

Insights into *Alternaria* in apple fruit causing mouldy core, external infection and mycotoxin production under retail and storage conditions

María Agustina Pavicich^{1,2,3*}, María Luisa Maldonado¹, Truong Nhat Nguyen², Marthe De Boevre^{2,3}, Sarah De Saeger^{2,3,4}, Andrea Patriarca^{1,3,5}

¹Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Química Orgánica, Laboratorio de Microbiología de Alimentos. CONICET, Instituto de Micología y Botánica (INMIBO), Buenos Aires, Argentina.

²Centre of Excellence in Mycotoxicology and Public Health, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium.

³MYTOX-SOUTH[®]

⁴Department of Biotechnology and Food Technology, Faculty of Science, Doornfontein Campus, University of Johannesburg, South Africa.

⁵Applied Mycology Group, MCAM, Faculty of Engineering and Applied Sciences, Cranfield University, College Road, MK43 0AL Bedford, UK

* Corresponding author

E-mail address: MariaAgustina.Pavicich@UGent.be

Abstract

Apple fruit is widely consumed worldwide, but fungal contamination in the postharvest stage presents a significant food safety concern. This study evaluates the production and accumulation of *Alternaria* mycotoxins, including alternariol (AOH), alternariol monomethyl-ether (AME), and the modified forms (AOH-3-S, AME-3-S, AOH-3-G, AME-3-G), altenuene (ALT), tenuazonic acid (TeA), tentoxin (TEN), altertoxin I and II (ATX-I, ATX-II), in Red Delicious apples under simulated retail and post-harvest conditions. Three *Alternaria tenuissima* strains (isolates 02, 31 and 36) were inoculated in apple fruit at two sites separately (core and exterior) and incubated at two temperatures (25 °C and 4 °C) for 1 and 9 months. Mycotoxin production was quantified using LC-MS/MS, revealing significant variability across strains and conditions. Isolates 02 and 36 exhibited significant temperature and site-dependent variability in mycotoxin production. Higher levels of AOH, AME, ALT, and ATX-I were produced at 25 °C and in the core. Long-term cold storage delayed fungal growth but did not prevent mycotoxin accumulation, raising concerns about the safety of processed apple products. These findings highlight the need for stricter monitoring of mycotoxins during post-harvest storage to mitigate health risks. The findings provide insights into their toxigenic capacity *in vivo* and highlight potential risks for food safety.

Keywords: Mycotoxins, food safety, postharvest, storage, pome fruit

1 Introduction

Apple is one of the most popular fruits in the world, with over 95 MM tons produced annually in 2022 (FAOSTAT, 2024). China is the largest producer, representing approximately 40% of world production, followed by the USA, Turkey, Poland, Iran, and Italy. It is also an economically important crop in many other countries, such as Australia, France, Greece, South Africa, Canada, Chile, and Argentina. Apple fruit is a seasonal crop, and it is consumed either fresh or processed. Currently, fresh apples can be stored for up to 9 or 12 months in refrigeration chambers, being available in the market all year long and preserving their sensory properties.

Nevertheless, some fruits do not comply with the quality standards for retail due to factors like unappealing appearance, firmness, or sensitivity to bruising and therefore, are used for apple products. The cosmetic quality of fruit destined for apple products is usually lower than for fresh consumption. However, less attention has been paid to other unwanted characteristics of these fruits, such as latent fungal infections without external visual signs (Heinmaa, 2020; Varming et al., 2013). Apple fruit is susceptible to several fungal diseases that can occur at preharvest or postharvest. Some fungi have been indistinctly identified as typical of one or another. In contrast, others can occur in both stages or, more commonly, begin the infection in the field and increase their incidence during the postharvest stage (Pavicich et al., 2020a). The Food and Agriculture Organization of the United Nations (FAO) estimated that between 15% and 50% of the world's production of fruits and vegetables is lost at postharvest stage, mainly due to pathogen spoilage (Gustavsson et al., 2019). In low and middle-income countries, postharvest loss is even higher, probably due to poor or low-tech availability during storage and transportation (Joardder and Masud, 2019). For apples, fungal diseases can cause significant losses; some are reported to reach up to 70% yield reduction (Jha et al., 2009) or 80% of decay in stored fruits (Sánchez-Torres et al., 2018). The mouldy

core (MC) is a frequent fungal apple disease that manifests as a rotten area inside the fruit, typically in the seed and carpel wall. Fungal genera like *Penicillium*, *Cladosporium* and *Fusarium*, but mainly *Alternaria* have been associated with MC (Basson et al., 2019; Elfar et al., 2018; Serdani et al., 1998; van der Walt et al., 2010).

In a previous work, the *in vitro* toxigenic potential of *Alternaria* strains isolated from apple fruit was studied, and metabolites such as alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), tentoxin (TEN), altenuene (ALT), altertoxin-I (ATX-I), altertoxin-II (ATX-II), altertoxin-III (ATX-III), altechromone A, isopropyl tetramic acid, pyrenochaetic acid A, and alterperyleneol, among others, were produced by these apple pathogens and therefore, are likely to accumulate in the fruits (Pavicich et al., 2022). Some of these metabolites are known mycotoxins and have been reported in naturally contaminated apple fruit and apple products (Pavicich et al., 2023; Puntischer et al., 2020). Nevertheless, the metabolomic capacity of *Alternaria* spp. in apples has yet to be deeply studied. The production of these mycotoxins might be influenced by several factors, such as the site of infection in the fruit or storage temperature. A report from the European Food Safety Authority (EFSA) established the need to increase the information on the toxicity, natural occurrence, and distribution of *Alternaria* toxins in the different food chains (Arcella et al., 2016). Moreover, the European Commission recently recommended monitoring *Alternaria* toxins in food and identifying the factors resulting in their high levels (Commission Recommendation (EU) 2022/553, 2022). Thus, regulating these mycotoxins will significantly impact international commerce, affecting the economies of both exporter and importer countries. Therefore, widening the knowledge on the metabolomic capacity of *Alternaria* strains in apples will represent a relevant contribution to food-producing countries and those depending on them by commerce.

The objective of this study was to evaluate the production and accumulation of known *Alternaria* mycotoxins, namely AOH, AME, ALT, TeA, TEN, ATX-I, and ATX-II, and the modified mycotoxins alternariol-3-sulphate (AOH-3-S), alternariol monomethyl ether-3-sulphate (AME-3-S), alternariol-3-glucoside (AOH-3-G), and alternariol monomethyl ether-3-glucoside (AME-3-G) in apple fruit simulating retail and storage conditions to determine their toxigenic capacity *in vivo*.

2 Materials and methods

2.1 Samples

A total of 72 ripened Red Delicious variety apples were acquired from a local retail market in Buenos Aires, Argentina. All apples had similar shape, size, colour and ripening state, and had no bruises, punctures, symptoms of fungal contamination or other diseases.

2.2 Standards and Reagents

AOH, AME (1 mg standard each), ATX-I and ALT (0.1 mg standard each) were obtained from Fermentek (Jerusalem, Israel) and dissolved in 1 ml of methanol (MeOH). Certified reference standards of TeA and TEN (101.3 and 100.5 mg respectively, dried down) were obtained from Romer Laboratories Diagnostic GmbH (Tulln, Austria) and dissolved in 1 ml of acetonitrile (ACN). ATX-II standard was kindly provided by Prof. Marko and Prof. Warth (TU Wien). Reference standards of conjugated *Alternaria* toxins (AOH-3-S, AOH-3-G, AME-3-S, AME-3-G) were synthesized as described by Mikula et al. (2013) and stock solutions were prepared at a concentration of 10 µg/ml in MeOH. The internal standard urolithin A (UR-A) (5mg) was purchased from Sigma-Aldrich (Bornem, Belgium) and dissolved in 5 ml of dimethyl sulphide (DMSO). Internal standard TeA ²H-13 (1 mg) was bought from Toronto Research Chemical (Toronto, Canada) and dissolved in MeOH. Ultra-

pure water was obtained from an Arium® pro system (Sartorius, Goettingen, Germany). ACN (absolute, LC-MS grade) and acetic acid (UPLC/MS) were obtained from BioSolve BV (Valkenswaard, The Netherlands), and ACN (HiPerSolv Chromanorm HPLC grade) was acquired from VWR International (Leuven, Belgium). Sodium chloride (NaCl) was purchased from Merck (Darmstadt, Germany), whereas magnesium sulphate (MgSO₄, anhydrous) from Sigma-Aldrich (Bornem, Belgium).

2.3 Strains and inoculation

Half of the apples (n=36) were subjected to infection with 3 different *Alternaria tenuissima* strains, and the rest served as control. Based on their secondary metabolite production determined in a previous work (Pavicich et al., 2022), three *Alternaria* strains isolated from apples and morphologically identified as *A. tenuissima*, were selected and inoculated in non-contaminated Red Delicious fruits (isolate ID: 02, 31 and 36). Isolate 02 was obtained from a lesion in the external part of the fruit, while 31 and 36 from MC. Apple fruits were disinfected by immersion in ethanol (70%) for 10 minutes. Half of the fruits subjected to fungal infection (n=18) were superficially wounded (1x1 cm) with a sterile scalpel for exterior inoculation, and the remaining 18 apples were inoculated in the core according to Gao et al., (2013). Suspensions of each strain in an aqueous solution of Tween 80 (0.05%) at a concentration of 10⁵ spores/ml were made. One microliter (µl) of the suspension was inoculated with a sterile calibrated loop in each inoculation site. Nine of the apples inoculated in the exterior and 9 inoculated in the core were incubated at 25 °C for 1 month to simulate retail conditions. The remaining inoculated apples were incubated at 4 °C for 9 months, to simulate storage conditions. Each assay was done in triplicate for each *A. tenuissima* strain and each apple was incubated in a separate sterile bag. Control fruits without inoculation were also incubated at both temperatures for the obtention of blank samples.

2.4 Extraction

The extraction procedure was made using an in-house validated method developed at the Centre of Excellence in Mycotoxicology and Public Health (CEMPH) for the detection and quantification of AOH, AME, ALT, TeA, TEN, ATX-I, AOH-3-S, AOH-3-G, AME-3-S, AME-3-G in apple products with some modifications (Pavicich et al., 2024; Walravens et al., 2016). Additionally, ATX-II was also detected and quantified. Briefly, samples consisting of the entire contaminated fruit and blanks were grinded in individual sterile bags and an aliquot of 2.0000 ± 0.0020 g was weighed in an extraction tube. For quantification, matrix-matched calibration curves (MMCC) were constructed on blank samples. Five blanks were fortified with the studied mycotoxins at concentration levels ranging from 5 to 100 $\mu\text{g/kg}$ and soaked for 15 min. Internal standards UR-A, and TeA $^2\text{H-13}$ were added in concentrations of 10 $\mu\text{g/kg}$ to the blanks and unknown samples. After 10 s of vortex-mixing, samples were kept in the dark for 15 min and extracted with 10 ml of ACN (HPLC grade) by shaking for 30 min. Sample extracts were briefly centrifuged (1 min, 3,200 g), and MgSO_4 anhydrous salt (2.00 ± 0.05 g) and NaCl (0.50 ± 0.05 g) were added. Afterwards, the tubes were vigorously shaken for 30 s, placed in an overhead shaker for 15 min, and centrifuged (10 min, 3,200 g). Six (6.00) ml of the supernatant were transferred to a tube and evaporated to dryness using a Turbovap LV module (Biotage AB, Uppsala, Sweden) maintained at 40 °C. Finally, the residue was redissolved in 100 μl of injection solvent (ultrapure water/ACN (LCMS grade), 70/30, v/v), vortex-mixed for 30 s, and centrifuged (Ultrafree-MC PTFE centrifugal filter units, 0.22 μm ; Merck Millipore, Darmstadt, Germany) for 10 min at 10,000 g prior to analysis.

2.5 Mycotoxin quantification by LC-MS/MS analysis

Determination and quantification of the studied mycotoxins was done on a Waters Acquity UPLC coupled to a XEVO TQ-S mass spectrometer (Waters, Milford, MA, USA) according

to Walravens et al. (2014). To achieve compound separation, an Acquity UPLC High Strength Silica trifunctional C18 Alkyl phase column (HSS T3, 1.8 μm , 2.1 x 100 mm) (Waters, Milford, MA) was used. The instrument was used in the negative electrospray ionisation (ESI⁻) mode. The temperature of the column was 35 °C, and the mobile phases were A: ultra-pure water/acetic acid (AA) (99/1, v/v) and B: ACN/AA (99/1, v/v). The flow rate was 0.4 ml/min and the total run time 7 min following the gradient described by Walravens et al. (2016) and Pavicich et al., (2020b). The capillary voltage was 30 kV, and nitrogen was applied as spray gas. The source and desolvation temperatures were set at 150 °C and 200 °C, respectively. The argon collision gas pressure was 9×10^{-6} bar, the cone gas flow, 50 l/h, and the desolvation gas flow, 500 l/h. Two selected reaction monitoring transitions with a specific dwell-time were optimised for each analyte, in order to increase the sensitivity and the selectivity of the mass spectrometric condition. The limits of detection (LOD) and quantification (LOQ), repeatability, reproducibility, specificity, linearity, apparent recovery, expanded measurement uncertainty, matrix effect, and precision for each mycotoxin are informed in Walravens et al. (2014) and in Pavicich et al., (2024). For data acquisition and processing, the MassLynx and QuanLynx[®] version 4.1. software (Micromass, Manchester, UK) were used. After data processing, samples with mycotoxin concentrations outside the working range of the corresponding MMCC were diluted appropriately and reanalysed.

2.6 Statistical analysis

For data analysis, mycotoxin concentrations below the limit of detection (LOD) were replaced by half the value of their respective detection limits. Significant differences in concentrations of mycotoxin production by the different isolates were tested using Kruskal-Wallis test, since the data was not normally distributed. The data was analysed using Infostat software and p -value < 0.05 was considered as statistically significant.

3 Results

At 25 °C the fungal growth of *Alternaria* isolates 02, 31 and 36 on the apples was more copious than at 4 °C. After one month of incubation at 25 °C, fungal development was abundant in the exterior and interior of the fruits for all the isolates. For the apples incubated at 4 °C, after 9 months of incubation, fungal lesions and disease symptoms were visible in the interior and exterior of the fruit. Control samples did not develop fungal spoilage and no mycotoxins were detected in these samples at any of the evaluated timepoints.

All the isolates were able to produce mycotoxins under the studied conditions. Table 1 shows the mean concentration of each mycotoxin under the tested conditions for each isolate. AOH, AME, ALT, TeA, TEN, ATX-I, ATX-II and AOH-3-G were detected at least at one of the assessed conditions. AOH-3-S, AME-3-S and AME-3-G were not produced under the tested conditions by any of the strains. TeA was produced in the highest concentrations by all the strains.

Figure 1 depicts mycotoxin concentrations for each strain. TeA is shown separately from the other toxins (Figure 2) due to the different scale required for its concentration. A Kruskal-Wallis test was conducted to evaluate the effect of different inoculation methods and temperature conditions on the production of mycotoxins by each fungal strain separately. The significance ($p < 0.05$) of inoculation site and temperature on mycotoxin production was mycotoxin- and strain-dependent.

3.1 Isolate 02

Temperature and inoculation site had a significant influence on AME production by isolate 02 ($p < 0.05$), being 25 °C and core inoculation the conditions that favoured mycotoxin accumulation (440 µg/kg). ATX-I was accumulated in significantly higher concentrations (32 µg/kg) in the core and at 25 °C than at 4 °C in any inoculation site, while ATX-II was only

produced at detectable levels at 25 °C and apple core, and AOH-3G at 4 °C and the external inoculation. For the rest of the mycotoxins, the effect was not significant, although large variations were observed between tested conditions. For example, the production of AOH by this isolate ranged from 98 µg/kg to 780 µg/kg, and this isolate produced the highest concentration of ALT among the three isolates used for this study (928 µg/kg) at 25 °C in the apple core, while much lower amounts were detected at the other conditions. Although the difference in concentrations detected were not significant for TeA production by isolate 02, it showed an unexpected pattern in relation to temperature (Figure 2). At 25 °C, higher amounts were produced in MC (mean 12,500 µg/kg), while at 4 °C the external lesion allowed a higher toxin production (6,572 µg/kg) than the core infection (38 µg/kg).

3.2 Isolate 31

The Kruskal-Wallis test did not reveal significant differences in mycotoxin production for isolate 31 across the different inoculation methods and temperature conditions ($p > 0.05$). However, ATX-II was detected at the highest concentration at 25 °C in the exterior of the fruit and AOH-3-G at 4 °C in the apple core. The production of the other mycotoxins tested remained relatively low and stable across the experimental conditions, indicating that mycotoxin production by this strain is similar under different storage temperature and inoculation site. Moreover, isolate 31 showed consistently lower levels of mycotoxin production compared to isolates 02 and 36 (Figures 1 and 2).

3.3 Isolate 36

Significant differences in mycotoxin production by isolate 36 in response to temperature and inoculation site ($p < 0.05$) were observed. AOH and TEN production displayed the largest variability, with the highest concentration observed in the core at 25 °C (618 µg/kg and 1388 µg/kg, respectively). The production of ALT and AME by this isolate was also significantly influenced by the conditions, which led to the highest levels of toxin accumulation at 25 °C

and core inoculation (153 µg/kg and 159 µg/kg, respectively). Although ATX-I production was not significantly different between the tested conditions, a pattern of higher accumulation at 25 °C compared to lower temperatures was observed, with the mouldy core at 25 °C yielding the highest concentration of 36 µg/kg. Interestingly, ATX-II was only detected at 25 °C in the apple core, and this isolate did not produce AOH-3-G at any of the tested conditions. For TeA, significant differences were found (Figure 2), with the external lesion at 25 °C producing the highest concentration (345,442 µg/kg), markedly higher than the production under other conditions. Overall, isolate 36 was particularly affected to the inoculation and temperature variations, with 25 °C consistently favouring higher mycotoxin production compared to other conditions.

4 Discussion

The metabolic differences of *Alternaria* strains from apple fruit were previously investigated, and the strains isolated from MC showed a broad *in vitro* metabolic capacity (Pavicich et al., 2022). The strains tested *in vivo* were isolated from the exterior (isolate 02) and MC (isolates 31 and 36) of apple fruit. All of the isolates, regardless of their isolation site, had the ability to infect the apples in the core and exterior of the fruit and synthesise a broad family of compounds. However, their source did not correlate with the site where maximum mycotoxin production was produced in the *in vivo* experiment. For example, isolate 02 (ext.) produced higher amounts of most of the mycotoxins when inoculated in the core. When tested *in vitro*, the three isolates selected for this study were able to produce all the tested mycotoxins except for the modified forms, and isolate 02 did not produce TeA. These strains could produce the same mycotoxins *in vivo*, at least under one of the tested conditions, except for ALT, which was not produced by strain 31 under any condition. Interestingly, isolate 02, which did not synthesise TeA *in vitro* at 25 °C, produced large amounts of this toxic metabolite when

270 colonizing the centre of the fruit at 25 °C for one month or the exterior at 4 °C for 9 months
271 and lower concentrations under the other conditions. Isolates 02 and 31 produced AOH-3-G, a
272 metabolite not detected *in vitro*. Although 25 °C was the most favourable temperature for the
273 majority of the toxins, AOH-3-G was only detected at 4 °C. Glucose conjugates are typically
274 formed by plant metabolism (Puntscher et al., 2019a); therefore, they were expected in the *in*
275 *vivo* analysis. The other modified forms of the alternariols were not detected under any
276 condition in the present study, but there are reports of the natural occurrence of these
277 metabolites (AOH-3-S, AME-3-S) in commercially available tomato products (Puntscher et
278 al., 2019b, 2018; Walravens et al., 2016). Whether they are produced by the tomato plant
279 metabolism or during food processing still needs to be evaluated. The *in vitro* testing revealed
280 differences in mycotoxin production compared to *in vivo* conditions, emphasizing the
281 importance of studying these fungi in their natural environment and suggesting a potential
282 influence of the fruit metabolism on the production of secondary metabolites.

283 The temperature and inoculation site only significantly influenced mycotoxin production for
284 isolates 02 and 36, demonstrating strain-specific responses. For these strains, mycotoxin
285 accumulation was higher when infecting the fruit in the core at 25 °C for one month. On the
286 other hand, strain 31 accumulated AOH and AME in higher concentration when infecting the
287 core at 4 °C than at the other conditions, but this strain produced significantly lower levels of
288 the toxins than isolates 02 and 36 and the difference between conditions was not significant.

289 TeA was the mycotoxin produced at the highest concentrations *in vivo* at all the conditions by
290 the 3 strains. Similarly, a report of naturally rotten apples in China also showed that TeA was
291 the toxin with the highest accumulation in rotten fruit in comparison with the other *Alternaria*
292 toxins (Li et al., 2020). Ozcelik et al. (1990) reported the production of AOH, AME, ALT and
293 TeA by an *Alternaria* strain artificially inoculated in the exterior of apple fruit at 25 °C. In
294 that study, the concentrations of AOH, AME, and ALT when storing fruit for 5 weeks at 25

295 °C were similar to those detected in the present one after one month of incubation at 25 °C.

296 More recently, Puntschner et al. (2020) investigated the presence of 17 *Alternaria* metabolites

297 in retail apples showing symptoms of *Alternaria* infection, but only TEN was detected and in

298 low concentrations. López et al. (2016) analysed fresh retail apples in the Netherlands for the

299 natural occurrence of AOH, AME, ALT, TEN and TeA, and only low concentrations of AOH

300 were detected in one sample (29 µg/kg); however, the fruit analysed did not presented

301 symptoms of the disease. In China, Li et al. (2020) analysed the presence of AOH, AME,

302 ALT, TEN and TeA on different parts of fresh retail naturally contaminated apples and

303 reported high concentrations of all these toxins in the areas closer to the lesion. Mao et al.

304 (2023) investigated mycotoxin production by *Alternaria* strains inoculated in apples and

305 observed variability between strains. The disparities reported in different studies highlight the

306 complex interplay of factors influencing mycotoxin production, including storage conditions,

307 strain variability, and fruit varieties.

308 After a prolonged cold incubation period, mimicking the storage commonly performed by

309 packhouses and apple processing industries, fungal growth was evident, and *Alternaria*

310 mycotoxin production occurred. At 4 °C, AOH, AME, ALT, TEN, TeA, ATX-I, ATX-II and

311 AOH-3-G were produced at least by one of the strains either in the exterior or the core of the

312 fruit stored for 9 months. Mycotoxin production was not reported by Ozcelik et al. (1990) at 4

313 °C, but the incubation time at low temperature was shorter (5 weeks) than the one proposed in

314 the current study. Puntschner et al. (2020) reported AOH, AME, AME-3-S and TEN in lower

315 concentrations than those from the present study in stored apples under professional storage

316 for 6 months. Nonetheless, in a sample with visual fungal growth after storage, they observed

317 high concentrations of AOH, AME, AOH-3-G, AOH-9-G, AOH-3-S, AME-3-S, ALT, its

318 isoform isoALT, ATX-I, ATX-II and alterperyleneol. Fungal infestation at 4 °C was

319 previously reported as inhibited after 30 days of incubation, with low or no mycotoxin

production (Mao et al., 2023). Nonetheless, in a previous work we demonstrated that cold storage selected *Alternaria* spp. over other apple fungal pathogens, and that *Alternaria* mycotoxins were frequent in long-term stored apples (Pavicich et al., 2020a). In this study, we showed that a longer storage period leads to the accumulation of multiple mycotoxins. Our results confirm that fungal growth and mycotoxin accumulation by *Alternaria* spp. is a slow but steady process at low temperature.

The statistically significant effect of temperature on the production of several *Alternaria* toxins indicates that changes in postharvest storage temperature would substantially impact the accumulation of these toxic metabolites. Elevated temperatures may enhance enzymatic activities involved in mycotoxin biosynthesis, leading to increased production. However, some of the metabolic biosynthetic pathways are still active at temperatures as low as 4 °C, suggesting the need to monitor the presence of these toxins after long-term storage. The complexity of mycotoxin regulation involves several factors which underscore the multifaceted nature of their biosynthesis (Tudzynski, 2014). Moreover, the *in vivo* production of TeA by a strain which did not produce it in an *in vitro* experiment suggests an essential role of the substrate and colonization factors involved in its biosynthesis. The fact that all three factors contribute to TeA production indicates a complex interplay of genetic, environmental, and host-specific factors in determining the levels of this mycotoxin. Together, these findings demonstrate that isolates 02 and 36 are more responsive to temperature and infection site, while isolate 31 exhibits lower and more stable toxin production across conditions.

5 Conclusions

In conclusion, a temperature of 25 °C favoured mycotoxin production, as expected, and higher levels of AOH, AME, TeA, TEN and ATXs accumulated when *Alternaria* strains from apple colonized the centre of the fruit, independently of their source (external lesion or MC). While cold storage slowed fungal growth and mycotoxin accumulation, it did not prevent

contamination entirely. Although visibly infested fruit is unlikely to reach consumers, the risk posed by mycotoxins in processed products remains significant, emphasizing the need for stricter monitoring in post-harvest handling. Since long-term cold-stored fruit is frequently destined for processing, it represents a risk from a food safety point of view, especially when affected with MC. These findings advance our understanding of the complex dynamics between fungi and their host environments, with potential implications for food safety and postharvest practices. Further studies into the underlying mechanisms and interactions will deepen the knowledge of *Alternaria* pathogenicity and mycotoxin production in apple fruit.

6 Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT in order to rephrase and improve the readability of the manuscript. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

7 References

- Arcella, D., Eskola, M., Gómez Ruiz, J.A., 2016. Dietary exposure assessment to *Alternaria* toxins in the European population. EFSA J. 14. <https://doi.org/10.2903/j.efsa.2016.4654>
- Basson, E., Meitz-Hopkins, J.C., Lennox, C.L., 2019. Morphological and molecular identification of fungi associated with South African apple core rot. Eur. J. Plant Pathol. 153, 849–868. <https://doi.org/10.1007/s10658-018-1601-x>
- Commission Recommendation (EU) 2022/553, 2022. Scientific Opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. Off. J. Eur. Union 9, 90–92. <https://doi.org/10.2903/j.efsa.2011.2407>
- Elfar, K., Zoffoli, J.P., Latorre, B.A., 2018. Identification and Characterization of *Alternaria* Species Associated with Moldy Core of Apple in Chile . Plant Dis. 102, 2158–2169. <https://doi.org/10.1094/pdis-02-18-0282-re>
- Gao, L.L., Zhang, Q., Sun, X.Y., Jiang, L., Zhang, R., Sun, G.Y., Zha, Y.L., Biggs, A.R., 2013. Etiology of Moldy Core, Core Browning, and Core Rot of Fuji Apple in China. Plant Dis. 97, 510–516. <https://doi.org/10.1094/PDIS-01-12-0024-RE>
- Gustavsson, J., Cederberg, C., Sonesson, U., van Otterdijk, R., Meybeck, A., 2019. Food loss and food waste: Causes and solutions, Food Loss and Food Waste: Causes and Solutions. Rome. <https://doi.org/10.4337/9781788975391>
- Heinmaa, L., 2020. Factors Affecting Apple Juice Quality And Mycotoxin Patulin Formation.
- Jha, G., Thakur, K., Thakur, P., 2009. The *Venturia* Apple Pathosystem: Pathogenicity Mechanisms and Plant Defense Responses. J. Biomed. Biotechnol. 2009, 1–10. <https://doi.org/10.1155/2009/680160>
- Joardder, M.U.H., Masud, M.H., 2019. Food Preservation in Developing Countries : Challenges and Solutions.
- Li, Y., Zhang, X., Nie, J., Bacha, S.A.S., Yan, Z., Gao, G., 2020. Occurrence and co-occurrence of mycotoxins in apple and apple products from China. Food Control 118, 107354. <https://doi.org/10.1016/j.foodcont.2020.107354>
- López, P., Venema, D., de Rijk, T., de Kok, A., Scholten, J.M., Mol, H.G.J., de Nijs, M., 2016. Occurrence of *Alternaria* toxins in food products in The Netherlands. Food Control 60, 196–204. <https://doi.org/10.1016/j.foodcont.2015.07.032>
- Mao, X., Chen, A., Qu, J., Luo, P., You, Y., Gao, Y., Dong, F., Wu, Y., Li, Y., 2023. New insights into in mycotoxins production in *Alternaria* infected apple during postharvest storage. Postharvest Biol. Technol. 198, 112238. <https://doi.org/10.1016/j.postharvbio.2022.112238>
- Mikula, H., Skrinjar, P., Sohr, B., Ellmer, D., Hametner, C., Fröhlich, J., 2013. Total synthesis of masked *Alternaria* mycotoxins—sulfates and glucosides of alternariol (AOH) and alternariol-9-methyl ether (AME). Tetrahedron 69, 10322–10330. <https://doi.org/10.1016/J.TET.2013.10.008>

399 Ozcelik, So., Ozcelik, N., Beuchat, L.R., 1990. Toxin production by *Alternaria alternata* in
400 tomatoes and apples stored under various conditions and quantitation of the toxins by
401 high-performance liquid chromatography. *Int. J. Food Microbiol.* 11, 187–194.
402 [https://doi.org/10.1016/0168-1605\(90\)90011-S](https://doi.org/10.1016/0168-1605(90)90011-S)

403 Pavicich, M.A., Cárdenas, P., Pose, G.N., Fernández Pinto, V., Patriarca, A., 2020a. From
404 field to process: How storage selects toxigenic *Alternaria* spp. causing mouldy core in
405 Red Delicious apples. *Int. J. Food Microbiol.* 322, 108575.
406 <https://doi.org/10.1016/j.ijfoodmicro.2020.108575>

407 Pavicich, M.A., De Boevre, M., Vidal, A., Iturmendi, F., Mikula, H., Warth, B., De Saeger,
408 S., Patriarca, A., 2020b. Fate of free and modified *Alternaria* mycotoxins during the
409 production of apple concentrates. *Food Control* 118, 107388.
410 <https://doi.org/10.1016/j.foodcont.2020.107388>

411 Pavicich, M.A., De Boevre, M., Vidal, A., Mikula, H., Warth, B., Marko, D., De Saeger, S.,
412 Patriarca, A., 2024. Natural Occurrence, Exposure Assessment & Risk Characterization
413 of *Alternaria* Mycotoxins in Apple By-Products in Argentina. *Expo. Heal.* 16, 149–158.
414 <https://doi.org/10.1007/s12403-023-00544-1>

415 Pavicich, M.A., Nielsen, K.F., Patriarca, A., 2022. *International Journal of Food*
416 *Microbiology*. Morphological and chemical characterization of *Alternaria* populations
417 from apple fruit. *Int. J. Food Microbiol.* 379, 109842.
418 <https://doi.org/10.1016/j.ijfoodmicro.2022.109842>

419 Puntischer, H., Aichinger, G., Grabher, S., Attakpah, E., Krüger, F., Tillmann, K., Motschnig,
420 T., Hohenbichler, J., Braun, D., Plasenzotti, R., Pahlke, G., Höger, H., Marko, D., Warth,
421 B., 2019a. Bioavailability, metabolism, and excretion of a complex *Alternaria* culture
422 extract versus altertoxin II: a comparative study in rats. *Arch. Toxicol.* 93, 3153–3167.
423 <https://doi.org/10.1007/s00204-019-02575-7>

424 Puntischer, H., Cobankovic, I., Marko, D., Warth, B., 2019b. Quantitation of free and
425 modified *Alternaria* mycotoxins in European food products by LC-MS/MS. *Food*
426 *Control* 102, 157–165. <https://doi.org/10.1016/j.foodcont.2019.03.019>

427 Puntischer, H., Kütt, M.L., Skrinjar, P., Mikula, H., Podlech, J., Fröhlich, J., Marko, D.,
428 Warth, B., 2018. Tracking emerging mycotoxins in food: development of an LC-MS/MS
429 method for free and modified *Alternaria* toxins. *Anal. Bioanal. Chem.*
430 <https://doi.org/10.1007/s00216-018-1105-8>

431 Puntischer, H., Marko, D., Warth, B., 2020. First determination of the highly genotoxic fungal
432 contaminant altertoxin II in a naturally infested apple sample. *Emerg. Contam.* 6, 82–86.
433 <https://doi.org/10.1016/j.emcon.2020.01.002>

434 Sánchez-Torres, P., Vilanova, L., Ballester, A.R., López-Pérez, M., Teixidó, N., Viñas, I.,
435 Usall, J., González-Candelas, L., Torres, R., 2018. Unravelling the contribution of the
436 *Penicillium expansum* PeSte12 transcription factor to virulence during apple fruit
437 infection. *Food Microbiol.* 69, 123–135. <https://doi.org/10.1016/j.fm.2017.08.005>

438 Serdani, M., Crous, P.W., Holz, G., Petrini, O., 1998. Endophytic fungi associated with core
439 rot of apples in South Africa , with specific reference to *Alternaria* species plant species.
440 *Sydowia* 50, 257–271.

- Tudzynski, B., 2014. Nitrogen regulation of fungal secondary metabolism in fungi 5, 1–16.
<https://doi.org/10.3389/fmicb.2014.00656>
- van der Walt, L., Spotts, R.A., Visagie, C.M., Jacobs, K., Smit, F.J., McLeod, A., 2010.
Penicillium Species Associated with Preharvest Wet Core Rot in South Africa and Their
 Pathogenicity on Apple. *Plant Dis.* 94, 666–675. <https://doi.org/10.1094/PDIS-94-6-0666>
- Varming, C., Petersen, M.A., Toldam-Andersen, T.B., 2013. Ascorbic acid contents in Danish
 apple cultivars and commercial apple juices. *LWT - Food Sci. Technol.* 54, 597–599.
<https://doi.org/10.1016/j.lwt.2013.06.024>
- Walravens, J., Mikula, H., Rychlik, M., Asam, S., Devos, T., Njumbe Ediage, E., Diana Di
 Mavungu, J., Jaxsens, L., Van Landschoot, A., Vanhaecke, L., De Saeger, S., 2016.
 Validated UPLC-MS/MS Methods to Quantitate Free and Conjugated *Alternaria* Toxins
 in Commercially Available Tomato Products and Fruit and Vegetable Juices in Belgium.
J. Agric. Food Chem. 64, 5101–5109. <https://doi.org/10.1021/acs.jafc.6b01029>
- Walravens, J., Mikula, H., Rychlik, M., Asam, S., Ediage, E.N., Di Mavungu, J.D., Van
 Landschoot, A., Vanhaecke, L., De Saeger, S., 2014. Development and validation of an
 ultra-high-performance liquid chromatography tandem mass spectrometric method for
 the simultaneous determination of free and conjugated *Alternaria* toxins in cereal-based
 foodstuffs. *J. Chromatogr. A* 1372. <https://doi.org/10.1016/j.chroma.2014.10.083>

7 Conflict of Interest

*The authors declare that the research was conducted in the absence of any commercial or
 financial relationships that could be construed as a potential conflict of interest.*

8 Author Contributions

MAP: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology,
 Writing – original draft; MLM: Formal analysis, Writing – review & editing; TNN: Formal
 analysis, Writing – review & editing; MDB: Conceptualization, Resources; SDS:
 Conceptualization, Resources, Project administration, Writing – review & editing; AP:
 Conceptualization, Resources, Funding acquisition, Supervision, Writing – original draft.

9 Funding

This work was supported by MYTOX-SOUTH® (<https://mytoxsouth.org>, Ghent
 University International Thematic Network), Universidad de Buenos Aires [UBACyT 2018,
 20020170100094BA], Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT),

Argentina [PICT-2017-0907] and Subsidio para investigadores en formación de la Universidad de Buenos Aires 2020.

Acknowledgments

Maria Agustina Pavicich is supported by an FWO (Research Foundation - Flanders) Junior postdoctoral fellowship (1240024N). The authors acknowledge Prof. Doris Marko & Prof. Benedikt Warth for the provision of altertoxin-II standard.

FIGURE LEGENDS

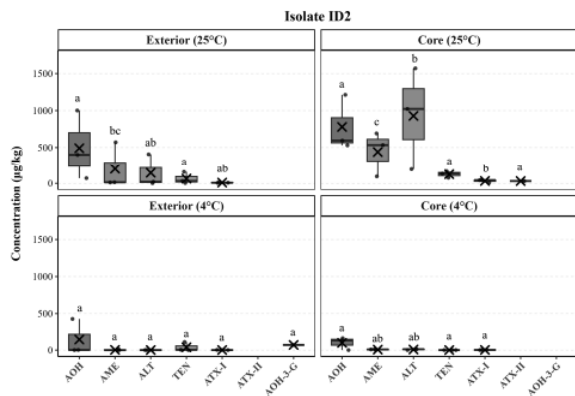
Figure 1. Box plots showing the production of *Alternaria* mycotoxins (excluding tenuazonic acid) by isolates 02, 31, and 36 under different inoculation sites and incubation conditions. EXT: external inoculation; CORE: core inoculation. Incubation temperatures are indicated in parentheses: 25 °C for 1 month and 4 °C for 9 months. Only detected mycotoxins are shown. Different letters above the boxes indicate statistically significant differences in mycotoxin levels across conditions for each isolate ($p < 0.05$). AOH: alternariol; AME: alternariol monomethyl ether; ALT: altenuene; TEN: tentoxin; ATX-I: altertoxin I; ATX-II: altertoxin II; AOH-3-G: alternariol-3-glucoside.

Figure 2. Box plots of tenuazonic acid (TeA) production by isolates 02, 31, and 36 under different inoculation sites and incubation conditions. EXT: external inoculation; CORE: core inoculation. Incubation temperatures are indicated in parentheses: 25 °C for 1 month and 4 °C for 9 months. Different letters above the boxes represent statistically significant differences in TeA production across conditions for each isolate ($p < 0.05$).

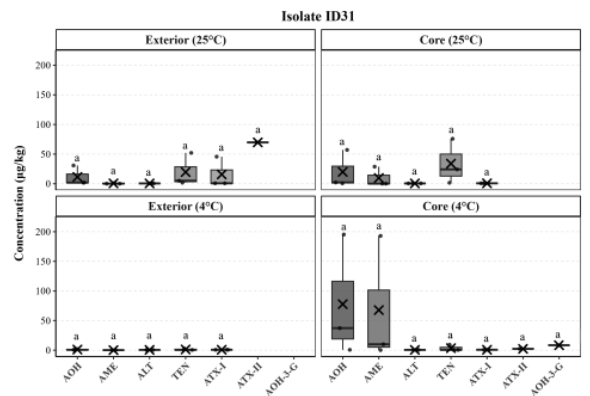
494 Table 1. Mycotoxin concentration (mean $\pm$ standard deviation) produced by isolates 02, 31, and 36 inoculated in the exterior or interior of apple
 495 fruit in $\mu\text{g/kg}$. Incubation period was one month at 25 °C and 9 months at 4 °C.
 496
 497
 498

Isolate ID	Inoculation site	Temperature (°C)	Concentration (µg/kg)										
			AOH	AME	ALT	TeA	TEN	ATX-I	ATX-II	AOH-3-S	AME-3-S	AOH-3-G	AME-3-G
02	Core	4	98 ± 86	7 ± 6	8 ± 7	39 ± 66	1 ± 0	2 ± 2	n.d.	n.d.	n.d.	n.d.	n.d.
		25	780 ± 377	440 ± 310	929 ± 694	12501 ± 21450	123 ± 45	32 ± 19	11 ± 14	n.d.	n.d.	n.d.	n.d.
	Exterior	4	143 ± 246	3 ± 5	0.8 ± 0.4	6572 ± 10181	38 ± 62	2 ± 1	n.d.	n.d.	n.d.	24 ± 33	n.d.
		25	491 ± 472	199 ± 323	143 ± 229	29 ± 27	64 ± 81	9 ± 5	n.d.	n.d.	n.d.	n.d.	n.d.
31	Core	4	78 ± 103	68 ± 109	1 ± 0	52226 ± 78813	3 ± 5	1 ± 0	1 ± 1	n.d.	n.d.	3 ± 4	n.d.
		25	20 ± 32	10 ± 17	1 ± 0	16403 ± 26612	34 ± 38	1 ± 0	n.d.	n.d.	n.d.	n.d.	n.d.
	Exterior	4	1 ± 0.8	1 ± 0	1 ± 0	1 ± 0	1 ± 1	1 ± 0	n.d.	n.d.	n.d.	n.d.	n.d.
		25	12 ± 17	1 ± 1	1 ± 0	6666 ± 11113	20 ± 28	16 ± 26	24 ± 33	n.d.	n.d.	n.d.	n.d.
36	Core	4	6 ± 8	1 ± 1	1 ± 0.7	761 ± 1318	3 ± 5	2 ± 1	n.d.	n.d.	n.d.	n.d.	n.d.
		25	618 ± 685	159 ± 140	153 ± 217	132726 ± 117368	1388 ± 2210	36 ± 27	33 ± 46	n.d.	n.d.	n.d.	n.d.
	Exterior	4	3 ± 4	1 ± 0	1 ± 0	295 ± 510	1 ± 0	3 ± 4	n.d.	n.d.	n.d.	n.d.	n.d.
		25	11 ± 10	1 ± 1	1 ± 0	345442 ± 305665	80 ± 74	4 ± 5	n.d.	n.d.	n.d.	n.d.	n.d.

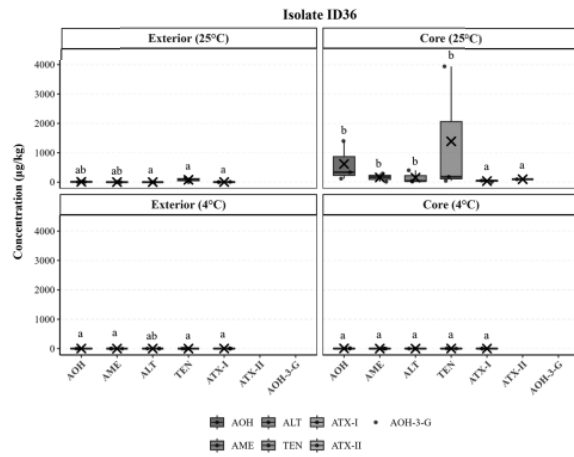
499 AOH: alternariol, AME: alternariol monomethyl ether, ALT: altenuene, TeA: tenuazonic acid, TEN: tentoxin, ATX-I: altertoxin-I, ATX-II:
 500 altertoxin-II, AOH-3-S: alternariol-3-sulphate, AME-3-S: alternariol monomethyl ether-3-sulphate, AOH-3-G: alternariol-3-glucoside, AME-3-
 501 G: alternariol monomethyl ether-3-glucoside. n.d.: not detected.



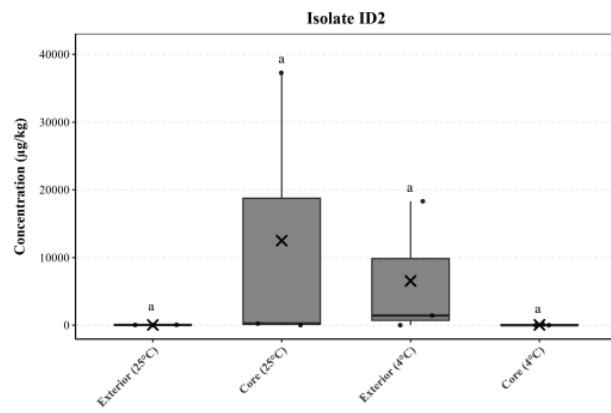
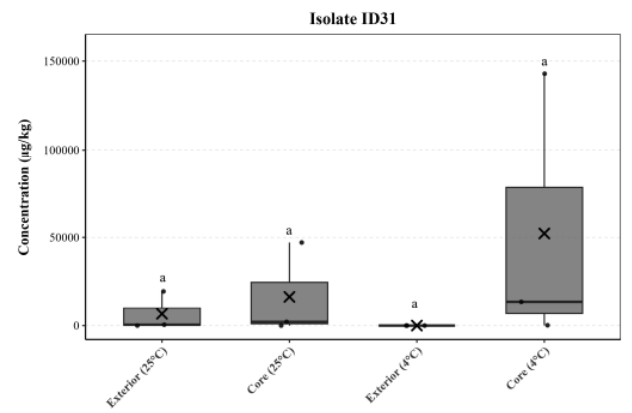
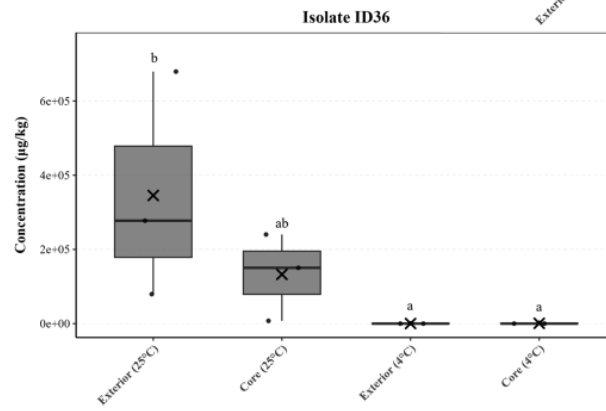
AOH ALT ATX-I AOH-3-G
 AME TEN ATX-II



AOH ALT ATX-I AOH-3-G
 AME TEN ATX-II



AOH ALT ATX-I AOH-3-G
 AME TEN ATX-II





DSPACE

<https://dspace.org/>

Insights into Alternaria in apple fruit causing mouldy core, external infection and mycotoxin production under retail and storage conditions

Pavicich, María Agustina; Maldonado, María Luisa; Nguyen, Truong Nhat; De Boevre, Marthe; De Saeger, Sarah ...

2025-08-02

Elsevier

<https://doi.org/10.1016/j.ijfoodmicro.2025.111272>

Pavicich MA, Maldonado ML, Nguyen TN, et al., (2025) Insights into Alternaria in apple fruit causing mouldy core, external infection and mycotoxin production under retail and storage conditions. International Journal of Food Microbiology, Volume 439, August 2025, Article number 111272