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Alternaria toxins—Still emerging?

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Abstract

Alternaria molds are known to cause the contamination of food with their secondary metabolites, a chemically very heterogeneous group of compounds. Yet, after decades of research on the occurrence and the toxicity of *Alternaria* toxins in academia, no regulation has been implemented yet, thus leaving these potential food contaminants in the status of so-called "emerging mycotoxins". However, research on this topic has been far from static, leading to the European Food Safety Authority repeatedly calling for more data on the occurrence and toxicity of genotoxic metabolites such as alternariol (AOH) and its monomethyl ether (AME). To give an overview on recent developments in the field, this comprehensive review summarizes published data and addresses current challenges arising from the chemical complexity of *Alternaria*'s metabolome, mixture effects and the emergence of novel biological targets like cell membranes or the interaction with different receptors. Besides toxicodynamics, we review recent research on toxicokinetics, including the first in vivo studies which incorporated the rarely investigated—but highly genotoxic—perylene quinones. Furthermore, a particular focus lies on the advances of liquid chromatography/tandem mass spectrometry (LC-MS/MS)-based analytical tools for determining a broader spectrum of *Alternaria* toxins including modified/masked forms and assessing exposure via human biomonitoring (HBM).

KEYWORDS
black mold, food safety, genotoxicity, mycotoxins, natural toxins, synergism

1 | INTRODUCTION

Black molds of the genus *Alternaria* occur ubiquitously and are able to grow under varying temperature and moisture condition as well as on a large diversity of substrates. They are known to infest moist buildings, where they are discussed as contributors to the "wet building syndrome". However, special relevance is given to their growth on plant matter, particularly on crops designated for animal or human consumption. There, they may produce a large variety of different secondary metabolites, so-called "*Alternaria* toxins", which are of relevance as potentially harmful food contaminants.

Research on those compounds dates back to the 1960s–1970s, when some metabolites produced by *Alternaria spp*. were reported for the first time to exert toxic effects (Pero et al., 1973). Since then, numerous studies were conducted on their toxicity and involved molecular mechanisms, predominantly using in vitro models. Analytical methods that allowed for the identification and

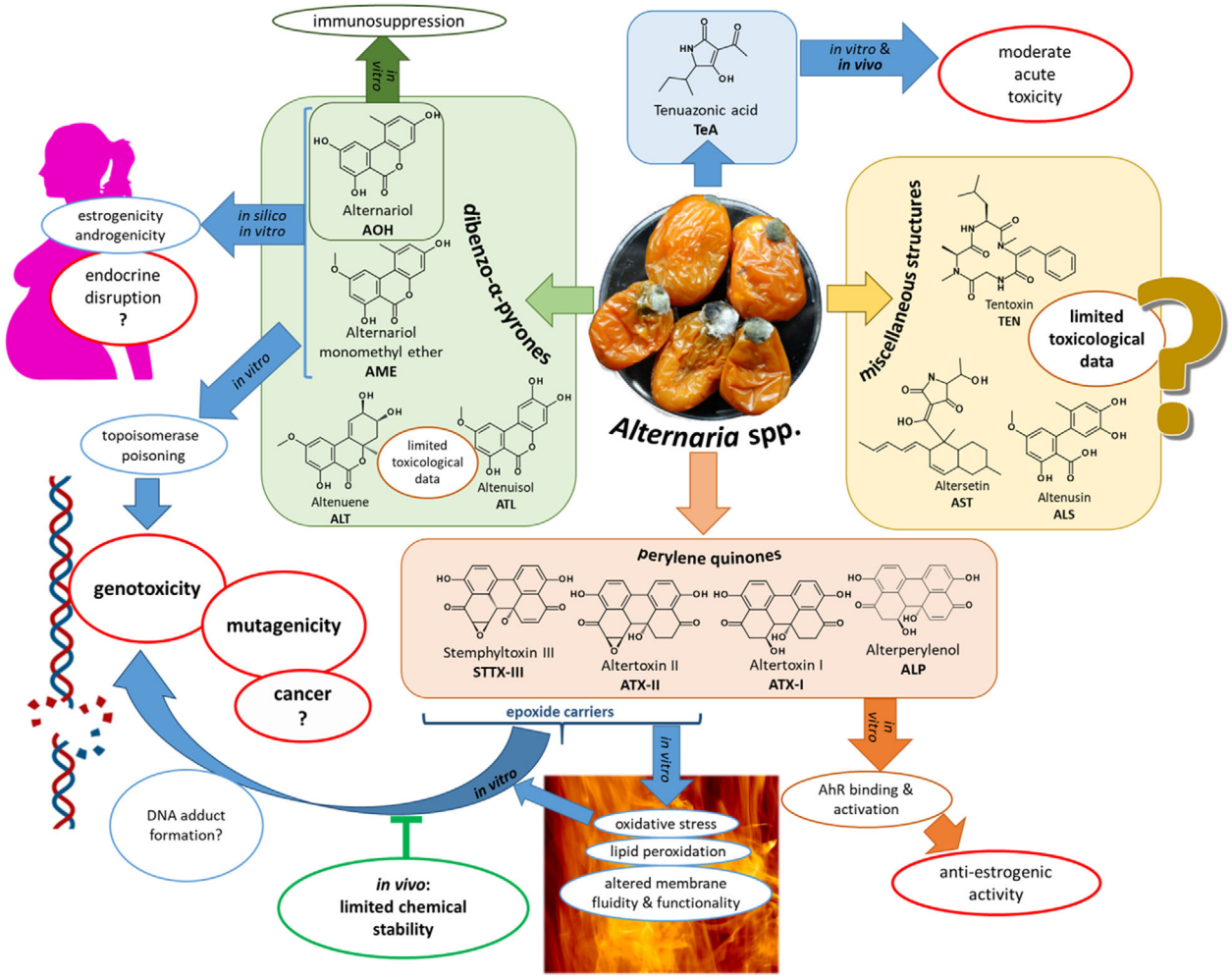

FIGURE 1 Chemical structures of known *Alternaria* toxins and a graphical display of associated biological activities

quantification of novel *Alternaria* toxins in different biological matrices were developed and used to gather food occurrence data to estimate human exposure.

Nevertheless, authorities around the globe still did not implement regulations for any *Alternaria* toxin. Thus, these compounds are still considered as "emerging mycotoxins", a term established to describe novel and yet unregulated fungal toxins (Gruber-Dorninger et al., 2017).

Yet, as it has been more than 50 years of research without generating the data needed for authorities to take action, one might ask the valid question whether *Alternaria* toxins are just not hazardous enough for requiring costly regulations. Should *Alternaria* toxins continue to be regarded as "emerging" contaminants or rather dismissed as irrelevant for human health?

To meet this concern issued to our research community, we hereby aim to give an overview on the state of the art, to provide insights into recent trends in *Alternaria* toxin research, to point out future challenges and the potentially upcoming developments within this scientific field.

2 | TOXICODYNAMICS

2.1 | Cytotoxicity and genotoxicity

Until 2012, toxicological research on *Alternaria* toxins largely focused on the two most abundant metabolites, namely tenuazonic acid (TeA, Figure 1) and alternariol (AOH, Figure 1). TeA is a phytotoxic compound and was shown to damage host plants and thus to ease plant infection for *Alternaria* molds (Kang et al., 2017). On mammalian cells, it exerts mild toxic effects that are mainly attributed to the inhibitory potential toward ribosomal activity (Shigeura & Gordon, 1963; Vejdovszky et al., 2016). Several publications addressed toxic effects of TeA in vivo. For example, a study on young chickens found the application of 1.25 mg/kg b.w. over 3 weeks to cause some adverse effects, although no increased mortality was observed (Giambrone et al., 1978). In mice and rats, LD_{50} values between 80 and 225 mg/kg b.w. were established in different studies, as reviewed by the European Food Safety

Authority (EFSA) scientific panel in 2011 (EFSA, 2011). Considering an estimated mean exposure of 127–177 ng/kg b.w. per day for humans, the EFSA (2016) concluded that TeA was highly unlikely to pose a threat to human health.

In 2016, the EFSA called for analytical data on *Alternaria* toxins, and stated in the subsequent evaluation that for AOH and its monomethyl ether (AME, Figure 1), "the estimated mean chronic dietary exposures at the upper bound and 95th percentile dietary exposures exceeded the toxicological threshold of concern (TTC) value" (EFSA, 2016). Thus, based on the estimated human exposure, AOH and AME are considered to be of significantly more concern than TeA. Furthermore, these dibenzo-α-pyrone derivatives are probably the best-studied *Alternaria* toxins with extensive data available on in vitro effects and occurrence. In vitro, both compounds have repeatedly been reported to possess cytotoxic (Bensassi et al., 2012) and—of particular concern—genotoxic properties in micromolar concentrations. Pfeiffer et al. (2007) first observed DNA damage caused by AOH and AME in different mammalian cell lines, starting from 6.25 μM in alkaline unwinding assays, which was later confirmed by different studies in other test systems (Schwarz et al., 2012a; Solhaug et al., 2015), which in part observed genotoxic effects in cells at concentrations as low as 1 μM (Fehr et al., 2009).

Concerning the underlying mechanism, genotoxic effects of AOH and AME are mostly attributed to their ability to inhibit and poison topoisomerases (TOPs), enzymes which are essential for maintaining DNA integrity, especially in proliferating cells. This mode of action was initially observed in cell free test systems and confirmed in human cancer cell lines for several TOP isoforms (primarily TOP IIa, to a lesser extent TOP I and TOP IIb) at concentrations that fit the doses where genotoxicity was observed (Fehr et al., 2009).

Additionally, the induction of oxidative stress might contribute to their DNA-damaging properties. Tiessen et al. (2013a) observed an increase in intracellular reactive oxygen species (ROS) levels after incubation of human colon cancer cells with AOH and AME, while Aichinger et al. (2017) confirmed the latter effect and first described the induction of sites sensitive to formamidopyrimidine-DNA glycosylase (FPG), a measure for oxidative damage of DNA bases, by these compounds. In line, AOH was reported to induce cell cycle arrest, apoptosis, and changes in cell morphology in different in vitro models (Solhaug et al., 2016).

However, in vivo data on the toxicity of dibenzo-α-pyrones are still scarce. The only relevant animal study so far found no genotoxic effects and only low acute toxicity after an oral administration of 3 × 2000 mg/kg BW (0, 24 and 45 hr) AOH to mice (Schuchardt et al., 2014). However, due to technical issues, toxicity was not assessed in the colon, which might represent the main target organ of the mycotoxin due to its apparent low systemic bioavailability (Solhaug et al., 2016). Thus, addressing health concerns requires additional in vivo testing or, alternatively, the development of respective computational models.

Besides TeA and the two major dibenzo-α-pyrones, *Alternaria* spp. may produce significant amounts of perylene quinone derivatives (Figure 1), such as alterperylenol (ALP), altertoxins (ATX) I-III and stemphyltoxin III (STTX-III). In 2012, two research groups independently discovered ATX-II to by far exceed the genotoxicity of AOH (Fleck et al., 2012), and to represent an important DNA-damaging component in an extract of *A. alternata* infested rice (Schwarz et al., 2012b). Its genotoxicity in cellular test systems was reported to start at concentrations as low as 0.05 μM (Tiessen et al., 2013b).

Since then, some research has been conducted on underlying modes of action and the toxicity of other perylene quinones. STTX-III was also found to act as a potent inducer of DNA damage (Fleck et al., 2016), which points to a mechanism involving a common structural feature of STTX-III and ATX-II, the functional epoxy moiety. Similar as planar epoxy-carrying metabolites of polyaromatic carbohydrates, the latter might be able to react directly with bio-macromolecules, as it was recently demonstrated for lipids (Del Favero et al., 2020a). Regarding nucleic acids, such reactions could result in the formation of persisting DNA adducts. This hypothesis, although underlined by the commonly observed induction of FPG-sensitive sites during genotoxicity testing (Aichinger et al., 2018; Schwarz et al., 2012b), was only recently confirmed by Soukup et al. (2020), who observed the formation of two guanine-ATX-II adducts and one cytosine-ATX-II adduct under cell-free conditions. Even as the exact chemical structure of the resulting adducts – and therewith the exact molecular site of the spontaneous chemical reactions – was not fully elucidated, the findings prove that epoxy-bearing perylene quinones are able to directly attack the DNA.

Furthermore, poisoning of TOPs was considered to play a role in the genotoxicity of perylene quinones. However, although ATX-I, ATX-II, ALP and STTX-III were found to inhibit the catalytic activity of TOP IIa in cell-free assays, the concentrations needed to achieve this effect were much higher than those sufficient to induce DNA damage in cell culture (Jarolim et al., 2016).

2.2 | Activation of the aryl hydrocarbon receptor

The aryl hydrocarbon receptor (AhR) is one of the key factors regulating phase I xenobiotic metabolism. It is activated by binding of planar ligands and—upon

dimerization with the aryl hydrocarbon receptor nuclear translocator (ARNT)—triggers the expression of various metabolic enzymes, particularly of the cytochrome P450 (CYP) 1 family (Badal & Delgoda, 2014). Although a functioning AhR system plays an important role in xenobiotic metabolism, it can also contribute to the metabolic activation of certain toxics (Mescher & Haarmann-Stemmann, 2018). Further, its chronic activation, being linked with cancer progression and its interference with other cellular signaling mechanisms (some of which are discussed further in section 2.3), make it an intriguing target in toxicology (Murray et al., 2014).

Schreck et al. (2012) found the *Alternaria* toxins AOH and AME to increase the transcription of cytochrome P450 (CYP) 1A1—one of the key enzymes involved in their metabolic detoxification—in murine hepatoma cells. Moreover, they described the effect to be clearly dependent on the presence of the AhR protein. Pahlke et al. (2016) confirmed the AOH-induced *CYP1A1* transcription in human cell lines originating from colon, esophagus and liver tissues, but also reported the perylene quinone ATX-II to exceed AOH in its potential to enhance *CYP1A1* transcription. Furthermore, a recent study described cumulative and even synergistic actions of *Alternaria* toxins, among them AOH, ATX-II and further perylene quinones, toward the activation of the AhR and related protein expression of CYP 1 enzymes in human breast cancer cells (Hohenbichler et al., 2020).

2.3 | Endocrine activity and related novel activities

According to the EFSA (2016), the genotoxic properties of AOH and AME are considered the primary concern for human health regarding *Alternaria* toxins. However, several novel aspects of bioactivity that risk assessors did not yet take into account were reported in recent years.

Among these, endocrine disruptive activities need to be mentioned. Lehmann et al. (2006) were the first to report the ability of AOH to bind to and activate estrogen receptors (ER), indicating a possible influence on endocrine pathways. In cell-free assays, the mycotoxin was shown to predominantly target the ER-β with an EC_{50} of 3.1 μM $\pm$ 2.9 μM (as compared to 30 μM $\pm$ 20 μM for ER-α), while in human endometrial cancer cells carrying both receptor forms, the lowest observed effect was at a concentration of 2.5 μM.

As these concentrations are quite high in comparison to other foodborne xenoestrogens, they were at first not considered to critically enhance the hazardous profile of the compound, particularly considering that genotoxicity is caused at similar levels. However, a combined *in silico*/in vitro approach predicted several naturally occurring metabolites of dibenzo- α-pyrones to possess endocrine activities (Dellafiora et al., 2018), thus raising concerns about cumulative or even synergistic contribution of different metabolites to endocrine disruption, a point which will be further addressed in section 2.6.

Additionally AOH was recently also described to act as a full androgen receptor agonist, albeit at very high concentrations with an EC_{50} of 269.4 μM (Stypuła-Trębas et al., 2017). Thus, the endocrine activity of the compound and structurally related metabolites could prove to be very diverse and complex.

On the contrary to AOH, complex extracts of *Alternaria* cultures have been observed to mediate anti-estrogenic rather than estrogenic activities. This counteractive, but nevertheless toxicologically relevant effect is probably related to the ability of perylene quinones to interact with the AhR, which may in turn lead to enhanced degradation of ERs or a modulation of ER-related signaling cascades (Aichinger et al., 2019).

Another recently described bioactivity is the ability of AOH, AME and, to a lesser extent, also ATX-II to inhibit cellular enzymes such as casein kinase 2 (CK2), possible influencing its endocrine disruptive potential as well as a number of distinct cellular responses (Aichinger et al., 2020a). As CK2 inhibitors are regarded a novel class of chemotherapeutic agents, those *Alternaria* toxins might even be used as basic scaffolds in future drug design.

2.4 | Inflammation

Solhaug et al. (2015) first described a potential influence of AOH on the immune systems, as the incubation with 30 μM of the mycotoxin induced morphological and phenotypical changes in RAW 264.7 mouse macrophages as well as human primary macrophages. In addition, a change in the transcription pattern of cytokines, particularly an enhanced secretion of tumor necrosis factor α (TNF-α) was reported.

Recently, AOH was found to suppress pro-inflammatory responses in human epithelial cells, human colon carcinoma cells as well as in murine and human macrophages (Grover & Lawrence, 2017; Kollarova et al., 2018; Schmutz et al., 2019). While a possible activation of the AhR could be excluded as a mechanism for immunosuppressive effects that manifested in morphological changes (Grover & Lawrence, 2017), the mycotoxin was found to target the NF-κB pathway and related cytokine transcription in THP1-derived macrophages (Kollarova et al., 2018). Such immunosuppressive activities might provoke a higher susceptibility to certain diseases in people with a high chronic intake of AOH (Schmutz et al., 2019). Furthermore, it

cannot be excluded that the mycotoxin leads to general adverse effects with decreased responsiveness and effectiveness of the adaptive immune system, which could hypothetically contribute to the so-called "sick building syndrome", an umbrella term for adverse effects observed with inhabitants of moist, moldy homes, which often involves problems with the immune system (Valtonen, 2017). In agreement with this hypothesis, we recently expanded the characterization of the immunomodulatory spectrum of *Alternaria alternata* toxins describing the potential of the perylene quinone ATX II to inhibit the activation of nuclear factor—kappa B (NF-κB) in THP-1 cells (Del Favero et al., 2020a). Thus, in addition to AOH and AME, also altertoxins appear to contribute to the immunomodulatory properties of native mixtures of *Alternaria* toxins.

2.5 | Impact on cellular membranes and the gastrointestinal barrier function

In addition to the above-mentioned studies, research activity in the field of *Alternaria* toxins expands toward multiple additional directions. Particularly, in order to provide models that with increasing accuracy reproduce the toxicological potential of the toxins in vivo, efforts were invested in the creation of experimental setups integrating functional readouts in the data acquisition panel. To this aim, the toxicological potential of ATX-II toward intestinal cells was investigated in combination with biomechanical stimulation mimicking the shear stress that is typical for the organ lumen (Del Favero et al., 2018). This approach allowed to describe how the toxin can directly impair the functional status of intestinal cells by altering membrane fluidity, as well as their migratory potential and membrane-cytoskeletal communication axes. Indeed, the presence of an epoxide moiety on the toxin scaffold confers a ROS inducing potential to ATX-II (Jarolim et al., 2017), which is in accordance with recent reports describing the perylene quinone to induce lipid membrane peroxidation and mitochondrial superoxide formation in THP-1 macrophages (Del Favero et al., 2020a). Intriguingly, despite the obvious differences between intestinal (HCEC and HT-29) and immune cells (THP-1 macrophages), in both tissue types, changes of the cell membrane were associated with a modulation of the toxicological potential of ATX-II, thus suggesting that the cell membrane could play a central role in mediating complex responses to *Alternaria* toxins. These conclusions are in line with previous studies describing relatively poor intestinal adsorption (Caco-2 monolayer) of epoxide containing toxins like ATX-II and STTX-III and better permeability for altertoxin (ATX) I and II, alteichin (ALTCH) (Fleck et al., 2014). Following this line of thoughts, we recently described how structural similarity between AOH and cholesterol accounts for the potential of the toxin to interact with the cell membrane as well as membrane proteins like caveolin-1 (Del Favero et al., 2020b). Since caveolin regulates fatty acids uptake (Pohl et al., 2002) and membrane proteins turnover (Shi & Sottile, 2008; Urra et al., 2012), this implies possible changes on transmembrane receptors distribution or uptake and confirms previous studies reporting the interaction between AOH and the GM1 plasma membrane raft ganglioside in RAW 264.7 macrophages (Solhaug et al., 2013). In addition, it was recently described how structure and biophysical properties (e.g., lipophilicity) of *Alternaria* mycotoxins seem to drive accumulation into microbial pellets, possibly via interaction with bacterial walls (Crudo et al., 2021). If this interpretation would be confirmed, the interaction mycotoxins-membranes could substantially affect toxins' bioavailability and biodistribution after oral intake.

2.6 | Toxicity of *Alternaria* metabolites in native mixtures

Alternaria toxins are not expected to appear in food as isolated compounds, but in chemical mixtures with further *Alternaria* toxins, other food contaminants and different bioactive food constituents. With this complex profile of co-occurring bioactives exerting different biological activities, classical risk assessment based on single compounds seems to be an outdated approach. In fact, recent research in both, analytical method development and toxicology, often focuses on cumulative or potential synergistic effects of *Alternaria* toxins in complex mixtures, as recently summarized by Crudo et al. (2019).

So far, several studies addressed combinatory effects of binary combinations of *Alternaria* toxins. For example, AOH and AME were found to impact cell viability of colon carcinoma cells in an additive way (Bensassi et al., 2015), and AOH and ATX-II lead to cumulative cytotoxic effects in HepG2, HT-29 and HCEC-1CT cells (Vejdovszky et al., 2017b). Furthermore, extracts of *Alternaria alternata* cultured on rice were recently found to exceed the cytotoxic and genotoxic effects of contained single compounds, pointing to an at least cumulative, if not synergistic, toxic activity (Aichinger et al., 2019). These findings should be addressed respectively by applying methods for cumulative risk assessment that are currently contemplated by the EFSA for assessing the cumulative risk of an exposure to multiple pesticides (EFSA Scientific Committee et al., 2019; Kruisselbrink et al., 2018).

Moreover, adverse effects of *Alternaria* toxins might be influenced by the co-occurrence of secondary plant

metabolites. For example, the polyphenols genistein and delphinidin were described to reduce oxidative stress and TOP poisoning caused by AOH and were found to antagonize its genotoxic effects on colon carcinoma cells in two-digit micromolar concentrations (Aichinger et al., 2017). In another study, oxidative stress induction and cytotoxic effects of AOH were found to be reduced by the phenol-rich fraction of olive oil in Caco-2 cells (Chiesi et al., 2015).

Furthermore, the DNA-damaging properties of the perylene quinone ATX-II were suppressed in the presence of delphinidin in a similar cell model (Aichinger et al., 2018). LC-MS/MS measurements revealing the disappearance of ATX-II even in cell-free mixtures with the anthocyanidin hint at a direct chemical reaction of the two compounds. Also, the fact that ATX-II seems extremely prone to undergo spontaneous detoxification reactions with co-occurring food constituents might put the toxicologic relevance of the compound into question. Even so, it is yet not clarified if the toxin might be released again at a later point, and one might speculate that it could be formed from other perylene quinones during phase I metabolism, following a similar activation pattern as the infamous aflatoxin B1 (EFSA, 2020).

Endocrine disruption by chemical mixtures is of particular interest in cumulative risk assessment, as it could serve as an explanation for epidemiologically observed biological activities which cannot be linked to effects of single EDCs, such as world-wide increases in infertility, early onset of puberty or raising breast cancer rates (Diamanti-Kandarakis et al., 2009). In human endometrial cancer cells, Vejdovszky et al. discovered sub-active nanomolar doses of AOH to potentiate the estrogenic activity of other xenoestrogens, such as the *Fusarium* toxins zearalenone and α-zearalenol (Vejdovszky et al., 2017a), or the soy isoflavone genistein (Vejdovszky et al., 2017b). In combination with the plasticizer bisphenol A, AOH did not act synergistically on the induction of ER-dependent gene transcription in the same cell line. Nevertheless, the two compounds exerted cumulative estrogenic effects that exceeded those of the single chemicals (Aichinger et al., 2020b).

Based on these data, further studies in OECD-approved test systems for EDCs are needed to allow the incorporation in risk assessment.

2.7 | Interactions with the gut microbiome

In addition to eukaryotic cells, after oral intake mycotoxins can potentially come into contact also with the gut microbiome. These interactions are very complex since on the one hand mycotoxins can be altered as result of microbial metabolism; vice versa according to mechanism of action, contaminated food commodities can theoretically modify the composition and functionality of the gut microbiome. On top, resident intestinal fungi may vary in composition in response to pathophysiological conditions contributing to dysbiosis in the intestinal compartment (Qiu et al., 2015, 2017).

In the case of *Alternaria* toxins, an initial study from Crudo et al. (2020) studied the impact of the gut microbiome on the composition and the genotoxic potential of a complex native *Alternaria* toxin mixture. An extract of *Alternaria alternata* infested rice was subjected to anaerobic fermentation with fecal samples of human donors. Already after 1 hr anaerobic fermentation with fecal slurry a strong concentration reduction of all the 17 monitored *Alternaria* metabolites was observed. Of note, the apparent toxin loss seemed rather to result from adsorptive phenomena to bacterial membranes and particulate matter than from microbial metabolism. In particular, epoxide-carrying perylene quinones were completely lost upon fecal fermentation. In line with this finding, the genotoxicity of the applied extract toward colonic cells was substantially decreased (Crudo et al., 2020).

A recently published follow-up study described *Alternaria* toxins to selectively impact the viability and growth of bacterial strains that colonize the gut, shedding light on the bidirectional interaction of the gut microbiome with mycotoxins (Crudo et al., 2021). Intriguingly, low concentrations (0.5 μg/ml) of the applied extract from an *Alternaria alternata* culture were shown to significantly inhibit biofilm formation by various microbes, including *Bacteroides caccae*, *Bacteroides thetaiotaomicron*, *Bacteroides vulgatus* and *Escherichia coli*. Furthermore, single strains were shown to impact the chemical composition of the applied extract.

More studies, examining the role of the gut microbiome on *Alternaria* toxin estrogenicity, but also the potential impact of *Alternaria* toxins on the composition of the microbial community in the intestine are expected to be published in the near future and might allow a more comprehensive understanding of the complex interaction between toxins and gut health.

2.8 | Partially characterized (minor) *Alternaria* metabolites

Only scarce toxicological data exists on bioactivities of a number of yet insufficiently studied *Alternaria* metabolites. The compounds altersetin (AST, Figure 1), macrosporin, altenusin (ALS, Figure 1) and pyrenophorol were reported to inhibit type II TOPs under cell-free con-

ditions (Jarolim et al., 2016). Several phase I and phase II metabolites of AOH and AME, as well as altertenuol, were predicted by a *in silico* approach to target human TOP I (Dellafiora et al., 2015). Furthermore, there are speculations that AST might be able to induce intracellular oxidative stress (Aichinger et al., 2020c). However, these hypotheses and their potential implications for genotoxicity are still awaiting experimental confirmation in human cells.

Additional secondary metabolites were predicted *in silico* to show affinity to TOPs (Dellafiora et al., 2015), including the catecholic dibenzo-α-pyrone altenuisol, which is—together with TeA, AOH, and AME—among the predominant compounds produced by different *Alternaria* strains under laboratory conditions (Zwickel et al., 2018). Again, experimental confirmation as well as food occurrence data are still lacking for this metabolite, but could—if confirming the predictions—open new research lines.

Furthermore, in a recent publication the pronounced genotoxicity of a complex extract of *Alternaria alternata* cultured on rice could not be fully related to already described mycotoxins (Aichinger et al., 2019). Thus, the mold appears to produce further genotoxic compounds, which await characterisation.

Additionally, *Alternaria* spp. are known to produce a number of host-specific toxins (HSTs), such as AF-toxins or AK-toxins, as reviewed by Tsuge et al. (2013) or Meena and Samal (2019). While those are described to be toxic for plants and thus play a pivotal role in plant infection and pathology, there is yet no data linking HSTs with effects on animal or human health.

3 | TOXICOKINETICS

Exploring absorption, distribution, metabolism and excretion (ADME) of toxins in the body is essential for estimating potential target organs and relevant concentrations for testing in cell-based systems.

Fraeyman et al. (2015) assessed toxicokinetics of TeA in pigs and broiler chickens, reporting complete oral availability of the compound and quantitative kinetic parameters for its absorption and elimination. As the data suggest the possibility of tissue accumulation, which could also lead to a transfer to the food chain, the authors encouraged studies taking into account tissue concentrations and biotransformation.

A few studies have dealt with the qualitative elucidation of metabolic reactions of AOH and AME, who have been demonstrated to be a substrate for phase I metabolic enzymes of the CYP450 family (Pfeiffer et al., 2008). In incubations with rat microsomal preparations as well as precision-cut rat liver slices, both toxins were reported to undergo transformation to several hydroxylated oxidative metabolites, as well as to methylated compounds originating from AOH being a substrate to the catechol-*O*-methyl transferase (COMT) (Burkhardt et al., 2011; Pfeiffer et al., 2007). As 4-hydroxylated products exerted less genotoxicity as compared to their parent compounds, probably due to decreased lipophilicity and thus cellular uptake, these reactions can be considered a detoxification step regarding DNA damage (Tiessen et al., 2017). On the other hand, these metabolites were shown to be rapidly converted by methoxylation, leading to methoxy-products that were recently predicted to exert estrogenicity in an *in silico* approach (Dellafiora et al., 2018). Thus, whether phase I metabolism can really be seen as detoxifying still has to be elucidated and is currently under examination.

What seems clear is that both, dibenzo-α-pyrones and their oxidative metabolites, undergo phase II metabolism, particularly glucuronidation and sulfation to enhance their excretion (Pfeiffer et al., 2009; Soukup et al., 2016).

With respect to the quantification of these processes and the corresponding determination of kinetic constants, there is a complete lack of data that urgently requires scientific attention.

However, kinetics were determined for the binding of AOH by serum albumin (Fliszar-Nyul et al., 2019), which is one of the key parameters in distribution and excretion of a compound.

So far, two in vivo studies included an assessment ADME of AOH. Schuchardt et al. (2014) conducted experiments administering extremely high concentrations (single oral doses of 200 or 1000 mg/kg BW) of radio-labeled AOH to mixed-sex NMRI mice, and reported a low systemic absorption of about 9% of the compound, mostly recovered from urine. Blood levels never exceeded 0.06% of the administered mycotoxin. Metabolization was then assessed by administering nonlabeled AOH in doses of 200 or 2000 mg/kg BW as a single dose, or a triple (after 0, 24, and 45 hr) administration of 2000 mg/kg BW. Therewith, four hydroxylated metabolites of AOH (with functional groups added at positions 2, 4, 8, or 10) were confirmed in vivo.

Puntscher et al. (2019a, 2019c) administered a single dose (50 mg/kg BW, bolus) of a complex mixture of toxins (extracted from an *A. alternata* DSM 62010 culture on rice) to male Sprague Dawley rats, and largely confirmed the low bioavailability of most contained toxins, taking into account phase I and II metabolism. TeA, which is considerably more hydrophilic than other *Alternaria* toxins, present in the applied extract in very high amounts, was recovered from urine (87% after 24 hr), whereas all other toxins were mostly recovered from collected feces. Intriguingly, the high doses of perylene quinones present

in the ingested toxin mixture were not recovered from feces, urine or plasma, indicating either an accumulation of these compounds in tissues or a metabolic degradation. Both options should be further investigated, as perylene quinones have long been described as mutagenic (Stack & Prival, 1986) and are thus of potentially high toxicological relevance.

For assessing still pending questions with toxicokinetics of *Alternaria* toxins, which could ultimately allow for an accurate estimation of ADME in humans, researchers are encouraged to carry out further animal studies or preferably to apply computational methods such as physiologically-based pharmacokinetic (PBPK) modelling and in vitro to in vivo extrapolation (IVIVE). These are part of international efforts (e.g., NIH's "Tox21" initiative, the EU's 3R's agenda) to reduce the dependency on animal experiments by providing rapid, high-throughput toxicity testing tools (Bouvier d'Yvoire et al., 2008; Krewski et al., 2010). However, as a necessary first step toward that goal, the quantitative elucidation of metabolic processes in state-of-the-art in vitro test systems is urgently required.

4 | ADVANCES IN ANALYTICAL CAPABILITIES AND EXPOSURE ASSESSMENT

4.1 | Analysis of *Alternaria* toxins in food

According to the EFSA (2016), comprehensive occurrence data for *Alternaria* toxins is still lacking and a reason for the preliminary exposure estimate and risk assessment. With AOH, AME, TeA, and TEN, only four *Alternaria* toxins were included in the most recent dietary exposure assessment (EFSA, 2016), thus not comprising the highly mutagenic perylene quinones. Consequently, there is still need to develop and validate more comprehensive analytical methods and apply them in large-scale food monitoring programs.

Similar to other classes of food contaminants, also in the field of *Alternaria* toxin analytics a clear trend toward multi-analyte LC-MS/MS methods can be seen. These mass spectrometry-based methods typically offer high selectivity and sensitivity in addition to the benefit of assessing multiple toxins simultaneously. During the last couple of years, a number of matrix-specific LC-MS/MS assays were developed, typically involving different mixtures of *Alternaria* toxins and sometimes applying QuEChERS or SPE extraction/cleanup/concentration. Most methods so far do not go beyond the most prominent *Alternaria* toxins (typically 4–6 analytes) and do not include modified forms, that is, metabolic products of plants, microbes, animals, or humans. Analytical methods for the determination of *Alternaria* toxins in food and other matrices were reviewed by Man et al. (2017) and more recently, amongst other mycotoxins, by Tittlemier et al. (2020). Hence, no comprehensive overview of all published analytical methods is repeated here but rather topics of current interest are highlighted for drawing attention to current research trends but also outstanding questions and current limitations.

4.2 | Modified *Alternaria* toxins in food

The term "modified mycotoxins" incorporates all molecules derived from a parent mycotoxin that have been either "biologically" or "chemically" (includes thermally formed variations) modified (Rychlik et al., 2014). According to current definitions, the term "masked mycotoxins" should therefore be used exclusively for biologically modified mycotoxins produced by plants (Berthiller et al., 2013).

Modified *Alternaria* toxins have been a neglected area of research for a long time. This is reflected by the two above cited articles on masked/modified mycotoxins from 2013 and 2014, in which no conjugated forms of any *Alternaria* toxin were reported. An important milestone was the first total synthesis of AOH and AME sulfates and glucosides by Mikula et al. (2013). Subsequently, these NMR-confirmed reference standards were used for the quantitative analysis of modified forms of those toxins in different foodstuff. A first method including conjugated AOH and AME was developed by Walravens et al. (2014), which allowed for the simultaneous determination of 10 *Alternaria* toxins including sulfated and glucosylated forms of AOH and AME conjugated at the C-3 position. When this method was applied in a pilot survey investigating commercially available rice ($n = 31$) and oat flake ($n = 16$) only TeA and TEN were found, while no modified toxins were detected. A similar method was also validated for tomato and fruit/vegetable juices by the same authors and tested in 129 samples purchased in Belgium (Walravens et al., 2016). Here, low levels (<LOQ to 9.9 μg/kg) of AOH-3-sulfate and AME-3-sulfate were detected.

In 2016, Lopez et al. investigated in total 81 samples of dried figs, sunflower and tomato products from the Netherlands. However, none of the seven conjugated *Alternaria* toxins included in the study (AOH-3-glucoside, AOH-9-glucoside, AOH-9-diglucoside, AME-3-glucoside, AME-3-malonylglucoside, AOH-3-sulfate, AME-3-sulfate) was detected above their respective LOQ value (López et al., 2016). The authors stated that the method was not validated for the analysis of *Alternaria* toxin conjugates due to limited standard availability. An LOQ of 2.5 μg/kg

for the conjugates was derived from the signal-to-noise ratio of injected standards in solvent. In South African sunflower seeds ($n = 159$), no AME-3-glucoside was detected while the parent AME was positive in >11% of the samples (Hickert et al., 2017). No other modified forms were included in the applied LC-MS/MS method. Puntscher et al. (2018a) developed a comprehensive LC-MS/MS method covering 17 *Alternaria* toxins for investigating the natural occurrence of free and modified *Alternaria* toxins in tomato sauce, sunflower seed oil, and wheat flour. This included AOH-3-glucoside, AOH-9-glucoside, AOH-3-sulfate, AME-3-glucoside, and AME-3-sulfate. Interestingly, AOH-9-glucoside and AME-3-sulfate were found in concentrations similar to their parent toxins in a naturally contaminated tomato sauce sample, an observation highlighting the importance to include modified *Alternaria* toxins in analytical methods for food surveillance. In an expanded follow-up survey, tomato sauces (n = 56), sunflower seed oils (n = 39) and wheat flours (n = 100) were collected in retail markets in Austria (n = 110), Croatia (n = 26) and Italy (n = 6) or ordered from online shops (n = 53). 61% of all products were coming from conventional agriculture, while the others (39%) were grown organically. AOH-3-sulfate (up to 2.1 ng/g) and AME-3-sulfate (up to 17.5 ng/g) were detected in 9% and 34% of tomato sauces accounting for 7–100% of their parent toxin concentrations (Puntscher et al., 2019b). In a recent study, the fate of free and modified *Alternaria* mycotoxins was investigated during six stages of apple concentrate production (grinding, turbos, decanter muds, preconcentration, concentrate and rejection)(Pavicich et al., 2020). The contamination levels of 10 *Alternaria* toxins/metabolites including AOH-3-sulfate, AOH-3-glucoside, AME-3-sulfate, and AME-3-glucoside were tested. Quantifiable levels of AOH, AME, TeA and TEN were observed in the ground apples but only AME-3-sulfate was found in the raw material with AOH-3-sulfate and AOH-3-glucoside detected during other processing steps.

Already in 2015, Hildebrand et al. reported that both, AOH and AME, can be efficiently conjugated, especially with glucose but also malonyl glucose, by cultured plant cells (Hildebrand et al., 2015). Moreover, it was demonstrated in follow-up experiments that AOH and AME sulfates can be produced directly by *Alternaria alternata* and that tomato tissue is able to convert AOH and AME to glucosylated metabolites. Moreover, sulfoglucosides were found in tomatoes inoculated with *A. alternata*, making this the first report of a mixed sulfate/glucoside diconjugate of a mycotoxin (Soukup et al., 2016). In 2015, Kelman et al. isolated 148 Canadian strains of *Alternaria* spp. and screened agar extracts by LC/data-dependent tandem mass spectrometry (MS/MS) (Kelman et al., 2015). The generated HRMS data was then processed by post-acquisition neutral loss filtering. Seven isolates were reported to produce sulfoconjugates of known *Alternaria* toxins. In total, 108 of the 148 isolates produced sulfoconjugated metabolites on agar plates. The analysis of seven isolates, additionally grown in liquid culture, on rice and Cheerios, resulted in discovering six new, two previously reported and 30 so far unidentified sulfoconjugated compounds. Interestingly, some of these conjugated metabolites had no known 'free' *Alternaria* precursor metabolite, suggesting that they are potential new *Alternaria* metabolites. In 2016, Zwickel et al. investigated the influence of temperature, substrate (rice, wheat kernels) and incubation time on the production patterns of 13 *Alternaria* toxins and three sulfoconjugates by *A. tenuissima* and *A. infectoria* isolates by LC-MS/MS (Zwickel et al., 2016). Additional LC-HRMS experiments were performed for exploring potentially occurring other modified forms for which no reference standards were available. These included precursor ion scans, neutral loss scans and collision induced mass spectra (MS/MS). AOH-, AME- and altenuisol-sulfates could be tentatively detected in rice and wheat extracts whereas sulfates of ALT, isoALT, ATX-I, ATX-II or STTX-III as well as glucose conjugation were not detected. The same group later isolated 93 *Alternaria* strains belonging to the four species *Alternaria tenuissima*, *A. arborescens*, *A. alternata*, and *A. infectoria* from winter wheat kernels harvested in Germany and Russia and incubated under equal conditions (Zwickel et al., 2018). Interestingly, LC-MS/MS analysis revealed that 95% of the strains were able to form at least one of the targeted 17 (modified) toxins. Sulfoconjugated modifications of AOH, AME, altenuisol and altenuene were frequently determined. Unknown perylene quinone derivatives were additionally detected.

All these relatively new reports suggest that modified *Alternaria* toxins are of relevance for exposure and risk assessment. Consequently, further studies should aim at identifying additional, so far unknown conjugates, for example, by new stable isotope based technology (Bueschl et al., 2017). Importantly, efforts on characterizing their occurrence should be intensified and become a standard in multi-mycotoxin LC-MS methods. Moreover, exploring their fate during food processing and digestion should be a priority.

4.3 | Determination of *Alternaria* toxins beyond the "big five"

Perylene quinones, including ATX-I, ATX-II, ATX-III, alterperylenol (ALP), and STTX-III, gained some interest lately, although the inclusion in analytical methods, and thus also occurrence data, remains limited to date.

This is partly due to the absence of commercially available reference standards but also caused by the high reactivity of the molecules bearing an epoxide group, namely ATX-II, ATX-III and STTX-III. So far it is not clear if ATX-II is indeed hardly present in *Alternaria* infested agricultural goods or if it is lost during food processing and/or analytical sample preparation. Potentially, ATX-II could be bound to food-related chemicals and/or be chemical instable because of the enhanced reactivity. Of note, ATX-II was recently detected for the first time in a naturally contaminated apple sample (Puntscher et al., 2020).

Walravens et al. (2014) included ATX-I in a multimethod assessing 10 analytes ("big 5", four modified mycotoxins and ATX-I) but did not detect it in any grain sample ($n = 47$). When testing for the same panel of analytes in 129 tomato and fruit juice samples, no ATX-I was detected as well (Walravens et al., 2016). The detection of low concentrations of ATX-I and ALP has been reported by Liu and Rychlik (2015) for cereal-based food in the range of 0.04–4.7 ng/g and 0.2–5.4 ng/g, respectively. ATX-I was determined in single samples of Nigerian rice (n = 2 of 38) at a mean of 1.6 ng/g (Rofiat et al., 2015) and also Nigerian wheat grains (n = 5 of 14) at a mean of 1.8 ng/g (Egbontan et al., 2017), in a single maize sample at 43 ng/g and one sunflower seed oil sample (<LOQ) (Hickert et al., 2016). Zwickel et al. (2018) detected unknown PQ derivatives when examining 93 *Alternaria* strains from German and Russian winter wheat by LC-HRMS. In a 2019 survey investigating European foods on 17 *Alternaria* toxins, including PQs, Puntscher et al. (2019b) did not detect ATX-II. However, ATX-I and alterperylenol were determined in the investigated matrices (wheat flour, tomato products, sunflower seed oil) with concentrations of up to 6 and 48 ng/g, respectively. The same analytical method was also applied to quantify *Alternaria* toxins in infant foods (infant formulae and cereal-based products) from the Austrian and Czech market besides a high number of other mycotoxins, including the regulated ones that were determined by a second method (Braun et al., 2020). Overall, 17 of the investigated 46 mycotoxins were detected at low levels. This included AME, TeA, TEN, and alterperylenol at maximum concentrations of 1.1 ng/g, 124 ng/g, 1.5 ng/g, and 20 ng/g, respectively. Gotthardt et al. (2019) determined ATX-I and alterperylenol rarely in infant food samples ($n = 25$) purchased in Germany.

4.4 | Pitfalls in *Alternaria* toxin analysis

The stability of the epoxide-bearing ATX-II was recently examined in a lab experiment simulating different storage and food processing steps of intact and blended tomatoes after experimental addition of the toxin for exploring its fate (Puntscher et al., 2018b). A significant decrease in ATX-II concentrations was observed in samples stored at room temperature but even more in those undergoing thermal treatment. At room temperature about 90% of ATX-II was recovered after 1.5 hr in raw tomato purees and purees heated prior to the addition of the ATX-II standard. After 24 hr only approximately 50% remained. Interestingly, recoveries were even lower in intact tomato fruits (23% after 1.5 hr and <1% after 24 hr). The reduction of the epoxide moiety to the alcohol (i.e., the formation of ATX I), was shown in intact tomato fruits (7–12%), suggesting enzymatic biotransformation by the plant. Aichinger et al. (2018) demonstrated that co-incubation of ATX-II with the anthocyanidin delphinidin or its degradation product phloroglucinol aldehyde significantly decreased ATX-II in aqueous solutions. This suggested a direct chemical reaction of ATX-II with these chemicals. To explore potential reaction products LC-HRMS full scan and MS/MS experiments of the ATX II solutions with or without delphinidin were compared applying untargeted metabolomics. However, for the 15 features identified to be of potential interest, no tentative structures based on fragmentation patterns could be elucidated.

Another potential analytical issue that might have resulted in serious underreporting of certain *Alternaria* toxins in the past could arise from the widespread use of microfiltration during food sample preparation. Aichinger et al. (2020c) assessed different membrane filters for their interaction with an *Alternaria* culture extract and found some membrane materials to be prone to adsorption of lipophilic toxins. In particular, filters using membranes out of nylon, polyethersulfone, glass fiber/cellulose acetae and (to a lesser extent) polyvinylidene fluoride were found to significantly reduce the concentrations of toxins from the perylene quinone family, but also AOH and AME. Furthermore, literature research revealed that food surveys using PVDF filters often reported lower concentrations and numbers of positive samples for AME, while finding AOH in amounts comparable to other studies. In the light of the result that PVDF shows a much higher affinity to bind AME than AOH, the authors speculate that AME exposure might have been frequently underestimated and suggest a critical re-evaluation of published surveys.

4.5 | Human biomonitoring via exposure biomarker analysis

The trend of analyzing multiple *Alternaria* toxins and metabolites simultaneously by sensitive LC-MS/MS methods can also be clearly seen in the area of human biomonitoring (HBM). Here, biomarkers of exposure, that is, the parent toxin or a metabolic product thereof, are measured

in biological fluids including urine, blood/plasma/serum, and breast milk. While the first published method in the area of *Alternaria* research was a single analyte LC-MS/MS method focusing on the rather abundant TeA, nowadays a number of potentially relevant *Alternaria* toxins are being incorporated in broad multi-mycotoxin assays (Table 1). To date, it is not entirely clear if the included analytes are in fact the once most indicative for exposure and toxicity as HBM for *Alternaria* toxins is lagging behind the regulated mycotoxins (Vidal et al., 2018; Warth et al., 2013). It seems that their implementation is rather related to the commercial availability of reference standards and the performance of certain toxins during LC-MS/MS analysis. For example, AME is ionized extremely well in electrospray ion sources and thus over-proportionally detected due to comparably very low limits of detection. In a recently reported method for quantifying mycotoxins in human breast milk, the LOD of AME was in the sub-ppt (fg/ml) range (Braun et al., 2020) and also in some urinary assays this ultimate sensitivity might be reached in the near future. Glucuronides, which were Suggested as the major metabolites of AOH and AME in humans based on microsome and cell model-based experiments (Burkhardt et al., 2009, 2011; Pfeiffer et al., 2009), are not commercially available and typically show lower ionization efficiencies. Hence, there direct quantitative determination in human urine is not established to date.

The only *Alternaria* toxin frequently detected at higher levels (ng/ml range) in urine is TeA, while AOH, AME, and TEN are typically in the pg/ml range in urine and breast milk (Table 1). This was also confirmed in a recent Chinese study investigating urine samples of 60 healthy volunteers from the Beijing province (Liu et al., 2020). The authors stated that particularly the high occurrence and concentration levels of TeA, AME, and ochratoxins warrant attention in future studies. While this sheds light on the potential high (low-dose) prevalence of some *Alternaria* toxins, it should be mentioned that most regulated mycotoxins (aflatoxins, trichothecenes, zearalenone) were not included in the study. A second Chinese study by Qiao et al. (2020) reported TeA, AME and AOH to be the predominant *Alternaria* mycotoxins with detection rates of 85.9%, 96.3% and 51.9%, respectively in 135 volunteers from healthy donors in Beijing. This group was tested for five *Alternaria* toxins (TeA, AOH, AME, ALT, TEN) before and after enzymatic hydrolysis exclusively (Table 1).

5 | CONCLUSION: FUTURE TRENDS—Quo vadis *Alternaria*?

In this article, we addressed doubtful considerations about a lack of relevance of *Alternaria* toxins as food contaminants. The incompleteness of available data should not be falsely attributed to a lack of toxicity or occurrence, but rather to the extraordinary complexity of the chemicals involved—and the attendant diversity concerning chemical stability, bioactivities, ADME and human exposure levels, which makes research in the field so challenging. Therefore, with resources being limited as they are, research on *Alternaria* toxins is encouraged to be as effective as possible, and to be focused on the most important knowledge gaps.

With respect to toxicity, the situation in vivo is not fully clarified even for comparably well-studied compounds such as AOH and AME (Schuchardt et al., 2014), not even to speak of perylene quinones. In addition, published data suggests considerable mixture effects. Closing respective data gaps for proper risk assessment should be a top priority.

Similarly, toxicokinetics of *Alternaria* toxins are poorly understood and call for additional testing *in silico*, *in vitro*, and in vivo. This should involve innovative stable isotope-assisted metabolomics workflows for unraveling toxin metabolism in detail (Flasch et al., 2020) but also the application of computational methods such as PBPK modelling to allow for interspecies extrapolation to humans. Newly developed LC-MS/MS methods being able to simultaneously detect traces of multiple *Alternaria* toxins are expected to be widely applied in testing food and feed for currently insufficiently assessed metabolites with potential toxicological relevance. Additionally, the implementation of such methods in biomonitoring and exposome approaches seems promising for complementing food occurrence data toward a more accurate estimation of human exposure. Particularly, with the existence of PBPK models, reverse dosimetry could be used to quantitatively link already existing biomonitoring data (e.g. Martins et al., 2019) with exposure assessment and TTCs.

A novel line of research hint toward substantial interplay between *Alternaria* toxins and the gut microbiome (Crudo et al., 2020) which on one hand might affect the microbial composition but, on the other hand, is likely to impact toxicodynamics and toxicokinetics.

Furthermore, both, the testing of toxicologically characterized *Alternaria* toxins for novel bioactivities as well as assessing the toxicity of yet insufficiently studied metabolites is far from over. For example, the discovery of the estrogenic and androgenic potential of AOH and related chemicals, and the contrast to anti-estrogenic effects described for naturally occurring *Alternaria* toxin mixtures (Aichinger et al., 2019), has sparked research on the activity of *Alternaria* toxins as endocrine disruptors (EDCs) (Dellafiora et al., 2018; Stypuła-Trębas et al., 2017). Again, the complex pattern of bioactivities, which involves interactions with estrogen (Lehmann et al., 2006), androgen

TABLE 1 Overview of LC-MS methods for human biomonitoring (HBM) of *Alternaria* toxins (historical order) and associated exposure biomarker levels in the according pilot studies. Multiple methods involve the analysis of additional mycotoxins/xenobiotics besides *Alternaria* toxins (see "Comments" column)

Investigated toxins	Biological matrix	LOQ (ng/ml)	Mean concentration and range (ng/ml)[a]	Comments	Reference
TeA	Urine	0.60	TeA: 6.8 (1.3–17.3)	b-glucuronidase, derivatization, SPE, SIL, LC-MS/MS24-hr urine of six German volunteers	Asam et al. (2013)
TeA, *allo*-TeA	Urine	0.11	TeA: 6.6 (0.16—44.4) Allo-TeA: 1.25 (0.11—5.7)	SPE, SIL, LC- MS[c];48 samples from Germany	Hovelmann et al. (2016)
AOH	Urine	0.03	AOH: 0.06 (0.03–0.20)	Multi-mycotoxin method (12 mycotoxins); SPE cleanup; b-glucuronidase treatment; 120 samples from Nigerian volunteers	Šarkanj et al. (2018)
AOH, AME	Urine	0.9–1.0	AOH: 0.21[c] (<LOQ— 9.9)	Multi-mycotoxin method (34 analytes); QuEChERS UPLC-MS/MS; 94 participants from Portugal; 1st morning and 24-hr urine; here only 1st morning urine reported	Martins et al. (2019)
AOH, AME	Urine SerumBreast milk	0.15–2.0	Urine: AME: <LOQ	Multi-estrogen method (75 analytes); li-li extraction, evaporation & reconstitution; low number of Austrian urine, serum, and milk samples tested	Preindl et al. (2019)
AOH, AME, ALT, TeA, TEN	Urine	0.003–0.5	AOH: 0.34 (<LOD—7.68) AME: 0.059 (<LOD—0.17) TeA: 4.4 (<LOD—54.1) TEN: 0.024 (<LOD—0.19)	Multi-mycotoxin method (18 mycotoxins); SPE cleanup; b-glucuronidase treatment; 60 samples from healthy individual (4–70 years).	Liu et al. (2020)
AOH, AME, ALT, TeA, TEN	Urine	0.001–0.05	AOH: 0.60 (0.06–2.64) AME: 0.015 (<LOQ—0.17) ALT: 0.02 (0.02) TeA: 0.95 (<LOQ—26.9) TEN: 0.002 (<LOQ—0.004)	Targeted *Alternaria* HBM method; liquid-liquid extraction β-glucuronidase/ arylsulfatase; UPLC-MS/MS	Qiao et al. (2020)
AOH, AME, TEN	Breast milk	0.001–0.046	AME: 0.002[b]	Multi-mycotoxin method (34 analytes); SPE cleanup; SIL; one pooled sample from Austrian milk bank donors	Braun et al. (2020)
AOHAOH, AME, TEN	UrineBreast milk	0.030.001–0.046	n.d.AME: 0.003 (<LOQ—0.012)	Breast milk ($n = 22$), complementary food and infant urine tested on multiple mycotoxins	Ezekiel et al. (2020)
AOHAOH, AME, TEN	UrineBreast milk	0.030.001–0.046	n.d.AME: 0.0048 (<LOQ—0.056)AOH & TEN: n.d.	Case study in mother-infant pairs; $n = 75$ for breast milk	Braun et al. (2020)

[a]As reported in the respective publications.
[b]First determination of AME in breast milk.
[c]Median value; mean not reported.

(Stypuła-Trębas et al., 2017) and aryl hydrocarbon receptors (Aichinger et al., 2019; Hohenbichler et al., 2020) as well as kinase inhibition (Aichinger et al., 2020a), is yet poorly elucidated and in the focus of ongoing research.

In all these areas, research is ongoing, thus fortifying the overall impression: *Alternaria* toxins have never been more "emerging".

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All authors contributed writing-original draft; writing-review editing. These authors Georg Aichinger, Benedikt Warth, and Doris Marko were involved conceptualization.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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