Food Control, Volume 191, January 2027, Article number 112418
DOI: 10.1016/j.foodcont.2026.112418

Alternaria and *Fusarium* across malting barley process: a comprehensive study of mycotoxins and quality effects

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The final published version (version of record) is available online at DOI:10.1016/j.foodcont.2026.112418. Please refer to any applicable publisher terms of use.

Abstract

This study aimed to evaluate the incidence and biomass of *Alternaria* and *Fusarium* and their mycotoxins during the malting process, as well as their impact on barley grains and malt quality. Barley samples from different geographical regions of Argentina and two genotypes were subjected to micro-malting. Mycotoxins were quantified in grains and subsamples collected at different stages of the malting process using HPLC-MS/MS, while fungal biomass was determined by qPCR. Tenuazonic acid and deoxynivalenol were the main mycotoxins produced. However, alternariol and 15-acetyl-deoxynivalenol were also present in high proportion. The results show low mycotoxin content in the early stages of the process, followed by an increase in later stages, with higher concentrations in malts compared to grains. *Alternaria* mycotoxins exhibited the highest concentration in malt, while *Fusarium* mycotoxins were most abundant in the rootlets. Fungal biomass displayed a similar pattern to mycotoxins, increasing as malting progressed, but with a decrease in *F. graminearum* biomass in malts. The incidence decreased for *Alternaria* across the malting process but increased for *Fusarium* in malt, equalling the incidence observed in grains. A negative statistically significant correlation was noted between *A. tenuissima* sp.-grp. and wort extract. Moreover, significant positivecorrelations were identified between both protein percentage and soluble protein and tenuazonic acid and alternariol. B-hordein was the hordein fraction with higher degradation in malt and with a high negative association with fungal biomass and incidence in grains. These findings are particularly relevantgiven the high mycotoxin concentrations detected in malt, surpassing the legislative limits or indicative values established for grains.

Keywords: Food safety, Tenuazonic acid, Deoxynivalenol, Fungal biomass, grains and malt quality parameters

1 Introduction

Barley (*Hordeum vulgare* L.) is one of the most widely cultivated cereals globally, with a production of 142 million tons in the 2024 growing season, during which Argentina accounting for 3 % of total production (FAO, 2025). In Argentina, the observed increase in the production of malting barley in the latest decades was in relation to the rise in the beer world production. In the 2023/2024 growing season, the cultivated area was 1.4 million hectares with a production of 5.1 million tons, of which around 40 % is for internal consumption, mainly malting, and the rest for export (BCR, 2025).

One of the difficulties faced by crops and malt producers is fungal infection, with *Alternaria* and *Fusarium* being prevalent worldwide. These fungi affect grain quality and imply food safety risks due to mycotoxin production, which has been detected in raw barley, malt, as well as beer and its by-products (Bauer et al., 2016; Geisinger et al., 2017; Oliveira Lago et al., 2022; Piacentini et al., 2019; Scheibenzuber et al., 2022). In addition to mycotoxin contamination, these fungi could also have implications for grain quality such as the reduction of grain size, germinative energy, and malt extract, degradation of storage protein, and increasing the gushing potential are the most studied (Bretträger et al., 2025; Martinez et al., 2020; Oliveira et al., 2012; Sarlin et al., 2005; Schwarz, 2017). However, fungal colonisation could promote beneficial factors to malt quality such as contributing to the grain enzyme activity needed for the degradation of barley endosperm cell walls (Justé et al., 2011; Laitila et al., 2007).

The main *Fusarium* mycotoxins, based on their prevalence in cereal crops, are deoxynivalenol (DON) and its acetyl derivatives 15-acetyl-deoxynivalenol (15-ADON) and 3-acetyl-deoxynivalenol (3-ADON), as well as nivalenol (NIV). These mycotoxins are the great concern because they have been linked to acute and chronic toxicity, resulting in the inhibition of protein, DNA, and RNA synthesis, as well as mitochondrial function, and promoting cell senescence (EFSA, 2013; Li et al., 2024; Rocha et al., 2005; Yang et al., 2025). Although mycotoxins represent a potential hazard to human health,

regulations are still scarce and vary among countries. For *Fusarium* mycotoxins, the European Union (EU) has established a maximum DON limit of 1000 µg/kg in unprocessed cereal grains other than durum wheat, oats, and maize (European Commission- EC N° 2024/1022). Regarding *Alternaria* mycotoxins, they are still considered emerging mycotoxins due to insufficient toxicity and human exposure data for the Food Safety authorities to conduct a risk assessment. Genotoxic, mutagenic, cytotoxic, estrogenic among other effects that represent a potential hazard to human consumers have been reported, which encourage the need for urgent regulation (Aichinger et al., 2020; EFSA, 2016; Nagda and Meena, 2024). However, no regulations have been established regarding mycotoxins in malted barley until date.

Malting is a biological process that aims to break down grain macromolecules into soluble compounds through the action of enzymes. This process involves three key stages: steeping to increase grain humidity; germination, which activates and synthesises new enzymes that disaggregate the endosperm and expose starch for future enzymatic degradation; and kilning, which reduces grain humidity to halt enzymatic activity. In turn, the conditions present during these processes promote the proliferation of filamentous fungi that were already colonising the grains. Among these, *Alternaria* and *Fusarium* are commonly found coexisting in barley. The diversity of this fungal community in barley grains could be related to weather conditions, barley genotype, geographical locations, and agricultural practices (Beccari et al., 2016; Castañares et al., 2021; González et al., 2008; Medina et al., 2006). Preliminary studies have shown that *F. graminearum sensu stricto* (*F. graminearum s.s.*) is the most frequent *Fusarium* species affecting barley grains in Argentina, while in relation to the *Alternaria* genus, *A. tenuissima* species-group (*Alternaria* section) and *A. infectoria* sp.-grp. (*Infectoriae* section) are the most prevalent (Castañares et al., 2014, 2021; Manno et al., 2024; Nogueira et al., 2018). In the same way, mycotoxins can coexist in barley grains, and since many are heat-stable, they can be transferred throughout processing to the final products. Research has shown that steeping favours the reduction of water-soluble

mycotoxin concentrations. Conversely, the conditions of the following germination stage encourage fungal growth and *de novo* synthesis of mycotoxins (Habler et al., 2016; Lancova et al., 2008; Oliveira et al., 2012; Pascari et al., 2018; Prusova et al., 2022). Many of these studies have focused on the advancement of *Fusarium* mycotoxins in malting, mainly DON, in trials with inoculated barley. On the other hand, there are scarce studies that have assessed the behaviour of *Alternaria* along the malting process or the co-occurrence of mycotoxins from both fungal genera (Brettrager et al., 2025; Prusova et al., 2022).

Our primary objective in this study was to assess the development of *Fusarium* (represented primarily by *F. graminearum)* and *Alternaria* (represented primarily by *A. tenuissima* sp.-grp. and *A. infectoriae* sp.-grp.) and their mycotoxins during the malting of naturally contaminated barley grains. We also evaluated the impact of fungal development and mycotoxin contamination on the quality parameters of both barley and malt. Additionally, we explored how barley genotype and geographical origin influenced mycotoxin dynamics, potentially linked to initial contamination levels. This comprehensive study, which included evaluating fungal biomass, mycotoxin concentrations, and key quality parameters, will provide valuable insights into the behaviour of these fungal genera during barley grain processing.

2 Materials and Methods

2.1 Samples analysed

Barley grains were supplied by the National Network of Brewery Barley of the National Institute of Agricultural Technology (INTA). Barley grains were conventionally grown under zero-tillage practices and controlling foliar disease during all periods and harvested in December 2018 at physiological maturity with around 12 % humidity. Grain samples were stored at 4 °C until analysis.

Barley grains were obtained from three different geographical areas of Argentina's main producing region (Bordenave 37°48'00.0"S 63°03'00.0"W-southwest of Buenos Aires province-, Chillar 37°18'00.0"S 59°59'00.0"W-south of Buenos Aires province-, and Bigand 33°23'00.0"S 61°11'00.0"W -south of Santa Fe province-) from 2018 growing season and two of the most widely cultivated barley genotypes in Argentina at the time of the analysis (Andreia and Shakira). Both barley genotypes were highlighted in a previous study, where Andreia exhibited the highest frequency of *A. tenuissima* sp.-grp., and Shakira the highest frequency of *A. infectoria* sp.-grp., both of which were the main *Alternaria* sp.-grps. isolated (Castañares et al., 2021). Similarly, geographical origins were chosen based on the results of a previous study in which Bigand was one of two localities showing TeA occurrence in one of the sampling seasons; Bordenave exhibited co-occurrence of the three mycotoxins analysed (AOH, AME, and TeA) in high mean concentrations in one of the growing seasons; and Chillar was notable for having one of the lowest concentrations of mycotoxins (Castañares et al., 2020).

2.2 Malting process

Barley grain samples (2 kg) were reduced to 500 g and malting was performed in duplicate in the micro-malting belonging to the INTA Bordenave Experimental Station in 2019, according to the following conditions: washing for 30 min; conditioning for 30 min; 2 periods of steeping for 4 h each with an aeration period of 9 h after each of them; and germination for 96 h. The moisture content of the grains was measured during germination, adjusting to a value of around 46% during the first 18-22 h of the stage and of 42-43% at the end of this stage (at 96 h). All these stages were performed at 15 °C with agitation at 15 rpm. The germinated barley grains were then kilned for 18 h, which increased the temperature from 50 to 82 °C. At the end of the dry stage, rootlets were removed from the malting grain.

Sub-samples (70-100 g) were taken from different stages of the malting process (Fig. 1): at the beginning (barley grains-Sub-sample 1), at the end of the steeping (Sub-sample 2), at 18-22 h of germination (Sub-sample 3), at the end of the germination (Sub-sample 4) and finally, at the end of the kilning (malt-Sub-sample 5 and rootlets-Sub-sample 6). Rootlets were mixed and analysed as a whole, and then divided into two to have a replicate. Samples collected were freeze-dried and stored at -20 °C until use. The quantity of each sample after freeze-drying was around 60 g. Mycotoxin concentrations, fungal incidence, and fungal biomass were evaluated in each sub-sample.

2.3 Mycotoxin analysis

Four *Alternaria* mycotoxins (AOH, AME, TeA, and TEN) and three *Fusarium* mycotoxins (DON, 15-ADON, and NIV) were analysed. The analytical standards of *Alternaria* mycotoxins were obtained from Fermentek (Jerusalem, Israel), while those for *Fusarium* mycotoxins were obtained from Sigma-Aldrich (Bornem, Belgium). For mycotoxin analysis, 50 g of each malting sample was finely ground in a laboratory mill and homogenised. Mycotoxins were extracted according to Arroyo Manzanares et al. (2015). Briefly, a volume of 20 mL of extraction solvent ($CH_3CN/H_2O/CH_3COOH$; 79:20:1) was added to 10 g of the sample. The samples were homogenized and sonicated. Then, a volume of 10 mL of extract was transferred into glass vials and evaporated to dryness at 45 °C under a stream of N_2. Samples were resuspended in methanol/water (70:30) and filtered through a 0.22 mm nylon filter before analysis. Mycotoxin identification and quantification were performed using high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). The analyses were conducted in a Thermo scientific ™ system consisting of a degasser, quaternary pump, column oven and an LTQ XL™ ion trap mass spectrometer. Chromatographic separations were performed with a C18 100 x 2.1 mm Hypersil™ ODS (5 µm particle size) column. A solution of ammonium formate (10 mM) and acetonitrile was used as a mobile phase. Samples (10 µL) were analysed at a flow rate of 0.2 mL/min at 45 °C. The method

159 detection limits (LD) and quantification limits (LQ) were 20 µg/kg and 60 µg/kg,
160 respectively, for AME, and 7 µg/kg and 20 µg/kg, respectively, for the other mycotoxins
161 analysed. The validation parameters of the analytical method are provided in
162 Supplementary Table 1.

163 *2.4 Fungal incidence and biomass*

164 A total of 100 grains per sub-sample of the malting process were placed on potato
165 dextrose agar with 0.05 g chloramphenicol/L (PDA) (10 grains per plate) and incubated
166 at 25 °C for 7 days. The resulting *Alternaria* and *Fusarium* colonies were enumerated
167 and identified at the genus level according to Simmons (2007) and Leslie and Summerell
168 (2006), respectively. The incidence was calculated as the percentage of infected grains
169 for each genus per sample.

170 The fungal biomass of*A. tenuissima* sp.-grp., *A. infectoria* sp.-grp. and *F. graminearum*
171 was evaluated with a qPCR assay using the primers detailed in Table 1. The DNA of
172 each malting sample, as well as from the mycelium of *Alternaria* and *Fusarium* isolates
173 (M44-a5, M33-1f, 1-1)(Castañares et al., 2014; 2021), was extracted according to Atoui
174 et al. (2012). Briefly, DNA from 200 mg of ground sample was extracted with 1 mL of
175 SDS extraction buffer (200 mM Tris–HCl pH 7.5, 25 mM EDTA pH = 8, 288 mM NaCl,
176 0.5% w/v SDS), vigorously vortexed and centrifugated for 5 min. DNA was quantified
177 using a Fluorometer (Qubit Fluorometer, Invitrogen) and adjusted to 10 µg/mL. A
178 standard curve of each pure isolate was made using DNA dilutions from 10 to 0.0001
179 µg/mL. Independently, qPCR assays were carried out in a total volume of 10 µL
180 containing 2 µL of DNA at 10 µg/mL, 0.1 µL of each primer at 0.1 µM, 5 µl of 2X
181 SsoAdvanced™ Universal SYBR® Green Supermix (BIO-RAD), and Milli-Q water to
182 complete the final volume. PCR reactions were performed in duplicate in an Applied
183 Biosystems 7500 real-time PCR system (Thermo Fisher Scientific) using the following
184 cycling protocol: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60

°C for 1 min followed by dissociation curve analysis at 60-95 °C. The results were expressed as ng of fungal DNA per 10 ng of total DNA.

2.5 Quality parameters of barley and malt

The quality of barley grains used in the malting process was analysed. Protein content and humidity were measured with a NIT analyser with a double-face monochromator (Agricheck, Bruins Instruments, Salem, NH, USA). Besides, thousand kernel weights (TKW) and the ratio of grains retained on a 2.5 mm sieve were evaluated (screening percentage).

On the other hand, the quality parameters of the malt obtained were also evaluated. Total protein content and soluble protein content were measured by near-infrared spectroscopy (NIR- Infratect^{TM} 1241). Wort extract ratio, saccharification time, filtration time, and pH were determined according to the methodology proposed by the European Brewing Convention (EBC, 1998). Total hordein content and hordein fractions (D, C, and B) were determined by the method of Salgado-Albarrán et al. (2015). Hordeins were extracted from 50 mg of milled sample with the extraction buffer (12.5 mM of sodium borate, 1 % of sodium dodecyl sulfate and 2 % of β-mercaptoethanol at pH 10) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with 0.05 % Coomassie Brilliant Blue R250, scanned, and analysed using TotalLab (v1.10 demo) software to measure pixel intensity as an abundance indicator.

2.6 Statistical analysis

The software INFOSTAT version 2012 (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina) was used for exploratory statistical analysis. The Shapiro-Wilk test was used to evaluate the normal distribution of the data. The variables *F. graminearum* biomass, *A. tenuissima* sp.-grp. biomass, *A. infectoria* sp.-grp. biomass, and the mycotoxins DON, 15-ADON, TeA, AOH, and TEN were transformed with Ln to achieve

normal distribution. For mycotoxin analysis, only positive samples were used. ANOVA analysis with positive samples evaluated statistically significant differences between means along the malting process: from grains to malt, without rootlets, using the DGC test with a confidence higher than 95 % (p-value < 0.05). Besides, the relationship between variables was evaluated with the Spearman correlation coefficient with untransformed variables. Incidences of *Fusarium* and *Alternaria* were evaluated with Kruskal-Wallis test because the data could not be transformed to be normal. Since rootlets from all samples were pooled for biomass and mycotoxin quantification, they were subsequently removed for ANOVA and Spearman analyses.

3 Results

3.1 Initial mycotoxin concentration, fungal incidence, and biomass according to geographical origin and genotype of the samples

The malting process was carried out using grain samples from three distinct geographical origins and two barley genotypes. Statistically significant differences among localities concerning the initial mycotoxin levels (in barley grains) were observed for AOH, where Bordenave and Bigand had significantly higher levels than Chillar. Bigand showed a higher concentration of TEN than Bordenave, while Chillar exhibited no contamination with this mycotoxin. Additionally, statistically significant differences among genotypes were observed only for TEN, with Shakira showing a higher TEN concentration than Andreia (Fig. 2).

Alternaria incidence (42-61 %) in barley grains was higher than *Fusarium* (3-18 %) in all samples analysed. With respect to the geographical origin of the samples, Bigand showed the highest *Alternaria* incidence, followed by Chillar, while Chillar and Bigand showed the highest *Fusarium* incidence. Biomass of *Alternaria* was similar among localities, while *F. graminearum* biomass was higher in Chillar and Bigand than in

236 Bordenave. Similar *Alternaria* and *Fusarium* incidences and biomass were observed in
237 the genotypes evaluated (Supplementary Table 2).

238 *3.2 Mycotoxin concentration along the malting process*

239 TeA was found in all 56 samples analysed, followed by DON (55/56 samples), 15-ADON
240 (44/56), and AOH (35/56), while TEN was only detected in three samples. NIV and AME
241 were not detected in any sample. Mycotoxin levels fluctuated during the malting process
242 regardless of initial mycotoxin content. In general, a reduction in mycotoxins at steeping
243 followed by a subsequent increase in the later stages was observed for all mycotoxins
244 analysed. The concentrations of *Alternaria* and *Fusarium* toxins in each stage of the
245 malting process are shown in Table 2. TeA showed the highest mean concentration in
246 all stages except the rootlets, followed by DON. In turn, the highest concentration was
247 observed for TeA (19636 µg/Kg) in a malt sample, followed by DON (8975 µg/Kg) in the
248 rootlets.

249 All these five mycotoxins except TEN were detected in each step of the malting process,
250 from grains to malt. However, only 15-ADON, DON, and TeA were detected in the
251 rootlets. Moreover, the highest concentration of DON was detected in the rootlets. On
252 the contrary, rootlets showed the lowest concentration of the *Alternaria* mycotoxin TeA
253 while AOH and TEN were undetected (Table 2). Around 39 % of the samples exceeded
254 the legislative limit established of 1000 µg/kg for DON. All these samples corresponded
255 to the end of the germination stage and after the kilning stage (malt and rootlets).
256 However, concentrations in barley grains were below the legislation.

257 The ANOVA analysis for TeA showed statistically significant differences alongside the
258 malting process, with a notable rise in concentration in malts compared to the other
259 stages ($p < 0.05$) (Table 2). For DON, a reduction at the steeping stage followed by a
260 subsequent increase was observed. Similarly, an increase at the end of the germination

followed by a decrease after kilning was observed for 15-ADON. AOH did not show statistically significant differences among stages of the malting process (Table 2).

3.3 Mycotoxins co-occurrence along the malting process

The beginning of the malting process (grain samples) was the only stage where the five mycotoxins (15-ADON, DON, TeA, AOH, and TEN) co-occurred in 50 % of the samples analysed. The co-occurrence of four mycotoxins (15-ADON, DON, TeA, and AOH) was the most frequent in the total number of samples analysed. It was low in the early stages of the malting process (25-33 % of the samples) but increased at the end of germination and in malt (66.7 % and 75 %, respectively). By contrast, combinations of only three or two mycotoxins were less frequent. Only one sample belonging to barley grains was contaminated with only one mycotoxin (TeA) (Supplementary Table 3).

3.4 Fungal Biomass and Incidence

In terms of fungal biomass, the ANOVA analysis for *A. infectoria* sp.-grp. showed statistically significant differences for stages of the process ($p < 0.05$). A decrease at the steeping stage and an increase during the subsequent stages were observed. The biomass levels in grains were similar to the germination stages. Finally, an additional increase was observed in malts, which was significantly higher than the rest of the stages ($p < 0.05$) (Fig. 3A). A similar trend was observed for *A. tenuissima* sp.-grp. A significant decrease at the steeping followed by a slight increase at the germination stages, without significant differences in these three stages, was observed. Then, the biomass increased in malts, resulting statistically similar to grain biomass (Fig. 3A). In contrast, the biomass of *F. graminearum* showed a decrease after steeping, followed by an increase during germination, and a subsequent significant decrease in malt.

Conversely, the incidence of *Alternaria* decreased in malts, with respect to the grains and other stages (Fig. 3B). *Fusarium* incidence decreased during the steeping stage,

286 then increased towards the end of germination. The incidence in grains was not
287 significantly different from the one at the end of germination or malts (Fig. 3B).

288 *3.5 Correlations between Fungal biomass, incidence and mycotoxins*

289 Based on the Spearman coefficient, the biomass of the *A. infectoria* sp.-grp.
290 demonstrated a moderate and positive correlation with TeA ($r = 0.58$, $p < 0.05$).

291 The biomass of the *A. tenuissima* sp.-grp. showed only a significant but low correlation
292 with AOH ($r = 0.31$, $p < 0.05$). Likewise, *F. graminearum* biomass showed only a
293 moderate correlation with 15-ADON ($r = 0.51$, $p < 0.05$). Otherwise, *Fusarium* incidence
294 showed a positive but low correlation with DON ($r = 0.42$, $p < 0.05$), while *Alternaria*
295 incidence showed a low and negative correlation with TeA ($r = -0.35$, $p < 0.05$).

296 *3.6 Barley and malt quality parameters*

297 Barley and malt quality parameters are shown in Table 3. All but one barley sample,
298 corresponding to Chillar/Andreia, exceeded the legislative limit of 13 % protein in barley
299 grains, while three corresponding to Bordenave/Andreia/Shakira and Chillar/Shakira
300 surpassed the 12.5 % humidity threshold. All barley samples complied with the legislation
301 regarding screening percentage. A significant negative correlation was observed
302 between C-hordeins and *Alternaria* incidence ($r = -0.89$, $p < 0.05$), and B-hordeins with
303 *A. tenuissima* sp.-grp. biomass ($r = -0.94$, $p < 0.05$), *F. graminearum* biomass ($r = -0.94$,
304 $p < 0.05$), and *Fusarium* incidence ($r = -0.89$, $p < 0.05$).

305 In terms of industrial malt quality, the wort extract was over 80 %, as expected in all
306 samples except those from the Shakira genotype. Additionally, the filtration time
307 surpassed the reference value in both genotypes from the Bigand locality. Besides, in
308 this locality, the Shakira genotype showed a total protein percentage in malt above 13
309 %. The remaining parameters conformed to the reference values. All hordein fractions
310 showed degradation in malts compared to grains, as expected. B-hordein showed the

311 highest percentage of degradation. The correlation among malt quality variables, fungal
312 biomass or incidence and mycotoxins was statistically non-significant, except for the
313 relationship between D-hordeins with *A. tenuissima* sp.-grp. biomass ($r = -0.85$, $p < 0.05$)
314 and with *Fusarium* incidence ($r = -0.65$, $p < 0.05$); B-hordeins with *Fusarium* incidence (r
315 $= -0.61$, $p < 0.05$); total hordeins content with *Fusarium* incidence ($r = -0.73$, $p < 0.05$);
316 protein percentage with TeA ($r = 0.74$, $p < 0.05$) and with AOH ($r = 0.79$, $p < 0.05$); soluble
317 protein with AOH ($r = 0.81$, $p < 0.05$) and TeA ($r = 0.68$, $p < 0.05$); as well as between
318 wort extract with *A. tenuissima* sp.-grp. biomass ($r = -0.71$, $p < 0.05$).

319 **4 Discussion**

320 Mycotoxins pose a significant risk to human health, and numerous studies have
321 confirmed their presence in end products like beer. This has driven research into their
322 incidence in barley grains and their behaviour during processing to find strategies for
323 their control. However, few studies have focused on the co-occurrence and behaviour of
324 *Alternaria* and *Fusarium* mycotoxins specifically during the malting process. The current
325 study revealed that mycotoxins from the *Alternaria* and *Fusarium* genera were present
326 in most of the analysed samples, with varying frequencies and concentrations, with TeA
327 and DON being the most predominant. As in this study, Prusova et al. (2022) also found
328 that TeA was found in higher concentration in barley grains (585 µg/kg). AOH and TEN
329 were also detected, although in lower concentrations. In addition, TeA was the
330 predominant mycotoxin found in malt but its progress along the process was not
331 evaluated. Type A trichothecenes were also detected. However, *F. graminearum* and
332 DON were not observed, although an initial artificial inoculation with *Fusarium* species
333 was done. Similarly, Beccari et al. (2018) found that, after infectopyrone, TeA was the
334 predominant *Alternaria* mycotoxin and the one present in the highest concentration (26.4
335 µg/Kg), followed by AOH and AME, although the mean concentrations were lower than
336 those observed in the current study (1.8 and 0.3 µg/Kg, respectively). Otherwise, the
337 same author found NIV in higher concentrations and incidence than DON. These results

are correlated with the fungal species isolated, where *F. poae* producing NIV was the predominant. Other studies focused on *Fusarium* or *Alternaria* mycotoxins in barley grains, and results are diverse, but many of them also showed concentrations below recommended limits (Biehl et al., 2024; Bolechová et al., 2015; Gavrilova et al., 2021; Nathanail et al., 2015; Oliveira et al., 2012; Scheibenzuber et al., 2022) or higher, with, for example, an average of 3835 µg/Kg for DON (Piacentini et al., 2019). Orina et al. (2021) found TeA in 85 % of cereal samples and with maximum concentrations of 14963 µg/Kg detected in wheat grains. In this report, NIV and AME were not detected. In other investigations carried out on barley samples from Argentina, the concentration of NIV and DON have been variable, as well as the incidence of the main species associated with these mycotoxins (*F. poae* and *F. graminearum s.s.*, respectively) (Castañares et al., 2014; Manno et al., 2026; Martinez et al., 2020; Nogueira et al., 2018). With respect to AME, a previous study showed that AME was present only in five samples out of 60 from barley grains in Argentina, while AOH followed by TeA, were the most frequent *Alternaria* mycotoxins (Castañares et al., 2020).

Differences among *Fusarium* and *Alternaria* mycotoxin concentrations in malts and rootlets were observed. Other studies have shown similar results for DON in trials with artificially inoculated barley in which higher concentrations were observed in rootlets than malts (Biehl et al., 2024; Habler et al., 2016; Lancova et al., 2008). Similarly, higher concentrations in rootlets compared to malts were observed for type A trichothecenes (Habler et al., 2016) and enniatins (Hu et al., 2014). However, Biehl et al. (2024) found a reduction in the DON mean concentration from grains to rootlets in the control malting trial (without inoculation). The high mycotoxin concentrations found in the rootlets by several studies cannot be ignored since this by-product is used not only for animal feed but, given its nutritional value, has potential applications for other uses such as food ingredients, substrates for fermentations, among others (Neylon et al., 2020).

Current scientific literature shows that the malting process involves specific, crucial stages where the level of mycotoxins can vary significantly. In general, a decrease in mycotoxin concentration during the steeping stage is observed, mainly due to the water solubility of mycotoxins. The humidity and temperature conditions during the germination stage could allow the fungal development and thus the synthesis *de novo* of mycotoxins, with a consequent increase in mycotoxin concentrations. Subsequently, during the kilning stage, high temperatures are reached (from 50 °C to 82 °C). Despite this, the thermostability of many mycotoxins, such as DON, allows their concentration to be maintained or even increased while reducing fungal development (Habler et al., 2016; Jin et al., 2021; Lancova et al., 2008; Prusova et al., 2022; Zachariasova et al., 2012). Besides, fungal stress caused by exposure to high temperatures can promote the production of mycotoxins and their increase during kilning (Oliveira et al., 2012). However, many researchers have observed a reduction in mycotoxins after malting process due to mycotoxin degradation, by the synthesis of masked forms during germination, i.e. DON-3-glucoside synthesis by glycosylation, or by the separation of the rootlets from the malted grains (Habler et al., 2016; Hu et al., 2014; Maul et al., 2012; Piacentini et al., 2019). Biehl et al. (2024) found different results after kilning depending on whether samples were artificially inoculated (an increase in mycotoxin concentration) or naturally contaminated samples (a reduction in mycotoxin concentration).

In the present work, a joint analysis of fungal biomass and mycotoxin concentration in naturally infected barley samples was carried out throughout the process. The results obtained are partially in agreementwith the literature; the malting process led to an increase in *Alternaria* and *Fusarium* mycotoxins, with final concentrations in malts exceeding those in the starting barley grains (Habler et al., 2016;. Lancova et al., 2008; Prusova et al., 2022; Zachariasova et al., 2012). Despite the high temperatures, the *Alternaria* biomass continued to increase, at least in the initial stage of kilning, reaching higher levels in malts than grains. In contrast, *F. graminearum* biomass was reduced to

values lower than the initial. Similar results were obtained by Biehl et al. (2024), who also found lower DNA concentrations in malts than in grains in naturally contaminated samples, and showed different results depending on initial spore concentration and malting temperature. A reduction of fungal biomass (*A. alternata*, *Cladosporium spp*., and *Epicoccum nigrum*) after malting was also observed by Brettrager et al. (2022). Habler et al. (2016) also found a decrease of *F. sporotrichioides* biomass after the malting process, suggesting a possible biological degradation during kilning. Nevertheless, in this study, the viability of the spores was reduced, leading to a lower *Alternaria* incidence in malts, while the similar *Fusarium* incidence in grains and malts may be due to the presence of resistant structures such as chlamydospores (Leslie and Summerell, 2006). However, it is necessary to emphasise that the technique used for biomass quantification measures the total fungal DNA, from both viable and non-viable colonies, whereas the incidence only considers viable colonies; thus, both techniques are not comparable. Additionally, the methodology for biomass determination was simplified to the analysis of a single species, *F. graminearum*, which is the main *Fusarium* species in barley grains, while *Fusarium* incidence accounts for all species within the genus and may include species other than *F. graminearum*. Besides, an underestimation of *Fusarium* incidence in barley grains caused by a lower growth rate on agar plates compared to other fungi that grow faster could also explain the similar incidence found after malting. Brettrager et al. (2022) also found an increase of *Fusarium* abundance and a reduction of *Alternaria* abundance after malting , varying according to the harvest year evaluated, by isolating fungi from grains placed on PDA. Other authors also found a reduction of fungal growth after malting using conventional methods (Ackerman et al., 1998; Douglas and Flanningan, 1988; Kocic-Tanachov et al., 2005).

Significant correlation with fungal biomass were observed only for some mycotoxins. Besides, a low and significant correlation between fungal incidence and mycotoxins was observed. An association between fungal biomass and mycotoxin production has been

observed in samples artificially inoculated (Biehl et al., 2024; Habler et al., 2016; Oliveira et al., 2012) or naturally contaminated (Nielsen et al., 2014). The lack of significant correlation in the present study may be due to the low fungal load present in the raw material as was observed by Biehl et al. (2024) in the trial carried out at low germination temperature. Likewise, the authors found positive correlations between fungal DNA and mycotoxins only in the experiments inoculated with higher initial spore concentrations. Besides, other studies have shown that genotype, sampling region and season could influence the relationship between fungal DNA and mycotoxin concentration (Hoeneder et al., 2022; Nielsen et al., 2014).

Quality parameters from barley grains and malt obtained were also evaluated to determine a correlation with fungal infection. The results showed an increase in protein percentage in barley grains overpassing legislative limits. Negative non-significant correlations were observed between protein percentage and fungal biomass and incidence, except for *Alternaria* incidence, the predominant genus infecting barley grains, which was positive although not significant ($p > 0.05$). A positive association between fungal infection and protein percentage was observed by Sarlin et al. (2005), but in a study with artificial inoculation with *Fusarium* species. A positive but non-significant correlation was also observed by Schwarz et al. (2006) for the DON mycotoxin. With respect to malt quality, the Shakira genotype from both sampling regions showed low wort extract. Besides, Shakira genotypes showed higher protein percentage values than Andreia in barley grains. In general, high protein content is related to low carbohydrate and wort extract percentage. Martinez et al. (2020) found that Shakira was the genotype most affected in terms of severity, germinative power, and screening percentage in an assay with artificial inoculation with *Fusarium* species, suggesting a genetic and environmental effect on the variables studied. However, quality malt parameters were not evaluated. High wort extract is one of the requirements necessary for beer production since it represents the extractable sugar for fermentation. In the present study, a negative

445 significant correlation between wort extract and *A. tenuissima sp.-grp.* biomass ($p < 0.05$)
446 and a negative but non-significant correlation with *A. infectoria* sp.-grp.and *F.*
447 *graminearum* biomass were observed. Similar results were shown by Nielsen et al.
448 (2014) in a study with *Fusarium* species. Brettrager et al. (2025) showed that inoculation
449 with *Alternaria* reduced β-glucan and wort viscosity, which is also crucial for the
450 lauthering and beer filtration, while increasing soluble nitrogen and free amino nitrogen
451 (FAN), both related with proteolytic activity. Moreover, although the relationship between
452 soluble protein and total protein (Kobalch index) was adjusted to recommended values,
453 these samples showed a filtration time higher than expected values. Nielsen et al. (2014)
454 found a negative association between fungal biomass and wort filtration volume.
455 Regarding hordein, the results showed that B-hordein fractions of the grains were the
456 main fraction affected by fungi infection, showing a negative correlation with *A.*
457 *tenuissima* sp.-grp. and *F. graminearum* biomass and with *Fusarium* incidence.
458 Additionally, C-hordein exhibited a negative correlation with *Alternaria* incidence. With
459 respect to hordein fractions in malt, D-hordein and B-hordein showed a negative
460 correlation with *Fusarium* incidence and D-hordein also correlated with *A. tenuissima*
461 sp.-grp. biomass. Martinez et al. (2020) found that D- and C-hordeins were mainly
462 affected by *F. graminearum* in an assay with artificial inoculation. Qi et al. (2005) found
463 that B-hordein was the main fraction associated with malt quality, showing a positive
464 correlation between B-hordein and grain protein and a negative correlation with malt
465 extract. A negative correlation with malt extract and a positive association with Kolbach
466 index was also observed by Šimić et al. (2007). However, a non-significant correlation
467 was observed between hordeins and malt quality parameters in the present study. Other
468 authors have proposed that a higher degradation of storage proteins (hordeins) is
469 associated with better malt quality for beer production and contributes to foam formation
470 in beer (Oliveira et al., 2013; Šimić et al., 2007).

471 **5 Conclusion**

The results of this study, carried out on naturally contaminated barley grains, are noteworthy due to the high mycotoxin concentrations detected in malts, surpassing the legislative limits established for DON, while concentrations detected for *Alternaria* mycotoxins were high. Moreover, the rootlets, by-products of the malting process, must be monitored, given the high contamination with *Fusarium* mycotoxins. The identification of the key stages in the production chain where mycotoxin reduction is most effective will provide industries with proven strategies to ensure the safety and quality of their final products.

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727

728 **Figure legends**

729 **Figure 1**. Stages of the malting process.

730 **Figure 2**. Mycotoxins mean concentrations (µg/kg) in barley grains according to
731 geographical origin (A) and genotype (B). Columns represent the mean ± standard
732 deviation (SD) of two replicates for each geographical origin (A) and three replicates for
733 each genotype (B). Columns with different letters are statistically different according to
734 DGC test ($p < 0.05$). *Not detected.

735 **Figure 3**. Fungal development along the malting process. (A) Fungal biomass,
736 expressed as LN of fungal DNA per 10 ng of total DNA. Mean ± standard error of the
737 mean (SEM). (B) Fungal incidence, expressed as the mean of percentage of infected
738 grains for each genus per sample. Malting was performed in duplicate. Different letters
739 are statistically different according to DGC test ($p < 0.05$). Grains ($n = 6$), Steeping ($n =$
740 12), 18-22 h of germination ($n = 12$), End of germination ($n = 12$), Malts ($n = 12$).

741

742

Table 1. Primers sequences used to quantify fungal biomass.

Target fungal biomass	Primer name	Sequence (5′-3′)	Reference
Alternaria spp. (*A. tenuissima* sp.-grp.)	Alt4	CTTTTGCGTACTTCTTGTTTCC	Crespo-Sempere et al. 2013
	Alt5	CAGGCATGCCCTTTGGATAC	
A. infectoria sp.-grp,	Ain3F	CTCGATGTCCGCCTCAGTAG	Gannibal and Yli-Mattila 2007
	Ain4R	GAGGATAGCACGGCTGGTAG	
F. graminearum	FgramB379 fwd	CCATTCCCTGGGCGCT	Nicolaisen et al. 2009
	FgramB411 rev	CCTATTGACAGGTGGTTAGTGACTGG	

Table 2. Percentage of positive samples (%) and mycotoxin concentrations (μg/kg) in each stage of the malting process. Letters show significant differences among mean mycotoxin levels in different stages of the process according to ANOVA and DGC test ($p < 0.05$).

Mycotoxin		Grains (*n* = 6)	Steeping (*n* = 12)	18-22 h of germination (*n* = 12)	End of germination (*n* = 12)	Malts (*n* = 12)	Rootlets (*n* = 2)
TeA	% positive	100	100	100	100	100	100
	Mean*	2349[a]	1646[a]	2204[a]	2493[a]	7920[b]	406
	Median*	2283	1458	1983	1897	6218	406
	Min	839	717	881	1076	2658	318
	Max	4200	4681	5069	5709	19636	495
AOH	% positive	83	50	58	75	67	0
	Mean*	206[a]	204[a]	955[a]	416[a]	577[a]	ND
	Median*	205	189	517	401	357	ND
	Min	92	92	92	92	191	ND
	Max	312	374	4164	951	1230	ND
TEN	% positive	50	0	0	0	0	0
	Mean*	172	ND	ND	ND	ND	ND
	Median*	105	ND	ND	ND	ND	ND
	Min	82	ND	ND	ND	ND	ND
	Max	327	ND	ND	ND	ND	ND
DON	% positive	83	100	100	100	100	100
	Mean*	780[b]	460[a]	497[a]	1467[c]	1344[c]	7349
	Median*	748	504	488	1494	1296	7349
	Min	667	247	203	682	929	5723
	Max	930	635	807	2648	2067	8975
15-ADON	% positive	67	83	42	92	100	100
	Mean*	115[a]	68[a]	70[a]	307[b]	126[a]	303
	Median*	122	56	58	197	101	303
	Min	58	30	35	55	73	295
	Max	156	115	114	948	277	310

* Based on positive samples.

ND: not detected.

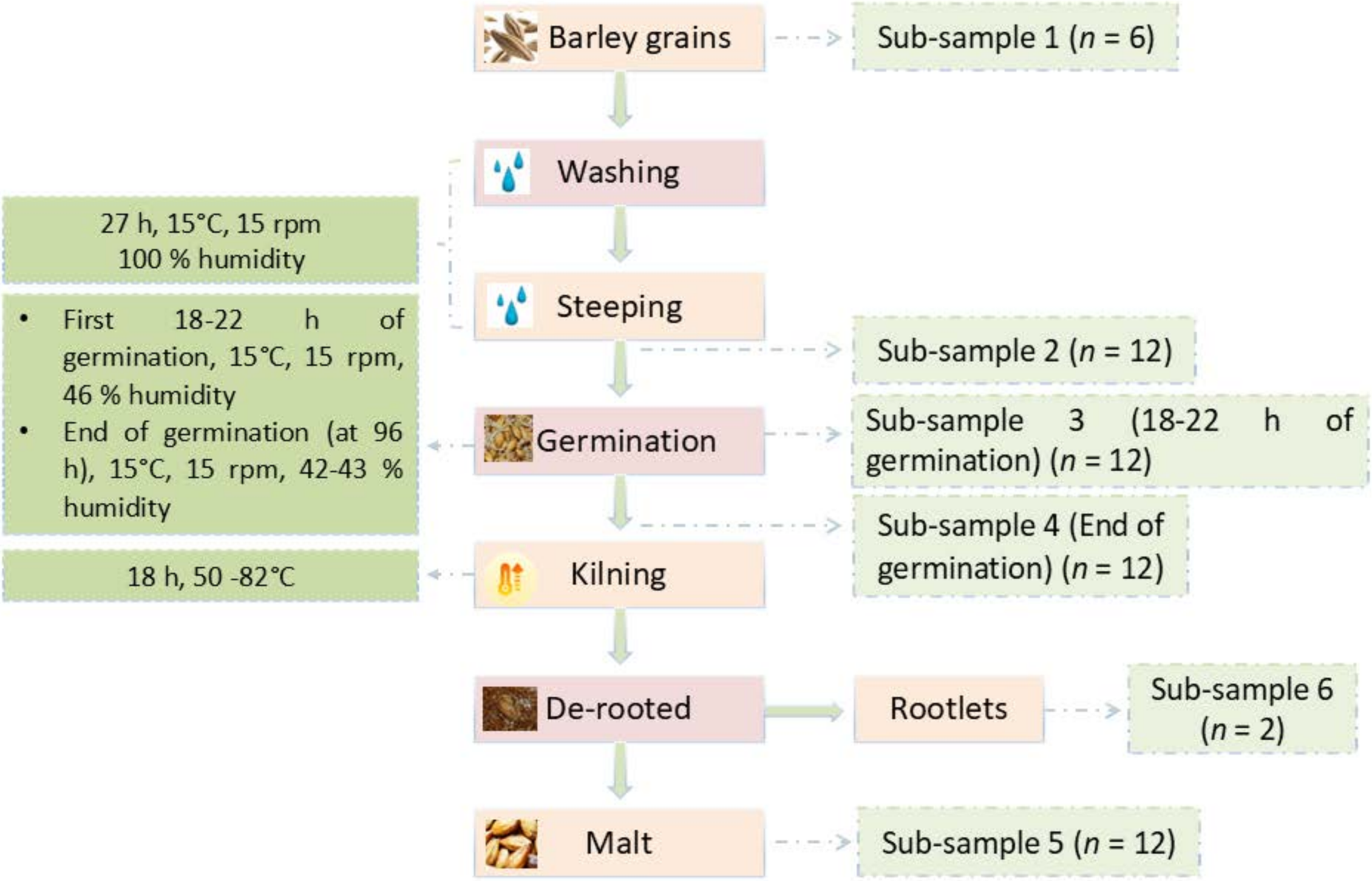
Table 3. Quality parameters in barley grains (A) and malts (B)

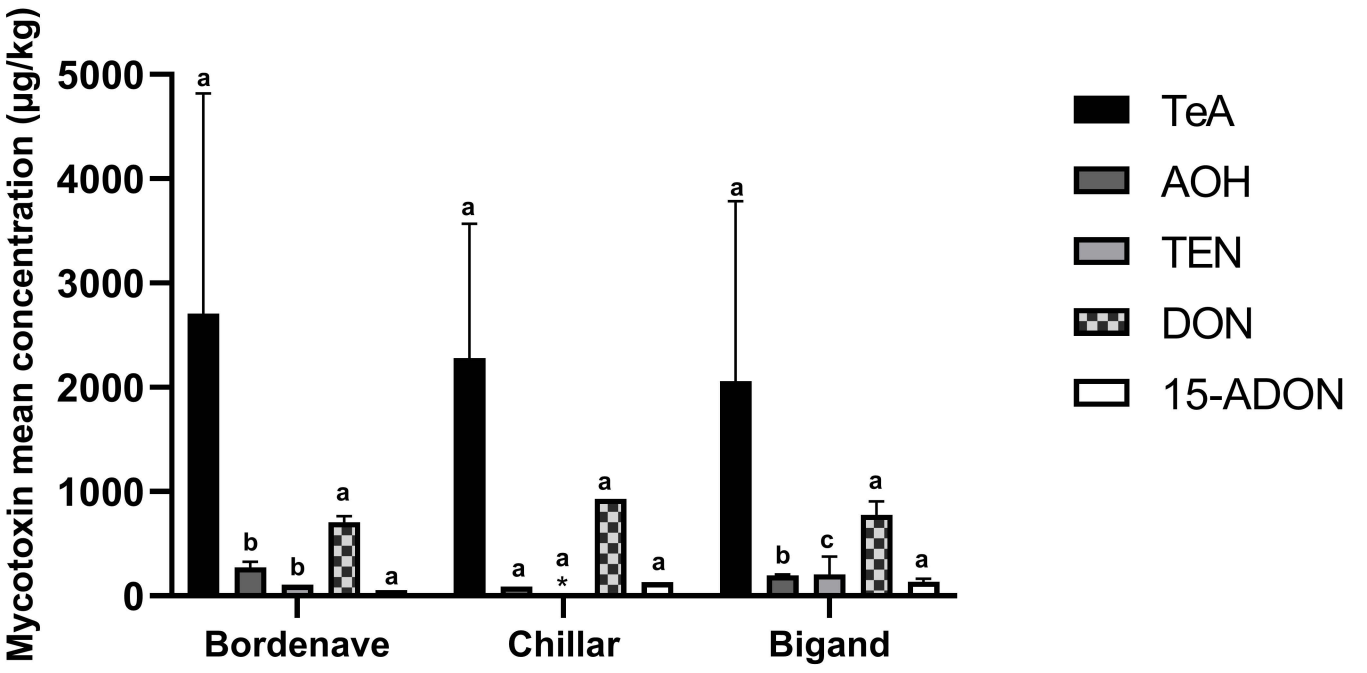
A - Grains									
Geographical origin	Genotype	% Humidity	TKW	% Protein	% Screening	D-hordein	C-hordein	B-hordein	Total hordein
Bordenave	Andreia	11.8	48	13.5	94.4	52.8	182.7	190.1	425.6
Bordenave	Shakira	12.3	46	13.8	96	45.2	170.0	220.7	435.8
Chillar	Andreia	12.8	45	13	93.7	40.2	174.6	113.0	327.8
Chillar	Shakira	12.3	51	13.4	95.4	36.9	167.0	108.0	311.9
Bigand	Andreia	12.8	48	13.9	93.9	36.6	135.0	127.3	299.1
Bigand	Shakira	12.8	48	14.0	96.3	41.1	166.4	184.4	391.9

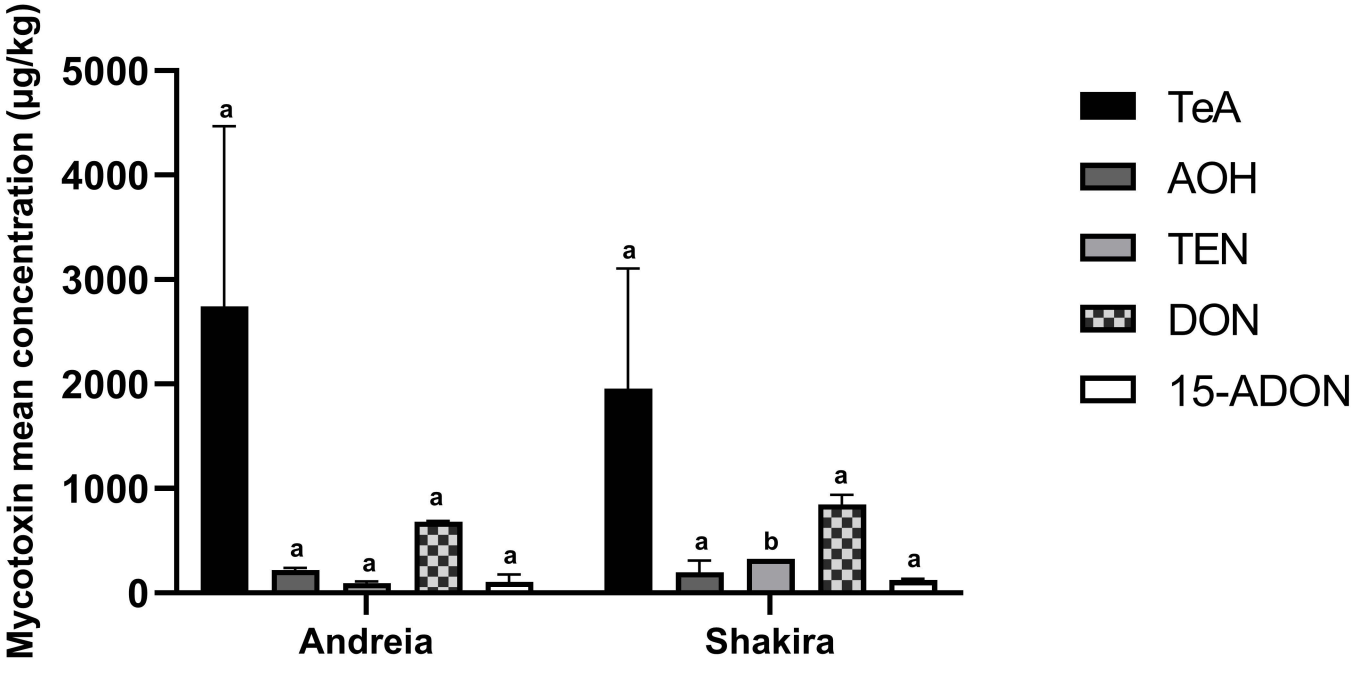
B - Malts											
Geographical origin	Genotype	% Total Protein	% Soluble Protein	Index Kolbach	Wort extract	Saccharification time	Filtration time	D-hordein[a]	C-hordein	B-hordein	Total hordein
Bordenave	Andreia	12.4	4.7	37.9	81.0	10-15	Regular	37.68	135.07	113.2	285.95
Bordenave	Andreia	12.1	4.7	38.8	80.5	10-15	Regular	35.87	126	108.5	270.37
Bordenave	Shakira	12.5	4.6	36.8	80.1	10-15	Regular	32.52	127.47	106.37	266.36
Bordenave	Shakira	12.2	4.7	38.5	79.9	10-15	Regular	36.58	116.15	100.98	253.71
Chillar	Andreia	12.0	4.7	39.2	80.4	10-15	Regular	28.15	121.35	70.7	220.2
Chillar	Andreia	11.0	4.5	40.9	80.3	10-15	Regular	26.86	114.17	64.48	205.51
Chillar	Shakira	11.9	4.5	37.8	79.5	10-15	Regular	31.39	112.25	76.07	219.71
Chillar	Shakira	12.1	4.7	38.8	79.1	10-15	Regular	32.24	117.1	89.9	239.24
Bigand	Andreia	12.9	5.0	38.8	80.3	10-15	>60	35.92	130.36	52.92	219.2
Bigand	Andreia	12.7	4.8	37.8	80.4	10-15	>60	29.23	131.07	52.85	213.15

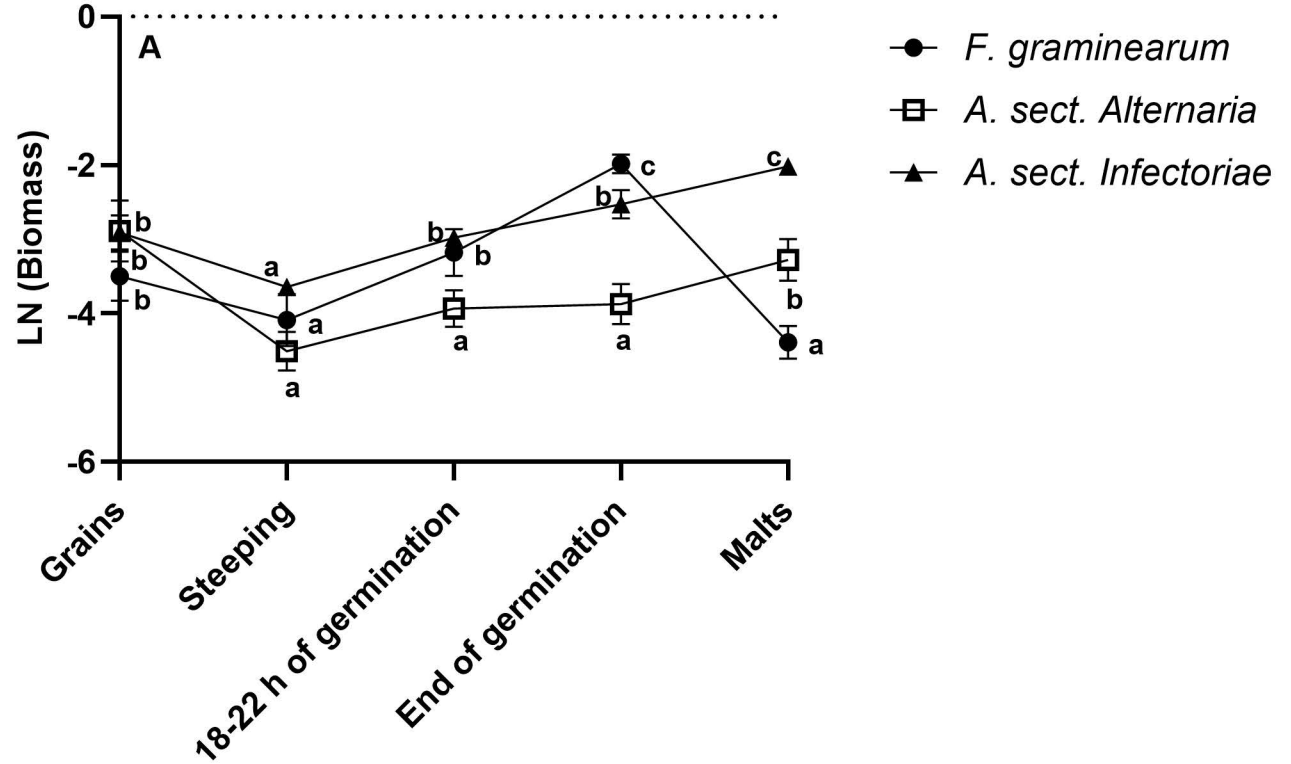
Bigand	Shakira	13.0	4.9	37.7	79.1	10-15	>60	29.53	117.87	89.95	237.35
Bigand	Shakira	13.3	5.0	37.6	79.6	10-15	Regular	45.48	140.54	100.96	286.98

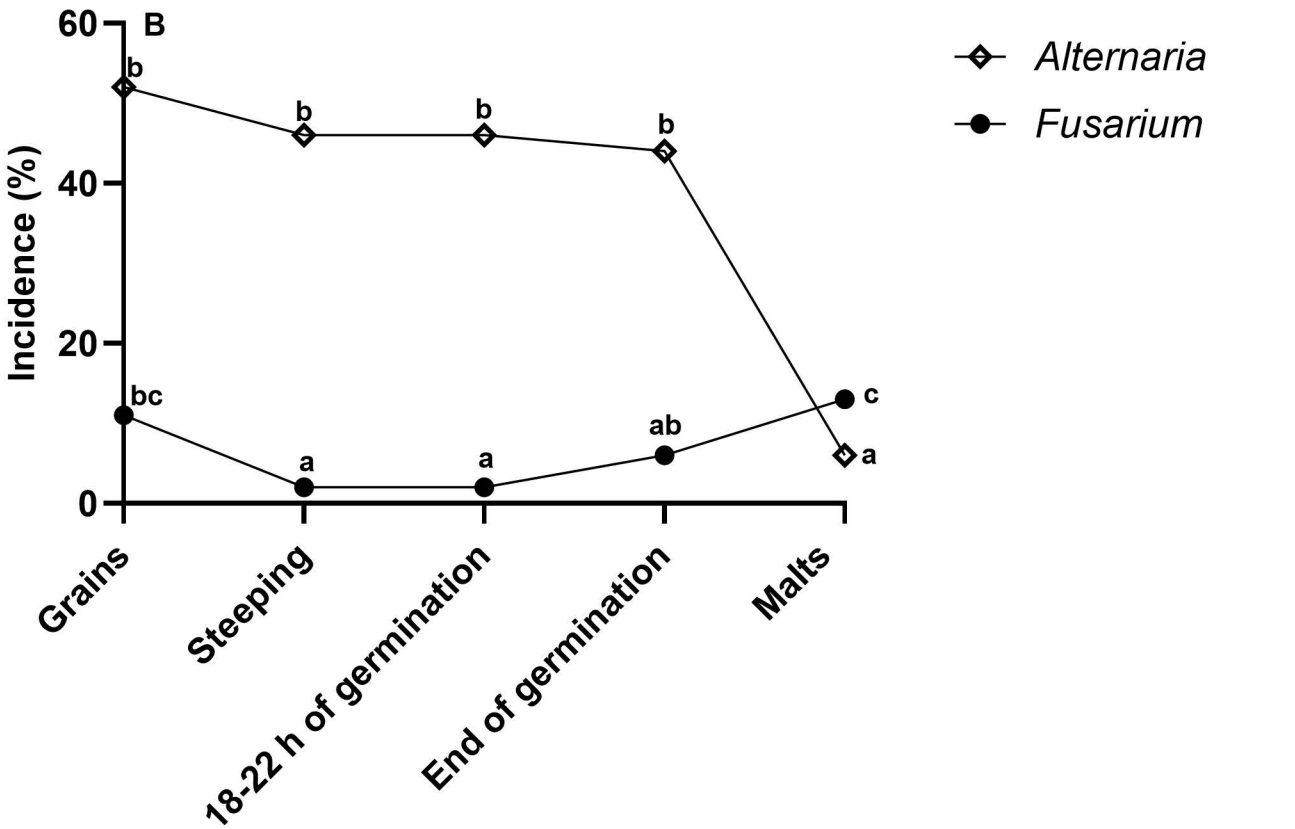
[a] Hordeins are expressed as absolute abundance.


Barley grains
Sub-sample 1 (n = 6)
Washing
27 h, 15°C, 15 rpm
100 % humidity
Steeping
Sub-sample 2 (n = 12)
First 18-22 h of germination, 15°C, 15 rpm, 46 % humidity
End of germination (at 96 h), 15°C, 15 rpm, 42-43 % humidity
Germination
Sub-sample 3 (18-22 h of germination) (n = 12)
Sub-sample 4 (End of germination) (n = 12)
18 h, 50 -82°C
Kilning
De-rooted
Rootlets
Sub-sample 6 (n = 2)
Malt
Sub-sample 5 (n = 12)


Mycotoxin mean concentration (µg/kg)
5000
4000
3000
2000
1000
0
a
b
b
a
a
a
a
a
*
a
a
a
b
c
a
a
Bordenave
Chillar
Bigand
TeA
AOH
TEN
DON
15-ADON


Mycotoxin mean concentration (µg/kg)
5000
4000
3000
2000
1000
0
a
a
a
a
a
a
a
b
a
a
Andreia
Shakira
TeA
AOH
TEN
DON
15-ADON


A
LN (Biomass)
0
-2
-4
-6
F. graminearum
A. sect. Alternaria
A. sect. Infectoriae
Grains
Steeping
18-22 h of germination
End of germination
Malts


B
Incidence (%)
60
40
20
0
Alternaria
Fusarium
Grains
Steeping
18-22 h of germination
End of germination
Malts



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Alternaria and Fusarium across malting barley process: a comprehensive study of mycotoxins and quality effects

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2027-01
Elsevier
https://doi.org/10.1016/j.foodcont.2026.112418
Castañares E, Dinolfo MI, Manno C, et al., (2027) Alternaria and Fusarium across malting barley process: a comprehensive study of mycotoxins and quality effects. Food Control, Volume 191, January 2027, Article number 112418

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