EFSA CONTAM Panel, 2016). The simultaneous activation of both α and β estrogen receptor isoforms in the ZEN-GEN and ZEN-AOH binary mixtures may explain the stronger synergistic effects observed. Although some GEN-AOH combinations showed synergistic effects, other combinations at very low doses led to antagonistic effects. Indeed, anti-estrogenic effects were found testing the combination 0.001 μM GEN-0.1 μM AOH and observing a reduction of AlP activation (10.9%) compared to the control (vehicle). A subsequent more-in-depth analysis of the combinatory effects, performed through the combination index and the isobologram method, allowed to determine the type of interactions occurring in the different combinations. Both methods showed that the combinatory effects of GEN and ZEN in the constant ratio of 1000:1 were mainly synergistic and, only at very low or very high effect levels, additive or antagonistic effects were observed. In the constant ratios of 100:1 and 10:1, the substances led to a strong antagonism at low effect levels, and to a strong synergism at higher effects. Comparable outcomes were reported for the 1:10 GEN:AOH ratio (which showed antagonistic or synergistic effects at low or high effect levels, respectively), while the 1:5 combination ratio determined mainly antagonistic effects. Additionally, the 1:1 GEN:AOH ratio resulted in the onset of synergistic effects up to about 65% of the maximum AlP activation observed (E2 1nM). Above, additive or antagonistic effects were observed depending on the concentrations tested. Thus, the nature of the interactions seemed to depend on both the ratio of substances and the specific concentrations tested.

It was also established that AOH is able to cause oxidative stress and to exert genotoxic effects in different cellular models, mainly by acting as a topoisomerase poison (Fehr et al., 2009). Aichinger et al. investigated the effects of AOH in combination with the two polyphenols GEN and delphinidin (DEL) (Aichinger et al., 2017). These two compounds are known for their antioxidant effects at specific concentrations, although pro-oxidant

effects at certain concentrations were also demonstrated (Chen et al., 2014; Fukumoto and Mazza, 2000). Both GEN and DEL were found to interact, albeit with different mechanisms, with topoisomerases: while GEN usually acts as a topoisomerase poison, turning the enzyme into a DNA-damaging agent, DEL acts as a catalytic inhibitor of topoisomerase hindering the formation of the TOP-DNA intermediate. Therefore, considering both the antioxidant effects and the interaction with the topoisomerases, a modification of the effects induced by AOH may be expected when the mycotoxin is combined with these two polyphenols. Preliminary investigations on the combinatory cytotoxic effects were conducted in HT-29 colon carcinoma cells with concentrations ranging from 1 to 100 μM (1:1 ratio): cytotoxicity was observed starting from 25 μM for AOH and GEN, and from 50 μM for DEL. Both AOH/GEN and AOH/DEL combinations led to cytotoxic effects starting from 25 μM (1:1 ratio) and the type of interactions was described as synergistic, with a tendency to lose synergism when increasing cytotoxic effects. DNA strand breaks and oxidative DNA damages of the combinations of AOH (50 μM) with DEL (10–100 μM) or GEN (25–250 μM) were evaluated by performing an alkaline comet assay with or without treatment with formamidopyrimidin-DNA-glycosylase (FPG). When combined, DEL and AOH showed marked antagonistic effects at 50 μM in the FPG-untreated samples, while lower oxidative DNA damages were observed at 25 and 100 μM. Similar results were found for the combination AOH/GEN at 25 and 100 μM, which showed a lower oxidative damage than AOH tested individually. The authors also evaluated the influence of the co-incubations on the stabilization of the topoisomerases/DNA intermediate (the so-called "cleavable complexes"), which is typically due to the action of topoisomerase poisons (such as AOH). The AOH/GEN combination did not increase the formation of cleavable complexes, rather an antagonistic effect was found at the highest GEN concentration tested (100 μM). Antagonistic effects were also found in AOH/DEL combinations starting from 25 μM. These results were partially attributed to the dual anti-oxidant or pro-oxidant properties of the polyphenols. In this respect, simultaneous short-time incubations with AOH and DEL led to a reduction of AOH-induced ROS generation at

concentrations of DEL starting from 1μM. On the contrary, GEN induced oxidative stress per se and did not suppress the pro-oxidative effects induced by AOH. Moreover, 24-h pre-incubations with polyphenols followed by incubation with AOH, did not result in any change in pro-oxidant effects of AOH. This evidence led to exclude any possible modulations of anti-oxidant defense systems as a mechanism underlying the observed antagonistic effects. Therefore, direct anti- or pro-oxidant activities are reasonably as the base of the effects observed during the co-incubations with DEL and GEN. On this basis, DEL could help in preventing the genotoxic effects of AOH, but, considering the low systemic bioavailability of DEL, these protective effects may be limited to the gastrointestinal tract only (Bitsch et al., 2004; Braga et al., 2018).

The same authors also investigated the effects of DEL in combination with ATX-II, one of the most genotoxic *Alternaria* toxins (Aichinger et al., 2018b). As reported for the combination with AOH, DEL reduced both DNA strand breaks and oxidative damage in HT-29 cells after short-time co-incubation with ATX-II. The type of interaction was found to be antagonistic according to the applied "independent joint action" model. The production of ROS induced by 10 μM ATX-II was also reduced by DEL in concentrations from 1 μM to 100 μM, but these reductions cannot fully explain the huge reduction of genotoxic effects observed following the co-incubation with DEL. Indeed, no increase of ROS production was observed at the concentration of ATX-II used in the comet assay (1 μM). In cell-free conditions, a reduction of the concentration of ATX-II was found upon co-incubation with DEL. The authors suggested that DEL, after being degraded to phloroglucinol aldehyde (PGA) and gallic acid (GA), might react with ATX-II neutralizing its epoxy group, which is the reactive chemical moiety presumably responsible for genotoxicity. Considering the hypothesis that PGA can react with ATX-II, it is important to underline that the reduction of adverse effects of this mycotoxin may actually occur in subjects that follow diets with a high content of anthocyanins as they are prone to release PGA during digestion.

In the context of the evaluation of combinatory effects between *Alternaria* toxins and bioactive compounds of food origin, polyphenols represent a class of compounds of great interest since they are widely distributed in

those food categories which are prone to contamination with *Alternaria* mycotoxins. Quercetin (QUE) is one of the most abundant flavonoids in human diets. QUE has been previously associated to several potential health benefits mainly related to its antioxidant properties, although pro-oxidant effects at certain concentrations have also been described (Jan et al., 2010). The potential ability of QUE to reduce the cytotoxicity of AOH and AME was investigated by Fernàndez-Blanco et al. (Fernández-Blanco et al., 2016a). Although cytoprotective effects were attributed to QUE (Jeong et al., 2005), simultaneous exposure of Caco-2 cells to AOH and QUE (at concentrations ranging from 3.125 to 100 μM) did not result in any cytoprotective effect. In particular, no significant differences were found between the QUE-AOH combination and AOH tested alone after 48 h of exposure. However, the combination significantly affected cell viability at 24 h of treatment in comparison to AOH tested alone. Similarly, no difference between the binary combinations QUE + AME and AME tested alone were detected and, additionally, no cytoprotective effect was found in the tertiary combination AOH + AME + QUE at any of the tested concentrations. Therefore, QUE was not effective in reducing the effects of AOH and AME.

Possible cytoprotective properties of food components against the effects of AOH were also evaluated by Vila-Donat et al. in Caco-2 cells (Vila-Donat et al., 2015). Keeping in mind that AOH may contaminate legumes (including soybeans and lentils), the authors investigated the effects of AOH in combination with soy saponin I (Ss-I), which was previously found to possess antioxidant activity, or with a lentils extract. In particular, the authors used two different approaches to evaluate the effects of Ss-I and lentil extract: (i) the first one consisted in pre-treating cells with Ss-I (6.25 μM) or with the extract, and then refreshing the growth medium and testing different dilutions of AOH (ranging from 3.125 to 50 μM); (ii) the second approach aimed at evaluating the combinatory effects and the type of interaction co-incubating AOH with Ss-I or lentil extract. By using the first approach, no differences were found between samples pre-treated with Ss-I and samples treated only with AOH. As an exception, the highest AOH concentration tested (50 μM) caused an increase in cell viability in the pre-treated samples. In contrast, co-treatments with AOH + Ss-I (1:1 ratio) above

6.25 μM resulted in an increase in cell viability compared to AOH tested alone. These results suggested that Ss-I likely acted via a direct interaction rather than modulating intracellular defence systems. With regard to cytoprotective effects of the lentils extract, only one single combination was tested and about 30% increase in cell viability was found in comparison to AOH tested alone.

4. The Key Role of Bioactive Compounds

Food is a complex matrix composed by macro- and micro-nutrients, containing also a huge number of non-nutrient compounds that may exert several biological activities. These compounds can interfere at different levels with mycotoxin activities. For instance, they can: (i) activate or inhibit enzymes involved in the metabolism of xenobiotics; (ii) act as anti-oxidant or pro-oxidant compounds; (iii) act as receptor agonists or antagonists targeting, in some cases, the same biological targets of mycotoxins; (iv) modify the expression of genes encoding proteins involved in the regulation of important physiological functions. On this basis, bioactive compounds of food origin may determine the onset of additive, synergistic or antagonistic effects when combined with *Alternaria* mycotoxins. Keeping in mind that the application of mitigating strategies along the food chain are supposed to progressively reduce the dietary exposure to toxicants, the assessment of combinatory effects of mycotoxins with other food constituents will be the most accurate and realistic, but also highly challenging tasks to achieve in the next decades. The challenge will be even harsher taking into account that many food constituents potentially interplaying with mycotoxins are generally recognized as health promoting (i.e., polyphenols) and the consumption of foods rich in such compounds is typically recommended in healthy diet habits. In this framework, this section focuses on the modulation of *Alternaria* mycotoxins toxicity by bioactive compounds.

One of the best characterized toxicological endpoints of *Alternaria* mycotoxins likely affected by food constituents is the estrogenic activity. As a matter of fact, estrogenic and anti-estrogenic effects of bioactive compounds might markedly modify the overall estrogenicity of the *Alternaria* mycotoxins AOH and AME. In terms of risk characterization, this

might change the toxicological relevance of such mycotoxins case by case, though they show a weak estrogenicity per se, depending on the composition of chemical mixtures in given foods. In this respect, foods prone to *Alternaria* contamination with a high content of potentially interfering constituents (e.g., polyphenolic phytoestrogens) are legumes (especially soy) and some alcoholic beverages (especially wine and beer). In particular, soybeans and derived products are among the richest dietary sources of phytoestrogens, and many of the isoflavones of soy (including genistein, daidzein, glycitein, and coumestrol) induce estrogen-receptor dependent estrogenic stimuli (Rosenblum et al., 1993). As a matter of fact, combinations of GEN-AOH at specific concentrations have been demonstrated to determine synergistic or antagonistic effects in Ishikawa cell line (Vejdovszky et al., 2017c). Similarly to soybeans, hops used to produce beer is characterized by the presence of some prenylflavonoids (e.g., naringenin, 8-prenylnaringenin, 6-prenylnaringenin, 6,8-diprenylnaringenin, and 8-geranylnaringenin) that are potent phytoestrogens with a dual effect being able to bind both estrogen receptor isoforms and to inhibit specific enzymes involved in the estrogenic cellular responses (Karabín et al., 2016; Milligan et al., 2000). In this context, Aichinger et al. (Aichinger et al., 2018a) demonstrated the ability of the phytoestrogens from hops xanthohumol and 8-prenylnaringenin to antagonize the estrogenic effects of the *Fusarium* mycotoxins ZEN and α-ZEL. Therefore, possible interactions can be expected also in combination with the estrogenic *Alternaria* toxins AOH and AME. Other important food constituents able to modulate estrogen receptor activity are resveratrol and β-sitosterol, whose primary dietary sources are peanuts, grapes, and wine. Resveratrol, in particular, may exhibit a super-agonist activity inducing a stimulation higher than the endogenous ligand 17β-estradiol in estrogenic gene report assay, even if anti-estrogenic effects were found in the MCF-7 cell line (King et al., 2006). Although evidences have been not yet collected, these compounds are likely to affect the estrogenicity of *Alternaria* mycotoxins.

Another focal point of the cross-talk between mycotoxins and food components that requires further investigations is the modulation of the aryl

hydrocarbon receptor (AhR) (De Waard et al., 2008). The cascade of events following the activation of AhR is of particular interest in toxicological investigations as it modulates the expression of genes involved in detoxification and transport of various xenobiotics, including the expression of cytochrome P450 family members. Interestingly, AOH and AME were able to bind and activate AhR, causing the increase of CYP1A1 expression and promoting their own metabolism (Schreck et al., 2012). This process was not affecting the mycotoxin-dependent production of ROS in murine hepatoma cells (Hepa1c1c7). In addition, the authors showed that mycotoxins reduced the number of cells via an AhR-independent process, although the apoptotic phenotype was found only in cells with functional AhR and ARNT (Schreck et al., 2012). With regard to the ability of AOH to suppress the lipopolysaccharide-induced inflammation previously mentioned, Grover & Lawrence did not find any correlation between AOH-mediated AhR activation and the suppression of the inflammation found in BEAS-2B cells (Grover and Lawrence, 2017). Thus, despite the increased metabolism of AOH and AME, AhR activation does not seem to raise much concern for ROS production, cytotoxic and immunosuppressive effects, further studies are needed to determine the toxicity of hydroxylated metabolites (e.g., estrogenic properties). In particular, Dellafiora and co-workers showed that hydroxylated forms of AOH and AME cannot interact with estrogen receptors in vitro, pointing to the relevance of phase-I metabolism to modify the toxicodynamic these mycotoxins. However, methylation of respective catecholic metabolites might reactivate the estrogenic potential (Dellafiora et al., 2018).

Besides AOH and AME, many food constituents have been described to activate or inhibit AhR. Thus, they are likely to interfere with the ability of AOH and AME to bind AhR and/or with the metabolic processes following the activation of AhR. Foods consumed worldwide such as potatoes, cruciferous, bread, hamburgers, and citrus juices were investigated for the presence of natural AhR-agonists (NAhRAs) (De Waard et al., 2008). Among these, indole-3-carbinol, and many polyphenols and furocoumarins were found to be responsible for the activities shown by cruciferous vegetables (Brussels sprouts, broccoli, cabbage) and citrus juices, respectively. On the

contrary, the activation of AhR induced by the baked or fried foods tested is thought due to secondary chemicals originating from the high-temperature processing, such as polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines or Maillard products (De Waard et al., 2008). In addition, many dietary flavonoids showed a significant context–dependent AhR agonist or antagonist activities, depending on the concentration and cell types tested (Zhang et al., 2003). As an example, galangin, GEN, daidzein, and diosmin were found to be AhR agonist only in Hepa-1 cells, while cantharidin acted as an agonist only in human HepG2 and MCF-7 cells. On the contrary, AhR antagonist activities were shown both in MCF-7 and HepG2 cells by luteolin, while the antagonistic activity of kaempferol, quercetin and myricetin was strictly dependent on the cell context (Zhang et al., 2003). Many other flavones, flavonols, flavanones, isoflavones, and catechins also showed a high affinity to the AhR at dietary exposure levels (Ashida et al., 2000): apigenin, luteolin, quercetin, kaempferol, and myricetin were found to inhibit the activation of AhR induced by the most potent AhR activator identified so far (2,3,7,8-tetrachlorodibenzo-*p*-dioxin at 5 nM in MCF-7 cells) (Ashida et al., 2000). Taken together, these findings suggest that the AhR-dependent effects of food constituents strongly depend on both the chemical environment (which may significantly change among the different type of food) and on the cell type tested. Therefore, both the metabolism of *Alternaria* mycotoxins in vivo and their ability to modulate AhR could change depending on the food-specific chemical mixture.

An additional noteworthy activity of AOH and AME common with a number of food components is the capability to poison topoisomerases. In particular, many food bioactives naturally occurring in fruits, vegetables and legumes have been shown to affect the activity of both topoisomerase I and II. Taking into consideration that some *Alternaria* mycotoxins exert important genotoxic effects via either the inhibition or poisoning of these enzyme (see Section 3 for further details), the co-occurrence of other compounds targeting topoisomerases may reasonably change the overall topoisomerase-dependent genotoxic effects of *Alternaria* mycotoxins. Several studies demonstrated the ability of some polyphenols to poison topoisomerase I and/or topoisomerase II, albeit their specific mechanism of

action has been poorly investigated. Kaempferol and quercetin were reported to be, at specific concentrations, non-covalently binders of topoisomerase IIα, while myricetin showed the ability to covalently bind to topoisomerase IIα and cleaving DNA in a redox-dependent way (Bandele et al., 2008). Additionally, the flavonoids quercetin, myricetin, fisetin, and apigenin were highlighted by other authors as poisons of topoisomerase I (López-Lázaro et al., 2010), whilst genistein, daidzein, biochanin A, chrysin, have shown poisoning effects also against topisomerases II (Bandele and Osheroff, 2007). Interestingly, genistein and especially delphinidin (that acts as catalytic topoisomerase inhibitor) were found to protect cells from AOH-induced genotoxicity (Aichinger et al., 2017). Grapes and red wines are characterized by a large amount of resveratrol, belonging to polyphenols' stilbenoids group, which has always been regarded to have beneficial effects thanks to its manifold activity. Nevertheless, the capability to establish non-covalent cross-linking interactions with both topoisomerase II and DNA leading to cell death was described too (Leone et al., 2012). An influence of these compounds on poisoning and/or inhibition of topoisomerases by *Alternaria* mycotoxins, also diversifying the outcomes in vivo in a mixture-dependent way, appears therefore to be possible.

On the basis of the data reported above, *Alternaria* mycotoxins and a wealth of food constituents may interfere to each other, mutually influencing their final effects. Moving further steps toward a more precise molecular-oriented understanding of the food-specific and mixture-dependent outcomes in vivo will allow mapping those categories of food might pose a higher risk for specific toxicological endpoints. In the near future, adopting such an approach will effectively pave the ground to set personalized risk/benefit assessment studies of food prone to be contaminated by *Alternaria* mycotoxins.

5. Conclusions and Future Perspectives

Alternaria mycotoxins are frequently occurring in various fresh and processed foods such as cereals, fruits, vegetables, nuts, fruit and vegetable juices, seeds and oils. In many cases, contaminated foods have been found to simultaneously contain more than one *Alternaria* mycotoxin. In addition, the

co-occurrence of *Alternaria* mycotoxins along with *Fusarium*, *Penicillium* and *Aspergillus* mycotoxins is also well documented, though not routinely checked. In addition, mycotoxins co-occur with the huge number of food constituents inherently present in contaminated foods. Notably, a growing number of data pointing to significant effects of chemical mixtures of mycotoxins in combination with each other or with food components is available. On this basis, a more precise description of mycotoxin contamination in food, detailing both the co-occurrence of mycotoxins and the types of co-contaminated food categories, is urgently required to better support risk assessment studies.

In this respect, the current risk assessment of mycotoxins is mostly based on human exposure data and animal toxicity evidences of individual compounds, while the evaluation of possible effects due to chemical mixtures is only occasionally assessed. Studies on the combinatory effects of different *Alternaria* mycotoxins, also in combination with other mycotoxins, have already shown that the co-exposure may result in either additive, synergistic, or antagonistic effects, depending on the doses, time of exposure or type of combinations assessed. In addition, recent findings have shown that mycotoxins may interplay with other food constituents, with different outcomes depending on the nature of combinations tested. Taken together, these results show that the toxicity of mycotoxins may significantly change depending on the composition of chemical mixtures, whereby not only co-contaminants but also food bioactives might act as contributors. This evidence pointed out the need to carefully check the multiple co-occurrence of mycotoxins, also in combination with the other food constituents. On the other side, it is crucial to characterize the effects the various combinations with the other food constituents may cause on the toxicity of mycotoxin mixtures. However, the evaluation of combinatory effects is not easy to perform since the toxic action exerted by individual mycotoxins is often strictly dependent on the cellular model and the concentrations tested. In addition, the use of different cellular models and different tested concentrations makes the inter-laboratory comparison of results difficult. Moreover, from a practical point of view, the number of food constituents possibly co-occurring with mycotoxins and potentially able to modulate

their toxicity is so huge to make the systematic assessment of any possible combination unaffordable. Therefore, the definition of a consensus to define the combinations that really deserve investigations is strongly suggested. From a toxicological point of view, the use of the Adverse-Outcome-Pathway (AOP) approach or the adoption of grouping criteria, such as read-across methodologies or other computational-based categorizing methods, might provide a convincing rational to support the early definition of combinations to be tested. Moreover, in order to improve the interpretability of the data, homogeneity in the expression of the results, as well as in the tested concentrations, used cellular models, and applied methods, should become a common objective for researchers dealing with these issues in the future.

In summary, *Alternaria* toxins in food are not yet regulated mainly as a consequence of the shortage of toxicological occurrence and exposure data. A more in-depth elucidation of their toxicity, taking into account the effects of chemical mixtures, will ensure a more precise evaluation of their effects on human health eventually resulting in a more reliable assessment of risks with an overall lower degree of uncertainty. In this framework, this review collected the main data available so far in terms of occurrence and combined actions of *Alternaria* mycotoxins and it highlighted that chemical mixture may significantly change the individual toxicity of mycotoxins. Notably, most of the combinations found naturally in food still need to be tested in terms of toxicity. Therefore, it is hard to infer with precision the actual toxicological effects due to the consumption of food contaminated by *Alternaria* mycotoxins. Nonetheless, the data presented here may serve as a ground to design further studies to deepen the knowledge about the toxicity of this class of mycotoxins and to support the assessment of risk taking into account the actual role of chemical mixtures. The proposed paradigm can be logically extended to the risk assessment of other mycotoxins, as the relevance of mixtures has been described also for other classes of mycotoxins.

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2

Chapter

Research Article

Gut microbiota and undigested food constituents modify toxin composition and suppress the genotoxicity of a naturally occurring mixture of *Alternaria* toxins in vitro

Francesco Crudo[1,2], Georg Aichinger[1], Jovana Mihajlovic[3], Luca Dellafiora[2], Elisabeth Varga[1], Hannes Puntscher[1], Benedikt Warth[1], Chiara Dall'Asta[2], David Berry[1,3,4] and Doris Marko[1,2]

[1] Department of Food Chemistry and Toxicology, Faculty of Chemistry, University of Vienna, Währinger Str. 42, 1090 Vienna, Austria

[2] Department of Food and Drug, University of Parma, Area Parco delle Scienze 27/A, 43124 Parma, Italy

[3] Department of Microbiology and Ecosystem Science, Centre for Microbiology and Environmental Systems Science, University of Vienna, Althanstr. 14, 1090 Vienna, Austria

[4] Joint Microbiome Facility of the Medical University of Vienna and the University of Vienna

*Correspondence: doris.marko@univie.ac.at

Adapted from

Crudo, F., Aichinger, G., Mihajlovic, J., Dellafiora, L., Varga, E., Puntscher, H., Warth, B., Dall, C., David, A., Marko, D., 2020. Gut microbiota and undigested food constituents modify toxin composition and suppress the genotoxicity of a naturally occurring mixture of Alternaria toxins in vitro. Arch. Toxicol. https://doi.org/10.1007/s00204-020-02831-1

Abstract

Molds of the genus *Alternaria* produce several mycotoxins, some of which may pose a threat for health due to their genotoxicity. Due to the lack of adequate toxicological and occurrence data, they are currently not regulated. Interactions between mycotoxins, gut microbiota and food constituents might occur after food ingestion, modifying the bioavailability and, therefore, overall toxicity of mycotoxins. The present work aimed to investigate the impact of in vitro short-term fecal incubation on the in vitro DNA-damaging effects exerted by 5 μg/mL of an *Alternaria alternata* extract, containing, among others, 15 nM alternariol, 12 nM alternariol monomethyl ether, 241 nM altertoxin II and 301 nM stemphyltoxin III, all of which are known as genotoxic. The involvement of microorganisms, undigested food constituents and soluble substances of human fecal samples in modifying the composition and the genotoxicity of the extract was investigated through the application of LC–MS/MS analysis and comet assays in HT-29 cells. Results showed that the potential of the mycotoxins to induce DNA strand breaks was almost completely quenched, even before anaerobic incubation, by contact with the different fractions of the fecal samples, while the potency to induce formamidopyrimidine DNA glycosylase (FPG)-sensitive sites was only slightly reduced. These effects were in line with a reduction of mycotoxin concentrations found in samples analyzed by LC–MS/MS. Although a direct correlation between the metabolic activity of the gut microbiota and modifications in mycotoxin contents was not clearly observed, adsorptive phenomena to bacterial cells and to undigested food constituents might explain the observed modifications.

1. Introduction

Alternaria mycotoxins are low-molecular-weight compounds produced by different *Alternaria* species, among which *Alternaria alternata* is the most important mycotoxin-producing species of this genus of molds (EFSA, 2011). Alt*ernaria* species can infect various fruits and vegetables and can produce, under favorable conditions of temperature and humidity, more than 70 structurally characterized toxins (EFSA, 2011; Ostry, 2008). Some *Alternaria* mycotoxins are thought to represent a threat for human and animal health since they have shown a wide number of adverse effects in vivo (teratogenic and fetotoxic effects) and in vitro (genotoxic, clastogenic, mutagenic, estrogenic and androgenic effects) (EFSA et al., 2016). Regarding toxicity, alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tenuazonic acid (TeA) and tentoxin (TEN) are the most studied of these compounds. However, few toxicological and occurrence data are available for other *Alternaria* mycotoxins of potential high relevance for food safety, such as alterperylenol (ALP), altersetin (AST), altertoxins (ATXs) I, II, and III, stemphyltoxin III (STTX-III), altenuisol, altenuic acid and the *A. alternata* f. sp. *lycopersici* toxins (AAL-TA and AAL-TB).

Among the substances which were most frequently reported in food and feed, both AOH and AME were found to act as topoisomerase poisons, leading to single- and double-DNA strand breaks, while ALT and iso-ALT did not show any genotoxic properties (Fehr et al., 2009). The ability of AOH and AME to poison topoisomerases is thought to be a consequence of their planar structure, which would favor DNA pair-base intercalation (Dellafiora et al., 2015). Interestingly, the perylene quinones ATX-II and STTX-III have been described to be even more genotoxic than AOH, acting probably through the formation of mycotoxin-DNA adducts as the main mechanism (Fleck et al., 2016; Schwarz et al., 2012b). Besides the genotoxic effects, AOH and AME have also been reported to act as possible endocrine disruptors (Frizzell et al., 2013). As a matter of fact, both mycotoxins have been found to activate estrogen receptors (Dellafiora et al., 2018; Lehmann et al., 2006), and AOH can act as a full androgen agonist (Stypuła-Trębas et al., 2017).

Despite the potential threat to human and animal health posed by some *Alternaria* mycotoxins, no specific regulations are currently in place. In

this context, the risk assessment on chemical contaminants is currently based on the evaluation of the effects of the single substances, without taking into account that often food can be simultaneously contaminated by more than one substance and that the contemporary exposure to mycotoxin mixtures can change the final effect exercised by the individual compounds (Benford, 2017). The frequent co-occurrence of many *Alternaria* mycotoxins and the onset of additive, synergistic, and antagonistic effects have been reported in a recent study, which highlighted the need to reconsider the current risk assessment method (Crudo et al., 2019). In addition, several studies have shown that the effects exerted by a mixture of mycotoxins may vary with the concentrations of the individual mycotoxins in the mixture (Aichinger et al., 2019; Vejdovszky et al., 2017). Consequently, any factor able to modify the chemical composition of an occurring mixture could potentially modify the final toxic effect.

In this scenario, different authors reported the ability of bacteria from the human gut microbiota to metabolize mycotoxins: the *Fusarium* mycotoxin deoxynivalenol (DON) and its masked form (deoxynivalenol-3-glucoside) were found to be metabolized to de-epoxy DON and DON, respectively (Gratz et al., 2018; He et al., 1992). In addition, bacterial-mediated hydrolysis was also reported for the masked mycotoxins nivalenol-3-glucoside, T-2 toxin-glucoside, zearalenone-14-glucoside, and α- and β-zearalenol-14-glucoside (Gratz et al., 2017). The gut microbiota, as well as some food constituents, seem to be involved in xenobiotic removal processes (including mycotoxins) also through binding to the specific compounds, thus determining the reduction of their bioavailability (Boroujerdi, 2015; Liew and Mohd-Redzwan, 2018) . Also, a recent study of our group demonstrated the ability of potentially co-ingested food constituents to spontaneously react with epoxide-carrying *Alternaria* toxins (Aichinger et al., 2018).

Considering that the gastrointestinal tract is directly exposed to mycotoxins in food, studies investigating the effects induced by *Alternaria* mycotoxins after incubation with fecal samples could provide useful information about their potential impact on health.

Therefore, the aims of the present study were to: (1) evaluate potential modifications of the genotoxic effects of an *Alternaria* mycotoxin extract on

the human colorectal adenocarcinoma HT-29 cell line after 3 h of anaerobic incubation with fecal samples; (2) correlate the modifications of the genotoxic effects to changes in mycotoxin composition and concentration; (3) identify the fractions of the fecal samples mainly involved in these modifications.

2. Materials and Methods

2.1. Materials

For LC–MS/MS analysis and sample preparation, methanol and acetonitrile (all LC–MS grade) were purchased from Honeywell (Seelze, Germany), while 25% ammonia solution in water and ammonium acetate (both LC–MS grade) were obtained from Sigma–Aldrich Handels GmbH (Vienna, Austria). LC–MS grade water was purchased from VWR International GmbH (Vienna, Austria). Reference materials of *Alternaria* toxins were obtained from several suppliers or were kindly provided by other researchers. For details, the interested reader may refer to Puntscher et al. (2019a).

For cell culture experiments, Dulbecco's modified Eagle medium (DMEM), heat inactivated fetal calf serum and penicillin/streptomycin solution were purchased from Invitrogen™ Life Technologies (Karlsruhe, Germany). The human colorectal adenocarcinoma HT-29 cell line and the formamidopyrimidin-DNA-glycosylase enzyme (FPG) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and from New England Biolabs (Frankfurt, Germany), respectively.

The complex extract of *Alternaria* mycotoxins (CE) used in the present study was obtained by growing the *Alternaria alternata* DSM 62010 strain on long grain rice for 21 days, as previously described (Aichinger et al., 2019; Puntscher et al., 2019a, 2019b). Concentrations of the *Alternaria* mycotoxins in the extract are shown in Table 1.

Tab. 1 Characterization of the used *Alternaria* extract by liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis (adapted from Aichinger et al., 2019)

Alternaria mycotoxins	Concentration (mg toxin / g extract)
Alternariol	0.79
Alternariol monomethyl ether	0.65
Altenuene	0.78
isoAltenuene	<LOD
Tenuazonic acid	597
Tentoxin	0.02
AOH-3-glucoside	<LOD
AOH-9-glucoside	<LOD
AOH-3-sulfate	<LOD
AME-3-glucoside	<LOD
AME-3-sulfate	<LOD
Altertoxin I	9.92
Altertoxin II	14.1
Alterperylenol	12.6
Stemphyltoxin III	21.0
Altenuic acid III	<LOD
Altenusin	0.28

Abbreviation: "< LOD" = below the limit of detection

2.2. In vitro *fecal incubation*

Fresh fecal samples were provided by four healthy omnivorous volunteers (i.e. two males and two females) aged 26–34 years and with a normal body mass index (18.5–24.9 kg/m^2). No donor had previous intestinal diseases, none was treated with antibiotics, probiotics and prebiotics for the previous three months, and all signed the informed consent for the fecal sample donation. Each fecal sample was sampled with a spoon from feces on clean sampling paper, collected in a sterile plastic tube and placed in an anaerobic jar together with one anaerobiosis generator sachet (AnaeroGen™ 3.5L, Oxoid Ltd.) to keep the anaerobic conditions until their delivery to the laboratory. Samples were then transferred into an anaerobic tent (10% CO_2, 5% H_2 and 85% N2) and used within 3 h of collection.

The fecal samples were individually diluted to a concentration of 2% (w/v) with sterile phosphate-buffered saline solution (PBS, 0.1 mol/L, pH 7.4) which was previously placed into the anaerobic tent overnight to allow

deoxygenation. The 2% fecal slurries (FS) obtained were then individually used for the preparation of a FS spiked with 1% dimethyl sulfoxide (FS + DMSO, used as a control) and a FS spiked with 50 μg/mL of the CE (FS + CE). The concentration of 50 μg/mL of CE was chosen to reach a final concentration of 5 μg/mL after the dilution in DMEM for producing incubation solutions, a dose which was previously reported to induce DNA damage in human cells (Aichinger et al., 2019). Five additional samples were prepared starting from the FS. Briefly, to test the effects of the soluble (sterile) part of the fecal slurry on mycotoxin stability, a filtered fecal water spiked with 1% DMSO (FW + DMSO, used as a control) and a filtered fecal water spiked with 50 μg/mL of the CE (FW + CE) were prepared by high-speed centrifugation of the FS (5200 rcf; 10 min) followed by filtration of the supernatant with 0.2 μm cellulose acetate filters and spiking. To test the effects of the particulate matter of the fecal sample, another sample (PM + CE) was obtained by low-speed centrifugation (175 rcf; 10 min) of an aliquot of the FS to pellet the particulate matter and keep most of the microorganisms in suspension (Yen et al., 2015); the pellet was then re-suspended in PBS at the same original volume and spiked with 50 μg/mL of the CE.

To investigate the contribution of living and dead microorganisms on the fate of the mycotoxins, two different aliquots of the FS were additionally centrifuged at low speed (175 rcf; 10 min) to remove the particulate matter and the supernatants were re-centrifuged at high speed (5200 rcf; 10 min) to isolate the pellet of microorganisms. One pellet was re-suspended in PBS (original volume) and spiked with 50 μg/mL of the CE (sample containing living microorganisms, LM + CE). The other pellet was re-suspended in 70% ethanol, incubated at room temperature for 5 min and centrifuged at high speed (5200 rcf; 10 min). The pellet was then re-suspended in PBS (original volume) and spiked with 50 μg/mL of the CE (sample containing dead microorganisms, DM + CE). The method of killing microorganisms by re-suspension in ethanol was adapted from Taddese et al. (2018), who demonstrated the maintenance of the cell wall integrity.

One sample of PBS spiked with 50 μg/mL of the CE was prepared for each fecal incubation and used to compare results obtained from samples

containing fecal material (PBS + CE). The final concentration of DMSO was 1% (*v*/*v*) for all the samples prepared. Samples were then placed in glass tubes, sealed with rubber stopper to maintain anaerobic conditions, and incubated for 3 h at 37 °C and 150 strokes/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.

2.3. Comet assay and cell viability test

Single-cell gel electrophoresis ("comet assay") was carried out according to the guidelines of Tice et al. (2000) with slight modifications. Briefly, 1.5×10^5 HT-29 cells were seeded in Petri dishes with a diameter of 3.5 cm and grown for 48 h at 37 °C, under humidified conditions and with 5% CO_2. Before cell treatment, the incubated fecal samples were centrifuged at 17,000 rcf for 10 min to remove the pellet, the supernatants were diluted 1:10 with DMEM and the resulting solutions were used for incubations. PBS containing 1% (*v*/*v)* DMSO was diluted 1:10 with DMEM and used as a solvent control. Thus, after the dilution, the final CE concentration tested on cells was 5 μg/mL and all samples contained 0.1% DMSO. Cells were then incubated for 1 h, at the end of which a positive control was prepared by exposing cells (incubated with 0.1% DMSO) to UV-B radiation for 1 min. Then, each Petri dish was washed twice with PBS, trypsin was added to detach and singularize the cells, and cell counting and viability was carried out by trypan blue exclusion. After that, four aliquots of 30,000 cells for each sample were re-suspended, after centrifugation (420 rcf, 10 min), in 0.8% low-melting agarose and embedded on object slides (two slides for each sample). Cell lysis was carried out overnight at 4 °C by immersing the slides in a lysis buffer containing triton X, DMSO and N-lauryl sarcosine. One slide for each sample was treated with formamidopyrimidine–DNA glycosylase (FPG). After 30 min of incubation at 37 °C, slides were allowed to equilibrate in an alkaline buffer (pH > 13) for 20 min, followed by electrophoresis (20 min, 25 V, 300 mA). Both, equilibration and electrophoresis were carried out on ice. Finally, the slides were washed with a neutralization buffer and stained with ethidium bromide. Microscopic analysis of DNA damages was performed using a Zeiss Axioskop (ex = 546 ± 1 nm; em = 590 nm) and the

"Comet Assay IV" software (Perceptive Instruments, Suffolk, UK) was employed to score 100 cells per object slide.

2.4. Sample preparation for MS analysis

Samples collected at the two time points were centrifuged (17,000 rcf, 15 min, 4 °C) and the respective supernatants were diluted 1:5 with an ice-cold extraction solvent (ACN/MeOH, 1:1, v/v). Afterwards, these samples were kept at − 20 °C for 1 h and centrifuged again (17,000 rcf, 15 min, 4 °C). The supernatants were then diluted 1:1 with a dilution solvent (ACN/water, 1:1, v/v) and immediately analyzed.

2.4. LC-MS/MS analysis

Samples were analyzed using a high-performance liquid chromatographic system (HPLC, UltiMate3000, Dionex Thermo Fisher Scientific, Vienna, Austria) coupled to a TSQ Vantage triple quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI) interface (Thermo Fisher Scientific). The LC–MS/MS method used in this study has been originally developed and validated for the analysis of complex food matrices (Puntscher et al., 2018) and has also been applied for the analysis of rat urine and feces (Puntscher et al., 2019b). Briefly, ammonium acetate in water solution (5 mM, pH adjusted to 8.7 with a 25% NH_4OH solution) and MeOH were used as eluents A and B, respectively. For the chromatographic separation, a Supelco Ascentis® Express C18 column (100 × 2.1 mm, 2.7 μm) equipped with a pre-column (SecurityGuard™, C18, 2 mm, Phenomenex, Torrance, CA) was used. The flow rate and the temperatures of the autosampler and the column oven were set to 0.4 mL/min, 10 °C and 30 °C, respectively. The gradient was as follows: 0–1 min: 10% B, 1–1.5 min: linear increase to 38% B, 1.5–6 min: linear increase to 40% B, 6.0–6.1 min: increase to 58% B, 6.1–7.5 min: linear increase to 61%, 7.5–.0 min: linear increase to 85%, 9.0–9.1 min: increase to 100% B, 9.1–13 min: 100% B, 13.0–13.5 min: linear decrease to 10% B, 13.5–15.5 min: 10% B.

Data were acquired in multiple-reaction monitoring (MRM) mode applying negative electrospray ionization and the specific transitions for each analyte were reported by Puntscher et al. (2018). Samples were randomly analyzed,

and injections of solvent blanks were routinely performed to verify the overall performance of the instrument and to avoid carry-over phenomena. Quantification of the analytes was performed by external calibration and the calibration set was injected after every 20–22 samples. Chromeleon™ Chromatography Data System Software (v. 6.80 SR13 Build 3818) and Xcalibur™ Software (v. 3.0, Thermo Scientific) were used for instrument control and data acquisition. Data evaluation was performed with TraceFinder™ (v. 3.3) Software (Thermo Scientific).

2.5. Statistical analysis

Independent Student *t* test and analysis of variance (one-way ANOVA) with Bonferroni post hoc tests were performed using SPSS software (v. 23.0, SPSS inc., Chicago, IL, USA) to determine significant differences in relation to the two different time points of analysis and among the various samples. Samples were considered significantly different for $p \leq 0.05$ or $p \leq 0.01$.

3. Results

3.1. Genotoxicity

Genotoxic effects of the collected samples were evaluated in HT-29 colon carcinoma cells after a dilution 1:10 with DMEM, to reach a final concentration of *Alternaria* extract on cells of 5 μg/mL. For this purpose, comet assays with or without enzymatic treatment with FPG were performed. Upon cell treatment with samples prior to anaerobic incubation, statistically significant differences ($p < 0.01$) were observed in FPG-untreated samples between the extract dissolved in PBS (PBS + CE), showing the highest tail intensity (14.7 ± 3.29%), and all the other samples containing fecal material, except for CE dissolved in fecal water (FW + CE) (Fig. 1). The mean tail intensity reached after incubation with FW + CE was slightly lower (11.2 ± 4.89%), but not significantly different compared to PBS + CE. In contrast, the other FPG-untreated samples showed tail intensities (0.72–2.30%) almost comparable to that of the negative control (0.78 ± 0.38%) (Fig. 1). FPG treatment showed the same trend, since no statistically significant differences were found between PBS + CE and FW + CE, while

significantly lower tail intensities were found for all the other FPG-treated samples ($p < 0.01$) compared to PBS + CE. In this case, the recorded tail intensities of PBS + CE and FW + CE were 33.9 ± 1.88% and 30.4 ± 4.67%, respectively (Fig. 1). In contrast to what was observed for FPG-untreated samples in which the DNA-strand-break ability of mycotoxins was almost completely quenched by the presence of the fecal material, the extent of FPG-sensitive sites was mitigated but not eliminated, with tail intensities ranging from 24.1 ± 4.70% (PM + CE) to 13.5 ± 2.47% (FS + CE). Incubation with extract added to dead microorganisms (DM + CE) resulted in a lower level of FPG-sensitive sites compared to those recorded for samples containing living microorganisms, although without reaching statistical significance after Bonferroni correction.

Treatment of HT-29 cells with samples subjected to 3 h anaerobic incubation and not treated with FPG clearly showed a significant difference in mean tail intensities between the PBS + CE (13.4 ± 4.46%) and all the other samples, including FW + CE (5.82 ± 1.97%) ($p < 0.01$). Moreover, the statistical differences observed for FPG-treated samples after anaerobic incubation were similar to those observed before anaerobic incubation, although the tail intensities were different and varied from 14.3 ± 6.08% to 8.53 ± 4.75% for PM + CE and FS + CE, respectively.

Nevertheless, after fecal incubation, the tail intensities of FPG-treated samples containing dead microorganisms were found to be similar to those recorded for samples containing living microorganisms. However, a significant reduction ($p < 0.01$) of the tail intensity after 3 h of fermentation was only found in samples of living microorganisms compared to the respective samples before fermentation.

To exclude any intrinsic genotoxic activity of the fecal material, which could have distorted the results obtained from the samples containing the extract, FS + DSMO and FW + DMSO control samples from each donor were also analyzed whereby no enhanced levels of DNA strand breaks or FPG-sensitive sites were observed.

With regard to differences observed before and after anaerobic incubation, a significant decrease ($p < 0.05$) of the DNA-strand break properties was only observed for complex extract in fecal water (FW + CE). With respect to FPG-

sensitive sites, anaerobic incubation in the presence of particulate matter or living microorganisms showed significant mitigative effects ($p < 0.05$ and $p < 0.01$, respectively), while samples prepared by the use of dead microorganisms (DM + CE) did not affect the level of DNA damage.

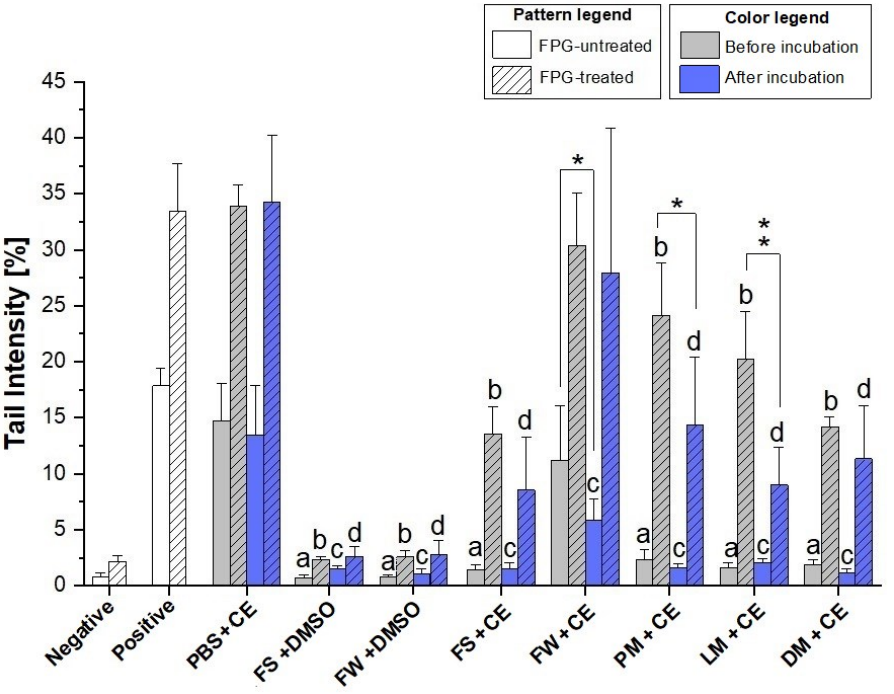

Figure 1 DNA strand breaks (-FPG) and additional FPG-sensitive lesions (+FPG) measured with the comet assay in HT-29 cells after 1 h treatment with the samples collected before (0 h) and after (3 h) anaerobic incubations with fecal fractions. Cells were exposed to samples diluted 1:10 with DMEM to reach an extract concentration of 5 µg/ml. All values are expressed as mean + SD of four independent biological experiments. Significant differences between the extract in PBS ("PBS+CE") and the other samples were calculated by one-way ANOVA, followed by Bonferroni post hoc test ($p<0.01$), with "a", "b", "c", and "d", indicating a significant difference to the FPG-untreated PBS+CE samples before incubation, to the FPG-treated PBS+CE samples before incubation, to the FPG-untreated PBS+CE samples after incubation, and to the FPG-treated PBS+CE samples after incubation, respectively. Differences between tail intensity values found before and after anaerobic incubation for each sample were calculated by Student's t-test (* = $p < 0.05$; ** = $p < 0.01$).
Abbreviations: "Negative"= 0.1% DMSO; "Positive"= UV light treatment; "PBS+CE"= extract dissolved in PBS; "FS+DMSO"= fecal slurry + DMSO; "FW+DMSO"= filtered fecal water + DMSO; "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.

3.2. Cell viability

To exclude artefacts deriving from cytotoxic effects, potentially induced by the mycotoxins or by the fecal material, cell viability was determined by trypan blue exclusion test prior to the comet assay. After 1 h of treatment of HT-29 cells with the collected samples, the resulting cell viabilities were all above 80% and ranged from 89.8 ± 1.87% to 97.1 ± 2.22% for the samples before anaerobic incubation and from 90.5 ± 1.36% to 94.5 ± 0.98% for the samples collected after 3 h of anaerobic incubation. Therefore, these results confirmed that the genotoxic effects observed were directly ascribable to the genotoxic properties of the mycotoxins contained in the extract and not to cytotoxicity phenomena (Supplementary Fig. S1).

3.3. Impact on mycotoxin prevalence

Mycotoxins contained in the original *Alternaria* extract were quantified by LC–MS/MS in the samples collected after 0 h and 3 h of anaerobic incubation to identify any possible modification induced by the presence of fecal material. In particular, eight different mycotoxins were quantified: AOH, AME, TeA, TEN, ATX-I, ATX-II, AST, and ALP. The mycotoxin STTX-III was also monitored, but not quantified due to limitation of reference material.

The blank control samples (FS + DMSO and FW + DMSO) were analyzed to ensure the absence of pre-existent *Alternaria* mycotoxins and they were found to be negative (< LOD) for all the mycotoxins under investigation. With regard to the extract-containing samples, the mean concentrations of the mycotoxins that were strongly affected by the presence of fecal material are reported in Fig. 2, while the data from the unaffected mycotoxins are shown in Table 2.

Among the various *Alternaria* toxins, AOH concentrations were not affected by the presence of the soluble fraction of the feces (fecal water, FW), since no difference in its content was found between FW + CE and PBS + CE samples. Nevertheless, AOH concentrations decreased considerably already without anaerobic incubation by the addition of fecal particulate matter, living or dead microorganisms ($p < 0.01$). Additionally, AOH concentrations were

significantly higher in the samples containing living microorganisms after 3 h of incubation than those recorded before incubation ($p < 0.01$).

AME concentrations in samples before anaerobic incubation showed the same behavior as AOH, without any modification in FW + CE compared to PBS + CE and with a significant decrease ($p < 0.01$) in the other samples. In contrast to what was observed for AOH, the anaerobic incubation period did not affect the AME content of all the samples tested.

Among the quantified mycotoxins, ATX-II showed the highest reduction in samples containing fecal material. This was probably because of its high reactivity deriving from the reactive epoxy group present in its chemical structure which made the mycotoxin prone to react with the substances present in fecal samples. Although its presence in PBS + CE and FW + CE was the same before anaerobic incubation (Fig. 2), the concentrations were strongly diminished ($p < 0.01$) in all the other samples, with a maximal reduction of 80.6% in FS + CE compared to PBS + CE. A similar percentage of reduction was observed by the addition of dead microorganisms (DM + CE), whereas addition of living microbes reached only a reduction of 50.2%. After anaerobic incubation, ATX-II was not detected (< LOD) any more in FS + CE, PM + CE, LM + CE and DM + CE, and a reduction of its concentration (47.8%) was found in fecal water samples compared to PBS + CE. In addition, comparing the concentrations of ATX-II found in FW + CE samples at the two time points, a reduction of 41.0% of its content was found after anaerobic incubation. ALP was one of the most stable mycotoxins before anaerobic incubation, since reductions in its concentration were found only in FS + CE (− 31.6%; $p < 0.05$) and DM + CE (− 39.7%; $p < 0.01$). Different results were obtained for the samples subjected to anaerobic incubation: ALP concentrations remained stable in FW + CE, while reductions of 48.1–83.2% were observed in the other samples. Differences between the two time points were found in all the samples tested except for FW + CE. Although the quantification of STTX-III was not possible, the graph reported in Fig. 2 highlights the tendency of the mycotoxin to decrease, already before anaerobic incubation, in all the samples containing fecal material compared to the control PBS + CE. In contrast to the other measured mycotoxins, an evident reduction of the STTX-III peak size was found also in FW + CE and,

at the same time, its instability was even more evident in the other samples. After 3 h incubation, nearly a total loss of STTX-III was detected in all the samples, and a significant reduction of its content ($p < 0.05$) was also found in the control PBS + CE.

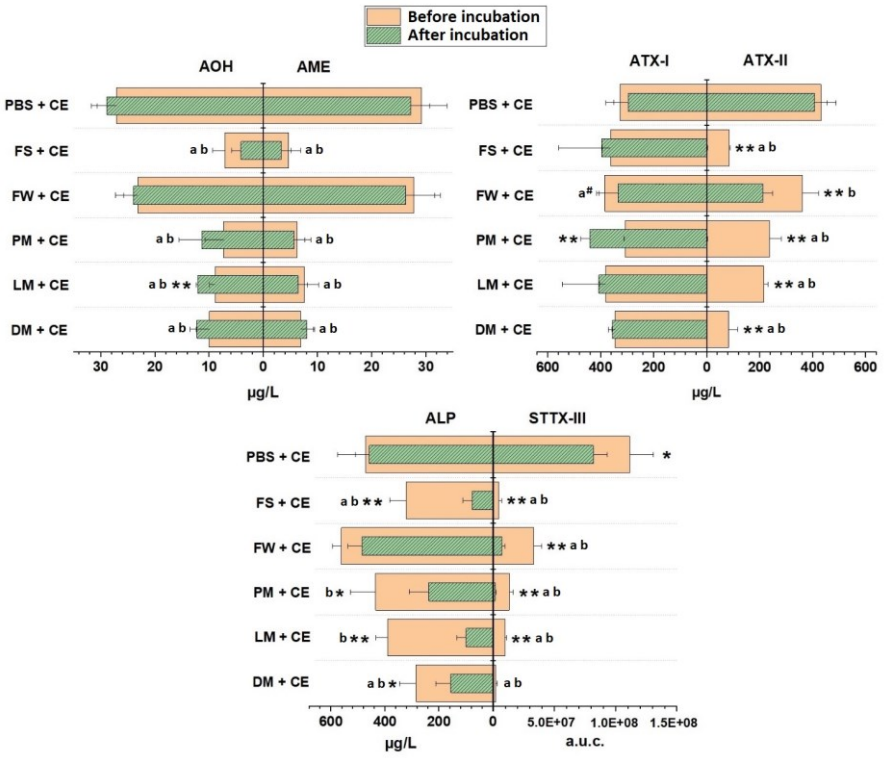

Figure 2 Bar charts highlighting mycotoxins affected by contact with fecal material. All values are expressed as mean + SD of four independent biological experiments. Differences between concentrations found before and after anaerobic incubation for each sample were calculated by Student's *t*-test (* = p < 0.05; ** = p < 0.01). Significant differences between mycotoxin concentrations found in the extract-containing PBS control ("PBS+CE") and the other samples were calculated by one-way ANOVA, followed by Bonferroni post hoc test (p<0.01). (a): significant difference between the mycotoxin concentration found in samples containing fecal material before incubation and the PBS+CE control before incubation; (b): significant difference between the mycotoxin concentration found in samples containing fecal material after incubation and the PBS+CE control after incubation. Letters followed by "#" indicate a significant difference at p<0.05.
Abbreviations: "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; "a.u.c." = area under the curve.

Among all the mycotoxins measured, TeA, TEN and AST showed the highest stability (Table 2), since their concentrations were mostly unaffected by the addition of fecal material and by anaerobic incubation. In particular, no difference in TeA concentrations was found in samples containing fecal material compared to the control PBS + CE. However, TeA concentrations were slightly lower ($p < 0.05$) in PM + CE samples before anaerobic incubation compared to LM + CE at the same time point. In addition, TeA content in the sample set did not show any variation over the time. Similarly, TEN and AST concentrations were unaffected by the presence of the fecal material before incubation and their stability was even more evident in the samples after 3 h of incubation, since no significant difference was found comparing the two time points of each sample. However, AST concentrations in FW + CE samples before and after anaerobic incubation were higher than those found in PBS + CE samples.

Unlike for ATX-II, ATX-I concentrations were almost the same in all the tested samples before anaerobic incubation, except for FW + CE samples that were characterized by a higher amount of ATX-I compared to PBS + CE ($p < 0.05$). Interestingly, although after 3 h of anaerobic incubation, no difference was found in ATX-I concentrations among the samples, a higher concentration of ATX-I was determined in PM + CE samples compared to the respective concentrations before incubation ($p < 0.01$). In addition, the FS + CE samples of 3 out 4 donors showed comparably high levels of ATX-I after the anaerobic incubation (457–507 μg/L), in contrast to a low concentration found in one donor only (155 μg/L). This low concentration of ATX-I found in the sample of the fourth donor was responsible for the failure to achieve a statistically significant difference between the mean ATX-I concentrations found before and after the anaerobic incubation of the FS + CE samples.

Table 2 Mean concentrations of *Alternaria* mycotoxins unaffected by fecal incubation in samples spiked with 50 μg/mL of the extract.

Sample	Mean concentration									
	TeA [mg/L]		TEN [μg/L]		AST [μg/L]	
	T 0 h	T 3 h	T 0 h	T 3 h	T 0 h	T 3 h
PBS+CE	20.8 ± 4.26	24.0 ± 0.69	0.91 ± 0.48	0.65 ± 0.42	227 ± 71.9	149 ± 36.9
FS+CE	21.5 ± 2.80	23.8 ± 6.84	0.61 ± 0.18	1.01 ± 0.21*	125 ± 23.0	92.6 ± 49.5
FW+CE	24.4 ± 1.50	18.5 ± 9.73	0.68 ± 0.28	0.71 ± 0.21	357 ± 64.1	350 ± 78.3
PM+CE	17.3 ± 4.97	24.6 ± 3.58	0.63 ± 0.28	0.89 ± 0.13	195 ± 43.3	153 ± 53.3
LM+CE	26.7 ± 1.58	21.9 ± 5.02	1.34 ± 0.42	0.75 ± 0.43	151 ± 32.7	127 ± 36.2
DM+CE	24.6 ± 1.64	23.1 ± 3.07	1.11 ± 0.31	0.95 ± 0.46	189 ± 32.1	180 ± 26.6

[1] All values are expressed as mean ± SD of data obtained from the four donors.
[2] * indicates a significant difference ($p<0.05$) compared to the respective time point 0.
[3] Abbreviations: "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.

4. Discussion

Alternaria mycotoxins are frequently found to co-occur in different food matrices and their potential to exert harmful effects has been already reported in several in vitro and in vivo studies. However, limited information is available about the possible influence of both the gut microbiota and co-digested food constituents on the bioavailability and bioactivity of the mycotoxins, and therefore about the possible modifications of the overall toxicity. To evaluate whether the different fractions of fecal samples modify the genotoxicity of a complex mixture of *Alternaria* mycotoxins, comet assays were performed. A preliminary investigation to exclude possible intrinsic genotoxic properties of the fecal material was carried out and data confirmed the direct genotoxic activity of the extract, whereas in the absence of the extract, no DNA strand breaks or enhanced levels of FPG-sensitive sites were induced by samples of fecal slurry or fecal water in the applied concentrations. With regard to the samples containing the *Alternaria* extract, results from the comet assay clearly showed a limited impact of the presence of fecal material or even anaerobic incubation on the induction of FPG sensitive sites. These results

might argue for the persistence of pro-oxidative properties of the *Alternaria* extract, but might also reflect other FPG-sensitive lesions. Among the mycotoxins contained in the extract, the *Alternaria* mycotoxins AOH and AME were found to induce oxidative DNA damage and reduce intracellular levels of the antioxidant glutathione in HT-29 cells already after 1 h of incubation (Tiessen et al., 2013). However, authors reported the disappearance of oxidative DNA damage after 3 h of incubation, probably due to inactivation of the compounds or enhanced DNA repair. In contrast to what was observed for the FPG-sensitive sites, the DNA strand breaks induced by the extract were completely quenched by the presence of microorganisms, particulate fecal matter or the whole fecal slurry. Genotoxic properties were only maintained in the presence of fecal water supernatants (Fig. 1). These results are supported by LC–MS/MS analysis, since the concentrations of most of the mycotoxins were reduced in samples containing the particulate matter of the fecal samples, living or dead microorganisms (Fig. 2). On the contrary, fecal water spiked with the extract did not show any significant difference in the mycotoxin pattern compared to the extract in PBS, thus excluding a possible role of the soluble proportion of the fecal samples in the observed modifications. Only STTX-III showed a very high instability even in the samples containing fecal water. In addition, this instability was also clearly evidenced by the significant reduction of STTX-III observed in the extract-containing PBS samples ("PBS + CE") after 3 h of anaerobic incubation (Fig. 2). These results may be explained by the presence of the reactive epoxy group in its chemical structure. STTX-III, in fact, was previously reported to be instable even in solvent solutions (Zwickel et al., 2016). However, the involvement of STTX-III in esophageal cancer pathogenesis cannot be completely excluded, as suggested before (Zwickel et al., 2016). Furthermore, there is a lack of knowledge on potential degradation products of STTX-III or closely relate perylene quinones, such as ATX-II, which might exert toxicological effects themselves.

Among the tested *Alternaria* mycotoxins, TeA, TEN, AST and ATX-I showed the highest stability in samples containing the various fecal fractions. Thus, considering the nearly unmodified concentrations of these mycotoxins in the samples containing fecal fractions compared to the extract in PBS and the

decrease of DNA-strand-breaking properties observed already without further anaerobic incubation, TeA, TEN, AST and ATX-I seem to be not involved in DNA damage induced by the complex extract. Our results are in accordance with literature describing TeA and TEN as not genotoxic (EFSA, 2011; Schwarz et al., 2012a). On the contrary, ATX-I, albeit being less potent than ATX-II, was reported to induce DNA strand breaks in human and animal cancer cell lines (Fleck et al., 2014), while, no information is currently available about possible genotoxic properties of AST. However, in the present work, no DNA strand breaks were associated with ATX-I presence and this may be a consequence of the very low final concentration the HT-29 cells that were exposed to in the comet assay (0.08 μM). Nevertheless, it is important to highlight that the presence of substances exerting other kinds of genotoxic effects, e.g. oxidative DNA damage, cannot be completely excluded (e.g. bioactive compounds deriving from the rice used for the growth of the *Alternaria* alternata strain or yet undiscovered mycotoxins).

Considering our results, the soluble part of the fecal slurry played a marginal role in attenuating the genotoxicity of the extract. This observation was directly related to the unmodified concentrations of most of the mycotoxins prior and after anaerobic incubation (Table 2 and Fig. 2). This applies to both DNA strand breaks and FPG-sensitive sites before anaerobic incubation, while it applies only to the FPG-sensitive sites for the samples after 3 h of incubation. As a matter of fact, a significant reduction of DNA strand breaks was observed in samples of fecal water after anaerobic incubation and it was mainly related to the significant losses of ATX-II and STTX-III, whose abilities to induce DNA strand breaks have been recently demonstrated (Fleck et al., 2016; Jarolim et al., 2017). The loss of ATX-II was probably due to reactions of the mycotoxin with constituents present in the fecal water samples, while the loss of STTX-III might be attributed both to reaction phenomena with soluble unabsorbed substances and to a lack of stability of the mycotoxin over time, since a decrease of its concentration was observed even in the extract dissolved in PBS ("PBS + CE") after 3 h of anaerobic incubation.

The decrease of ATX-II content could be, in turn, a consequence of its reduction to ATX-I. The epoxy bearing moiety of ATX-II was previously

reported to be prone to reactions with thiol moieties, which might result in the formation of ATX-I (with dithiols) or monothiol adducts of ATX-II (with monothiols) (Fleck et al., 2014). The proposed mechanism for the reduction of ATX II to ATX-I by dithiols was based on the initial formation of a monothiol adduct (with one of the two thiol groups of the dithiol compound), followed by the formation of five- or six-membered rings (depending on the type of dithiol compound). Thus, the second thiol group would be responsible for an intramolecular reduction reaction that leads to the formation of ATX-I and the oxidized form of the dithiol. Transformation of ATX-II in ATX-I was also reported in tomatoes during storage, as well as in biological samples of rats after oral application of ATX-II (Puntscher et al., 2019a, 2019c). Despite not yet elucidated, this mechanism could at least contribute to the loss of STTX-III observed in the present study.

The slightly higher levels of ATX-I found in samples of fecal water (FW + CE) before anaerobic incubation compared to the respective control in PBS could be the consequence of the previously described mechanism involving the reaction of ATX-II with the soluble substances of the feces. Additionally, the absence of microorganisms and insoluble fecal matter in these samples, which might have partially adsorbed the toxin, prevented from underestimating the real content. Higher concentrations of ATX-I, together with a total loss of ATX-II, were also observed after anaerobic incubation in samples containing the particulate matter of the fecal samples ("PM + CE"). Despite the presence, in this case, of the particulate matter, the increase in ATX-I was not completely masked by adsorption phenomena.

Adsorptive effects exerted by different compounds and structures and involving xenobiotics have been widely reported in literature. The *Alternaria* mycotoxins AOH, AME and ALT (Lemke et al., 2016), ochratoxin A (Kabak et al., 2009) and the aflatoxins B1, B2, G1 and G2 (Kabak and Ozbey, 2012) were reported to be adsorbed to the bacterial cell surface. In addition, other studies highlighted the ability of many agricultural products and by-products, especially when rich in nondegradable dietary fibers, to adsorb xenobiotics introduced with the diet, thus preventing the onset of toxicosis. As an example, grape pomace was found to sequester different mycotoxins, including aflatoxin B1,

zearalenone, ochratoxin A, fumonisin B1, and, moderately, also deoxynivalenol (Avantaggiato et al., 2014). Similarly, different food plants were able to adsorb mycotoxins potentially occurring in the normal diet and the ability of indigestible fibers to contrast the toxic effects induced by some mycotoxins was also reported in various in vivo studies (Greco et al., 2019). All these findings support the results obtained in the present work; already before anaerobic incubation, the concentrations of most of the mycotoxins tested (AOH, AME, ATX-II, STTX-III and ALP) were lower in the samples containing microorganisms, particulate fecal matter (which is supposed to be composed also by indigestible fiber fractions), and both of them ("FS + CE"), compared to the matrix-free toxin mixture in PBS (Fig. 2).

The maximum level of mitigation of the genotoxic properties of the studied complex *Alternaria* extract, as well as the maximum reduction of the original mycotoxin concentrations, was recorded in samples prepared with the whole fecal slurry ("FS + CE"). This finding suggests a cooperation of the different fractions of the feces (particulate matter and microorganisms) in the "detoxification" processes. However, it has to be pointed out that the reduction of genotoxicity of this natural mixture of *Alternaria* toxins predominantly related to an effective suppression of the direct DNA-strand-breaking potential, while the potency to induce FPG-sensitive sites remained largely unaffected, raising the question on the relevant extract constituents. Since a natural extract of an *Alternaria* culture was applied, it has to be underlined that not all constituents have been elucidated yet and it cannot be excluded that so far unknown constituents play a role for the persistent induction of FPG-sensitive sites. From the already identified constituents, ALP is a potential candidate with its concentrations largely unmodified before anaerobic incubation in almost every samples, and showing a reduction only after 3 h of incubation (except in samples of fecal water). Anyway, the presence of a considerable amount of ALP even after the incubation may pose the question whether this mycotoxin might be involved in the overall genotoxicity of the *Alternaria* extract, since no information concerning its genotoxic properties is available to date.

With regard to the effects exerted by living microorganisms, in the present study, no direct correlation between the metabolic activity of the gut

microbiota and the A. mycotoxin patterns with their genotoxic potentials was observed. The only data suggesting a possible role of microorganisms in the modifications of mycotoxin concentrations are related to the mycotoxin AOH. In fact, AOH concentrations were significantly higher in samples containing living microorganisms after 3 h of incubation than those recorded before incubation ($p < 0.01$). However, it has to be pointed out that the significant difference found was due to the very low standard deviation value of samples containing living microorganisms after 3 h of anaerobic incubation. In our opinion, this finding cannot be related to the metabolic activity of microorganisms, also considering that no difference was found in AOH concentrations among samples containing particulate matter, living and dead microorganisms.

To date, only one study focused on the evaluation of the ability of gut microbiota microorganisms to modify the *Alternaria* mycotoxins AOH, AME and ALT (Lemke et al., 2016), and no information is currently available about the other *Alternaria* toxins contained in the extract used in this work. Considering that results related to not-yet-investigated *Alternaria* mycotoxins have been also reported for the first time in the present work, and that these results refer to the effects induced by short-term fecal incubations, further studies dealing with the evaluation of a possible involvement of gut microbiota in mycotoxins metabolization in long-term fecal incubations should be encouraged for further clarification. However, it is difficult to design such experiments since they would require the use of growth media to keep the microorganisms of the gut microbiota alive and metabolically active. Growth media might in fact alter the initial microbial composition of the feces, favoring the growth of some microorganisms at the expense of others. Anyway, the results obtained in the present work represent a starting point, suggesting that adsorptive phenomena of genotoxic *Alternaria* mycotoxins to bacterial cells may have occurred.

5. Conclusion

This is the first *in vitro* study investigating the effects of an anaerobic fecal incubation on the genotoxic effects induced by a complex mixture of *Alternaria* mycotoxins. We observed the ability of both, microorganisms and undigested food constituents to suppress the DNA-strand-breaking potential induced by the applied extract, while the potency to induce FPG-sensitive lesions remained largely unaffected. These results were related to an observed reduction of mycotoxin concentrations, especially those of alternariol and its monomethyl ether, altertoxin II, stemphyltoxin III and alterperylenol.

Although a direct correlation between the metabolic activity of the gut microbiota and the modifications in mycotoxin content was not found, possible adsorptive phenomena of mycotoxins to the bacteria cells and food constituents may explain these results.

In the light of this, the present study provides useful data for assessing the risk related to the multitude of tested *Alternaria* mycotoxins, suggesting that their toxic effects might be quenched or reduced by the digestive process and by the intestinal environment. Subsequent studies are needed to explore the fate of toxins by untargeted high-resolution MS approaches to investigate the type of interactions determining the loss of mycotoxins, as well as the actual role of the gut microbiota in the metabolization of the *Alternaria* mycotoxins.

SUPPLEMENTARY MATERIAL

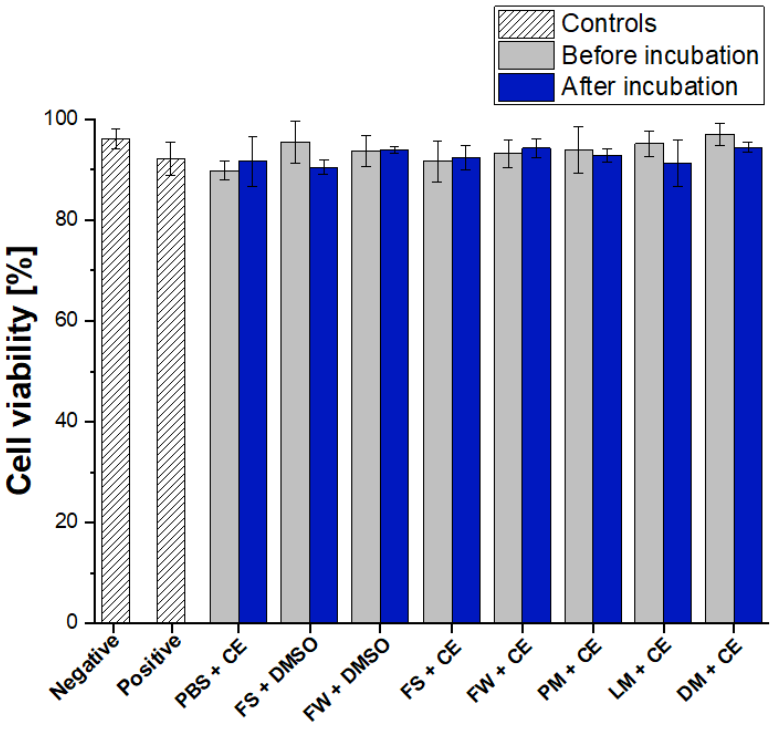

Figure S1 Impact of the samples collected before (0 h) and after (3 h) anaerobic incubation with fecal fractions on the viability of HT-29 cells, as measured through trypan blue exclusion test after 1 h incubation. Cells were exposed to samples diluted 1:10 with DMEM to reach an extract concentration of 5 μg/ml. All values are expressed as mean ± SD of data obtained from the four donors.
Abbreviations: "Negative"= 0.1% DMSO; "Positive"= UV light treatment; "PBS+CE"= extract dissolved in PBS; "FS+DMSO"= fecal slurry + DMSO; "FW+DMSO"= filtered fecal water + DMSO; "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.

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3

Chapter

Research Article

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

Francesco Crudo[1,2], Georg Aichinger[1], Luca Dellafiora[2], Giorgia Del Favero[1,3], Jovana Mihajlovic[4], David Berry[4,5], Chiara Dall'Asta[1] and Doris Marko[1,2]

[1] Department of Food Chemistry and Toxicology, Faculty of Chemistry, University of Vienna, Währinger Str. 42, 1090 Vienna, Austria;

[2] Department of Food and Drug, University of Parma, Area Parco delle Scienze 27/A, 43124 Parma, Italy;

[3] Core Facility Multimodal Imaging, Faculty of Chemistry, University of Vienna, Währinger Str. 42, 1090 Vienna, Austria

[4] Department of Microbiology and Ecosystem Science, Centre for Microbiology and Environmental Systems Science, University of Vienna, Althanstr. 14, 1090 Vienna, Austria;

[5] Joint Microbiome Facility of the Medical University of Vienna and the University of Vienna.

* Correspondence: doris.marko@univie.ac.at

Submitted

Abstract

Several *Alternaria* mycotoxins are supposed 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 are in direct contact with food constituents and 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 waters, even after 3h of fecal incubation. Mechanistic studies using an *in vitro/in silico* approach, revealed the ability of the extract to decrease the ER-α/ER-β nuclear ratio, while a possible action of the mycotoxins as ER-antagonists was excluded. Our results provide an important proof of principle suggesting that *Alternaria* mycotoxins might have the potential to act as EDCs even *in vivo.*

1. Introduction

The contamination of food commodities by naturally occurring environmental contaminants is a global food safety issue. Food contaminants 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, bioactivity of endogenous hormones, thus modifying body homeostasis, developmental and reproductive physiology (Diamanti-Kandarakis et al., 2009). To date, several classes of compounds, either natural or synthetic (e.g. polychlorinated biphenyls, phthalates, dioxins, bisphenol A, genistein and coumestrol), have been reported to act as EDCs. Among the physiological pathways potentially targeted by these molecules there are those activated by progesterone, androgens and/or estrogens (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 were reported to produce secondary toxic metabolites, named "mycotoxins", which were reported to interfere with estrogenic pathways (Dellafiora et al., 2018a; Vejdovszky et al., 2017). In particular, contamination of food by mycoestrogens can occur not only in fields, but also during other stages of the food chain, including the storage in domestic environment (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 (CONTAM, 2011; 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, 2011), but also to potentially act as EDCs

(Frizzell et al., 2013; Stypuła-Trębas et al., 2017). As a matter of fact, AOH was found to increase the expression of the progesterone receptor, to increase *in vitro* the production of estradiol and progesterone (Frizzell et al., 2013), as well as to induce androgenic effects (Stypuła-Trębas et al., 2017). 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 ER-β over ER-α (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., 2018b). 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)

As recently summarized, 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). 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) (Crudo et al., 2019). 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 fecal components (i.e. gut microorganisms and undigested food constituent) to reduce the bioavailability of some *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 3h of co-incubation with fecal samples (collected from several human donors) and defined fractions thereof. Furthermore, in mechanistic studies, we investigated the possible impact on the immunolocalization of α- and β-ERs in nucleus and cytoplasm of Ishikawa cells supported by *in silico* studies.

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) are shown in Table 1. Further information about the extract preparation can be found in Puntscher et al. (2019).

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 who signed the informed consent for the fecal sample donation.

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

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

Fecal samples were transferred into an anaerobic tent with 10% CO_2, 5% H_2 and 85% N_2 and processed within 3 hours 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 strokes/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% CO2) 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 48h incubation of Ishikawa cells with the samples collected before and after fecal incubation was determined 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 48h into a humidified incubator (37°C; 5% CO_2). After 48h incubation, the collected fecal samples were diluted 1:10 with assay medium, to reach a final DMSO or CE concentration of 5 μg/ml or 0.1%, respectively. Control media containing 0.1% DMSO (with 10% PBS; PBS+DMSO) or 1nM E2 (with 10% PBS and 0.1% DMSO; PBS+E2) were also prepared and used as controls. Thus, the spent medium of each well was replaced with the test and control

media, and cells were incubated for a further 48h. 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. Every condition for each donor was measured in triplicate. Results were expressed as mean + standard deviation of results obtained from the four donors and related to the positive control (1 nM E2).

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 48h and, finally, incubated with the samples collected and controls for a further 48h (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 E_2).

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 activity of the AlP enzyme. 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 48h 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., 2018a). After the cell membrane permeabilization (0.2 % Triton X, 10 min, room temperature-RT), cells were incubated for 1h 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 2h 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 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 trough 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 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 crystallographic 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 state. 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 structure of ALP, AST and ATX-I was 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 number 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) and OriginPro (v. 9.5, OriginLab® Corp., Northampton, MA, USA) software.

3. Results

3.1. Estrogenicity

Estrogenic properties exerted by the *Alternaria* extract before (0h) and after (3h) incubation with the fecal materials and corresponding fractions (i.e. fecal water, particulate matter, microorganisms) were assessed in the endometrial Ishikawa cell line by measuring the AlP activity. As shown in Figure 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 3h of anaerobic incubation, none of the collected samples showed changes in its estrogenic/antiestrogenic properties, except for the samples of FS+DMSO. To avoid a possible misinterpretation of the estrogenicity data due to cytotoxicity, quantification of the cellular protein content was carried out by the sulforhodamine B assay. Results of the assay confirmed that the antiestrogenic effects observed could not be ascribed to cytotoxicity phenomena (Figure 1B).

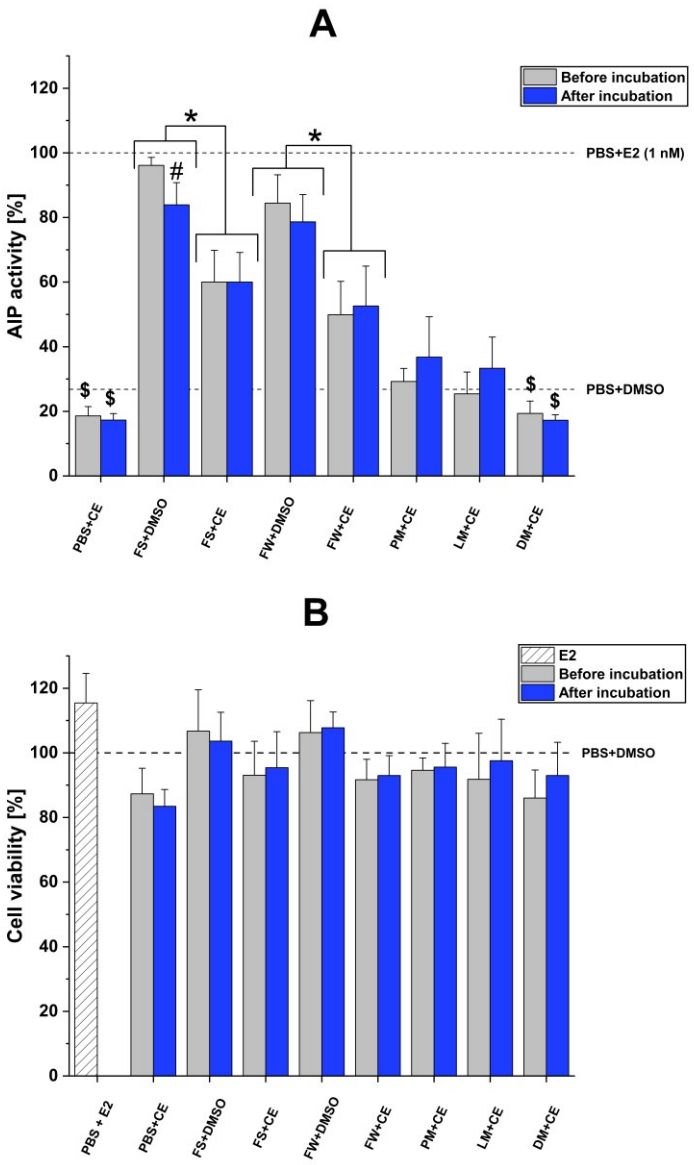

Figure 1 Effects on AlP activity (A) and cell viability (B) resulting from 48h incubation of Ishikawa cells with the collected samples and controls. Feces-containing samples were obtained by incubating the fecal materials (or their fractions) with the *Alternaria* extract for 0h (before incubation) and 3h (after incubation). 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.

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.

Figure 2 shows representative images of the central cross section of an Ishikawa cell island, in which ERα (Fig. 2A) are ERβ (Fig. 2B) are colored in red and green, respectively.

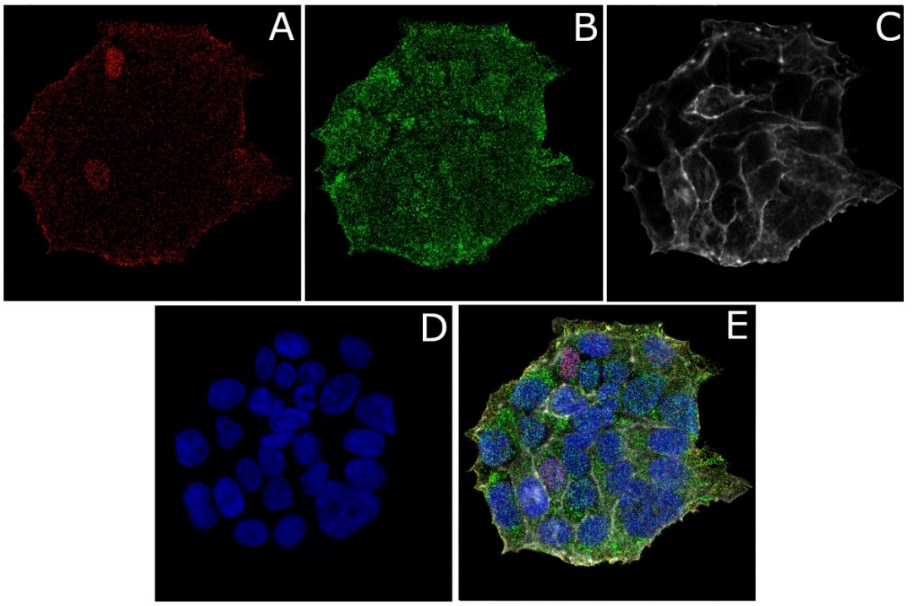

Figure 2 Representative images of the central planar section of an Ishikawa cell island, showing the localization of the ERα and ERβ. A. ERα (red); B. ERβ (green); C. Cytoskeleton (grey); D. Nucleus (Blue); E. Merged image.

Results of the ERs quantification (Fig. 3) clearly showed increased nuclear/cytoplasmic ratios, for both types of ERs, in cells treated with E2, FS+DMSO, FS+CE, and CE, compared to the solvent control (PBS+DMSO). The increased ratios observed were determined by reductions of the number of ERs into the cell cytoplasm, while no difference in the number of nuclear receptors was observed 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.

Despite no significant difference in the number of nuclear or cytoplasmic ERs was found between cells exposed to FS+DMSO and FS+CE, which might have partially explained the anti-estrogenic 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 3C). On the contrary, this difference was not observed at the cytoplasmatic level, where all samples led to a decreased ERα/ERβ ratio compared to the solvent control (PBS-DMSO; $p<0.05$) (Fig. 3C).

3.3 In silico analysis

The *in silico* analysis aimed at assessing the capability of ATX-I, ALP 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 and AST recorded negative scores in al 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 and AST were actually due to their incapability to fit the ER pocket.

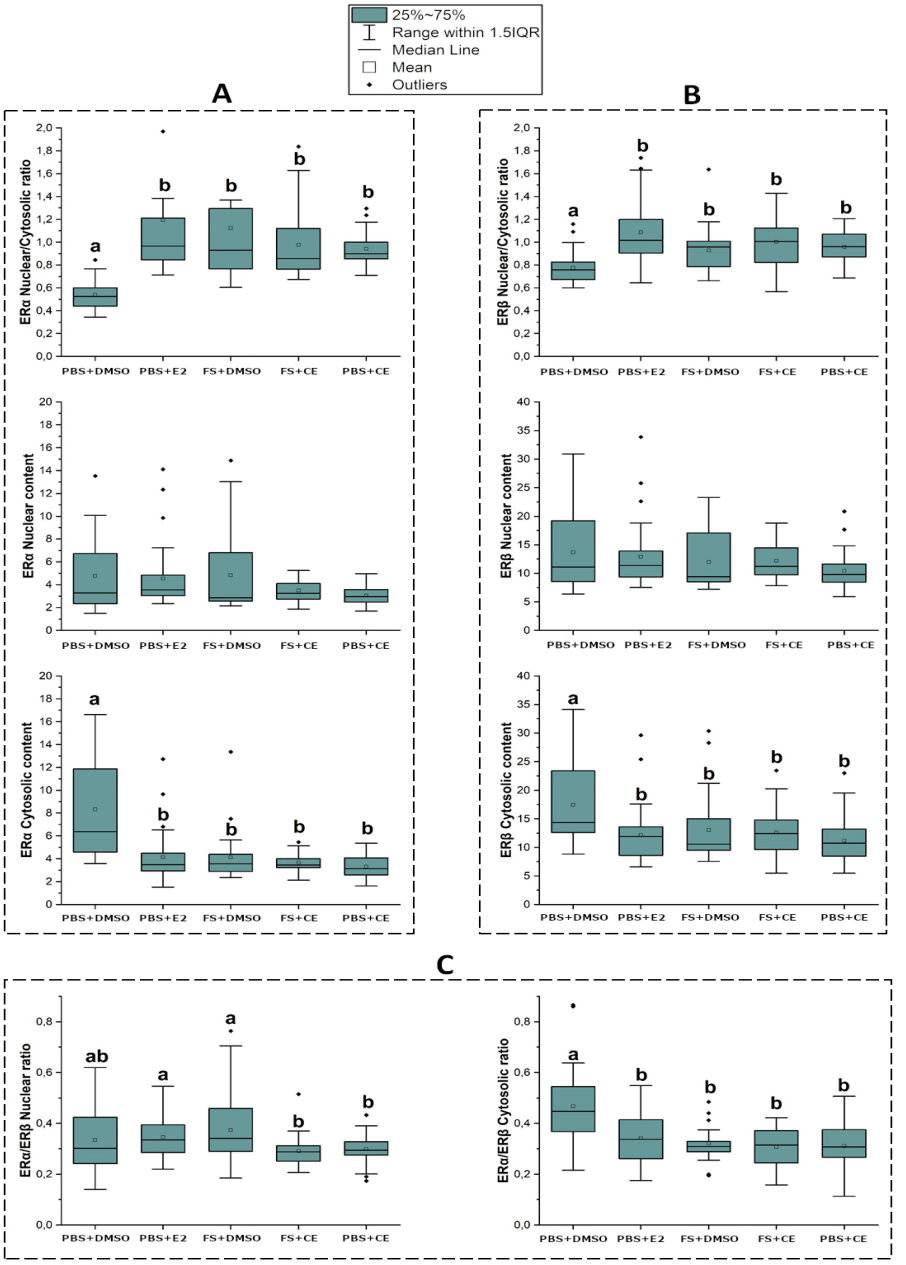

Figure 3 Effects of CE-containing samples and controls on the number and distribution of ER*α* and ERβ in Ishikawa cells. Box and whisker plots show the mean and median values, the interquartile range (IQR) and the 1.5 IQR. Outliers were defined as the values above or below the 1.5 IQR. "A" and "B" show the nuclear/cytoplasmic ratio, the nuclear content and the cytoplasmic content of ER*α* and ERβ, respectively. "C" reports the calculated ER*α*/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 ($p < 0.05$).

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

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

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 (Figure 4A). In the two antagonistic states, ATX-I, ALP 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 and AST did not match the pharmacophoric requirements of pockets since they arranged most of their polar groups in regions markedly hydrophobic (Figure 4B) causing hydrophobic/polar interferences to the binding event. Therefore, ATX-I, ALP 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.

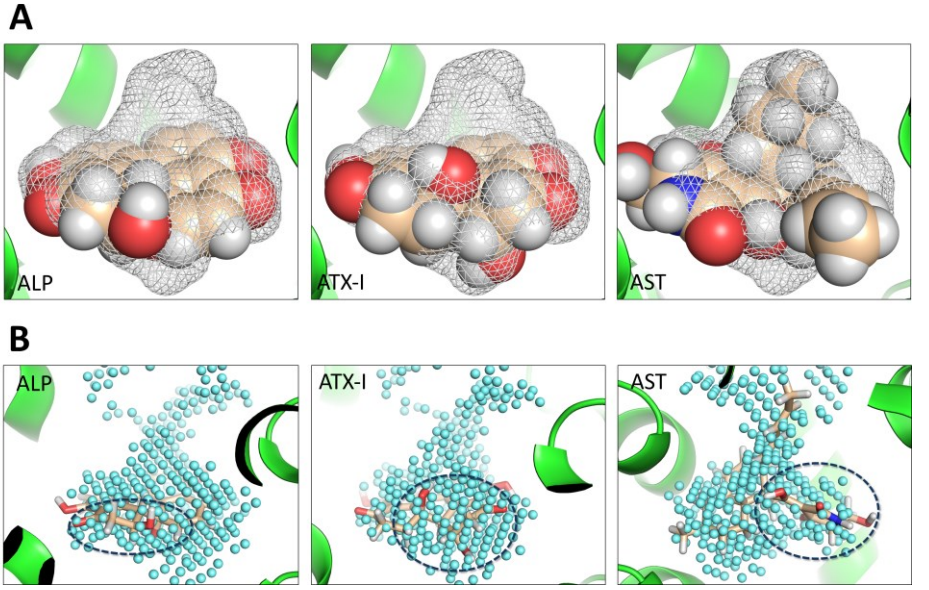

Figure 4. Computated pose of ATX-I, ALP and AST within the agonistic or antagonistic conformation of ER. A. Binding poses within the agonistic conformation of ER. The protein is represented in cartoon, while ligands are represented in van der Waals spheres. The shape of the binding site is represented by mesh. In all the computed binding architectures, portions of ligand exceeded the boundaries of binding site. 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 rings indicate the improper arrangement of ligands polar groups within the hydrophobic space of the pocket.

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 Figure 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 1nM 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 (Tab.1). These mycotoxins were previously reported to act as estrogenic compounds starting from higher concentrations (Dellafiora et al., 2018b).

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 3h of incubation. The absence of differences in the anti-estrogenic effects of the extract between the fecal slurry samples collected before 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 anti-estrogenic effects observed in FS+CE and FW+CE might be attributable to one or more of the more persistant 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 1nM 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 anti-estrogenic effects could

not be excluded, considering the employment of a different cell line and the enhanced CYP1A1 expression 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 on the protein level 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 proteosome-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. 3, 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 3h 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. 3A-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 found 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. 3A-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, reductions of α- and β-ERs were also observed in the cytoplasm of cells exposed to samples containing the extract, which induced anti-estrogenic

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. 3C, 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 anti-estrogenic 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 ER-β to affect the gene-regulation activity of ER-α, 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 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., 2018b), whereas TeA was not considered as a possible candidate due to the lack of an aromatic system that has been largely documented as

crucial to interact with the ER pocket (Ng et al., 2014). Of note, also AST lacks of an aromatic portion, but it was included in the analysis since its overall molecular shape could be compatible with the shape of the ER pocket. However, none of the mycotoxins considered in the *in silico* analysis was found able to favorably interact with the ER. Therefore, the computational outcome did not support the existence of ER-dependent mechanisms at the basis of the anti-estrogenic effects described in this work.

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 3h 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, TeA and TEN as the most likely candidates for subsequent studies. With respect to the underlying mechanisms of action, despite no antagonistic activity of the mycotoxins of the extract toward the ERs was observed during the *in silico* analysis, the ability of the extract to decrease *in vitro* the ERα/ERβ nuclear ratio might partially explain the anti-estrogenic effects observed in the Ishikawa cell line. Based on these results, 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) will be addressed in subsequent in-depth studies.

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4

Chapter

Research Article

Interactions of *Alternaria* mycotoxins, an emerging class of food contaminants, with the gut microbiota: a bidirectional relationship.

Francesco Crudo[1,2], Georg Aichinger[1], Jovana Mihajlovic[3], Elisabeth Varga[1], Luca Dellafiora[2], Benedikt Warth[1], Chiara Dall'Asta[2], David Berry[1,3,4], Doris Marko[1,2,*]

[1] Department of Food Chemistry and Toxicology, Faculty of Chemistry, University of Vienna, Währinger Str. 42, 1090 Vienna, Austria;

[2] Department of Food and Drug, University of Parma, Area Parco delle Scienze 27/A, 43124 Parma, Italy;

[3] Department of Microbiology and Ecosystem Science, Centre for Microbiology and Environmental Systems Science, University of Vienna, Althanstr. 14, 1090 Vienna, Austria;

[4] Joint Microbiome Facility of the Medical University of Vienna and the University of Vienna.

* Correspondence: doris.marko@univie.ac.at

Submitted

Abstract

The human gut microbiota plays an important role in the maintenance of human health. Factors able to modify the composition of the gut microbiota might predispose the host to the development of pathologies. Among the various xenobiotics introduced through the diet, *Alternaria* mycotoxins are gaining recent interest due to the potential harmful effects on human health. However, limited data is currently available about the bidirectional relation between gut microbiota and *Alternaria* mycotoxins. In the present work we investigated the effects of different concentrations of a complex extract of *Alternaria* mycotoxins (CE) on human gut bacterial strains, as well as the ability of the latter to metabolize or adsorb these compounds. Results from the minimum inhibitory concentration assay showed the scarce ability of CE to inhibit the growth of the tested strains. However, the growth kinetics of most of the strains were negatively affected by exposure to the various CE concentrations, mainly at the highest dose (50 μg/mL). Mycotoxins included in the extract were also found to antagonize the formation of biofilms, already at CE concentrations of 0.5 μg/mL. LC-MS/MS data analysis of the mycotoxin concentrations found in bacterial pellets and supernatants after 24 h incubation showed the ability of bacterial strains to adsorb some *Alternaria* mycotoxins, especially the key toxins alternariol, alternariol monomethyl ether, and altersetin. The tendency of these mycotoxins to accumulate within bacterial pellets, especially in those of Gram-negative strains, was found to be directly related to their lipophilicity.

1. Introduction

Through its metabolic activities and interactions with the host, the complex microbial community that inhabits the human intestine, termed the "gut microbiota", plays a key role in the well-being of humans (Clemente et al., 2012; Jandhyala et al., 2015). Any factor able to modify the composition and activity of the gut microbiota might influence both normal physiology and disease susceptibility of its host (Hasan and Yang, 2019). Among the various factors influencing the gut microbial composition, diet is the most important one (Scott et al., 2013). In addition to the nutritional composition of ingested foods, the presence of food contaminants, such as mycotoxins, can affect the balance within the intestinal microbiome (Liew and Mohd-Redzwan, 2018).

Mycotoxins are fungal toxic metabolites that may enter the food chain due to the ability of toxigenic fungi to infest a wide variety of crops and food commodities (Berthiller et al., 2013; Drejer Storm et al., 2014). Out of the roughly 400 mycotoxins known to date, only a few are regulated (Righetti et al., 2016). Among the compounds currently under consideration for new regulatory limits in the European Union, toxins produced by the genus *Alternaria* are gaining increasing interest due to the high level of occurrence in fresh and processed foods (e.g., fruits, vegetables, cereals, nuts, seeds and oils) and because of the toxic effects observed *in vivo* (e.g., teratogenic and fetotoxic effects) and *in vitro* (e.g., androgenic, estrogenic, genotoxic, mutagenic, and clastogenic effects) (Crudo et al., 2019). *Alternaria* species can produce more than 70 toxins, among which, alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tenuazonic acid (TeA), tentoxin (TEN), alterperylenol/alteichin (ALP), and the altertoxins (ATXs) I and II, are the best characterized (Crudo et al., 2019). Of note, the dibenzo-α-pyrones AOH and AME, and the epoxide-carrying compounds ATX-II and stemphyltoxin III (STTX-III) were reported to induce DNA strand breaks *in vitro* and to act as topoisomerase poisons and topoisomerase inhibitors, respectively (Fehr et al., 2009; Fleck et al., 2016). AOH and AME also showed clastogenic, mutagenic and estrogenic effects (Brugger et al., 2006; Dellafiora et al., 2018; Lehmann et al., 2006), as well as the ability to modulate innate immunity (Grover and Lawrence, 2017; Kollarova et al., 2018). Information

about the absorption, distribution and excretion of *Alternaria* toxins is currently available only in mice (Schuchardt et al., 2017) and rats (Puntscher et al., 2019a), while it is still scarce in humans. Consequently, the real impact on human health has yet to be clarified. A recent *in vivo* study in rats showed a fecal excretion > 89% for AOH and AME, while the perylene quinones ATX-I and ALP were recovered only at very low levels (<5%) (Puntscher et al., 2019a). Interestingly, the highly reactive epoxide-carrying compounds ATX-II and stemphyltoxin III (STTX-III) were not recovered at all (Puntscher et al., 2019a). On this basis, the high level of fecal excretion found for AOH and AME suggests that potential toxic effects are likely to occur predominantly at the level of the intestinal tract rather than systemically. Thus, possible inhibitory or growth-promoting effects on members of the gut microbiota cannot be excluded. On the other hand, the low recovery of ATX-I, ATX-II, STTX-III, and ALP might be the consequence of chemical transformation occurring at the gastro-intestinal level. Considering the ability of the human gut microbiota to participate in the detoxification processes of xenobiotic compounds (Collins and Patterson, 2020), the involvement of intestinal bacteria in the mechanisms of removal of ingested mycotoxins must be hypothesized, especially in view of a possible risk assessment of *Alternaria* mycotoxins.

A bi-directional interaction between mycotoxins and the gut microbiota has been reported by several authors, who investigated the effects exerted by mycotoxins on the gut microbiota and the ability of gut microbes to bind or metabolize mycotoxins ingested with the diet. As an example, germ-free rats inoculated with fecal microbiota from healthy humans showed a significant decrease of the concentration levels of *Escherichia coli* and an increase of the Bacteroides/Prevotella group after treatment with the *Fusarium* mycotoxin deoxynivalenol (DON) (Saint-Cyr et al., 2013). On the other hand, the ability of microorganisms from the intestines of broilers to transform DON to the less toxic de-epoxy-DON was also reported (He et al., 1992; Yu et al., 2010). Another mycotoxin that was shown to perturb the gut bacterial population is T2-toxin, which led to an increase of the aerobic bacteria in the intestines of rats and swine (Tenk et al., 1982). Microorganisms from human feces were found to release the parental compound from the glucoside metabolites of

T2-toxin, thus contributing to the increase of the amount of free absorbable mycotoxin (Gratz et al., 2017). The transformation of the mycotoxin zearalenone (ZEN) into unknown metabolites (Gratz et al., 2017), as well as its ability to modify the microbial diversity in porcine ascending colon contents, has also been reported (Piotrowska et al., 2014). A modulation of microbiota composition was also found to be induced by ochratoxin A and aflatoxin B1 (Baines et al., 2013; Guo et al., 2014; Ouethrani et al., 2013; Wang et al., 2016), as well as by mixtures of fumonisins B1+B2 (Burel et al., 2013) and ZEN+DON (Piotrowska et al., 2014).

We recently summarized the available data on the co-occurrence of *Alternaria* mycotoxins in food, highlighting the high frequency of co-occurrence of multiple metabolites (Crudo et al., 2019). Based on this, a complex extract of *Alternaria alternata* cultured on rice, containing eleven chemically characterized mycotoxins, was used in the present work to investigate the bidirectional relation existing between *Alternaria* mycotoxins and the gut microbiota. The aim of this work was to evaluate the effects exerted by the complex mixture of *Alternaria* mycotoxins on human gut bacterial strains belonging to five different phyla. In order to investigate the possible contribution of bacteria to the reduction of their harmful effects, the ability of the same bacterial strains to metabolize or adsorb *Alternaria* mycotoxins was studied.

2. Materials and Methods

2.1. Complex extract of Alternaria mycotoxins (CE)

The mycotoxin extract used in the present study is a complex mixture of *Alternaria* mycotoxins obtained in our laboratory by growing the *Alternaria alternata* strain DSM 62010 on long rice with a subsequent extraction procedure, as previously described (Puntscher et al., 2019b). The obtained extract was chemically characterized by LC-MS/MS. Mycotoxin concentrations in the extract are listed in Supplementary Table S1 and adapted from Aichinger et al. (2019)

2.2. Chemicals for sample preparation and LC-MS/MS analysis

Methanol and acetonitrile were acquired from Honeywell (Seelze, Germany), water was purchased from VWR International GmbH (Vienna, Austria), while ammonium acetate and 25% ammonia solution in water were obtained from Sigma–Aldrich Handels GmbH (Vienna, Austria). All solvents were of LC-MS grade. Reference materials of *Alternaria* toxins were kindly provided by other researchers or purchased from several suppliers. For further details refer to Puntscher et al. (2019b).

2.3. Bacterial strains and culture media.

The human gut bacterial strains were acquired from culture collections or isolated from human feces in our laboratory and identified through the 16S rRNA gene sequencing. Detailed information about the strains and media used in this study can be found in Supplementary Table S2. Fourteen bacterial strains were chosen in order to have at least one representative strain for each of the most dominant gut microbial phyla (i.e. Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Verrucomicrobia) and to test both Gram-negative and Gram-positive strains. *B. caccae* (BC), *B. eggerthii* (BE), *B. thetaiotaomicron* (BT), *B. vulgatus* (BV), *P. distasonis* (PD), *E. coli* (EC), and *C. innocuum* (CI), were cultivated in a supplemented brain heart infusion broth (BHI-S, ATCC Medium 1293), while for *A. finegoldii* (AF), *A. timonensis* (AT), and *R. bicirculans* (RB) a modified YCFA broth (Yeast extract-Casein hydrolysate-fatty acids) supplemented with glucose was employed (YCFA-g, DSMZ Medium 1611). For the growth of *A. muchiniphila*, brain hearth infusion broth (Oxoid; CM1135) supplemented with 0.05% pig gastric mucin type II (Sigma-Aldrich, M2378) was prepared (BHI-muc). A modified MRS broth (De Man, Rogosa, Sharpe; m-MRS) was used for the growth of *L. hominis, B. longum* and *Bifidobacterium sp.*. The m-MRS broth was composed as follow (per liter): 100 g Tryptone (Oxoid; LP0042), 10 g beef extract (Sigma-Aldrich; B4888), 5 g yeast extract (Oxoid; LP0021), 20 g glucose (Oxoid; LP0071), 1 g tween 80 (Fluka; 93780), 2 g K_2HPO_4 (Merck; 1.05104.1000), 5 g Na-acetate (Sigma-Aldrich; S8750), 0.2 g $MgSO_4$ (Sigma-Aldrich; M2643). Prior to performing an experiment, media

were left at least for 20 h in the anaerobic tent to allow deoxygenation.

2.4. Determination of the minimum inhibitory concentration (MIC)

Determination of the MIC values of the *Alternaria* mycotoxin extract was preliminarily performed on *B. thetaiotaomicron, B. vulgatus, B. caccae, C. innocuum* and *E. coli* with the aim to investigate the ability of the extract to completely inhibit the growth of the strains and define the concentrations to be tested for the subsequent analysis. Briefly, strains were grown overnight in BHI-S medium under anaerobic conditions. Then, the optical density at 600 nm (OD_{600}) was set to 0.1 and 50 µl of the adjusted cultures were pipetted in 96-well plates and diluted with 50 µl of the test media containing the various concentrations (2x concentrated) of the CE dissolved in DMSO. Thus, the final OD_{600} was 0.05. For each CE concentration tested, wells containing the corresponding percentages of DMSO were also prepared as controls. The 96-well plates also included growth controls (media plus bacteria, without DMSO) and a negative control (media). The final CE concentrations tested were 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56 µg/mL (maximum DMSO concentration of 0.4%) and each concentration was tested in triplicates. Plates were incubated under anaerobic and static conditions for 24 h at 37°C, followed by optical density measurements of the wells at 600 nm with a microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific). The lowest concentration of CE which reduced bacterial growth by 90% or higher was considered to be the MIC value.

2.5. Analysis of the growth curves

Determination of bacterial growth curves of the strains was carried out by measuring the optical density at 600 nm. Each strain was grown overnight in its appropriate medium at 37 °C under anaerobic conditions. Afterward, the overnight culture was diluted with the appropriate medium to a starting OD_{600} of 0.1, dispensed into wells of a sterile 96-well flat-bottomed microtiter plate (Costar 3595, Corning Inc., Corning, NY) and diluted 1:1 with the test media containing the different concentrations of CE or DMSO (final volume of 200 µl/well). The final CE and DMSO concentrations tested were 50

μg/mL, 25 μg/mL, 5 μg/mL, 0.5 μg/mL, and 0.1%, 0.05%, 0.01%, 0.001%, respectively. The final OD_{600} was 0.05. The prepared plates, which also included negative controls (media) and growth controls (media plus bacteria, without DMSO), were wrapped with parafilm to prevent dehydration and incubated for 24 h at 37°C in a microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific) placed in an anaerobic tent. Plates were shaken continuously at low speed (5 Hz, amplitude 15 mm) during the incubation time and for 10 s at medium speed (11 Hz, amplitude 3 mm) prior to each read. The OD_{600} was recorded automatically every 20 min for a total of 24 h. Each condition was tested at least in triplicates. Quantification of the area under the experimental growth curves was carried out by using the R package Growthcurver, available for installation from the Comprehensive R Archive Network (CRAN).

2.6. Quantification of biofilm biomasses

Evaluation of the ability of the extract to affect the formation of biofilms produced by *E. coli, B. vulgatus, B. thetaiotaomicron,* and *B. caccae* was carried out according to the microtiter-plate test described by Stepanović et al. (Stepanović et al., 2007) with slight modifications. Briefly, overnight cultures of the selected strains were diluted with their strain-specific media to an OD_{600} of 0.1. Then, 75 μl of the adjusted cultures were transferred into 96-well cell culture plates and diluted 1:1 with the test media containing the different percentages of DMSO (final concentrations of 0.1%, 0.05%, 0.01%, 0.001%) or concentrations of CE (final concentrations of 50 μg/mL, 25 μg/mL, 5 μg/mL, 0.5 μg/mL). The final OD_{600} was 0.05. Plates were incubated for 24 h or 48 h under anaerobic conditions and without shaking. Afterward, OD_{600} values were recorded to exclude bacterial growth inhibitions (Haney et al., 2018), plates were emptied, washed twice with tap water to remove planktonic cells and heat-fixed at 60 °C for 1 h. Biofilms were stained by adding 200 μl of 1% crystal violet solution to each well and incubating the plates at room temperature (RT) for 15 min. Then, plates were emptied, washed tree times with tap water and dried overnight at RT. Crystal violet was resolubilized by adding 200 μl 30% acetic acid.After 15 min incubation at RT, absorbance at 570 nm was measured by means of a

microtiter plate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific). Conditions for each strain were tested at least in triplicates. Definition of cut-off values, that allowed to distinguish biofilm-producing from non-biofilm-producing strains, was performed according to Stepanović et al. (Stepanović et al., 2007) as follow: $OD_{cut\text{-}off}$ = mean ODs of uninoculated medium + 3 times standard deviation.

2.7. Sample preparation for LC-MS/MS analysis

After 24 h incubation of the strains with the various concentrations of CE, the bacterial suspension of each well was collected and immediately stored at -80°C. To investigate whether the bacterial strains were able to metabolize or adsorb mycotoxins of the *Alternaria* extract, samples treated with 25 μg/mL of CE were chosen to be analyzed by LC-MS/MS analysis. The choice to analyze samples from this concentration was made by evaluating both the detection limits of mycotoxins and the results obtained through the analysis of the bacterial growth curves. The aim was to analyze the samples whose CE concentration had caused only a minor or no effect on bacterial growth, in such a way as not to affect the metabolic activity of the bacteria tested. For the extraction of mycotoxins from supernatants, treated and control samples were thawed and centrifuged at 20,000 rcf for 10 min (4 °C) to pellet the bacteria. Then, the supernatants were transferred into new tubes, diluted 1:5 with an ice-cold extraction solvent (ACN/MeOH, 1:1, v/v), vortexed and placed at -20 °C for 1 h to allow precipitation of proteins. Then, samples were centrifuged (20,000 rcf, 10 min, 4 °C) and the resulting supernatants were transferred into HPLC vials. As for the extraction from the bacterial pellets, after pelleting the bacteria through centrifugation (20,000 rcf, 10 min, 4 °C), a step of washing with phosphate buffer saline solution (PBS, 0.1 mol/L, pH 7.4) was performed to ensure the removal of mycotoxins present in the tube and deriving from the removed supernatants. After the PBS was removed, the washed bacterial pellets were resuspended with an extraction solvent (ACN/MeOH/water, 2:2:1, v/v/v), vortexed and sonicated for 15 min (on ice). After 1 h incubation at -20 °C, precipitated proteins and bacteria were pelleted by centrifugation (20,000 rcf, 10 min, 4°C) and supernatants transferred into HPLC vials. Extracted samples were immediately analyzed

or stored at -80 °C until analysis.

2.8. LC-MS/MS analysis

The extracted pellets, supernatants, and controls were analyzed by using a high-performance liquid chromatographic system (UltiMate3000, Dionex Thermo Fisher Scientific) coupled to a TSQ Vantage triple quadrupole mass spectrometer equipped with a heated electrospray ionization interface (Thermo Fisher Scientific).

The LC-MS/MS method used in the present study has been already used in our recent work for the quantification of *Alternaria* mycotoxins in human fecal slurries (Crudo et al., 2020). Briefly, a Supelco Ascentis® Express C18 column (100 x 2.1 mm, 2.7 μm) equipped with a pre-column (SecurityGuardTM, C18, 2 mm, Phenomenex, Torrance, CA) was employed for the chromatographic separation. An ammonium acetate in water solution (5 mM, pH adjusted to 8.7 with a 25% NH_4OH solution) and MeOH were used as eluents. MS data for the extracted samples were acquired in multiple reaction monitoring mode, applying negative electrospray ionization. Further information about the applied LC-MS/MS method can be found in Puntscher et al. (2018). External calibration was employed as a quantification technique and injections of the calibration set was performed after every 20-30 samples. Monitoring of the instrumental conditions and the data acquisition were carried out by using the software package Thermo Xcalibur™ (v. 4.0.27.42, Thermo Scientific), while TraceFinder™ software (v. 3.3; Thermo Scientific) was employed for data evaluation.

2.9. In silico *prediction of mycotoxins lipophilicity*

The prediction of mycotoxins lipophilicity was performed by using the freely available online tool SwissADME (http://www.swissadme.ch). The "consensus log $P_{o/w}$ value" for each mycotoxin, which is the arithmetic mean of the values obtained by five computational methods, was used for the LC-MS/MS data evaluation.

2.10. Statistical analysis

Significant differences ($p<0.05$ or $p<0.01$) between control and treated samples were evaluated by Independent Student *t*-test, performed by using SPSS software (v. 23.0, SPSS Inc., Chicago, IL, USA). Principal component analysis of the LC-MS/MS data was carried out by using OriginPro software (v. 2018, OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. Effects of Alternaria mycotoxins on bacterial strains

3.1.1. Minimum inhibitory concentration (MIC)

The complex *Alternaria* extract (CE), containing eleven characterized mycotoxins (See Supplementary Table S1), was tested against the human gut species *Bacteroides thetaiotaomicron, Bacteroides vulgatus, Bacteroides caccae, Escherichia coli,* and *Clostridium innocuum* to evaluate its ability to inhibit the growth of the strains. Results from the test showed the inability of the extract to inhibit the growth of *B. thetaiotaomicron, B. caccae,* and *E. coli* at any tested concentration of the *Alternaria* extract (ranging from 1.56 to 200 μg/mL). In contrast, MIC values of 50 μg/mL and 100 μg/mL were found for *B. vulgatus* and *C. innocuum*, respectively.

3.1.2. Influence on bacterial growth kinetics

The ability of the *Alternaria* extract to affect the growth kinetics of 14 reference human gut bacterial strains was evaluated by monitoring the optical density of microbial suspensions treated with 0.5, 5, 25, and 50 μg/mL of CE for 24 h. Detailed information about bacterial strains and media used are reported in Supplementary Table S2. Bacterial growth curves of all fourteen strains treated with the various concentrations of CE are shown in Supplementary Figure S1. As summarized in Figure 1, the exposure of bacterial strains to the various concentrations of CE led to an increase or decrease of area under the curve (AUC) values, depending on the strain under examination and on the specific CE concentration tested.

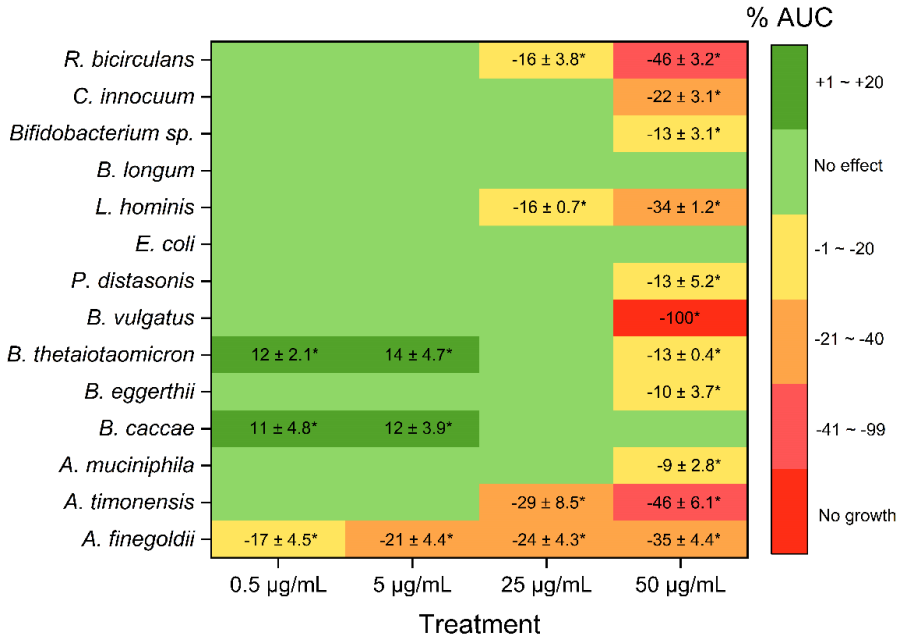


Figure 1. Modifications of the area under the curve (AUC) of growth curves induced by 24 h incubation of bacterial strains with different concentrations of the *Alternaria* extract. Reported numbers refer to mean ± SD of increase or reduction of AUC compared to the respective control (strain + DMSO). Percentages of DMSO varied from 0.1% to 0.001% (for treatments with 50 µg/mL or 0.5 µg/mL of CE, respectively). Each condition was tested in triplicate. Significant differences to the DMSO control were evaluated by Student's *t*-test (* $p<0.05$).

Of note, the extract did not show effects strictly dependent on the Gram-type but was active against most of the strains, especially at the highest CE concentration tested. Examples of the most diverse behaviors observed after exposure to the maximum and minimum concentrations of CE are reported in Figure 2, where the growth curves of *Alistipes finegoldii* (2A, 2B), *B. thetaiotaomicron* (2C, 2D), *B. vulgatus* (2E, 2F), *E. coli* (2G, 2H), and *Ruminococcus bicirculans* (2I, 2J) are shown.

Among all the strains tested, *B. vulgatus* was the most sensitive to the effects of CE, with its growth being completely suppressed at 50 μg/mL. Differently, the other tested strains belonging to the genus *Bacteroides*, namely *B. eggerthii, B. thetaiotaomicron,* and *B. caccae* were only slightly or not at all affected by an incubation with the maximum concentration of the CE (Fig. 1, Fig. 2A). Treatments of *B. thetaiotaomicron* and *B. caccae* with 5 and 0.5 μg/mL of CE led to an increase of the AUC values. Although no complete inhibition of the growth was detected after treatment of *A. finegoldii* (in-house isolated strain) with any of the CE concentrations tested, its growth was negatively affected by treatment with all CE concentrations. The growth of *Akkermansia muciniphila* and *Parabacteroides distasonis* (in-house isolated strain) was only slightly affected by treatment with the highest CE concentration, while *E. coli* was found to be the least sensitive Gram-negative strain to the *Alternaria* extract, as none of the incubations resulted in a change of growth kinetics. With regard to the growth of Gram-positive bacteria, none of the strains was affected by a CE-exposure to 0.5 and 5 μg/mL. Except for the type-strain of *Bifidobacterium longum*, all Gram-positive strains were affected at 50 μg/mL CE. A reduction of AUC values by 16±0.7% and 16±3.8% was also detected after treatment of *L. hominis* and *R. bicirculans* (in-house isolated strain) with 25 μg/mL of CE. As shown in Supplementary Figure S1, most of the detected AUC reductions were a consequence of the reduction of the stationary-phase growth yield, without any modification of the length of the lag phase. A reduction of the AUC caused by an extension of the lag phase together with a decreased growth yield was instead detected after treatment of *R. bicirculans* with the highest concentration of CE tested (Fig. 2I).

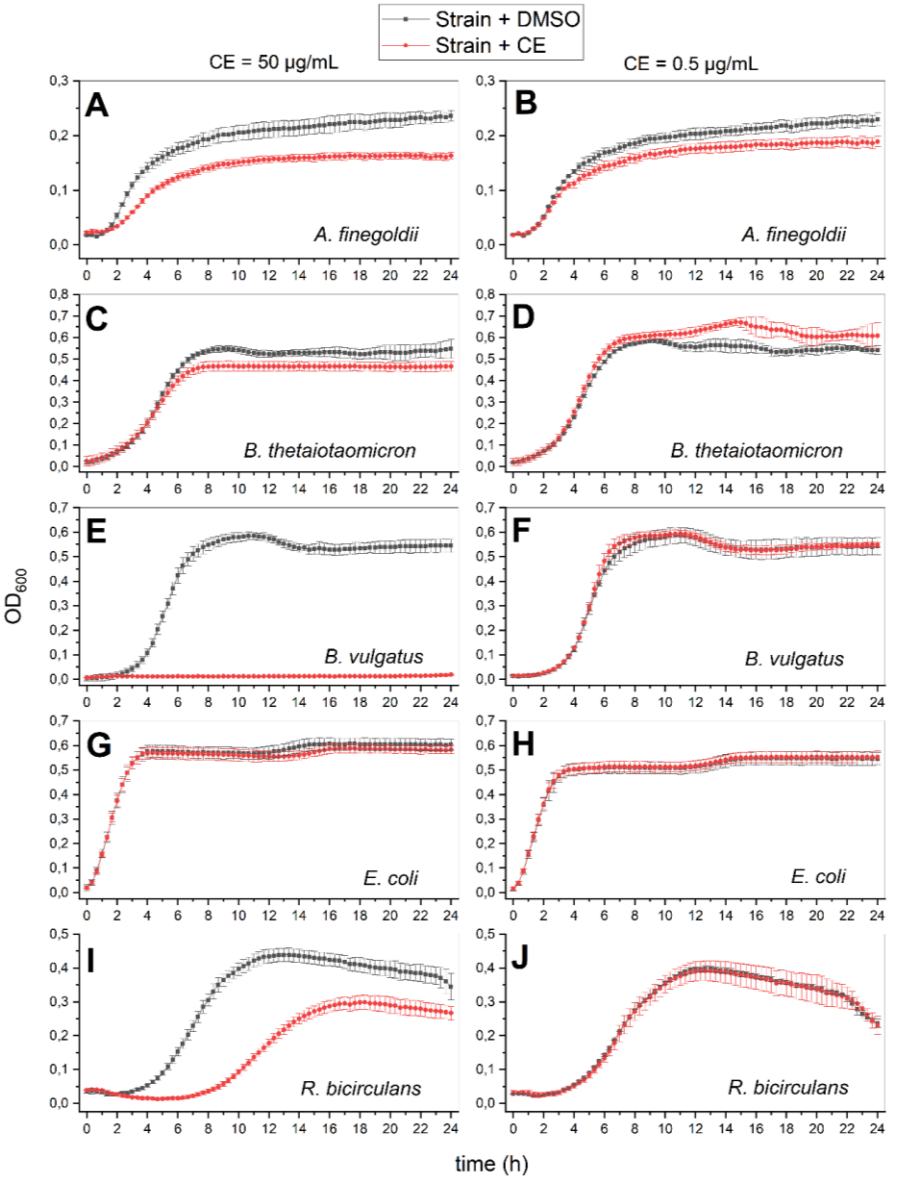


Figure 2. Representative growth curves expressed as optical density measured at 600 nm of different bacterial strains during treatment with the maximum (50 µg/mL, left column) and minimum (0.5 µg/mL, right column) concentration of the *Alternaria* extract (CE). Bacterial growth curves colored in black or red refer to the DMSO-treated or CE-treated strains, respectively.

3.1.3. Influence of *Alternaria* extract on biofilm production

The ability of the extract to affect biofilm production of gut bacteria was evaluated by employing the strains *E. coli, B. vulgatus, B. thetaiotaomicron,* and *B. caccae*. Except for the treatment of *B. vulgatus* with the maximum concentration of CE tested, all observed modifications in biofilm biomasses were attributed to the direct inhibition of biofilm formation by the CE, and not to a reduction of general bacterial growth, as confirmed through the analysis of the growth curves and the measurement of the OD_{600} values during each biofilm assay (See Supplementary Table S3). As shown in Figure 3, a concentration-dependent reduction of the biofilm biomass produced by *E. coli* was detected only after 48 h incubation of the strain with all CE concentrations (maximum reduction of 59±10% for treatments with 50 μg/mL). Similar results were obtained for *B. vulgatus*, whose biofilm-forming capacity was not affected by 24 h incubation with the various CE concentrations, except for the treatment with the highest concentration. However, the absence of biofilm mass at this concentration cannot be traced back to a direct effect of the extract on the biofilm-forming capacity of the strain but to a growth inhibition, as previously reported and showed in SI appendix, Tab. S3. After 48 h incubation, treatments of *B. vulgatus* with 25 μg/mL, 5 μg/mL, and 0.5 μg/mL led to reductions of the biofilm biomasses. The highest level of reduction (40±2%) was found for the treatment with the lowest CE concentration. Exposure of *B. thetaiotaomicron* and *B. caccae* to the various concentrations of CE led to reductions of the biofilm biomasses after 24 h. However, these effects were not persistent, as no difference between treated and control samples was found after 48 h incubation. Interestingly, treatment of *B. thetaiotaomicron* with the highest CE concentration did not result in any modification of biofilm production, while the treatment with 5 μg/mL led to the most pronounced reduction in biofilm biomass (48±8%). Similar results were obtained for *B. caccae*, whose strongest biomass reduction was reached after treatment with 5 μg/mL of CE (24 h). However, treatment with 50 μg/mL of CE resulted in a reduction of the biofilm biomass of 36±14%.

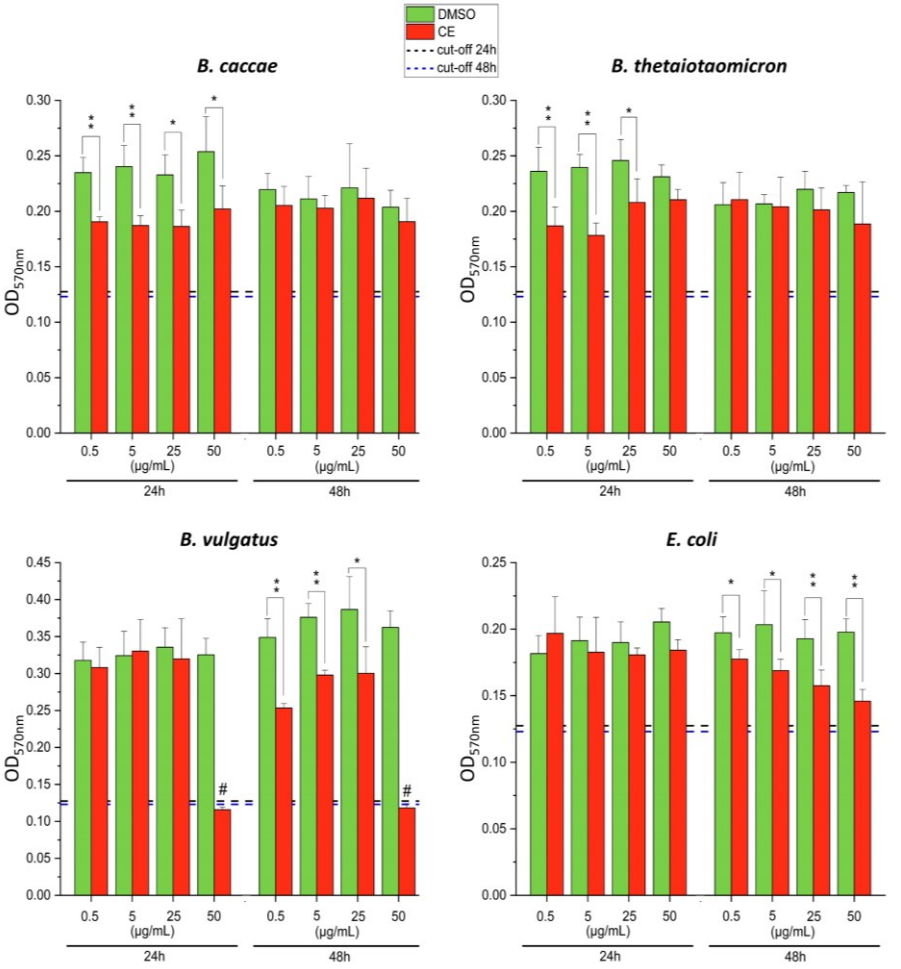


Figure 3. Effects on biofilm formation induced by 24 h and 48 h incubation of strains with different concentrations of the *Alternaria* extract. Differences between treated and control samples were evaluated by Student's *t*-test (*p<0.05; **p<0.01). Cut-off values were calculated as follow: $OD_{cut\text{-}off}$ = mean ODs of uninoculated medium + 3 times standard deviation. # indicates no growth.

3.2. Effects of bacterial strains on Alternaria *mycotoxins*

3.2.1. Total recovery of *Alternaria* mycotoxins

Quantification of mycotoxins in bacterial suspensions treated with 25 µg/mL of CE, carried out by liquid chromatography tandem–mass spectrometry (LC-MS/MS), revealed that among the mycotoxins originally present in the

extract, the highly reactive epoxide-carrying *Alternaria* toxins STTX-III and ATX-II, were found neither in bacteria-containing samples nor in the control media after 24 h. For this reason, they were excluded from the evaluation of the effects induced by the bacterial strains tested on the mycotoxins of the extract. The *Alternaria* mycotoxins AOH, AME, ALP, ALS, and AST were found to be the most affected ones in terms of total recovery (Fig. 4; Supplementary Table S4). Significant losses of AOH ($p<0.05$), ranging from 20±3% to 67±4% (*P. distasonis* and *E. coli*, respectively), occurred with all tested strains, except for *B. eggerthii, Bifidobacterium sp.* (in-house isolated strain) and *R. bicirculans*. The recovery of AME, which chemically differs from AOH by an additional methyl group, was slightly higher. As for the mycotoxin ALP, the highest levels of recovery were obtained for samples incubated with the lactic acid bacteria (LAB) *L. hominis, B. longum* and *Bifidobacterium sp.*, while very low levels of recovery were obtained for the other strains. Interestingly, samples containing the strain of *Bifidobacterium sp.* were characterized by higher amounts of ALP (+17±2%) compared to the control medium. An opposite trend was found in the total recovery of the *Alternaria* mycotoxin ALS, whose concentrations were significantly lower ($p<0.05$) only in the LAB strains. A total recovery of AST was not obtained after incubation of the extract with most of the strains tested. ALT, TeA, TEN, and ATX-I were the mycotoxins less affected by the incubation with the various strains (See Supplementary Fig. S2 and Tab. S4), since no significant difference between mycotoxin concentrations in media controls and in strain-containing samples was found for most of the strains after 24 h incubation. Notably, significant increased ATX-I concentrations ($p<0.05$) of 42±1%, 83±4%, and 36±6% were found in samples incubated with *B. eggerthii, P. distasonis,* and *Bifidobacterium sp.*, respectively.

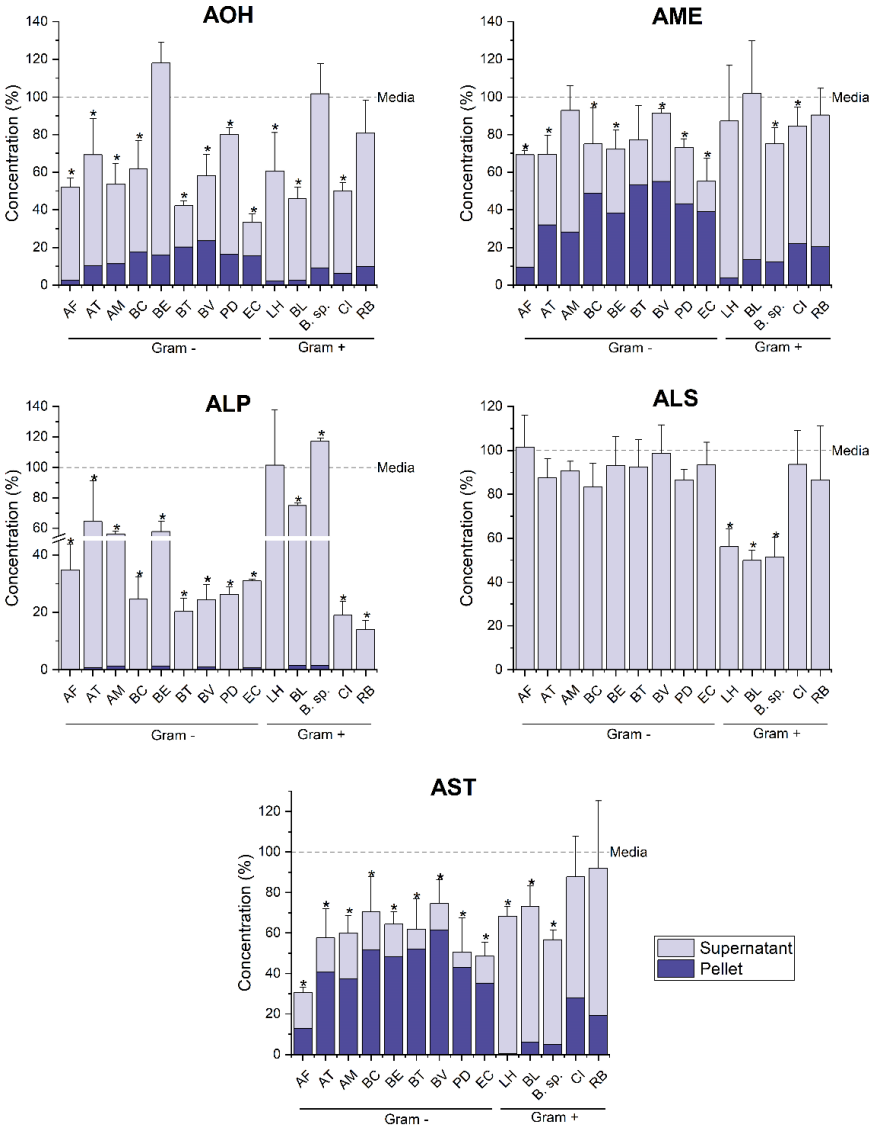


Figure 4. Bar charts showing the amount of the most affected mycotoxins recovered in pellets and supernatants of the tested strains after 24 h incubation with 25 μg/mL of the *Alternaria* extract. Data are reported as mean ± SD and differences between the total mycotoxin recovery in samples and media controls (media + CE) were evaluated by Student's *t*-test (*$p < 0.05$).

AF: *A. finegoldii*; AT: *A. timonensis*; AM: *A. muciniphila*; BC: *B. caccae*; BE: *B. eggerthii*; BT: *B. thetaiotaomicron*; BV: *B. vulgatus*; PD: *P. distasonis*; EC: *E. coli*; LH: *L. hominis*; BL: *B. longum*; B. sp.: *Bifidobacterium sp.*; CI: *C. innocuum*; RB: *R. bicirculans*.

3.2.2. Distribution of mycotoxins in pellets and supernatants of the tested strains and influence of mycotoxins lipophilicity.

LC-MS/MS analysis of the mycotoxin concentrations found in bacterial pellets and supernatants showed a different tendency of mycotoxins to bind to bacterial cells or to remain in solution (Fig. 4; Supplementary Fig. S2). Figure 5A shows the average amount of mycotoxins found in pellets of all strains tested after 24 h incubation with 25 μg/mL of CE compared to the total amount recovered (pellet + supernatant). The mycotoxins ALT, TeA, TEN, and ALS were only found in supernatants of the tested strains, while the other mycotoxins were also found in bacterial pellets. The perylene quinone ATX-I and the mycotoxin ALP were present only at very low concentrations in pellets of the bacterial strains tested and no difference between their concentrations in pellets of Gram-positive and Gram-negative strains was observed. Among the *Alternaria* mycotoxins of the extract, a rather marked tendency of adsorption of AOH, AME, and AST by the various strains was observed (Fig. 5A). The mean concentrations of AOH found in pellets of Gram-negative strains were significantly higher (26±15%; $p<0.05$) than in pellets of Gram-positive strains (8.7±4.1%), as shown in Figure 5B. Similarly, AME concentrations in pellets of Gram-negative bacteria (52±19%) were significantly different ($p<0.01$) from those found in Gram-positive strains (16±9%). The *Alternaria* mycotoxin showing the highest level of accumulation in bacterial pellets was found to be AST, whose mean recovery levels from pellets were higher ($p<0.01$) for Gram-negative (72±13%) than for Gram-positive bacteria (14±12%). The strong tendency of AOH, AME, and AST to accumulate within the Gram-negative bacterial pellets was a key factor contributing to the separation of Gram-positive from Gram-negative strains along the principal component (PC) 1 in the principal component analysis (PCA, Fig. 6). Gram-negative and Gram-positive bacteria distributed along the positive and negative axes of the PC1, respectively. However, four bacterial strains (*A. finegoldii*, *A. muciniphila, R. bicirculans, C. innocuum*) were overlapped and distributed along the negative axis of the PC2, thus showing mycotoxin distribution patterns similar to both Gram staining types.

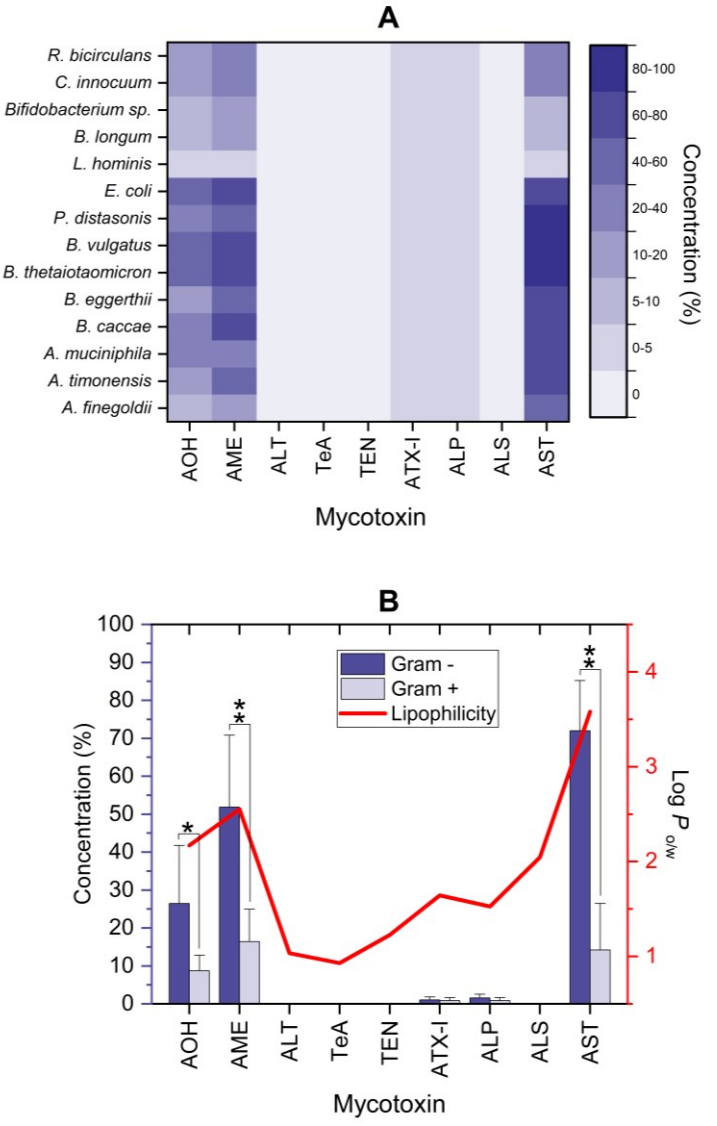


Figure 5. Recoveries of mycotoxins from bacterial pellets. (A) Heatmap showing the average amount (in % compared to the total amount recovered) of mycotoxins found after 24 h incubation with 25 μg/mL of CE in pellets of all strains tested. (B) Double-axis plot showing mean ± SD (in % compared to the total amount recovered) of mycotoxin concentrations found in pellets of Gram negative and positive strains (blue and light blue columns, respectively; left axis), and the mean value of theoretical lipophilicity (solid line; right axis) of mycotoxins. Significant differences between Gram negative and positive strains were evaluated by Student's *t*-test (*$p < 0.05$; **$p < 0.01$).

An in-depth investigation on the lipophilic properties of the *Alternaria* mycotoxins contained in the extract, performed by using the SwissADME online tool, revealed a direct correlation between the presence of mycotoxins in pellets and the *in silico* predicted values of lipophilicity of mycotoxins (Fig. 5B).The highest value for the n-octanol/water partition coefficient (log $P_{o/w}$) was obtained for AST (3.58±1.24), followed by AME (2.55±0.62), AOH (2.17±0.60), ALS (2.05±0.65), ATX-I (1.64±0.77), and ALP (1.53±0.66). Although these results are in accordance with the mycotoxin concentrations found in pellets, ALP was not found in any pellet of the tested strains. Of note, the mycotoxins not found in the pellets of the tested strains (i.e. ALT, TeA, and TEN) were characterized by the lowest log $P_{o/w}$ predicted levels (1.03±0.51, 0.93±0.76, and 1.23±1.36, respectively).

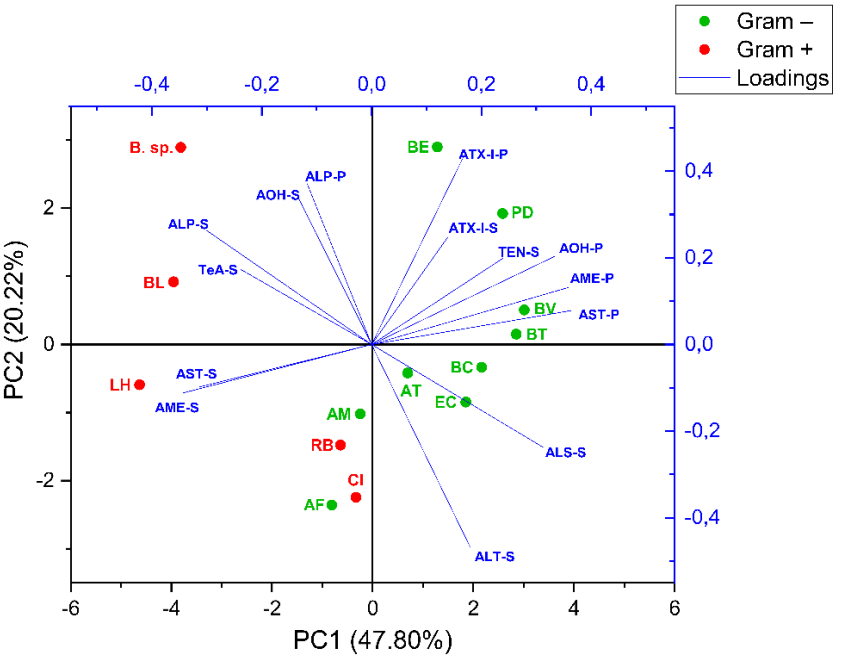


Figure 6. Score-loading plot of the first two principal components from PCA highlighting strain distribution in the bidimensional space and the contribution of mycotoxin concentrations found in pellets and supernatants.
AF: *A. finegoldii*; AT: *A. timonensis*; AM: *A. muciniphila*; BC: *B. caccae*; BE: *B. eggerthii*; BT: *B. thetaiotaomicron*; BV: *B. vulgatus*; PD: *P. distasonis*; EC: *E. coli*; LH: *L. hominis*; BL: *B. longum*; B. sp.: *Bifidobacterium sp.*; CI: *C. innocuum*; RB: *R. bicirculans*.

4. Discussion

A preliminary investigation examining the potential of the *Alternaria* extract to completely inhibit the growth of five selected strains (*B. thetaiotaomicron, B. vulgatus, B. caccae, C. innocuum* and *E. coli*) revealed no or low activity of the extract. Considerable MIC values were found only for *B. vulgatus* (50 μg/mL) and *C. innocuum* (100 μg/mL), while the other strains were not affected even at the maximum concentration of the CE (200 μg/mL). Despite this, most of the bacteria exhibited changes in their growth kinetics after exposure to specific concentrations of the extract (Fig. 1; Supplementary Fig. S1). A representative set of examples is reported in Fig. 2. In particular, while the AUC of *A. finegoldii* was reduced by the exposure to both maximum (Fig. 2A) and minimum (Fig. 2B) concentrations of CE, no changes in growth kinetics were found for *E. coli* (Fig. 2G, Fig. 2H) and the ATCC 15697 strain of *B. longum (Fig. 1)*, which were the most resistant bacteria against *Alternaria* toxins in study at hand. Strains belonging to the genus *Bacteroides* deserve particular attention because of the high intra-genus variability in their response to the presence of the *Alternaria* extract. Exposure of *B. vulgatus* to the maximum CE concentration resulted in growth inhibition (Fig. 2E), while exposure to the lowest CE concentration did not affect its growth (Fig. 2F). Exposure of *B. eggerthii* and *B. thetaiotaomicron* to the maximum CE concentration only slightly affected their growth, while no changes were found for *B. caccae* at this concentration. The strains *B. thetaiotaomicron* and *B. caccae* exhibited increased AUC values after exposure to the lowest CE concentrations tested (5 μg/mL and 0.5 μg/mL). This might be the consequence of a mycotoxin-induced modulation of intra-cellular pathways involved in bacterial growth, or the result of a possible utilization of some of the *Alternaria* mycotoxins as carbon and energy sources. Supporting this interpretation, bacteria were previously reported to metabolize xenobiotics, and the demethylation of compounds can provide a carbon source for their growth (Koppel et al., 2017). However, considering the lack of data in the literature, further studies are required to better clarify this phenomenon. All the observed modifications in bacterial growth discussed above were found to be mainly

attributable to modifications of the stationary-phase growth yield. *R. bicirculans* was the only strain for which an extended duration of the lag phase could be observed after treatment with 50 μg/mL of CE (Fig. 2I). The prolongation of the lag phase can be considered a defense mechanism that allows bacteria to survive unfavorable growth conditions, such as presence of antimicrobial compounds. As an example, the growth of a clinical isolate of *Enterococcus faecium* was delayed by the treatment with the fluoroquinolones ciprofloxacin and moxifloxacin, which act by inhibiting the DNA gyrase (Theophel et al., 2014). Interestingly, some of the *Alternaria* mycotoxins contained in the extract (i.e. AOH, AME, ATX-I, ATX-II, ALP, and STTX-III) were previously reported to target the bacterial gyrase (Jarolim et al., 2017). Consistently with the growth inhibitions and changes in the growth kinetics observed in the present study, the ability of *Alternaria* mycotoxins to exert antimicrobial effects was previously reported by several authors. AOH inhibited the growth of *Staphylococcus aureus* and *Corynebacterium betae* at micromolar concentrations, while the growth of an *E. coli* strain was only slightly reduced (Freeman, 1966). AME and ALT were active against both Gram-positive and Gram-negative bacteria (Lou et al., 2016; Pero et al., 1971). The mycotoxin ALS exhibited antimicrobial activity against several multidrug-resistant bacteria (Kjer et al., 2009), while AST only inhibited the growth of pathogenic Gram-positive strains (MIC values ranging from 0.12 to 2 μg/mL) (Hellwig et al., 2002). The concentrations of AST the strains tested in the present study were exposed to (see Supplementary Tab. S5) were in the MIC range reported above (Hellwig et al., 2002). Hence, this mycotoxin might have at least contributed not only to the changes of the growth kinetics of the Gram-positive bacteria, but also to the growth inhibition of *C. innocuum* (Gram-positive) after treatment with 100 μg/mL of CE. However, considering that the other *Alternaria* mycotoxins of the extract were present in significantly lower concentrations (see Supplementary Tab. S5) than those previously reported in literature to be able to exert antimicrobial effects (Freeman, 1966; Kjer et al., 2009; Lou et al., 2016; Pero et al., 1971) and that the growth of Gram-negative bacteria was also influenced, the observed effects might be the consequence of synergistic effects caused by the co-presence of multiple *Alternaria* mycotoxins.

Based on the results obtained in the present work, possible effect on the human gut microbiota may be assumed even in *in vivo* conditions. Gut dysbiosis, characterized by a reduced or increased presence of specific bacteria or groups of bacteria, have been linked both to the development of pathologies related to digestive tract and other organ systems (Carding et al., 2015). As an example, a possible involvement of some pathobiont strains of *E. coli* in the pathogenesis of inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), has been suggested (Perna et al., 2020). Patients with CD were found to have a higher abundance of *E. coli* at the level of the ileum than healthy people (Perna et al., 2020). On the contrary, the abundance of *A. muciniphila*, which is one of the most important mucolytic symbionts inhabiting the human intestine, was found to be reduced in IBD, autism, and obesity (Derrien et al., 2017). A lower abundance of the genus *Bacteroides*, compared to healthy controls, was also reported in CD and UC patients in active phase (Zhou and Zhi, 2016).

Although it has not yet been clarified whether these alterations in the abundance of intestinal bacteria are a cause or a consequence of IBD, the modifications in bacterial growth observed in the present work raises the question whether exposure to *Alternaria* toxins may actually represent a contributing factor to the development of diseases such as IBD.

An in-depth investigation with selected strains (*E. coli, B. vulgatus, B. thetaiotaomicron* and *B. caccae*) revealed also the ability of the *Alternaria* extract to affect biofilm formation. As already known, microorganisms inhabiting the human intestinal tract live as complex biofilm communities in close association with the outer layer of the host mucus (Buret et al., 2019). The disruption of the complex structure of the biofilm, which confers microorganisms increased tolerance to stress, may lead not only to diseases at the gastrointestinal level, but also in other organs (Buret et al., 2019). In the present study, the biofilm production by *E. coli* was reduced in a concentration-dependent manner after 48 h incubation with all concentrations of CE, without changes in the growth of the strain. Similar results were reported by Lee et al. (Lee et al., 2014), who reported the ability of coumarin and eight derivatives to inhibit the formation of biofilms produced by a pathogenic *E. coli* strain. Of note, treatment with 50 μg/mL of

ellagic acid (derivative), which is structurally related to some of the *Alternaria* mycotoxins (AOH, AME), led to the reduction of about 40% of the biofilm biomass produced by the strain (Lee et al., 2014). Although ellagic acid was not tested, coumarin and the two derivatives umbelliferone and esculetin were found to repress curli genes and motility genes. In addition, the observed reductions in fimbriae production and swarming motility were linked to the observed transcriptional modifications. On this basis, it cannot be excluded that, in the present study, some of the *Alternaria* mycotoxins might have triggered changes in the transcription of genes involved in biofilm formation. Unlike *E. coli*, the reduction in biofilm biomass observed for strains belonging to the genus *Bacteroides* did not follow a clear concentration-dependent pattern. In addition, while the biofilm production of *B. vulgatus* was affected after 48 h incubation, reduction of biomasses was found for *B. caccae and B. thetaiotaomicron* after 24 h, but not after 48 h. This suggests possible losses of the antibiofilm properties of the extract over time or the onset of adaptive cellular responses to mycotoxin-induced stress. The detailed elucidation of underlying mechanisms will be addressed in subsequent studies.

To evaluate the ability of the tested strains to chemically modify *Alternaria* toxins, quantification of mycotoxins in bacterial pellets and supernatants was performed by LC-MS/MS analysis. Results clearly showed no or low impact of bacterial strains on the total recovery of ALT, TeA, TEN, and ATX-I (Supplementary Fig. S2). An increased concentration of ATX-I after 24 h incubation was found in samples incubated with *B. eggerthii, P. distasonis*, and *Bifidobacterium sp.* compared to the controls without bacteria. These higher concentrations might be a consequence of de-epoxidation processes involving the highly reactive epoxide-carrying mycotoxin ATX-II. In fact, an *in vivo* study carried out by Puntscher et al. (2019a) reported the presence of ATX-I in plasma, urine and fecal samples of rats administered with the *Alternaria* mycotoxin ATX-II. This transformation was also reported in eukaryotic cell lines (Fleck et al., 2014a, 2014b). Moreover, in a recent study (Crudo et al., 2020) we found higher concentrations of ATX-I in samples of fecal slurries of 3 out 4 donors after a 3 h anaerobic incubation with the same *Alternaria* extract used in the present study. The ability of gut

microorganisms from broilers, pigs, and rats to transform the epoxide-carrying compounds DON into de-epoxy-DON was also reported (He et al., 1992; Kollarczik et al., 1994; Worrell et al., 1989; Young et al., 2007). Although in the present study ATX-II was found neither in samples containing bacteria nor in media controls after 24 h incubation, in our previous study we reported ATX-II to be still present after 3 h incubation (Crudo et al., 2020). Thus, it is probable that the transformation of ATX-II into ATX-I mediated by *B. eggerthii, P. distasonis*, and *Bifidobacterium sp.* occurred in the first hours after incubation with the *Alternaria* extract.

The transformation of the epoxide-carrying *Alternaria* mycotoxin STTX-III into ALP was also reported in literature (Fleck et al., 2014a, 2014b). In the present study, most of the bacteria mediated a reduction of the total content of ALP. Of note, ALP concentrations in samples incubated with *Bifidobacterium sp.* were higher than those present in the control media, suggesting a possible transformation of STTX-III to ALP by this bacterium. Among the other *Alternaria* mycotoxins, significant unrecovered amounts of AOH, AME, and AST were found in samples incubated with most of the strains, without any difference between Gram staining types. The incomplete recovery of AOH was also reported by Lemke et al. (2016) (Lemke et al., 2016) who recovered percentages of the mycotoxin ranging from 70 to 85% from pure cultures of *E. coli DH5α* and *L. plantarum* BFE5092. However, no data is currently available about the total recovery of the other mycotoxins after incubation with pure cultures, including ALS, for which we observed an incomplete recovery in samples from the three strains of LAB. As shown in Fig. 4 and Fig. 5A, LC-MS/MS data analysis revealed the tendency of AOH, AME, and AST to accumulate within bacterial pellets. Albeit to a much lesser extent, this phenomenon was also observed for ATX-I and ALP, while ALT, TeA, TEN, and ALS were only found in supernatants. This is in line with Król et al. (2018) who reported the ability of bacteria to bind mycotoxins.

Several parameters are considered to affect the ability of compounds to penetrate the bacterial cell walls (Macielag, 2012). Among these, the log $P_{o/w}$ value, which is a measure of lipophilicity of molecules, plays an important role because compounds having high hydrophilicity are excluded from the

passive passage through the lipid bilayer (Fost and John, 1997; Macielag, 2012). In the present work, a direct relation between mycotoxin concentrations in pellets and their theoretical log P values was observed (Fig. 5B). The accumulation of mycotoxins in or on bacterial cells was most pronounced for AST (up to 84.9% in relation to the total recovered amount, in *P. distasonis*; log P = 3.58±1.24), followed by AME (70,57%, *E.coli*; log P = 2.55±0.62), and AOH (47.91%, *B. eggerthii*; log P = 2.17±0.60). The mycotoxins ATX-I and ALP accumulated to considerably less amounts, which was accompanied with low log P values (1.64±0.77 and 1.53±0.66, respectively). The theoretical log P values of the other *Alternaria* mycotoxins not found in bacterial pellets were all below these values, except for ALS whose log P was between those of AOH and ATX-I (Fig. 5B).

Although in the present study both Gram staining types were able to adsorb AOH, AME, and AST, Gram-negative bacteria showed higher adsorptive capacities (Fig. 5B). This tendency was not observed for ATX-I and ALP, which were present in small comparable amounts within both Gram-negative and Gram-positive bacteria. The greater tendency of Gram-negative bacteria to accumulate AOH was also reported by Lemke et al. (2016), while no studies are currently available about the other *Alternaria* mycotoxins. The different levels of mycotoxin accumulation between the two Gram staining types might be explained by the different structure of the bacterial cell walls. Gram-negative bacteria possess a lipopolysaccharide-coated outer membrane and an inner cytoplasmic cell membrane which are separated by a thin layer of peptidoglycan. On the contrary, the cell wall surrounding Gram-positive bacteria is composed by a thick layer of peptidoglycan, but lacks the outer membrane (Denyer and Maillard, 2002). Thus, the high lipid content of the Gram-negative cell wall might have favored the accumulation of the mycotoxins showing the highest log P values. However, considering that the use of LC-MS/MS analysis is not suitable to determine the exact location of compounds within bacterial cells, further studies are required to elucidate this point. Interestingly, the ability of some *Alternaria* mycotoxins (i.e. ATX-II and AOH) to interact with eukaryotic cell membranes has already been reported (Del Favero et al., 2020b, 2020a, 2018). In particular, the mycotoxin AOH, which is structurally

similar to cholesterol, was found to affect the membrane fluidity of THP-1 macrophages and to intercalate in cholesterol-rich membrane domains (Del Favero et al., 2020b). Although sterols are not typically found in bacterial membranes and bacteria are unable to synthesize cholesterol (Huang and London, 2016), this sterol was previously found in membranes of strains belonging to the genera *Mycoplasma, Ehrlichia, Anaplasm, Brachyspira, Helicobacter* and *Borrelia*, probably due to the ability of these strains to acquire cholesterol from cell hosts (Huang and London, 2016). In addition, the bacterial hopanoids, which are structurally and functionally similar to sterols, were found to interact with glycolipids in bacterial outer membrane. All these findings support the possibility that some of the *Alternaria* mycotoxins contained in the extract were accumulated in the bacterial lipid bilayer (Sáenz et al., 2015).

5. Conclusion

In the present study we report a naturally occurring mixture of emerging food contaminants to affect the growth kinetics of bacterial strains of the human gut microbiota. Additionally, the extract's components were able to exert anti-biofilm properties, which could contribute to the appearance of deleterious effects on the human gut microbiota. On the other hand, gut strains were found to adsorb some of the *Alternaria* mycotoxins contained in the extract, thus exerting protective effects on human health by reducing the free absorbable portion of mycotoxins. This bidirectional interaction between food contaminants and the gut microbiota is likely to contribute to the former's effects on human health and demands appropriate scientific attention.

SUPPLEMENTARY MATERIAL

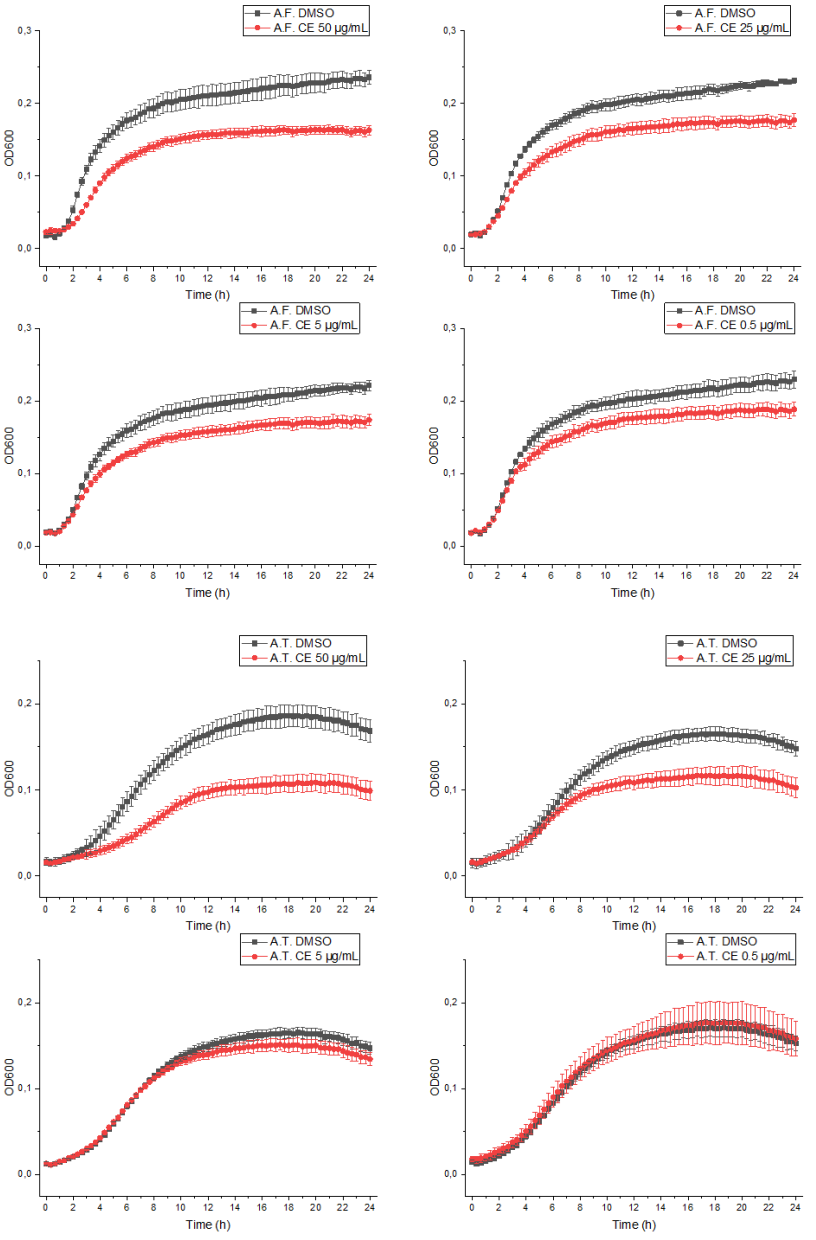
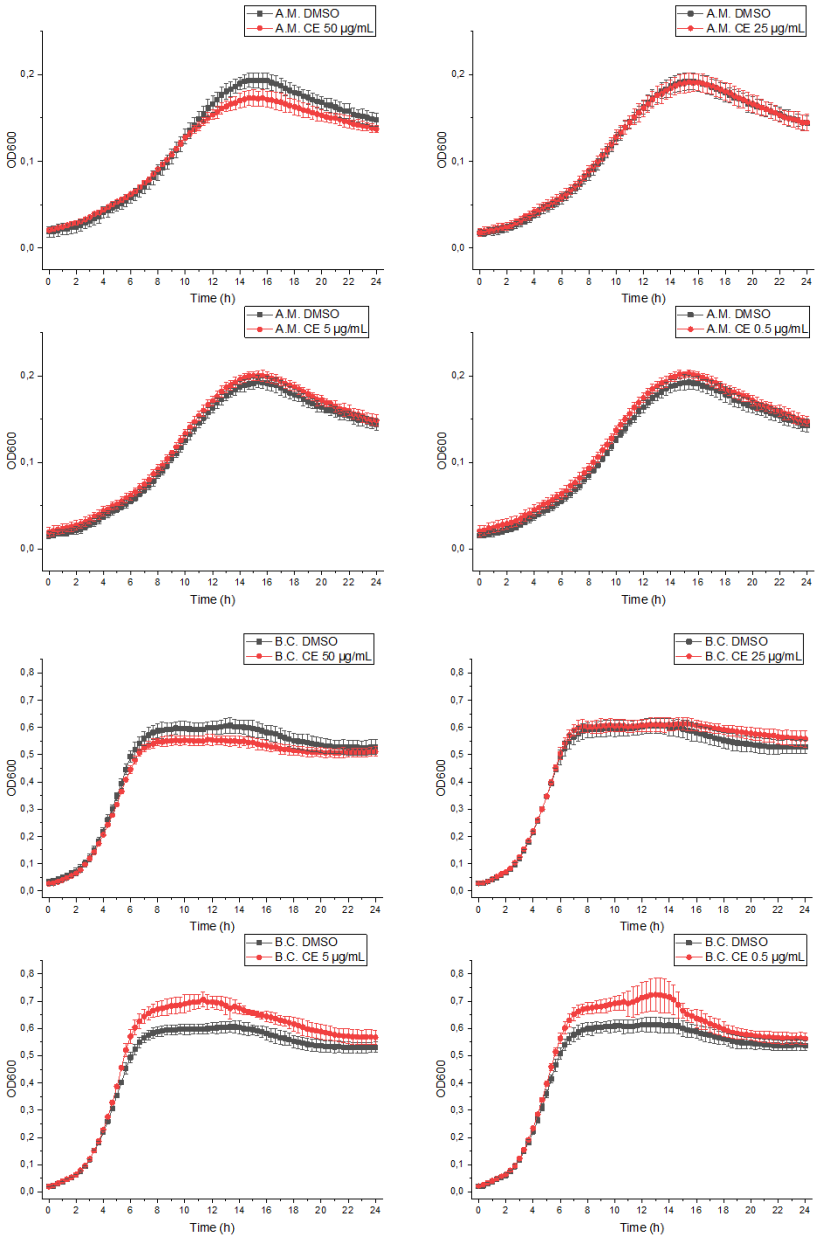


Figure S1. Growth curves of the strains exposed to various concentrations of the complex *Alternaria* extract (CE). The growth curves are expressed as mean values (tree replicates) ± SD of optical densities measured at 600 nm. DMSO indicate the growth curves of the respective controls (0.1% - 0.001% DMSO).
AF: *A. finegoldii*; AT: *A. timonensis*; AM: *A. muciniphila*; BC: *B. caccae*; BE: *B. eggerthii*; BT: *B. thetaiotaomicron*; BV: *B. vulgatus*; PD: *P. distasonis*; EC: *E. coli*; LH: *L. hominis*; BL: *B. longum*; B. sp.: *Bifidobacterium sp.*; CI: *C. innocuum*; RB: *R. bicirculans.*



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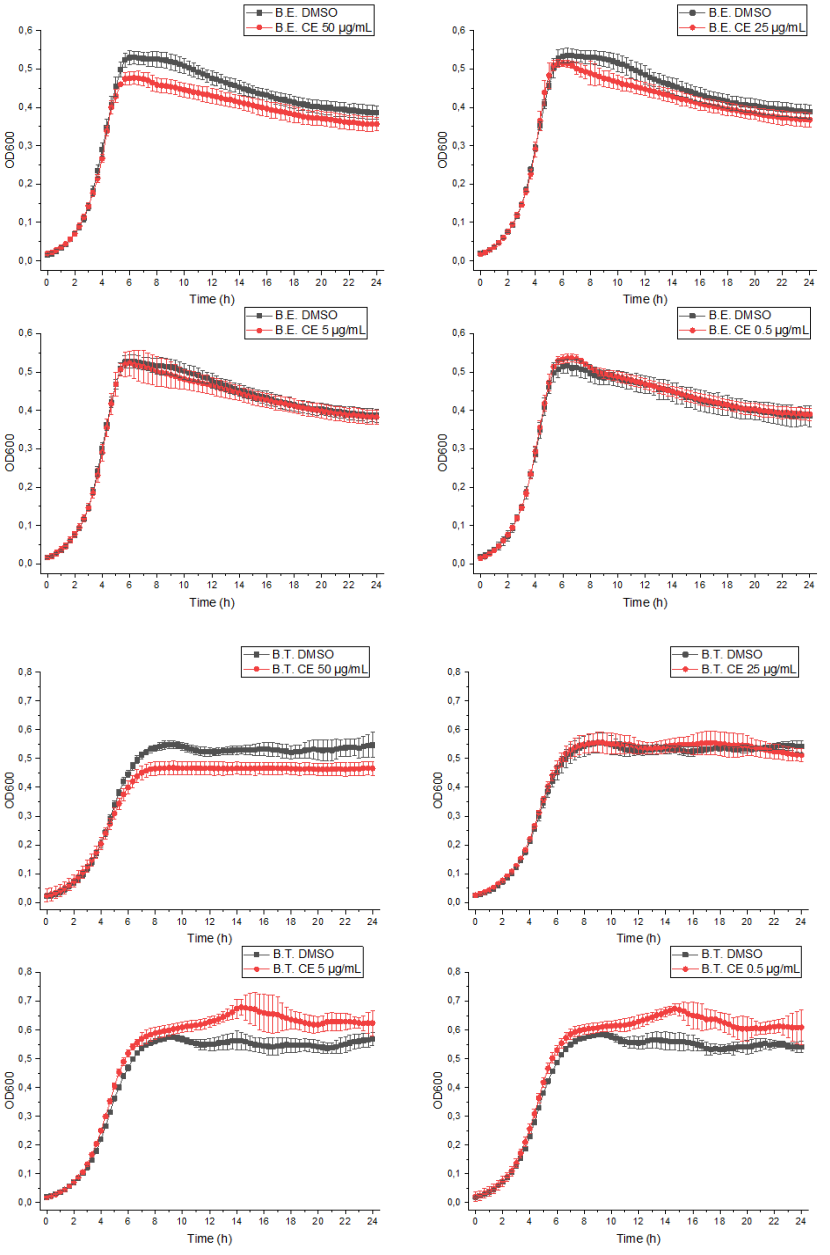

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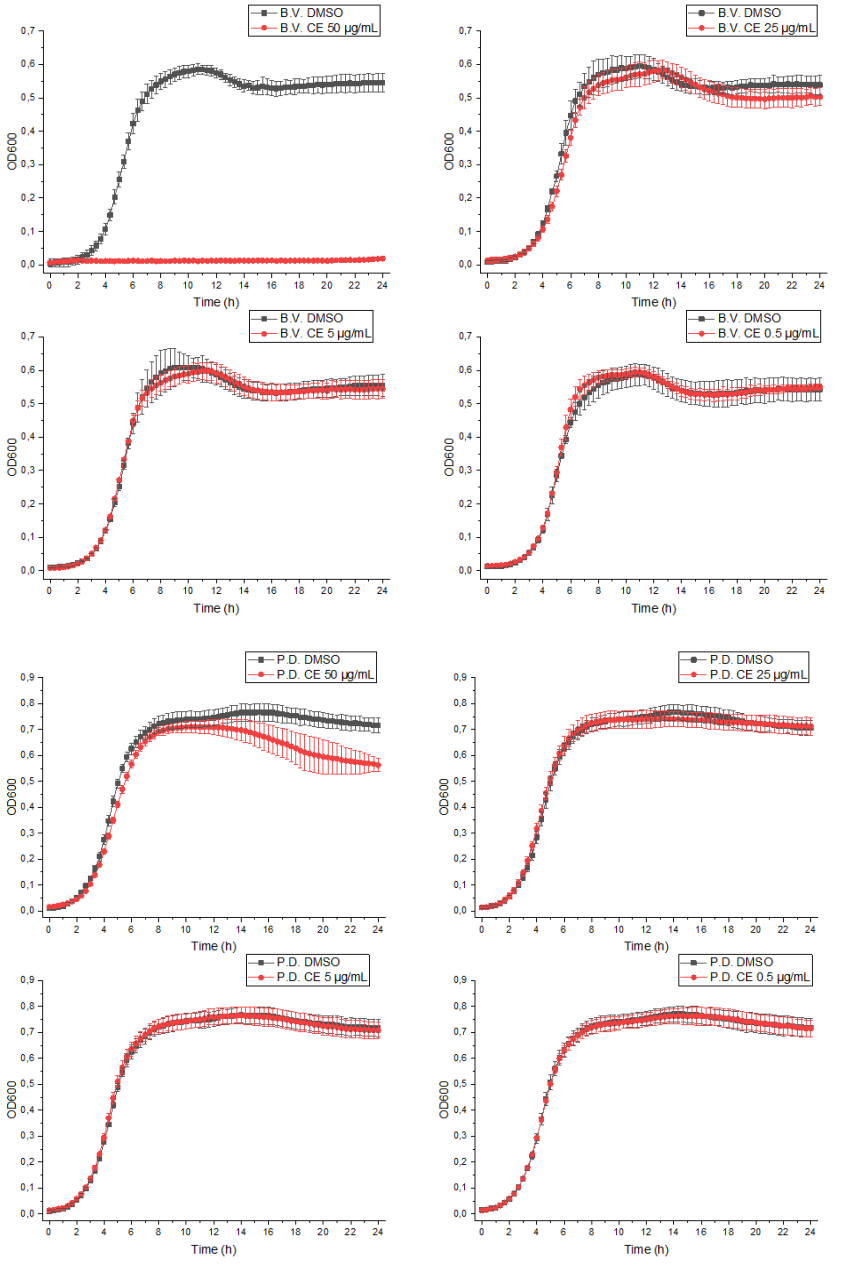

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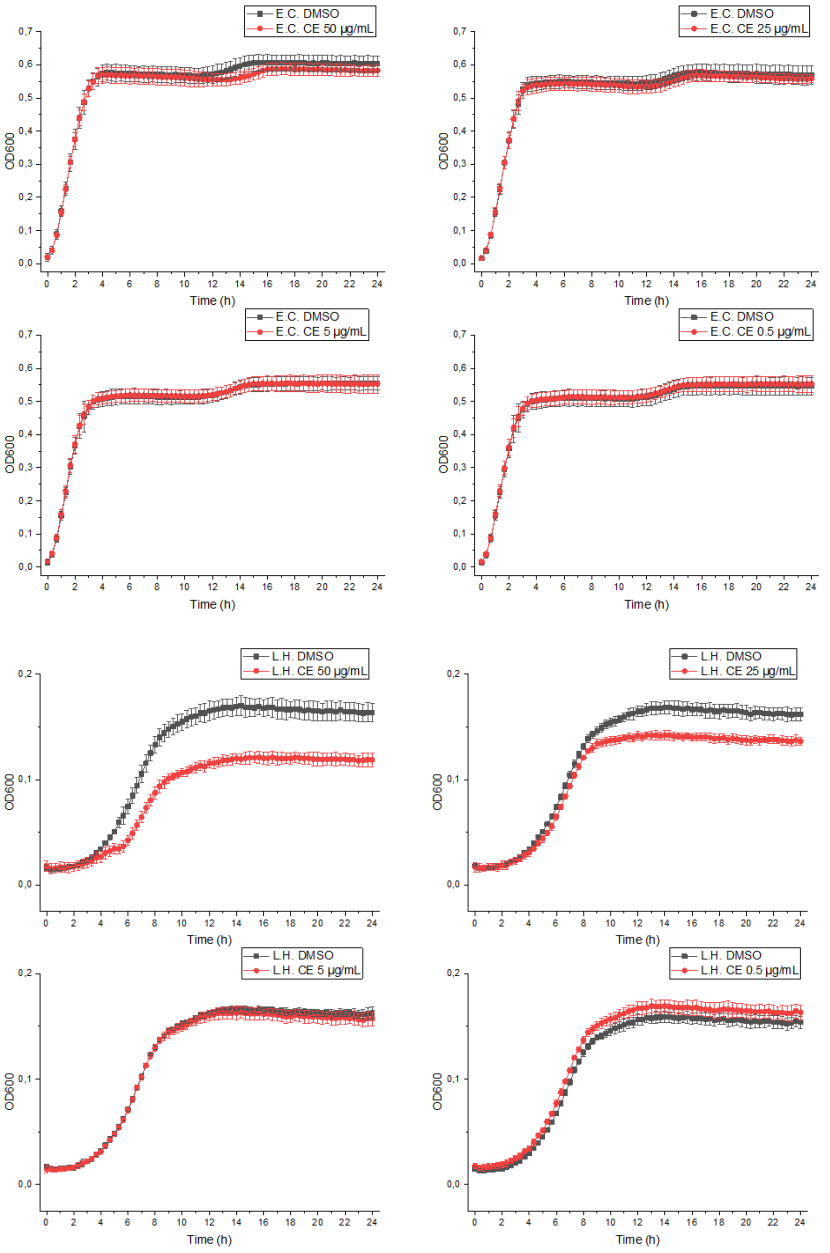

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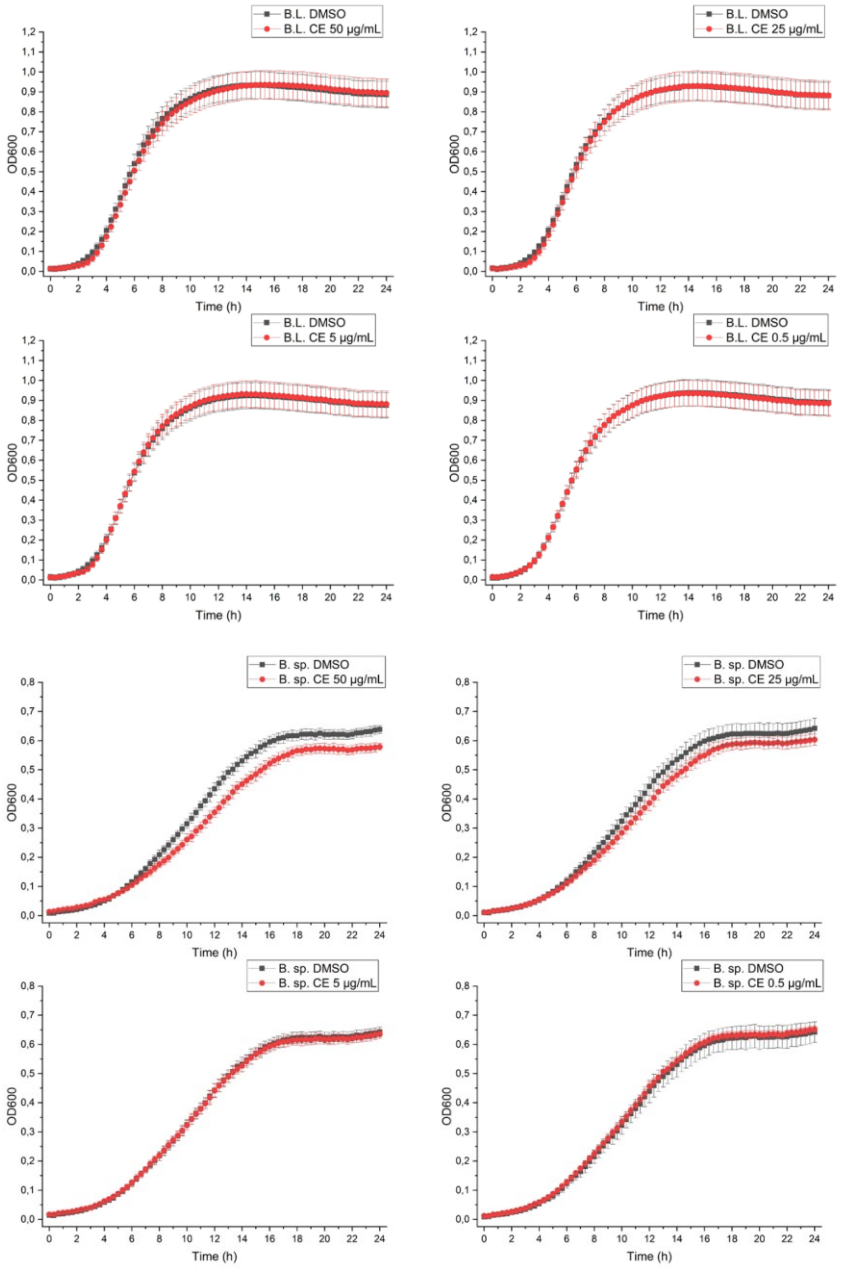

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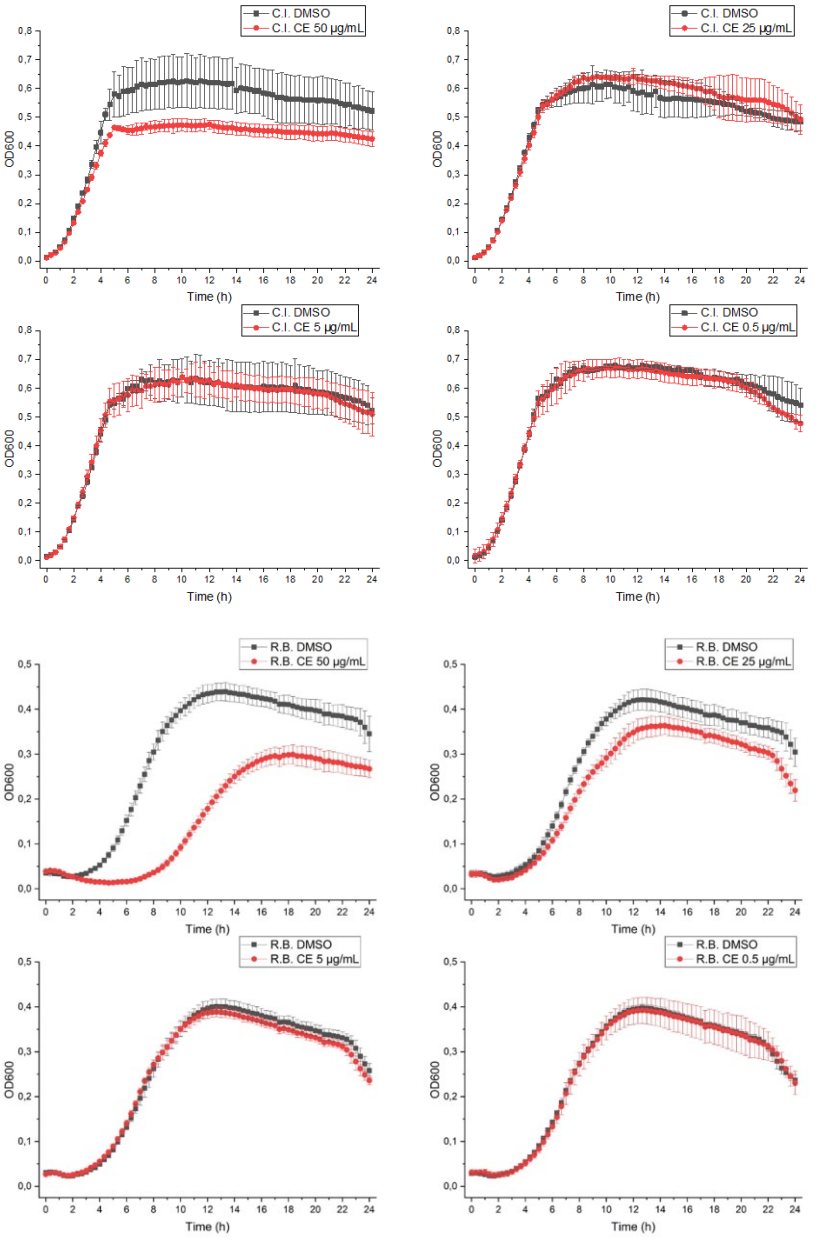

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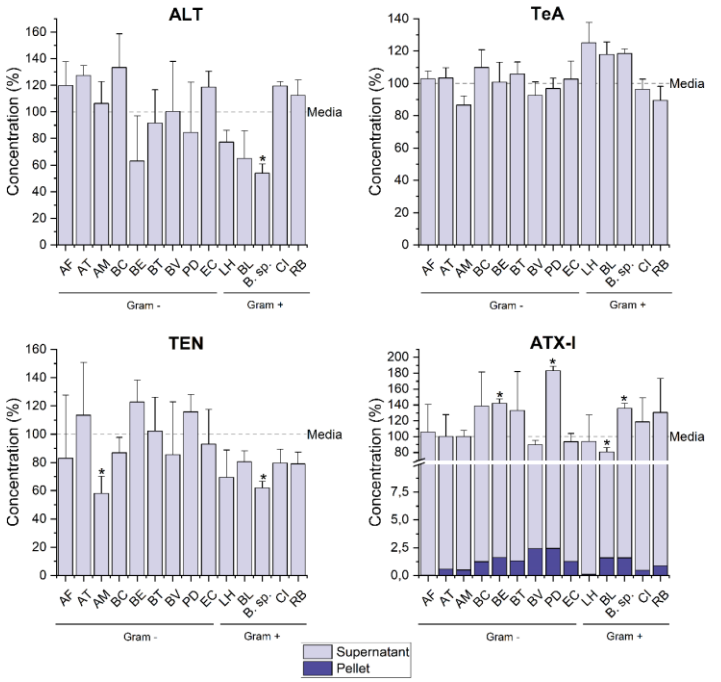

Figure S2. Bar charts showing the amount of the least affected mycotoxins recovered in pellets and supernatants of the tested strains after 24 h incubation with 25 μg/mL of CE. Data are reported as means + SD. Differences between the total mycotoxin recovery in samples and media controls (media + CE) were evaluated by Student's *t*-test (* = $p<0.05$).
AF: *A. finegoldii*; AT: *A. timonensis*; AM: *A. muciniphila*; BC: *B. caccae*; BE: *B. eggerthii*; BT: *B. thetaiotaomicron*; BV: *B. vulgatus*; PD: *P. distasonis*; EC: *E. coli*; LH: *L. hominis*; BL: *B. longum*; B. sp.: *Bifidobacterium sp.*; CI: *C. innocuum*; RB: *R. bicirculans*.

Table S1. Composition of the *Alternaria* mycotoxin extract characterized by LC–MS/MS analysis.

***Alternaria* mycotoxins (Abbreviations)**	**Concentration (mg toxin / g extract)**
Alternariol (AOH)	0.79
Alternariol monomethyl ether (AME)	0.65
Altenuene (ALT)	0.78
Tenuazonic acid (TEA)	597
Tentoxin (TEN)	0.02
Altertoxin I (ATX-I)	9.92
Altertoxin II (ATX-II)	14.1
Alterperylenol (ALP)	12.6
Stemphyltoxin III (STTX-III)	21.0
Altenusin (ALS)	0.28
Altersetin (AST)	18.4

Table S2. Bacterial strains and media employed in the study.

Strain	Abbr.	Received from	Phylum	Gram staining	Medium[2]
*Alistipes finegoldii*_AR1 (99% 16S rRNA gene sequence similarity with *A. finegoldii* DSM 17242[1])	*AF*	*Own isolation*	Bacteroidetes	–	YCFA-g
Alistipes timonensis DSM25383	*AT*	*DSMZ*	Bacteroidetes	–	YCFA-g
Akkermansia muciniphila DSM22959	*AM*	*DSMZ*	Verrucomicrobia	–	BHI-muc
Bacteroides caccae DSM19204	*BC*	*DSMZ*	Bacteroidetes	–	m-BHI
Bacteroides eggerthii DSM20697	*BE*	*DSMZ*	Bacteroidetes	–	m-BHI
Bacteroides thetaiotaomicron DSM2079	*BT*	*DSMZ*	Bacteroidetes	–	m-BHI
Bacteroides vulgatus DSM1447	*BV*	DSMZ	Bacteroidetes	–	m-BHI
*Parabacteroides distasonis*_AR2 (99% 16S rRNA gene sequence similarity with *P. distasonis* DSM 20701[1])	*PD*	*Own isolation*	Bacteroidetes	–	m-BHI
Escherichia coli ATCC23716	*EC*	*ATCC*	Proteobacteria	–	m-BHI
Lactobacillus hominis DSM23910	*LH*	*DSMZ*	Firmicutes	+	m-MRS
Bifidobacterium longum subsp. infantis ATCC 15697	*BL*	ATCC	Actinobacteria	+	m-MRS
*Bifidobacterium*_sp.AR3 (95% 16S rRNA gene sequence similarity with *B. longum*[1])	*B. sp*	*Own isolation*	Actinobacteria	+	m-MRS
Clostridium innocuum DSMZ1286	*CI*	*DSMZ*	Firmicutes	+	m-BHI
Ruminococcus bicirculans _AR4 (99% 16S rRNA gene sequence similarity with *Ruminococcus bicirculans 80/3*[1])	*RB*	*Own isolation*	Firmicutes	+	YCFA-g

[1]In-house isolated strains are identified to genus or species level based on the best BLAST (Morgluis *et al.* 2008, *Bioinformatic*s; https://pubmed.ncbi.nlm.nih.gov/18567917/) result for their 16S rRNA gene sequence similarity.
[2]YCFA-g: yeast extract-casein hydrolysate-fatty acids broth supplemented with glucose; BHI-muc: brain heart infusion broth supplemented with mucin; m-BHI: modified brain heart infusion broth; m-MRS: modified De Man, Rogosa, Sharpe broth.

Table S3. OD600 values recorded after 24 h and 48 h incubation with various concentrations of the Alternaria extract, each in comparison to the respective solvent control (0.1-0.001% DMSO).

* Significant different compared to control with DMSO. Differences were evaluated by Student's *t*-test ($p<0.05$).

Strain	Time	OD_{600} (mean ± SD)											
		Treatment with 50 µg/mL of CE						Treatment with 25 µg/mL of CE					
		Control			Treated			Control			Treated		
E. coli	24 h	0.59	±	0.04	0.58	±	0.03	0.59	±	0.03	0.58	±	0.03
	48 h	0.56	±	0.04	0.56	±	0.03	0.56	±	0.03	0.55	±	0.04
B. vulgatus	24 h	0.59	±	0.09	0.04	±	0.02*	0.60	±	0.07	0.52	±	0.05
	48 h	0.62	±	0.04	0.02	±	0.01*	0.63	±	0.08	0.56	±	0.04
B. thetaiotaomicron	24 h	0.64	±	0.04	0.63	±	0.03	0.64	±	0.03	0.63	±	0.04
	48 h	0.56	±	0.03	0.56	±	0.03	0.56	±	0.04	0.56	±	0.03
B. caccae	24 h	0.66	±	0.05	0.64	±	0.04	0.65	±	0.02	0.64	±	0.03
	48 h	0.66	±	0.04	0.61	±	0.04	0.67	±	0.06	0.61	±	0.03

Table S3. Continue.

Strain	Time	OD_{600} (mean ± SD)											
		Treatment with 5 µg/mL of CE						Treatment with 0.5 µg/mL of CE					
		Control			Treated			Control			Treated		
E. coli	24 h	0.58	±	0.03	0.58	±	0.04	0.58	±	0.04	0.57	±	0.02
	48 h	0.56	±	0.02	0.56	±	0.03	0.55	±	0.02	0.56	±	0.04
B. vulgatus	24 h	0.58	±	0.04	0.55	±	0.04	0.58	±	0.06	0.63	±	0.04
	48 h	0.60	±	0.03	0.58	±	0.06	0.61	±	0.03	0.60	±	0.06
B. thetaiotaomicron	24 h	0.62	±	0.04	0.63	±	0.03	0.62	±	0.03	0.63	±	0.03
	48 h	0.56	±	0.04	0.56	±	0.04	0.56	±	0.03	0.55	±	0.03
B. caccae	24 h	0.65	±	0.03	0.66	±	0.04	0.67	±	0.03	0.65	±	0.04
	48 h	0.63	±	0.02	0.65	±	0.03	0.72	±	0.03	0.66	±	0.03

Table S4. Total recovery of *Alternaria* mycotoxins after 24 h incubation of strains with 25 μg/mL of CE

Strain	AOH	AME	ALT	TeA	TEN
A. finegoldii	52.0* ± 5.0	69.3* ± 2.1	120 ± 18	103 ± 5	83.0 ± 44.6
A. timonensis	69.3* ± 19.2	69.6* ± 10.1	127 ± 8	103 ± 6	114 ± 37
A. muciniphila	53.7* ± 10.8	92.9 ± 13.3	106 ± 16	86.6 ± 5.6	58.1* ± 12.1
B. caccae	61.6* ± 15.3	75.0* ± 19.0	133 ± 25	109 ± 11	86.8 ± 10.9
B. eggerthii	118 ± 11	72.4* ± 10.0	63.1 ± 33.9	100 ± 12	122 ± 16
B. thetaiotaomicron	42.3* ± 2.5	77.3* ± 18.0	91.6 ± 25.1	106 ± 7	102 ± 24
B. vulgatus	58.1* ± 11.5	91.4* ± 2.3	100 ± 38	92.5 ± 8.5	85.5 ± 37.5
P. distasonis	80.2* ± 3.4	73.2* ± 4.7	84.4 ± 37.8	96.9 ± 6.4	116 ± 12
E. coli	33.5* ± 4.4	55.4* ± 12.0	119 ± 12	103 ± 11	93.0 ± 24.5
L. hominis	60.7* ± 20.3	87.2 ± 29.5	77.2 ± 8.9	125 ± 13	69.5 ± 19.4
B. longum	46.1* ± 6.0	102 ± 28	65.0 ± 20.9	118 ± 8	80.5 ± 7.6
Bifidobacterium sp.	102 ± 16	75.3* ± 8.6	53.8* ± 7.0	119 ± 3	62.0* ± 4.8
C. innocuum	50.1* ± 4.2	84.5* ± 10.3	120 ± 3	96.3 ± 6.4	79.7 ± 9.8
R. bicirculans	80.8 ± 17.4	90.4 ± 14.1	112 ± 12	89.4 ± 8.7	79.0 ± 8.4

* Significant different compared to control with DMSO. Differences were evaluated by Student's *t*-test ($p<0.05$).

Table S4. Continue.

Strain	ATX-I	ALP	ALS	AST
A. finegoldii	106* ± 35	34.8* ± 8.8	102* ± 15	30.6* ± 2.5
A. timonensis	100 ± 28	64.5* ± 26.7	87.5 ± 8.8	57.6* ± 14.6
A. muciniphila	100 ± 3	56.4* ± 1.6	90.8 ± 4.2	60.0* ± 8.5
B. caccae	138 ± 43	24.7* ± 7.6	83.5 ± 10.8	70.5* ± 17.2
B. eggerthii	142* ± 2	57.9* ± 6.7	93.1 ± 13.3	64.3* ± 6.3
B. thetaiotaomicron	133 ± 49	20.3* ± 4.6	92.4 ± 12.7	61.9* ± 15.0
B. vulgatus	89.8 ± 1.7	24.4* ± 5.3	98.7 ± 13.0	74.5* ± 11.7
P. distasonis	183 ± 4	26.3* ± 2.5	86.6 ± 4.9	50.7* ± 16.7
E. coli	93.7 ± 10.4	31.0* ± 1.7	93.5 ± 10.2	48.7* ± 6.9
L. hominis	93.8 ± 33.5	101 ± 36	56.1* ± 8.1	68.3* ± 4.6
B. longum	80.8* ± 2.5	75.0* ± 1.6	49.9* ± 4.6	73.2* ± 10.2
Bifidobacterium sp.	136* ± 6	117* ± 2	51.4* ± 8.9	56.8* ± 4.8
C. innocuum	119 ± 31	19.0* ± 5.0	93.6 ± 15.7	87.7 ± 20.0
R. bicirculans	131 ± 43	14.0* ± 3.3	86.4 ± 24.8	91.9 ± 33.4

* Significant different compared to control with DMSO. Differences were evaluated by Student's t-test ($p<0.05$).

Table S5. Concentrations of mycotoxins the bacterial strains were exposed to during treatments with various concentrations of the *Alternaria* extract.

Mycotoxins	Mycotoxin amounts (nM) per treatment			
	50 µg/mL	25 µg/mL	5 µg/mL	0.5 µg/mL
AOH	153	76.5	15.3	1.53
AME	119	59.7	11.9	1.19
ALT	133	66.7	13.3	1.33
TeA	151599	75799	15160	1516
TEN	2.41	1.21	0.24	0.02
ATX-I	1407	704	141	14.1
ATX-II	2012	1006	201	20.1
ALP	1798	899	180	18.0
STTX-III	3014	1507	301	30.1
ALS	48.2	24.1	4.82	0.48
AST	2300	1150	230	23.0

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5

Chapter

Research Article

An *in vitro* study on the transport and phase II metabolism of the mycotoxin alternariol in combination with the structurally related gut microbial metabolite urolithin C

Francesco Crudo[1], Amelia Barilli[2], Pedro Mena[3], Bianca Maria Rotoli[2], Daniele Del Rio[4], Chiara Dall'Asta[1], Luca Dellafiora[1]*

[1] Department of Food and Drug, University of Parma, Area Parco delle Scienze 27/A, 43124 Parma, Italy

[2] Department of Medicine and Surgery, University of Parma, Via Volturno 39, 43125 Parma, Italy

[3] Human Nutrition Unit, Department of Food and Drug, University of Parma, Via Volturno 39, 43125 Parma, Italy

[4] Human Nutrition Unit, Department of Veterinary Science, University of Parma, Via Volturno 39, 43125 Parma, Italy

* Correspondence: luca.dellafiora@unipr.it

Under Review in Toxicology Letters

Abstract

Alternariol is a mycotoxin produced by *Alternaria* spp. relevant to the food safety area due to its abundance in certain foods. The shortage of data on its toxicology, also as a part of chemical mixtures, prevents setting regulation to limit its abundance in food. To extend knowledge on the possible mechanisms underpinning alternariol toxicology in chemical mixtures, this work assessed the effects of urolithin C, a structurally related gut ellagitannin-derived metabolite, on its absorption and phase II metabolism in a monolayer of Caco-2 cells. A computational study was also used to provide a mechanistic explanation for the results obtained. Urolithin C influenced transport and phase II metabolism of alternariol with a late reduction of transport to the basolateral compartment. Moreover, it caused an early effect in terms of accumulation of alternariol glucuronides in the basolateral compartment, followed by a late reduction of glucuronides in both compartments. Concerning alternariol sulfates, the data collected pointed to a possible competition of urolithin C for the sulfotransferases, resulting in a reduced production of alternariol sulfates. Our results provide a compelling line-of-evidence pointing to the need to systematically tackle the evaluation of mycotoxin toxicity in the context of chemical mixture.

1. Introduction

The contamination of food with environmental pollutants, drug and chemical residues, microbial pathogens, and the wide array of other anthropogenic and natural types of contaminants represents a serious public health concern at a global scale. In this context, mycotoxins, a class of food contaminants of natural origin, are gaining an increasing interest due to their potential toxic effects on human and animal and to their high prevalence in food. In this respect, in 1998 the Food and Agriculture Organization estimated an overall global prevalence of 25% of mycotoxins in agricultural commodities, but recent findings suggest a possible much higher level (up to 80%, depending either on the mycotoxin or crop type) (Eskola et al., 2019). In addition, a recent work suggested the need to re-evaluate the occurrence of some mycotoxins due to the application of sample preparation procedures that may have underestimated their real content in food (Aichinger et al., 2020c). Mycotoxins are produced by several types of molds, mainly belonging to the genera *Alternaria, Fusarium, Aspergillus* and *Penicillium*. In particular, *Alternaria* spp. can produce more than 70 toxins, including some potentially harmful for humans and animals due to the high occurrence in both fresh and processed food and the adverse effects observed *in vitro* and *in vivo* (e.g. genotoxic, mutagenic, clastogenic, androgenic, estrogenic, fetotoxic and teratogenic effects) (Crudo et al., 2019). Among the best studied *Alternaria* mycotoxins, the dibenzo-α-pyrone alternariol (AOH) has been reported to induce DNA strand breaks and to poison topoisomerases, exerting genotoxic effects (Fehr et al., 2009). Several studies described AOH as an endocrine disruptor, being able to affect several hormonal pathways. In particular, AOH may exert estrogenic effects activating α and β estrogen receptors, although with a lower potency compared to its monomethyl ether derivative alternariol-9-methyl ether (AME) (Dellafiora et al., 2018; Lehmann et al., 2006). An increase of progesterone and estradiol level due to AOH treatment, as well as the ability of AOH to induce androgenic effects, were also reported in *in vitro* experiments (Frizzell et al., 2013; Stypuła-Trębas et al., 2017). Furthermore, AOH may suppress the lipopolysaccharide-induced innate immune

responses and increase and reduce the transcription of anti-inflammatory and pro-inflammatory cytokines, respectively (Grover and Lawrence, 2017; Kollarova et al., 2018).

Considering the scenario described so far, AOH has been referred to as an emerging mycotoxin and further studies on its toxicity are required to define a suitable and sufficiently informed background of knowledge for risk assessment (EFSA, 2011). Despite the human dietary exposure to AOH and AME can exceed the threshold of toxicological concern value (TTC) established for potential genotoxic compounds (2.5 ng/kg/day) (Arcella et al., 2016), no regulation is currently available for *Alternaria* mycotoxins in food due to the limited understanding of their occurrence and toxicity. In this context, the toxicological assessment of *Alternaria* mycotoxins in chemical mixtures is still rarely accounted although the *in vitro* data available so far describe that AOH in chemical mixtures can elicit a toxicological response different than that produced when tested alone (e.g. Aichinger et al., 2020b). In this light, the combined assessment of mycotoxin toxicity with chemicals derived from food components transformed by the gut microbiota should also be taken into account, considering that these metabolites may become part of the fraction potentially bioavailable and/or bioaccessible and able to interfere with mycotoxin toxicology.

Whit this respect, the human gut microbiota plays a key role in preserving human health due to its ability to modulate essential physiological functions (Clemente et al., 2012). As an example, it has a role in the metabolism of indigestible carbohydrates, in the synthesis of vitamins, in the modulation of the host's immune system, as well as in the detoxification processes of food-related xenobiotics, including mycotoxins (Clemente et al., 2012; Rowland et al., 2018). The capability of microorganisms to bind and/or modify mycotoxins, reducing their absorption or bioactivity, underlines the detoxification action (Crudo et al., 2020; Liew and Mohd-Redzwan, 2018). Of note, the gut microbiota interacts with a variety of other food-related phytochemical compounds that are introduced in concurrence with mycotoxins. In addition, several studies have reported that microbial metabolites derived from phytochemicals may have an enhanced intestinal absorbability or a higher biological activity compared to the respective

native compound (Corrêa et al., 2019). On this basis, it is interesting to investigate if some of the gut metabolites derived from food-related phytochemicals might interfere with AOH toxicology. In this framework, pomegranate, raspberries, strawberries and walnuts are example of foods rich of ellagitannins, a class of hydrolysable tannins (Del Rio et al., 2013). In the gut these compounds are metabolized to ellagic acid, which is further metabolized by the gut microbiota into a series of polyphenolic compounds referred to as urolithins. Urolithins are known to be more rapidly and easily absorbed compared to their precursors and to exert several protective effects on human health (Zanotti et al., 2015). Urolithin A, B, C, and D are those better characterized so far, and they differ in the number/position of hydroxy groups. In particular, urolithin C (UroC) has a chemical structure closely related to AOH (Figure 1) as they both have a trihydroxybenzo[c]chromen-6-one scaffold with a very similar pattern of hydroxylation.

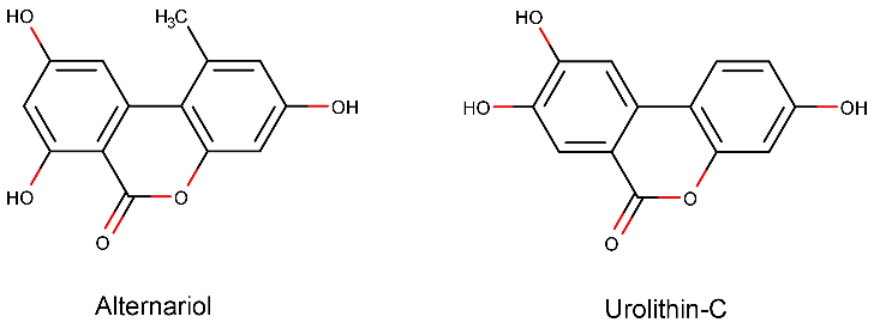

Figure 1. Chemical structures of alternariol and urolithin C.

The structural similarity provides the rational to hypothesize that AOH and UroC may share a certain degree of functional conservation in terms of mechanisms of action and biological targets, including a possible competition for phase I and II metabolism or for other cellular enzymes or transporters. Particularly, the competition for transporters and metabolizing

enzymes might change the production and the intestinal absorption of AOH and its metabolites.

The present work dealt with the mechanistic aspects of this possible competition, investigating whether UroC may influence the transport of AOH and the formation of its phase II metabolites (considered the most abundant forms reasonably circulating after AOH ingestion) in a polarized monolayer of Caco-2 cells (taken as a model system for the GI tract).

2. Materials and Methods

2.1. Chemicals

AOH and UroC (or 3,8,9-trihydroxy-urolithin, according to the nomenclature proposed by Kay et al. (2020) were purchased from Sigma-Aldrich (Milan, Italy) and Dalton Pharma Services (Toronto, ON, Canada), respectively. Methanol and acetonitrile (LC-MS grade), used for LC-MS/MS sample preparation and analysis, were supplied from Sigma Aldrich (Milan, Italy), while bi-distilled water was obtained in house by using an Alpha-Q system (Millipore, Marlborough, MA, USA). For cell culture experiments RPMI-1640, fetal bovine serum (FBS) and penicillin/streptomycin solution were purchased from Euroclone (Milan, Italy). Consumables for tissue culture were acquired from Microtech s.r.l. (Napoli, Italy).

2.2. Cell culture and experimental treatment

The human epithelial colorectal adenocarcinoma Caco-2 cell line was obtained from the Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia-Romagna (Brescia, Italy). Cells were routinely grown as a monolayer in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin solution (37°C; 5% CO_2) and passaged twice per week. For cell viability assays, cells were seeded at a cell density of 30000 cells/well into 96-well plates and allowed to grow for 24h. Afterward, the spent medium was removed and replaced with fresh medium containing 30 μM AOH, 30 μM UroC or the combination of AOH+UroC (30 μM + 30 μM). Since all compounds were dissolved in DMSO that, in all cases, reached the final percentage of 1%, the same concentration of the solvent was added to

control cells. Cell viability was measured after 6 and 24h incubation with the test media. To investigate the transport of AOH, UroC and corresponding metabolites through an intact monolayer of Caco-2 cells, polarized monolayers of cells were prepared by seeding 3×10^5 cells/ml cells on cell culture inserts (12 mm in diameter, pore size 0.4 μm; Falcon), with apical (AP) and basolateral (BL) fluid volumes of 350 and 900 μl, respectively. Growth medium was renewed every second day and formation of tight junctions was monitored by measuring the transepithelial electrical resistance (TEER) with an EVOM epithelial voltmeter (World Precision Instruments, Sarasota, FL, USA). Cell monolayers were used when TEER reached values >800 $\Omega\cdot cm^2$, thus indicating a tight monolayer (Narai et al., 1997). At this time, 350 μL fresh FBS-free medium containing 1% DMSO or 30 μM AOH or 30 μM UroC or the combination of AOH+UroC (30 μM + 30 μM) were added to the apical compartment, while 900 μL of fresh FBS-free medium were added to the basal one. Cells were incubated at 37°C in a humidified incubator (5% CO_2) for 24h. Aliquots (50 μL) of medium from the AP and BL sides were collected after 0h, 2h, 4h, 6h, and 24h of incubation and stored at -80°C until the time of analysis.

2.3. Cell viability assays

Cell viability was monitored after 6h and 24h incubation of Caco-2 cells with 1% DMSO or 30 μM AOH or 30 μM UroC or the combination of AOH+UroC (30 μM + 30 μM), as previously described (Barilli et al., 2008). Briefly, the test medium in each well was replaced with FBS-free medium containing 44 mM of the dye resazurin. After 2 h incubation, the fluorescence deriving from the intracellular reduction of resazurin to resorufin was measured at 572_{nm} by employing a Wallac 1420 Victor² Multilabel Counter (Perkin Elmer, Monza, Italy). Each condition was tested in triplicate and in at least three independent experiments. Data were expressed as mean ± SD (in percent compared to cells treated with DMSO).

2.4. Sample preparation and LC-MS^n analysis

For LC-MS^n sample preparation, samples collected from the AP and BL sides during the polarized monolayers assay (0h, 2h, 6h, and 24h) were diluted 1:1

(v/v) with ice-cold methanol and vortexed for 1 min. Then, samples were centrifuged at 20000 rcf (5 min; 4°C), supernatants were collected and diluted 1:1 (v/v) with bi-distilled water containing 0.1% formic acid. Finally, samples were transferred into vials and stored at -80°C or immediately analyzed.

Samples were analysed according to Mele et al. (2016) with minor modifications. Briefly, samples were run using an Accela UHPLC 1250 equipped with a linear ion trap-mass spectrometer (LTQ XL, Thermo Fisher Scientific) fitted with a heated-electrospray ionization probe (H-ESI-II). Separations were performed using a XSELECTED HSS T3 (50 × 2.1 mm), 2.5 μm particle size (Waters, Milford, MA, USA), with an injection volume of 5 μL, column oven temperature of 40 °C and elution flow rate of 0.5 mL/min. The initial gradient was 90% of 0.1% aqueous formic acid and 10% acetonitrile (in 0.1% formic acid), reaching 70% acetonitrile at 6 min. The MS conditions included: capillary temperature of 275 °C and source heater temperature of 250 °C, sheath gas flow of 60 units, auxiliary gas of 20 units, sweep gas of 3 units, source voltage of 3.0 kV, and capillary voltage and tube lens of −21 and −88 V, respectively. Analyses were carried out using specific MS^2 full scans corresponding to the parent compounds (AOH and UroC), the putative phase II metabolites glucuronide and sulfate of AOH, and the sulfate forms of UroC, with collision induced dissociation (CID) equal to 35 (arbitrary units). Pure helium gas was used for CID. Data processing was performed using Xcalibur software from Thermo Scientific. Quantification of AOH was performed with calibration curve of pure standard, while UroC and phase II metabolites were reported as the area under the curve (AUC) of the chromatographic peak.

2.5. Sulfotransferase enzyme (SULT) modeling and ligands preparation

In the present study SULT1A1 form was chosen as SULTs representative due to its ability to preferentially metabolize polyphenolic compounds (Ung and Nagar, 2007) and for being one of the most expressed SULT form in the Caco-2 cell line (Meinl et al., 2008). The model of human SULT 1A1 was derived from the crystallographic structure recorded in the Protein Data Bank (PDB; https://www.rcsb.org) having code 2D06 (Gamage et al., 2005).

The crystallographic structure was processed using Sybyl version 8.1 (www.certara.com) checking the consistency of atom and bond types assignment and removing water molecules and co-crystalized ligands, with the exception of the co-factor that was kept for the analysis, in agreement with previous studies (Dellafiora et al., 2015). The occasional missing coordinates of surface residue side-chains were computationally added using "Mutate Monomer" option of the Biopolymer-Composition tool. The chemical structures of AOH, UroC and 17β-estradiol (E2) were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov) (Kim et al., 2019) in the 3D structure-data file (sdf) format.

2.6. Docking simulation

Docking simulations were performed using GOLD software (Genetic Optimization for Ligand Docking, version 2020, (Cozzini and Dellafiora, 2012)) as it already showed reliability to compute protein-ligand interactions (e.g. ref. (Maldonado-Rojas and Olivero-Verbel, 2011; Rollinger et al., 2006)). The occupancy of the binding site was set within a 10 Å sphere radius around the centroid of the pocket. Software setting and docking protocol previously reported were used (Dellafiora et al., 2017) with minor modifications. The GOLD's internal scoring function GOLDScore was used to score docking poses as it succeeded in analyzing the reference compound (*vide infra*). Specifically, GOLDScore fitness considers the external (protein-ligand complex) and internal (ligand only) van der Waals energy, protein-ligand hydrogen bond energy and ligand torsional strain energy. In each docking study, the proteins were kept semi-flexible and the polar hydrogen atoms were set free to rotate. The ligands were set fully flexible.
GOLD implements a genetic algorithm that may introduce variability in the results. Therefore, each simualtions were performed in triplicates to avoid non-causative score assignments and results were expressed as mean ± standard deviation (SD).

2.7. Pharmacophoric analysis

The pocket was defined using GetCleft software (Gaudreault et al., 2015), while the respective pharmacophoric imagines were derived using the

IsoMIF software (Chartier and Najmanovich, 2015). Default parameters were used. As exception, the maximum distance value between the grid and residues atoms was set at 3, and a grid resolution of 1 Å was used.

2.8. Molecular dynamics

Molecular dynamic simulations were performed to investigate the dynamic of ligands interaction with the surface pocket at the protein-protein interface. The best scored binding poses calculated by docking simulations were used as input. MD simulations were performed using GROMACS (version 5.1.4) (Abraham et al., 2015) with CHARMM27 all-atom force field parameters support (Best et al., 2012). All the ligands have been processed and parameterized with CHARMM27 all-atom force field using the SwissParam tool (http://www.swissparam.ch). Input structures were solvated with SPCE waters in a cubic periodic boundary condition, and counter ions (Na^+ and Cl^-) were added to neutralize the system. Prior to MD simulation, the systems were energetically minimized to avoid steric clashes and to correct improper geometries using the steepest descent algorithm with a maximum of 5,000 steps. Afterwards, all the systems underwent isothermal (300 K, coupling time 2psec) and isobaric (1 bar, coupling time 2 psec) 100 psec simulations before running 50 nsec simulations (300 K with a coupling time of 0.1 psec and 1 bar with a coupling time of 2.0 psec).

2.9. Statistical analysis

Homogeneity of variance of *in vitro* data was preliminary evaluated with Levene's test. Differences between single and combined treatments were evaluated by Independent Student's *t* test (α = 0.05 or 0.01) and analysis of variance (one-way ANOVA) with Fisher's LSD post hoc test (α = 0.05). *In silico* data was analyzed by one-way ANOVA (α = 0.05), followed by post hoc Fisher's LSD test (α = 0.05). Statistical analyses were performed using SPSS software (v. 23.0, SPSS inc., Chicago, IL, USA).

3. Results

3.1. Cell viability

Possible cytotoxic effects of AOH and UroC at the concentrations under analysis were first assessed either in combination or as single treatment in Caco-2 cells through the resazurin cell viability test after 6 and 24h of incubation. As shown in Supplementary material, Figure S1, neither AOH or UroC at 30 μM nor their combination (30 μM + 30 μM) resulted in appreciable changes in cell viability compared to the solvent control (1% DMSO) after 6h or 24h.

3.2. Absorption and metabolism in Caco-2 monolayer

Once assessed the lack of cytotoxic effects for the concentrations and combinations under analysis, the absorption and metabolism of AOH was investigated in a polarized monolayer model of the human intestinal Caco-2 cell line after 2, 4, 6 and 24 h of incubation. The results deriving from the monitoring of the TEER values of cell monolayers exposed to 30 μM AOH, 30 μM UroC or their combination (30 μM + 30 μM) confirmed the maintenance of intact cell monolayers at all the time points and conditions under analysis. As a matter of fact, TEER values were all above 800 Ω·cm2 (Narai et al., 1997) even after 24h of incubation and no statistically significant modifications of the TEER values compared to the solvent control (DMSO 1%) were observed (Supplementary material, Figure S2). Aliquots of the AP and BL sides were collected after 2, 4, 6, and 24 h of incubation from the polarized monolayer either treated with single compounds or their combination to be analyzed in LC-MS^n.

3.2.1 Apical-to-basolateral transport of AOH and UroC

The LC-MS^n analysis of AOH in AP and BL compartments revealed a limited passage through the polarized monolayer up to 6h of incubation either after a single treatment or in combination with UroC (Figure 2). Nevertheless, a significant reduction of AOH level in the AP side was observed at all the time-points and conditions tested compared to the initial treatment time (0h; $p<0.05$), though no significant differences were observed

among samples collected at 2h, 4h and 6h either with AOH alone or in combination with UroC. Conversely, a significantly different level of AOH ($p<0.05$), upon single treatment and in combination with UroC, was observed after 24h of treatment in both AP and BL compartments compared to the other time-points. Of note, the co-treatment with AOH + UroC at 24 h resulted in a significantly higher level of AOH in the AP side compared to cells treated with AOH alone ($p<0.01$), while in the BL compartment the highest level of AOH was observed in cells treated with AOH alone. Although treatment with AOH alone resulted in a higher apical-to-basolateral transport of the mycotoxin, the total AOH recovery (AP + BL sides) was the same for the two treatments (Figure 2).

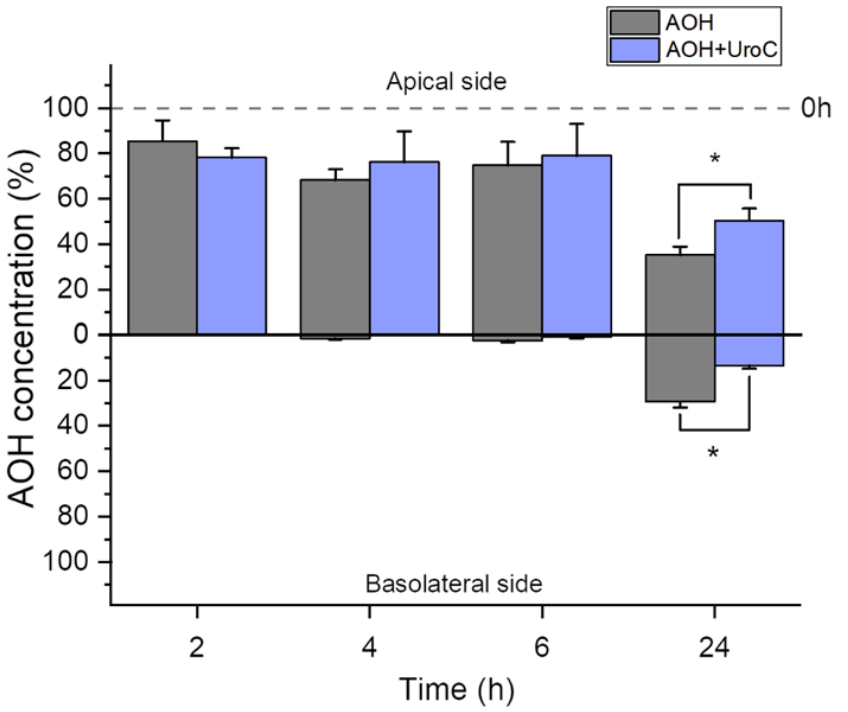

Figure 2. AOH concentrations in apical and basolateral sides after 2, 4, 6, and 24h of incubation of Caco-2 cells with 30 μM AOH or the combination with UroC (30 μM+30 μM). Results are reported as mean + SD of three independent biological experiments and expressed as % of AOH in the two compartments at the diverse time points compared to starting condition (i.e. AOH concentration in the apical side at timepoint 0h). Statistical differences between the two treatments at each time-point were calculated by Student's *t*-test (*$p<0.01$).

The possible mutual effect of AOH on UroC transport was also checked (Supporting material, Figure S3). Similarly to AOH, the level of UroC at all the time-points tested was significantly different from the initial treatment time (0h; $p<0.05$). However, differences among treatment times were observed from 6h treatment onward with an appreciable time-dependency as the amount of UroC in the AP compartment at 24h was significantly lower compared to that observed at 6h (Supporting material, Figure S3). Of note, the level of UroC in the AP compartment was not influenced by the co-treatment with AOH, since no significant differences between single and combined treatments were found. Concerning the BL compartment, UroC was detected only in samples co-incubated with AOH after 24h of incubation.

3.2.2 Formation of AOH metabolites and their passage through the polarized monolayer of Caco-2 cells

The formation and transport of AOH metabolites were also investigated by LC-MS^n analysis. The analysis of AP and BL compartments revealed two peaks associated to glucuronides and only one peak associated to sulfates. Specifically, due to the partial overlapping of the peaks associated with glucuronides, abundance of AOH-glucuronides in AP and BL samples was reported as the sum of the peak area of the two glucuronides (Supplementary material, Figure S4). The absence of certified reference materials for the AOH metabolites prevented to perform a quantitative analysis. However, the kinetic of formation and transport was assessed measuring the percentage variations of peak areas (expressed as AUC%), taking as reference (100%) in the respective compartment the amount of metabolites found after 24h h of incubation with AOH alone. As shown in Figure 3 (left panel), AOH-glucuronides were detected in the AP sides of samples treated with AOH alone or in combination with UroC already after 2h of incubation, with an apparent time-dependency being the level at 24h significantly higher than that at the others time-points ($p<0.01$). In addition, co-treatment with UroC was found influencing the formation of AOH

glucuronides only after 24h of treatment where the level of AOH glucuronides was lower in the case of co-treatment with UroC compared to the treatment with AOH alone ($p < 0.01$). Concerning the BL side, AOH-glucuronides were detected starting from 4h of incubation in samples co-treated with UroC, with a non-statistically different levels between 4h and 6h of treatment and a significantly higher level at 24h compared to the other treatment times ($p<0.01$). Of note, in the case of single treatment with AOH, AOH-glucuronides were present only after 24h of treatment with a significantly higher abundance compared to the co-treatment with UroC at the same time-point ($p<0.01$).

Concerning the production of AOH-sulfate, co-treatment with UroC resulted in a reduced production of AOH-sulfate at all the time-points tested (Figure 3, right panel). Also in this case, an apparent time-dependency of sulfate formation was observed as the level of AOH-sulfate at 24h was significantly higher than the other time-points in both the single and co-treated experiments ($p<0.01$). In addition, while AOH-sulfate was detected in the AP side of samples treated with AOH alone already after 2h of incubation, it was detectable in the AP side of co-treated samples after 4h. The effect of the co-treatment with UroC was even more marked after 24h of incubation, when the content of AOH-sulfate in the AP side of co-treated samples was significantly lower than in samples treated with AOH alone ($p <0.01$). With respect to the BL compartment, AOH-sulfate was detected only at 24h and in cells treated with AOH alone.

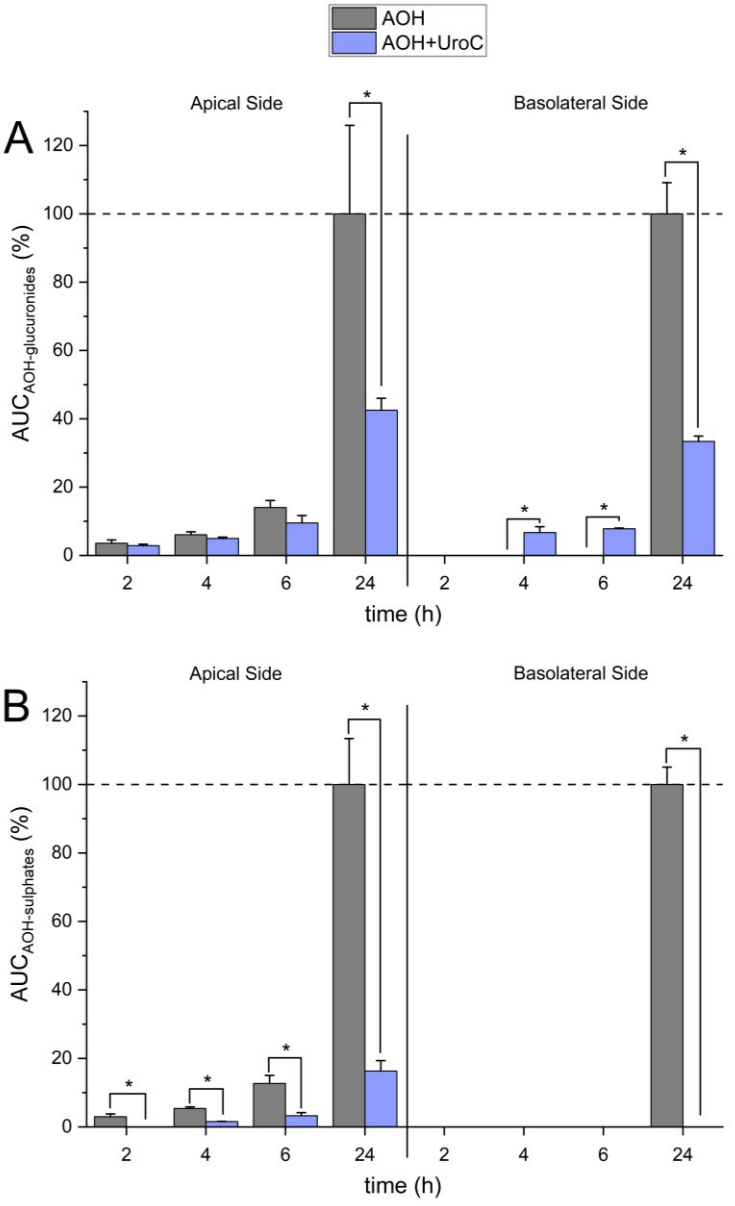

Figure 3. Area under the curve of glucuronides (A) and sulfate (B) of AOH in apical and basolateral sides after 2, 4, 6, and 24h of incubation of Caco-2 cells with 30 μM AOH or the combination of AOH+UroC (30 μM+30 μM). Significance according to Student's *t* test: * $p < 0.01$.

3.3. In silico analysis

Due to the lowering effects of UroC co-treatment on the production of AOH-sulfate at all the time-points considered, the theoretical capability of UroC to

competitively bind sulfotransferase enzymes (SULTs) was further assessed through a 3D *in silico* modeling approach that previously proved versatility to compute protein-ligand complex formation (Dellafiora et al., 2019; Nongonierma et al., 2018). Specifically, the interaction of UroC with SULT1A1 was calculated and compared to that of AOH by mean of a computational workflow relying on docking analysis and molecular dynamic simulation, in agreement with previous studies (Dellafiora et al., 2020). SULT1A1 was taken as SULTs representative considering its substrate preferentiality over (poly)phenolic compounds (Ung and Nagar, 2007) and its prevalent expression in Caco-2 cell line (Meinl et al., 2008). AOH and UroC were docked within the substrate pocket of human SULT1A1 and the collected binding poses were compared to that of E2, taken as reference for high-affinity SULT1A1 substrate (Gamage et al., 2005). As showed in Figure 4A, the calculated pose of E2 (160.3 ±0.1 GOLDScore units) was in agreement with the pocket occupancy reported in crystallographic study (Gamage et al., 2005) supporting the model reliability. AOH and UroC both showed a calculated binding pose resembling that of E2 (149.4±0.3 and 153.0±0.1 GOLDScore units, respectively), though UroC was found better overlaying the crystallographic pose of E2 compared to AOH. Of note, the GOLDScore units amount may be proportionally linked to the capability of ligands to bind a given pocket (Aichinger et al., 2020a). Considering the higher score of E2 compared to AOH and UroC, the docking study provided a consistent substrates rank supporting the model reliability and suggested the possible preferential interaction of UroC within the SULT1A1 pocket compared to AOH. Then, the capability of AOH and UroC to stably persist at the substrate pocket over the time was assessed to probe their theoretical capability to bind the pocket, in agreement with previous studies (Aichinger et al., 2020a). As showed in Figure 4B, the analysis of AOH and UroC trajectories revealed a more persistent capability of UroC to persist within the substrate pocket, while AOH left the binding site drawing an outward trajectory. These results supported the hypothesized higher affinity of UroC as a SULT1A1 substrate compared to AOH. The hypothesized higher affinity for SULT1A1 of UroC compared to that of AOH was further checked measuring the level of UroC-sulfate when cells were treated only with UroC

or in combination with AOH. AOH was found unable to reduce the level of UroC-sulfate, which was comparable in the case of single treatment or co-treatment with AOH, further supporting the higher affinity of UroC for SULTs (Supporting material; Figure S5).

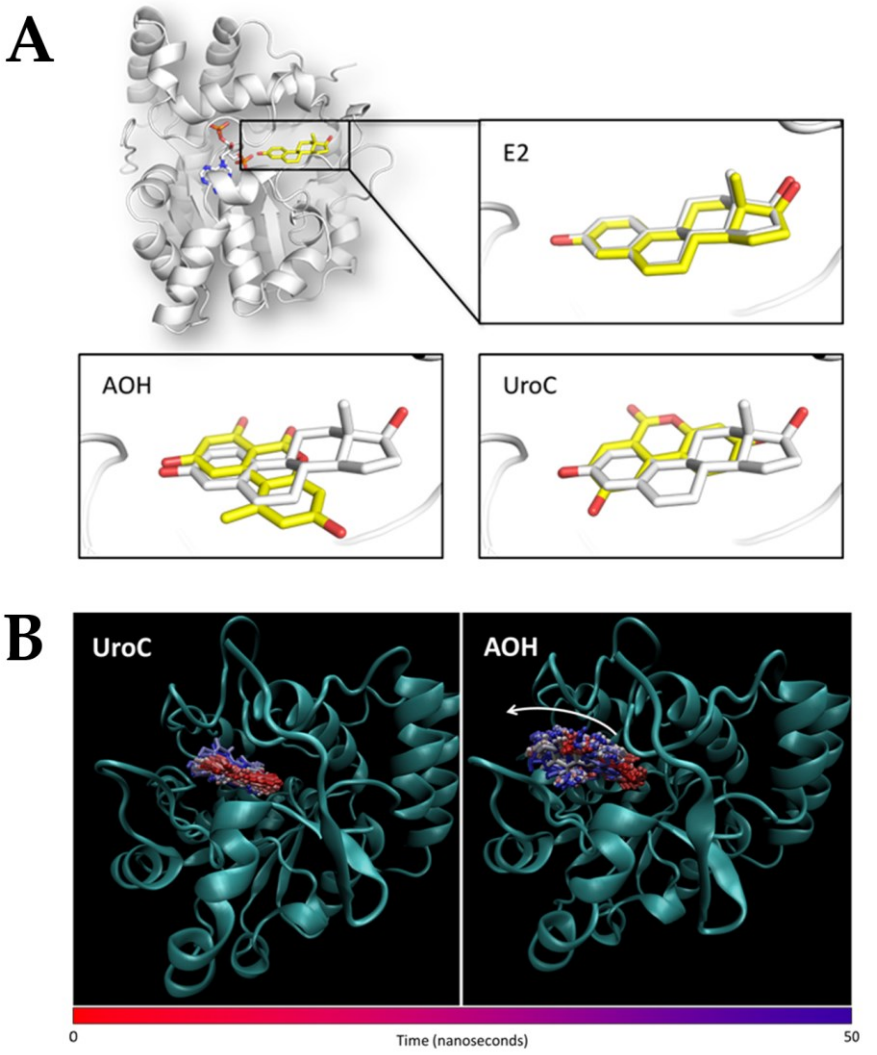

Figure 4. Computational results. A. Graphical representation of SULT1A1. Protein is represented in cartoon while ligands and cofactor are represented sticks. The calculated pose of E2, AOH and UroC (colored in yellow) are compared to the crystallographic pose of E2 (colored in white, PDB code 2D06). B. Time-step representation of AOH and UroC trajectories in molecular dynamic simulations. The from-red-to-blue color switch indicates the stepwise changes of ligand coordinates along the simulation. The white arrow indicates the outward trajectory of AOH.

4. Discussion

Recent findings have warned about the importance to assess the toxicology of mycotoxins in chemical mixtures rather than using single-substance tests. In this framework, the mechanistic analysis of mycotoxins in combination with other food-related compounds may concur to provide a suitable and informed background of knowledge to better characterize certain aspects of mycotoxin toxicity. Specifically, this work investigated whether the transport and phase II metabolism of AOH in a Caco-2 cell model system could be influenced by UroC, a structurally related and bioactive gut metabolite derived from ellagitannins. The analysis was focused on the phase II metabolism because: i) phase II conjugates are supposed to be the prevalent circulating forms upon AOH ingestion considering their already reported production in both cell and animal models (Burkhardt et al., 2009; Pfeiffer et al., 2009; Puntscher et al., 2019); ii) the structurally related urolithins are known to be glucuronidated and sulfated (Aragonès et al., 2017; Giménez-Bastida et al., 2012; González-Sarrías et al., 2014); iii) phase II conjugation is crucial for AOH toxicity since it was found to reduce the estrogenic potency of AOH in toxicodynamic terms, being able to prevent the interaction with the pocket of the estrogen receptors (Dellafiora et al., 2018).

The absence of cytotoxicity observed in this study is in line with those obtained by other authors reporting the lack of cytotoxic effects in Caco-2 cells up to AOH and UroC concentrations of 100 μM and 150 μM, respectively (Fernández-Blanco et al., 2016; Kojadinovic et al., 2017). Caco-2 cells are considered a valuable intestinal model thanks to the ability of Caco-2 cell monolayer to mimic the human intestinal epithelium (Tan et al., 2018) and they are largely used to study the epithelial transport of mycotoxins (e.g. Sobral et al., 2019). In this work, none of the experimental conditions affected the integrity of the cell monolayer (Figure S2), ensuring the consistence of data.

AOH in single-substance tests showed a limited and late passage through the polarized cell monolayer and the late accumulation in the BL side was accompanied by a reduction of AOH abundance in the AP side, suggesting a

slow-kinetic transport through the cells. Interestingly, the co-treatment with UroC at 24h determined a significantly lower abundance of AOH in the BL side compared to the treatment with AOH alone, accompanied by a higher abundance of AOH in the AP side. This data could suggest a late UroC-dependent effect in the trans-epithelial transport of AOH, rather than a change in AOH metabolism, also considering that the overall AOH recovery (AP + BL side) was similar in both treatments (i.e. AOH alone or in combination with UroC). In other words, the co-treatment with UroC reduced the transport of AOH, without modifying its overall abundance. Thus, UroC might exert protective effects worth of being analyzed further. This late effect is likely consequent to an influence on AOH transport which is reasonably due to an UroC-dependent change in the expression/turnover of transporters, rather than a competitive inhibition for transport, which should have been observed also at the earlier time-points tested. Nonetheless, the competition with membrane transporters and other metabolizing enzymes cannot be excluded.

The metabolic activity of Caco-2 cells on AOH, either upon single treatment or in combination with UroC, resulted in the production of two AOH-glucuronides and one AOH-sulfate. These results are in line with those reported by Burkhardt et al. (2009), who described the production of one AOH-sulfate (3-sulfate) and two AOH-glucuronides (3- and 9-glucuronide) after incubation of Caco-2 cells with AOH. Differently from the study reported above, a slower kinetic of transport was observed in this work. This difference could be partially due to the use of diverse chemical materials, as in this work a commercial AOH was used, while Burkhardt and coworkers used AOH synthesized in house containing a low percentage of AME. Also the diverse growth medium used could have had an effect as culture conditions may change monolayer permeability (Sambuy et al., 2005).

In the present work, AOH-glucuronides were detected in the AP sides of cells treated either with AOH alone or in combination with UroC already after 2h of incubation with an apparent time-dependency since the production at 24h was higher compared to the other time-points analyzed (Figure 3). Interestingly, co-incubation with UroC determined an early increase of AOH-glucuronides in the BL sides as they were detected at 4h

and 6h only in co-treated samples (Figure 3, left panel) as a possible consequence of an increased transport. The major impact of UroC on AOH-glucuronides accumulation was observed at 24h since a significant reduction of AOH-glucuronide(s) production was observed in both AP and BL sides. The lack of early effects of UroC on the reduction of AOH-glucuronides level led to exclude a substrate-like inhibition of UroC against the UGTs transforming AOH. Therefore, the reduction of AOH-glucuronides at 24h could be due to late UroC-dependent effects in terms of expression/turnover of UGTs or other enzymes possibly transforming glucuronidated compounds. In this respect, urolithins proved to modify the expression of some UGTs (González-Sarrías et al., 2009) and this phenomenon could partially explain the effect of UroC described in the present work.

Concerning the effects of UroC on the abundance of AOH-sulfates, UroC was found reducing and delaying their production. Indeed, a reduction of about 84% and a completely absence of AOH-sulfate was observed after 24h in the AP side and BL side of samples co-treated with UroC, respectively. This evidence might suggest that UroC could either compete or inhibit SULTs. The outcome of computational analysis along with the observed lack of effects of AOH on the UroC-sulfates production supported the hypothesized ability of UroC to competitively bind SULTs with a theoretically higher affinity compared to AOH. Of note, while glucuronides are typically considered inactive forms, sulfate derivatives of small molecules (such as AOH and UroC) may retain a degree of biological activity (Dellafiora et al., 2018, Ruotolo et al., 2013). Therefore, the UroC might exert a protective action *in situ*, which is worth of being analyzed in further studies, against the formation of metabolites retaining a certain degree of toxic activity.

4. Conclusions

The work investigated the effect of UroC on AOH transport and phase II metabolism in an intestinal *in vitro* cell model. The results described a late reducing action of UroC on AOH transport compared to the treatment with AOH alone. Moreover, UroC showed an early effect on the accumulation of AOH-glucuronides in the BL compartment, which was followed by a late

opposite effect with a significant reduction of AOH-glucuronides at both AP and BL sides at 24h. This could be due to a late effect in terms of expression/turnover of UGTs or other enzymes able to transform glucuronidated compounds. In addition, all the data collected pointed to a possible competition of UroC for the SULTs, that could explain the reduced production of AOH-sulfates.

Overall, our data suggest that AOH transport and metabolism are prone of being influenced by other structurally related compounds of food origin, thus pointing to a possible influence on AOH toxicity before entering the pre-systemic circulation. In this respect, AOH conjugates are supposed to have a lower toxicity compared to the parental compound. Therefore, the UroC-dependent reduction of AOH transport and conjugation may have possible consequences on dynamic and kinetic aspects of AOH toxicology that are worth of being studied further *in vivo*.

In agreement to recent findings reported in the literature, these data, taken together, suggest the need to investigate the toxicology of mycotoxins in chemical mixtures, being potentially relevant for a precise assessment of the risk related to their exposure through the diet.

SUPPLEMENTARY MATERIAL

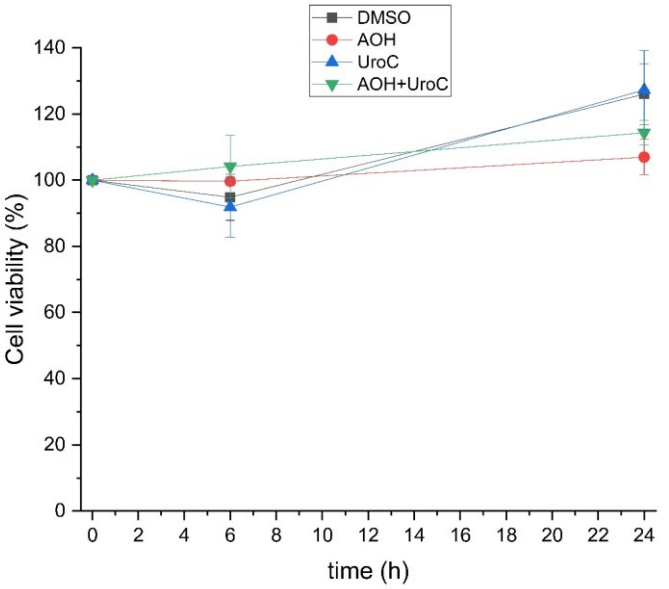

Figure S1 Effect of 30 μM AOH, 30 μM UroC or their combination (30 μM + 30 μM) on Caco-2 cell viability, as determined by the resazurin cell viability assay. Results are expressed as mean ± SD of three independent biological experiments.

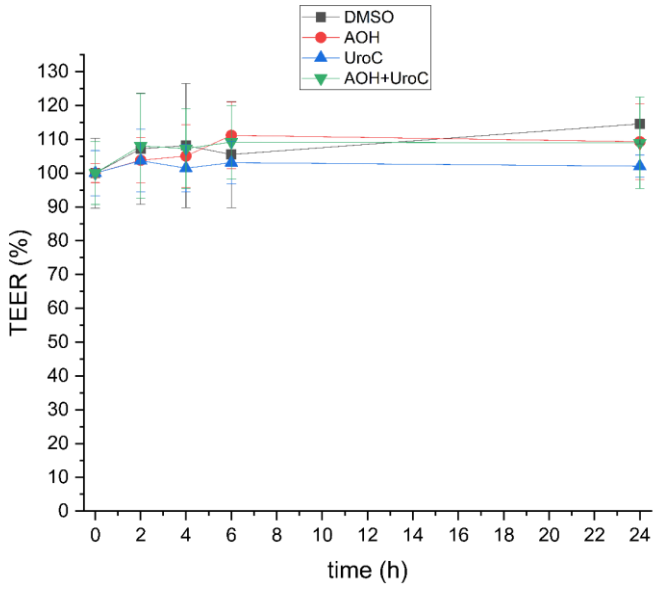

Figure S2 Transepithelial electrical resistance (TEER) of polarized Caco-2 monolayers exposed to 30 μM AOH, 30 μM UroC or their combination (30 μM + 30 μM). Results are expressed as mean ± SD of three independent biological experiments.

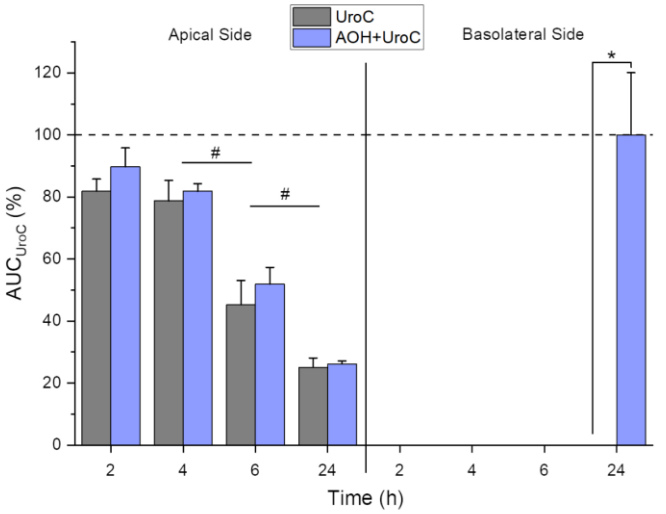

Figure S3. Level of UroC in AP and BL compartments (expressed as peak areas) after 2, 4, 6, and 24h of incubation of Caco-2 cells with 30 µM UroC or the combination of AOH+UroC (30 µM+30 µM). Results are reported as mean + SD of three independent biological experiments compared to UroC peak areas at the time-points 0h (for AP side) or 24h (for BL side). Significance according to Student's *t* test (*$p<0.01$) or Fisher's LSD post hoc test ($^{\#}p < 0.01$).

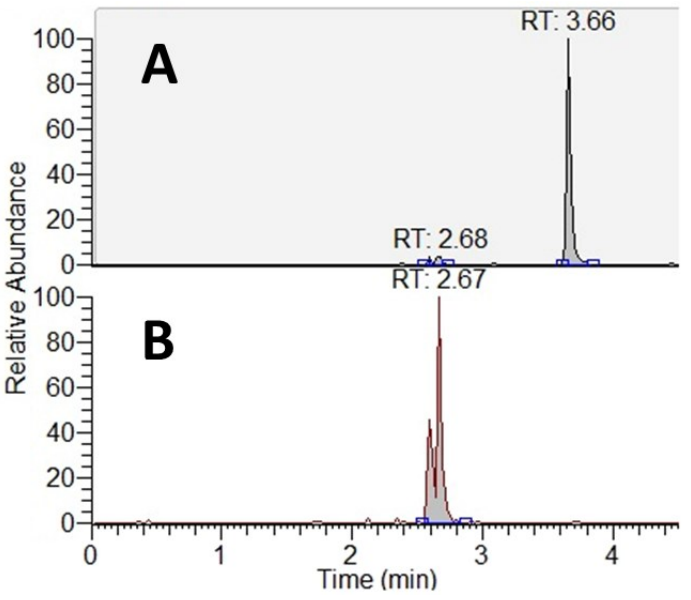

Figure S4. Representative chromatograms showing the peaks of AOH (A) and the peaks with a partially overlapped base of AOH-glucuronides (B).

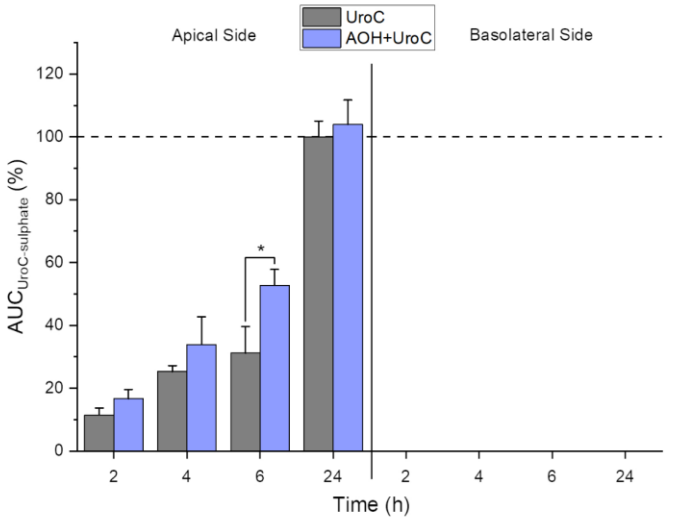

Figure S5. Level of UroC-sulfate in AP and BL compartments (expressed as peak areas) after 2, 4, 6, and 24h of incubation of Caco-2 cells with 30 μM UroC or the combination of AOH+UroC (30 μM+30 μM). Results are reported as mean + SD of three independent biological experiments compared to UroC-sulfate peak area at the time-point 24h. Significance according to Student's *t* test: * $p < 0.05$.

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GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The group of emerging mycotoxins produced by the genus *Alternaria* is still not regulated due to the shortage of toxicological and occurrence data. Despite many steps have been taken since the latest call for data on *Alternaria* mycotoxins inquired by EFSA in 2016, many efforts are still needed to clarify the actual risk related to this class of food contaminants.

The current risk assessment system is based on the evaluation of the potential of a given substance to individually cause harmful effects, without taking into account that food may be contaminated by multiple xenobiotics and the exposure to chemical mixtures may result in toxic effects other than those exerted by the single compound.

In this context, according to the literature reviewed (*Chapter 1)*, contaminated foods are often found to contain more than one *Alternaria* mycotoxin and, in some cases, these mycotoxins co-occur along with mycotoxins produced by other types of molds, such as those of *Fusarium*, *Penicillium* and *Aspergillus*. Of note, *Alternaria* mycotoxins in mixtures with other types of mycotoxins were reported not only in fresh and processed foods, but also in food supplements, in which a high number of mycotoxins was found (i.e. AOH, AME, TEN, 3-ADON, FUS-X, ENN-B, ENN-B1, ENN-A, ENN-A1, BEA, DON, HT-2, T-2, ZEN). These findings point out to the need to routinely check the co-occurrence of multiple mycotoxins in food, also considering that co-exposure to multiple *Alternaria* mycotoxins, as well as *Alternaria* mycotoxins with other types of mycotoxins may result in effects different than those exerted by the single mycotoxins. Additive, synergistic, or antagonistic effects were reported not only for mixtures of mycotoxins, but also for mycotoxins in combination with food bioactive compounds. Taken together, these data confirm the need to consider the effects resulting from mycotoxins in mixtures (also with bioactive compounds) rather than those caused by the single compounds in risk assessment of this class of food contaminants.

The experimental part of the present PhD thesis demonstrates that interactions of gut microbiota and food constituents with *Alternaria* mycotoxins may occur after food ingestion, resulting in modification of the bioavailability and, therefore, overall toxicity of mycotoxins. In particular, the ability of a complex extract of *Alternaria* mycotoxins to induce DNA

strand breaks was shown to be quenched by the contact with fecal samples, while its potency to induce formamidopyrimidine-DNA-glycosylase-sensitive sites was only slightly affected *(Chapter 2)*. These effects were related to the capacity of undigested food constituents and gut microorganisms to modify the concentrations of the mycotoxins of the extract, probably through binding to the mycotoxins. One of the most interesting results obtained from the quantification of *Alternaria* mycotoxins in feces-containing samples was the considerable loss of the epoxide-carrying *Alternaria* mycotoxins ATX-II and STTX-III, already before fecal incubation. This result indicates that these mycotoxins, probably due to their high reactivity, might not reach the intestine in their intact form, thus they may not represent a real risk for humans.

Despite the contact of the mycotoxins with the fecal materials resulted in complete or partial losses of the mycotoxins, the extract components were able to induce antiestrogenic effects in the Ishikawa cell line even after 3h of anaerobic incubation with the fecal materials (as shown in *Chapter 3*). Mechanistic studies revealed the inability of the single mycotoxins to act as ER-antagonists, but the complex extract was able to decrease the ER-α/ER-β nuclear ratio. These results suggest that some mycotoxins (or degradation products) could potentially act as endocrine disruptors even under *in vivo* condition, an hypothesis that deserves to be further investigated.

With regard to the role of gut microorganisms in modulating the toxic effects of *Alternaria* mycotoxins, results reported in *Chapter 4* clearly show the ability of human gut bacterial strains to adsorb the *Alternaria* mycotoxins AOH, AME, ATX-I, ALP, and AST, thus potentially exerting protective effects on human health by reducing the bioavailability of the mycotoxins. Interestingly, the tendency of these mycotoxins to accumulate within bacteria, especially in Gram-negative strains, was found to be directly related to their lipophilicity values. Although lipophilicity is not the only factor influencing the ability of compounds to penetrate the bacterial cell wall, these results suggest that some xenobiotics, having a degree of lipophilicity above a certain threshold, may be sequestered by the bacteria of the gut microbiota and, consequently, their effects on human health might be less pronounced under *in vivo* conditions. However, the incomplete

recovery of some mycotoxins from the bacteria-containing samples raises the question of whether gut bacteria could metabolize *Alternaria* toxins into metabolites retaining a certain degree of toxic activity, which could have an indirect impact on human health. Hence, subsequent studies based on untargeted high-resolution MS approaches are needed to better clarify the actual role of the gut microbiota in the metabolization of *Alternaria* mycotoxins.

The gut microbiota may contribute to the reduction of the harmful effects of *Alternaria* mycotoxins even through an indirect mechanism and, specifically, by producing compounds able to compete with the mycotoxins for the binding to cellular proteins such as transporters and enzymes. As a matter of fact, in *Chapter 5* the ability of urolithin C (a gut microbial metabolite derived from ellagitannins with structural similarities to AOH) to reduce the absorption of the *Alternaria* mycotoxin AOH in a Caco-2 cell model was demonstrated. Additionally, urolithin C impaired the phase II metabolism of AOH, especially in terms of AOH-sulfate production. The reduced formation of sulfates presumably results from the ability of urolithin C to competitively bind sulfotransferases with a theoretically higher affinity compared to the mycotoxin. These findings indicate that the structural similarity between gut-derived compounds (or other food constituents) and xenobiotics occurring in food might result in the modification of the toxicokinetic or toxicodynamic of the xenobiotic, with consequent modifications of the final toxicological outcome.

If on one hand the gut microbiota might reduce the direct negative effects exerted by *Alternaria* mycotoxins on human health, on the other hand the mycotoxins could act indirectly by altering the balance of the various bacterial species of the gut microbiota. To verify the hypothesis of a possible existence of a bidirectional relationship between *Alternaria* mycotoxins and the human gut microbiota, the *Alternaria* mycotoxin extract was tested for possible negative effects on bacterial species of the gut microbiota. Results reported in *Chapter 4* confirms that the *Alternaria* mycotoxins can modify the growth kinetics of human gut bacterial strains belonging to the most dominant gut microbial phyla (i.e. *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Verrucomicrobia*), regardless of the Gram-staining type.

While the growth of most of the bacteria was negatively affected by incubation with the mycotoxin extract, the growth of other bacteria was enhanced. In addition, the extract was also able to antagonize the formation of biofilms, even at the lowest concentration tested. Even if not evaluated in the present doctoral work, it should also be taken into account that *Alternaria* mycotoxins might also modify the bacteria – bacteria and bacteria – host communication processes, as well as they might target other microbial pathways. Based on this and considering that the microorganisms inhabiting the human intestine represent the body's first line of defense (together with the gut epithelium), the bidirectional relationship between *Alternaria* mycotoxins and gut microbiota deserves to be more-in-depth investigated.

In conclusion, the scientific works included in the present PhD thesis clearly highlight the need to consider the effects exerted by the *Alternaria* mycotoxins in complex mixtures (rather than singularly) during risk assessment on this class of food contaminants, given the high frequency of co-occurrence of multiple mycotoxins in food and the possible combined effects that could derive from exposure to chemical mixtures.

As a starting point for a better evaluation of the risk related to this class of contaminants, the routine control of multiple mycotoxins in food should become a common objective for researchers dealing with these issues in the near future. This would allow not only the definition of mycotoxin mixtures to be tested *in vitro and in vivo*, but also the identification of the foods or food groups more prone to contamination by multiple mycotoxins.

Furthermore, although pure mycotoxins have been tested in the present work (and not food contaminated by mycotoxins), the results obtained confirm the need for a change in trend in the evaluation of the effects exerted by xenobiotics. Specifically, considering that humans are exposed to *Alternaria* mycotoxins through the diet and that food constituents may modify the toxicokinetic and/or toxicodynamic of these compounds, the effects of xenobiotics should be evaluated in a context that considers these compounds as an integral part of the food and not as stand-alone units.

Although in the present work the gut microbiota showed protective effects on human health by reducing the free-absorbable proportion of mycotoxins or by producing metabolites able to interfere with mycotoxin toxicology, the

negative impact of mycotoxins on bacterial growth and biofilms production is the most critical point. Specifically, this poses the question whether *Alternaria* mycotoxins might also exert toxic effects indirectly, considering that modifications of the composition and/or activity of the gut microbiota might influence both normal physiology and disease susceptibility of the host. Thus, considering the known limitations deriving from an *in vitro* study and the importance of the gut microbiota for human health, the future *in vivo* investigation of the overall effects of the *Alternaria* mycotoxins on the gut microbiota would be desirable in order to pave the way for an all-round assessment of the actual risk related to this class of food contaminants.

AUTHOR

Francesco Crudo
Born on November 6, 1989 (Cetraro, Cosenza, Italy)

Contacts:
Email: che.francescocrudo@gmail.com
Linkedin: linkedin.com/in/francesco-crudo-629212a0
ResearchGate: https://www.researchgate.net/profile/Francesco_Crudo
Skype: che.francescocrudo

ORCID: https://orcid.org/0000-0002-4876-8057

STUDIES

Francesco Crudo got the bachelor's degree in Nutritional Sciences (University of Calabria, Italy) in October 2013, with a thesis titled *"Endocrine disorders during chronic hepatic diseases"*. In March 2016 he got the master's degree in Nutritional Sciences (110/110 with honors; University of Calabria, Italy), with a thesis titled *"Geographical characterization of Sicilian monovarietal olive oils by ICP-MS analysis of trace elements"*. In September 2016 he passed the State examination to practice the profession of biologist. After a six-months post-graduate internship at the Department of Microbiology and Nucleic Acids-Based Technology Applied to Food of the Istituto Zooprofilattico sperimentale della Lombardia e dell'Emilia Romagna (Brescia, Italy), he started the PhD in Food Science (University of Parma), under the supervision of Prof. Chiara Dall'Asta (University of Parma) and Prof. Doris Marko (University of Vienna). During the PhD program, which was focused on the toxicological evaluation of the interactions between *Alternaria* mycotoxins, food constituents and human gut microbiota, he carried out eighteen months of research at the University of Vienna, acquiring skills in the field of food toxicology.

SCIENTIFIC ACTIVITY

REVIEW

- *Co-Occurrence and Combinatory Effects of Alternaria Mycotoxins and Other Xenobiotics of Food Origin: Current Scenario and Future Perspectives*

  Authors: Francesco Crudo, Elisabeth Varga, Georg Aichinger, Gianni Galaverna, Doris Marko, Chiara Dall'Asta, Luca Dellafiora.
  Year: 2019
  Source: *Toxins (Basel)*, 11, 1–29. DOI: 10.3390/toxins11110640.
  Status: *Published*

ORIGINAL RESEARCH ARTICLES

- *Gut microbiota and undigested food constituents modify toxin composition and suppress the genotoxicity of a naturally occurring mixture of Alternaria toxins in vitro*

  Authors: Francesco Crudo, Georg Aichinger, Jovana Mihajlovic, Luca Dellafiora, Elisabeth Varga, Hannes Puntscher, Benedikt Warth, Chiara Dall'Asta, David Berry, Doris Marko.
  Year: 2020
  Source: Archives of Toxicology, 94, 3541–3552. DOI: 10.1007/s00204-020-02831-1
  Status: *Published*

- *Microfiltration results in the loss of analytes and affects the in vitro genotoxicity of a complex mixture of Alternaria toxins*

  Authors: Georg Aichinger, Natálie Živná, Elisabeth Varga, Francesco Crudo, Benedikt Warth, Doris Marko.
  Year: 2020
  Source: Mycotoxin Research, 36, 399–408. DOI: 10.1007/s12550-020-00405-9
  Status: *Published*

- *An in vitro study on the transport and phase II metabolism of the mycotoxin alternariol in combination with the structurally related gut microbial metabolite urolithin C*

  Authors: Francesco Crudo, Amelia Barilli, Pedro Mena, Bianca Maria Rotoli, Daniele Del Rio, Chiara Dall'Asta, Luca Dellafiora.
  Status: *Under review* in Toxicology Letters.

- *Interactions of Alternaria mycotoxins, an emerging class of food contaminants, with the gut microbiota: a bidirectional relationship.*

  Authors: Francesco Crudo, Georg Aichinger, Jovana Mihajlovic, Elisabeth Varga, Luca Dellafiora, Benedikt Warth, Chiara Dall'Asta, David Berry, Doris Marko
  Status: *Submitted*.

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

  Authors: Francesco Crudo, Georg Aichinger, Luca Dellafiora, Giorgia Del Favero, Jovana Mihajlovic, David Berry, Chiara Dall'Asta, Doris Marko.

  Status: *Submitted*

POSTER PRESENTATION

- *The effects of fecal incubation on the genotoxic properties of a complex extract of Alternaria mycotoxins*

  Francesco Crudo, Georg Aichinger, Jovana Mihajlovic, Chiara Dall'Asta, David Berry, Doris Marko.
  6th Theodor Escherich Symposium. Medical University of Graz (Austria). November 2019.

PARTICIPATION TO INTERNATIONAL CONFERENCES

- *Mycotoxin Summer Talks 2018*

  University of Natural Resources and Life Sciences (BOKU), Vienna (Austria). July 2018.

- *Joint AMICI and Theodor Escherich Symposium 2018*

  Medical University of Graz (Austria). November 2018.

- *Mycotoxin Summer Talks 2019*

  University of Natural Resources and Life Sciences (BOKU), Vienna (Austria). July 2019.

- *ASTOX Symposium 2019*

  Faculty of Chemistry, University of Vienna (Austria). April 2019.

- *6th Theodor Escherich Symposium*

  Medical University of Graz (Austria). November 2019.

PARTICIPATION TO SCHOOLS

- *Parma Summer School "One Health"*.

  School of Advanced Studies on Food and Nutrition, University of Parma, European Food Safety Authority and Università Cattolica del Sacro Cuore of Piacenza. June 2020.

I would like to thank all the members of my family (and in particular my mother), who have always encouraged and supported me in every important choice.

A special thanks is reserved to Olga, the fantastic person with whom I share my days, and to whom I will just say that "there is no better trip than that made with the right person".