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Changes in nutritional and hygienic quality due to storage of common native and processed grain cereals intended for horse feeding

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ABSTRACT

Horses are sensitive to feed hygiene change. Commercial products are expected to be clean and safe but sub-standard storage conditions could compromise the nutritional and hygienic quality. We assessed changes in the nutritional and hygienic properties of common cereals (oat, maize and barley) in native (whole) and processed (flaked) forms in response to storage conditions. Commercially bagged cereals for horses were subjected to 3 storage conditions: freshly opened (control), open-bag storage and sealed-bag storage for 42 d kept in a feed storage room of a privately owned horse farm. The temperature and relative humidity of the storage room ranged from 17 to 21 °C and 58–74%, respectively. Two samples were taken from the top and bottom positions of each bag (total n = 36) for analyses. Microscopic evaluations showed that oat samples contained 3–5 times higher numbers of impurities than maize and barley. Maize samples were highest in the number of flawed grains, while barley showed the greatest number of pest components. High counts of impurities, flawed grains and pest components were already found in the control samples, and the storage conditions did not promote more damage and contaminants or alter the chemical composition of samples. Sensory evaluation did not detect differences among samples. Processing enlarged the particle size of all grain sorts and promoted physical damage to maize in particular. Five samples analyzed for mycotoxins and other contaminants revealed the prevalence of secondary metabolites of *Fusarium* and *Alternaria* spp. (each group showing 80% frequency and >500 μg/kg). Ergot alkaloids were absent. Fumonisin B_1 and B_2 were exclusively detected in maize samples (max concentration = 267 and 36 μg/kg, respectively) and pesticide residues in barley samples. Our data indicate specific impurities, damages and contaminants associated with certain grain sort and processing with little impact of the test storage conditions.

1. Introduction

Oat, barley and maize are the main cereal grains used in horse feeding, mainly to supplement the equine diet with energy and some other relevant nutrients. Oat and barley are fed both in native and processed forms, whereas maize is commonly fed as flakes to increase the typically low precaecal digestibility of starch of unprocessed maize (Hymøller et al., 2012; Julliand et al., 2006). The grains and their products can be used either as a single feed or as mixed complementary feed, commonly offered to horses as muesli. Horse owners may buy grains and processed products from feed manufacturers, who sell their products in sealed bags to prevent deterioration and extend the shelf life.

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The manufacturers are expected to undergo various quality control checks and analyses. The manufacturing procedure commonly involves drying, cleaning and, optionally, hydrothermally processing the grains, which are then stored for an extended period either in the dealer's warehouse or, upon purchase, in the feed storage of a stable. Depending on the grain type (unprocessed vs. processed), and storage conditions (e.g., humidity, temperature, air exposure, presence of pests) the grains can change in terms of feed value and hygiene due to deteriorations by biotic factors (e.g., insects and microorganisms) and abiotic factors (e.g., enzymes and lipid peroxidation).

Deterioration processes, especially of biotic origin, have consequences not only for the shelf-life of the feed but also nutrition and health of the horse. For instance, a heavy infestation of pests and insects decreases the feed palatability, thereby lowering feed intake, and can trigger allergic reactions and colic (Kamphues, 2013). Field moulds such as *Fusarium* and ergot fungi infest cereals already in the field while storage moulds such as *Aspergillus* and *Penicillium* proliferate during storage (Fleurat-Lessard, 2017). Their infestation represents a major concern regarding horse health due to their ability to produce a wide range of metabolites called mycotoxins, some of which have been linked to health and fertility problems in horses (Kamphues, 2013). It is important to note that while the mould can be eliminated or deactivated during conventional feed processing such as thermal processing, the already-produced mycotoxins are not (Schmidt et al., 2018). Mycotoxin contamination can be prevalent in marketed products intended for animal feeding (Hassan et al., 2019; Liesener et al., 2010; Santos Pereira et al., 2019).

The majority of evaluations of the nutritional and hygienic quality of horse feedstuffs have been done for hays and some other forages including commercial products (Seguin et al., 2010; Wichert et al., 2008; Müller et al., 2011) since forages are the primary feed sources of horses. Cereal grains are feedstuffs prone to many biological contaminants like mould, yeast and pests and thus microbial toxins (Kamphues, 2013). Some studies have assessed for feeding values (e.g., Al Jassim, 2006; Särkijärvi and Saastamoinen, 2006). Fewer studies have dealt with the hygienic quality of cereals and concentrates for horses, but mainly focused only on microbial contaminations (Intemann et al., 2022; Sliwinsky et al., 2005). These studies showed that high levels of contamination of bacteria and moulds can be frequent. However, this does not address where and why these contaminations come from. While the frequency of increased microbial contamination of bought products was lower than home mix (Sliwinsky et al., 2005), it can be expected that substandard storage conditions of purchased products on farm can compromise the hygiene and safety of the grain due to exposure to pests and microorganisms. To fill this gap of knowledge, this work aimed to systematically investigate changes in the nutritional and hygienic properties of common cereals and their processed products for horses in response to storage and environmental conditions, up to 42 d. To do so, we performed analyses of sensory and hygienic properties, chemical composition, physical properties (particle size distribution), and a safety aspect (mycotoxin concentration) of commercial bags of native and processed oat, barley and maize under different storage conditions. A significant loss of quality in hygiene and chemical composition was expected for opened bags with extended storage time, while no deviations in quality were expected for closed grain bags kept under the same storage conditions.

2. Materials and methods

2.1. Experimental plans and conditions

Commercial bagged cereals ready to be sold were manufactured by local manufacturers, including native oat, barley and maize (all from manufacturer 1), flaked oat (manufacturer 2), flaked barley (manufacturer 3), and flaked maize (manufacturer 4). Two sealed bags of each sample type were randomly taken and used for the experiment. All processed cereals were hydrothermally treated. Test bags were in commercial size weighing 15–30 kg. Per sample type, one of the two bags was opened and was immediately sampled for analysis (Control d1). Subsequently, the opened bags were stored in a privately owned horse feed storage room for 42 d (Opened d42) for the next sampling and analysis. The unopened bags remained closed and stored for 42 d (Closed d42) in the same storage room for later sampling and analysis. The storage room was dark and equipped with a concrete floor, a stone wall and a small window facing north and with no air draft. All bags were stored on wooden pallets in an upright position commonly practised by horse owners. Data from the storage room on temperature and humidity were collected every third day using the wireless weather station WS 7394IT (Technoline Ltd., Wildau, Germany). During the experiment (August 18 – September 28, 2022), the temperature and relative humidity of the storage room ranged between 17.2 and 21.2 °C and 58–74%, respectively (Fig. 1). At each sampling, samples were taken from two locations within the bag: top and bottom (2 L of samples per location). The samples were sealed airtight until the investigation, which was carried out at the Centre for Animal Nutrition and Welfare, University of Veterinary Medicine Vienna, Austria between August–September 2022.

2.2. Sample analyses

2.2.1. Sensory evaluation

The assessment in terms of feed value and hygiene status was performed according to a standard protocol (Kamphues et al., 2014). The parameters were assessed in the order of texture, odour, taste, structure, colour, cross-section and visible contaminants. For each parameter, a score of 0–6 was rated according to the criteria of each sensory parameter. For instance, for the colour parameter, having red or blue-grey tinge indicating mould infestation would score 6, green indicating unripe grains 5, red-pinkish indicating seed treatment 4, greyish caused by dust 2, and typical grain colour but intense and bright 1. Therefore, broadly the scores represent the following conditions: 0 = Product typical, 1 = Very good quality free from defects, 2 = Good quality with minimal defects, 3 = Satisfactory quality with minor defects, 4 = Fair quality with moderate defects, 5 = Poor quality with major defects and 6 = Potentially harmful with massive defects. The investigation was carried out under the supervision of an experienced feed laboratory specialist. A subsampling of 100 ml volume was assessed on a white background in a brightly lit laboratory room. All samples were assessed within two consecutive days.

2.2.2. Bulk density

A volume of 1 L from the samples was measured using a 1-L measuring beaker, which was then compressed ten times from a distance of 10 cm to the tabletop on the smooth surface to compact the

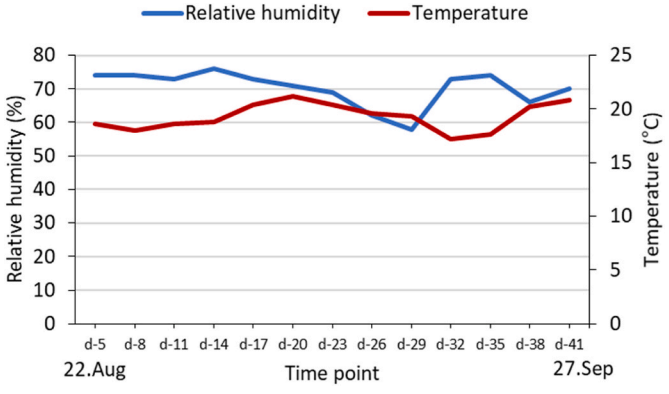

Fig. 1. Temperature and relative humidity of the storage room during the experiment.

grain. The grain sample was then weighed using an analytical balance (XS4002S, Mettler Toledo, Greifensee, Switzerland). This operation was done in triplicates and the arithmetic mean was used for data analysis.

2.2.3. Particle size distribution and detection of foreign components

The dry sieving method was used to determine the particle size proportions and foreign matter contained in the grain samples. The dry-sieving machine (AS 200 Digit, Retsch GmbH, Haan, Germany) was equipped with 4 sieves with mesh sizes of 8 mm, 5 mm, 4 mm, 2 mm and 0.5 mm. To carry out the dry sieving, a test sample of 250 g was weighed into the analytical sieve machine and then shaken through sieves of different sizes. The device setting for the dry sieving run was set with a duration of 1 min and a frequency of 50 Hz. After the end of the machine run, each sieve level was weighed on an analytical balance (XS4002S, Mettler Toledo, Greifensee, Switzerland) and the weight proportions on individual sieves were calculated.

The small components of the grain samples resulting from the dry sieving, which had collected on the sieve with a mesh size of 0.5 mm and on the floor, were examined for impurities and animal infestation using the stereo microscope (SMZ445, Nikon, Tokyo, Japan). The contaminants found were identified in a Petri dish using a spectrum from 8 to 35 × total magnification. The microscopic analysis was carried out for three consecutive days.

2.2.4. Chemical analysis

Samples were ground to pass a sieve mesh of 0.5 mm. Subsequently, about 100 g of the ground material was used for the proximate analysis using the protocol of VDLUFA (2002). Specifically, dry matter (DM) content was determined after oven-drying at 103 °C for 4 h and the ash content after combustion in the muffle furnace at 580 °C overnight. Crude protein (CP) was determined using the Kjeldahl method and ether extract (EE) using a Soxhlet extraction system (Extraction System B-811; Büchi, Flawil, Switzerland). Neutral detergent fibre (NDF) was analyzed using the Fibretherm FT12 (C. Gerhardt GmbH & Co. KG, Königswinter, Germany) according to the manufacturer's protocol with a heat-stable α-amylase. Contents of organic matter (100 - ash) and non-fibre carbohydrates (NFC = 100 - ash - CP - EE - NDF) were estimated.

2.2.5. Mycotoxin and other contaminant analysis

Those samples that showed abnormalities in the assessments in the previous analyses (atypical colour or high counts of visible contaminants) were subjected to analysis of mycotoxins and other secondary metabolites fungi, phytoestrogens, as well as pesticide residues using liquid chromatography-tandem mass spectrometry (LC–MS/MS). The method has been used for the analysis of various feed commodities and a detailed description of the validated method is described in Steiner et al. (2020). These included samples of Maize Control (d1), Maize Closed d42, Oat Opened d42, Barley Opened d42 and Flaked barley Opened d42. All selected samples were from the top bag position. As only a subset of samples were subjected to the contaminant analysis, the results were intended to support the sensory and microscopic evaluation findings of these samples in the present study. Furthermore, since we did not determine the microbial viability and growth of the samples, the detected mycotoxins and other contaminants can therefore reflect the presence of the compound-producing organisms at some certain point of the production and processing process. The LC–MS/MS analysis was carried out at the Department of Agrobiotechnology (IFA-Tulln) at the University of Natural Resources and Life Sciences Vienna (BOKU) in Tulln, Austria. Briefly, 5 g of finely milled samples were mixed with 20 ml of extraction solvent (acetonitrile/water/acetic acid in the ratio of 79:20:1) in a 250 ml flask and then homogenized for 90 min at 180 rpm in a rotary shaker (GFL 3017, Burgwedel). The method was validated for more than 1000 analytes according to the analytical standards (Steiner et al., 2020). The majority of the reference standards were obtained commercially. Results have been corrected for recoveries determined during method validation. This results in a limit of detection (LOD) and a limit of quantification (LOQ) for the respective contamination for the tested samples. Data below LOD were assigned as not detectable. Concentrations below LOQ were calculated as LOQ/2.

2.3. Statistical analysis

Variance analysis and boxplots of quantitative data were carried out using the Mixed and SGPLOT procedures of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA), respectively. Fixed factors of interest included grain sort (barley, oat, and maize), storage (control d1, opened d42, closed d42), grain processing (native and process) and bag position (top and bottom). Different mixed models including different sets of test factors and random factors were performed depending on the specific research questions. Accordingly, for microscopic analysis of impurities in grain samples, the model included fixed effects of grain sort, storage and their interaction with bag position and processing as the random factors. The counts of flawed grains/particles and pest components as well as particle distribution were analyzed for the effects of grain sort, grain processing and their interactions considering a random effect of storage nested within the bag in the model. For bulk density, the model included grain sort, storage, grain processing, and sort × processing with the random effect of bag position. For chemical composition, the data were analyzed for the effects of grain sort, bag position and their interaction with the distinction between native and processed grains. For sensory scores, which are categorical data, per grain sort the effect of storage was tested according to the Kruskal Wallis test using the NPAR1way procedure of SAS. Significant effects and differences are called when $P < 0.05$ and tendency when $0.05 \leq P \leq 0.10$. Data of LC-MS/MS analysis of selected five samples are presented but were not statistically analyzed.

3. Results

3.1. Foreign components and sensory scores

Microscopic analysis of samples showed that out of the 36 samples, 28 samples contained impurities, whereas 8 samples, mostly from maize, were free from impurities (Fig. 2). The impurities included foreign grains (e.g., rye, wheat, and wild oats), plant admixtures (e.g., chaff, husk) and earthy components (e.g., sand, earth, clumps of manure and sand). Grain sort ($P < 0.001$) and storage ($P = 0.024$) affected the counts of impurities in samples. Oat in general contained more impurities than barley and maize and unopened samples (Control, d1) contained higher counts of impurities than the d42 samples (Fig. 2). Notably, one oat sample showed the highest count reaching 130 detections. There was an interaction between grain sort and processing on the counts of flawed grains/particles and pest components ($P < 0.001$, Fig. 2). Specifically, processing increased the counts of flawed grains only in maize. All processed barley samples showed contamination of pest components, while pest components were sporadically found in native barley and some other grain samples. Pest components included moth larvae, meal mites, and booklice (Fig. 3). The effect of storage and its interaction with grain sort was not significant (data not shown). However, some of the control samples already contained flawed grains and pest components, with maize samples showing the lowest frequency for these parameters. Overall, the average sensory scores were within the satisfactory range and the storage condition did not affect the sensory scores (scores 0–3, Fig. 4). Openly stored bags of oat and barley had numerically elevated colour scores from those of the closed bags. A blue-greyish discolouration was noted for the native oat samples from both the top and bottom samples.

3.2. Bulk density and chemical composition

Bulk density of native barley samples ranged from 710 to 739 g/L, native maize samples from 756 to 815 g/L and oat samples from 582 to

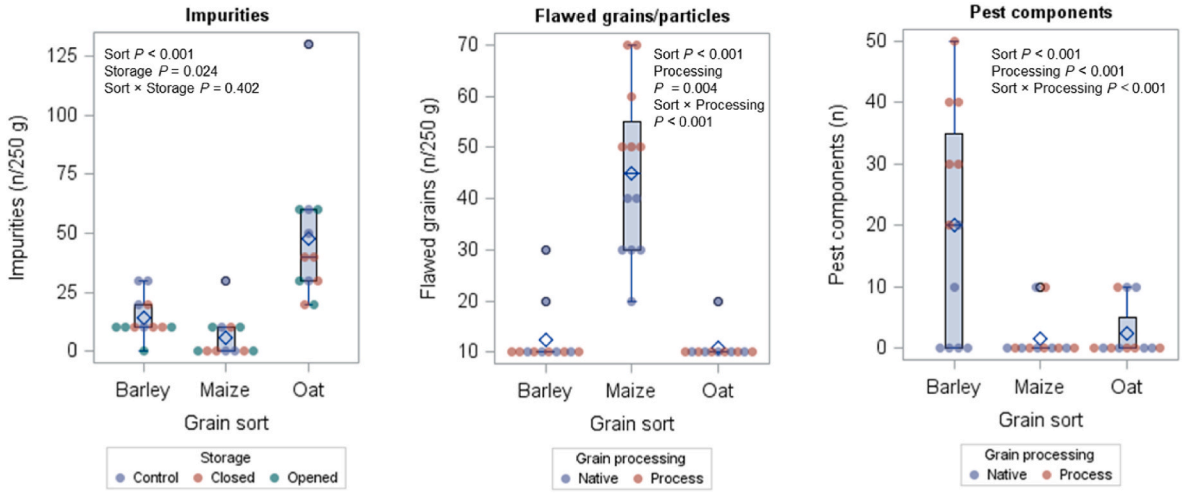

Fig. 2. Microscopic analysis of impurities, flawed grains and pest components in native or processed (flaked) cereal samples intended for horse feeding. Note: Per group of grain, there were three different storage conditions, namely Control (freshly opened), Closed (closed bag stored for 42 d) and Opened (open-bag stored for 42 d). Data of counts of impurities depict the effects of grain sort, storage and sort × storage interaction (n = 4 per treatment). Data of counts of flawed grains and pest components depict the effects of grain sort, processing and sort × processing interaction (n = 6 per treatment).

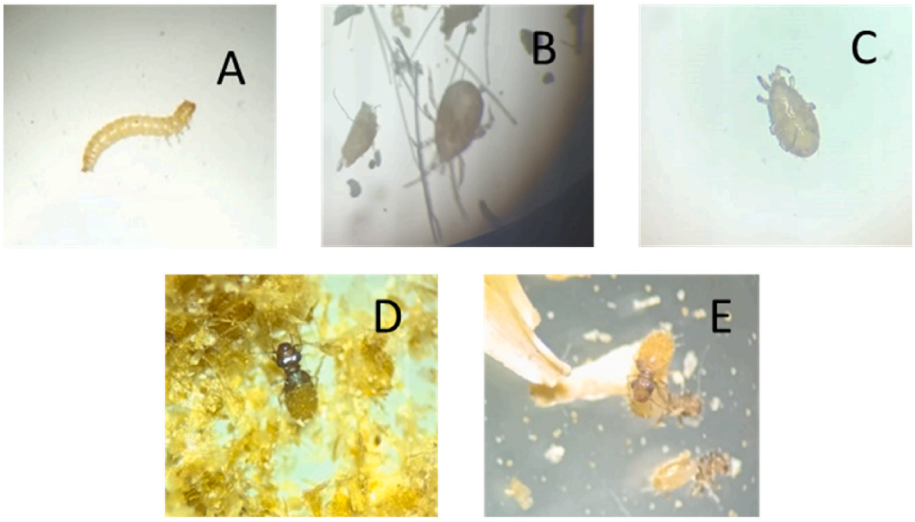

Fig. 3. Exemplars of pests detected in the native or processed grains samples intended for horse feeding (A: moth larva (*Plodia interpunctella*) in the 42-d open bag of flaked maize sample, B - C: mites (species not identified) in the Control, freshly opened bag of native oat, and D–E: psocids (*Liposcelis* spp.) in the 42d-closed bag of flaked barley). Note: Microscopic evaluation was done using Nikon SMZ445 (Tokyo, Japan) with an 8–35 × total magnification range.

644 g/L (Fig. 5). Processing lowered the bulk density of barley (532–578 g/L) and maize (400–425 g/L) but not oat (619–643 g/L), underlining the interaction between sort × processing. The effect of storage was significant (P = 0.002) but the differences were biologically minimal. The bag position of the sample did not show an influence on this parameter (data not shown).

It was our interest to investigate whether the chemical composition of grains and their products is homogenous within the bag. The homogeneity was confirmed by the current data (Fig. 6). Overall, no effect of bag position or its interaction with grain sort was observed on the chemical composition. Nevertheless, greater variations in the contents of CP, NDF and NFC were observed in oats, especially in the native form.

3.3. Particle size distribution

The particle size distribution did not differ between the top and bottom of the bag. Overall, particles larger than 8 mm were only found with maize and flaked maize. The strongest and most meaningful factor in the particle distribution was processing, whose effect intensity depended on the grain sort (Fig. 7). Native oat and barley showed relatively similar particle distribution with almost all particles (>90%) retained on the 2-mm sieve. Processing increased the particle sizes and thus about 60–70% of flaked barley was retained on the 5-mm sieve, while about 50% of flaked oat was retained on the 4-mm sieve. Flaked maize also contained greater proportions of large particles (>8 mm) of about 80% compared to 25% in the native form.

3.4. Mycotoxin and other contaminants

All tested samples showed concentrations of secondary metabolites of fungi. On an as fed basis, the sum of metabolites from *Fusarium* ranged 16–1746 μg/kg sample, *Alternaria* ranged 26–646 μg/kg, from *Penicillium* 2–60 μg/kg and unspecified sources 23–791 μg/kg (Fig. 8). Of note, 4 out of 5 samples (80%) showed concentrations of secondary metabolites from *Fusarium* and *Alternaria* above 500 μg/kg sample. Fumonisin B_1 (FB_1) and B_2 (FB_2) were only detected in the maize samples. None of

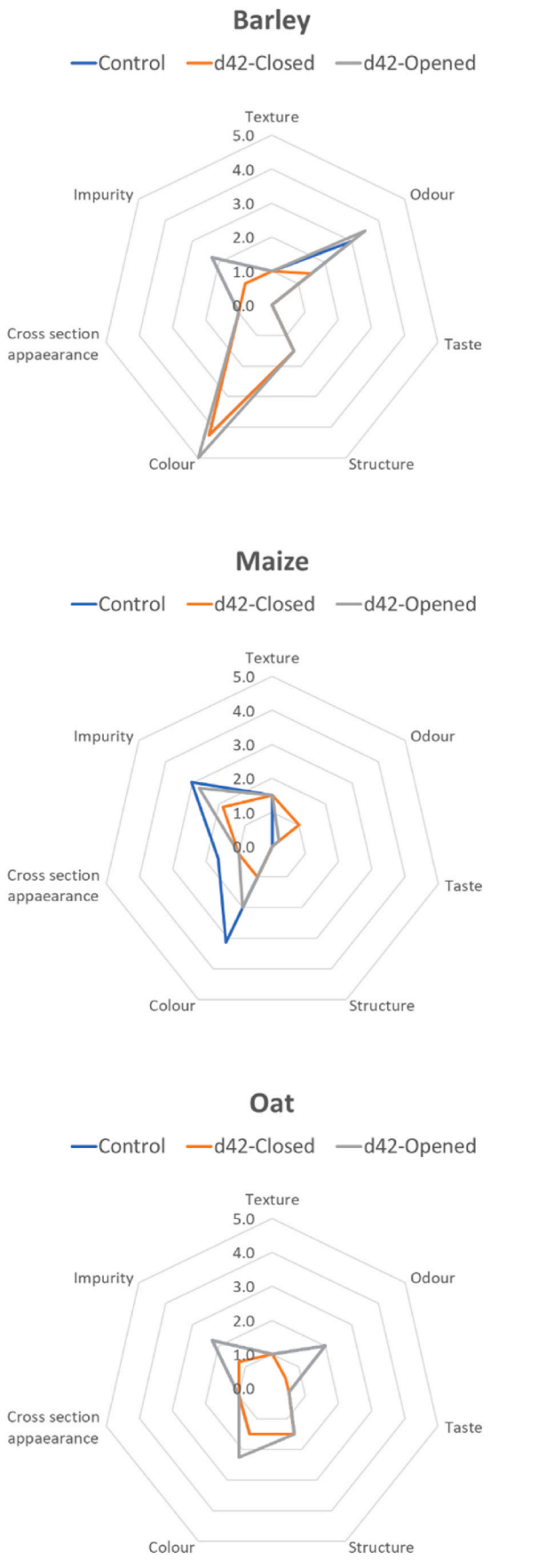

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Fig. 4. Average scores from sensory evaluation of cereal grains under different storage conditions. Note: Lower scores indicate favourable conditions: 0 = Product typical, 1 = Very good quality free from defects, 2 = Good quality with minimal defects, 3 = Satisfactory quality with minor defects, 4 = Fair quality with moderate defects, 5 = Poor quality with major defects and 6 = Potentially harmful with massive defects. Per grain sort, there were three different storage conditions (n = 4 per treatment), namely Control (freshly opened bag), Closed (closed bag stored for 42 d) and Opened (open-bag stored for 42 d). No significant differences among storage conditions in all scores, except for the odour score in maize ($P < 0.05$) according to Kruskal-Wallis Test.

the samples was positive for ergot alkaloids. Both barley samples showed noticeable concentrations of residues of various pesticides. The sum concentrations were 92 and 303 μg/kg for flaked barley and native barley, respectively. Piperonylbutoxide was a dominant component in both samples with concentrations of 75 and 66 μg/kg, respectively.

4. Discussion

Nutrient composition and in particular the hygienic value of feeds are key to horse health and performance. Previous research on the nutritional and hygienic qualities of horse feed has focused mainly on forage sources (Seguin et al., 2010; Wichert et al., 2008; Müller et al., 2011). In terms of hygiene, cereal grains can be risky feed sources due to pest infestation and microbial toxin contamination (Kamphues, 2013; Intemann et al., 2022; Sliwinsky et al., 2005). Many horse owners use commercial horse cereal grains in native or processed forms. These purchased bags are usually kept in a stable or barn and so the quality can be affected when the storage condition is substandard. We expected commercial cereal grains to be flawless, being free or very low in toxic contaminants and impurities, and therefore representing excellent hygienic quality. However, the quality can be compromised over the time of storage, especially when bags are stored open, thus being exposed to biotic factors like pests and microorganisms as well as abiotic factors such as air, heat, light and oxidation. With that, the physical and nutritional properties of the grains would change as well.

As opposed to our expectation, it is striking to see that our analysis of the commercially sold grains contained considerable amounts of impurities, flawed grains and, more importantly, pest components, despite having passed the formal quality checks of the manufacturers or dealers. However, the 42-d storage under stable condition did not further increase these contaminants or change the composition of the nutrients studied, even when bags were kept open. Respectively, the sensory scores of all samples were within the satisfactory range. Temperature, relative humidity and the water content of cereal grains are among the most important factors affecting the quality of stored grains (Fleurat-Lessard, 2017). In our experiment, these factors were in the range considered safe (Fleurat-Lessard, 2017), with the storage temperature of 17–21 °C and relative humidity always below 75%. Dry matter contents can indicate high risk of aerobic spoilage and the values below 85% are unacceptable (Kamphues, 2013). In our study, all samples showed DM contents well above 88%. Our data underline the importance of these environmental factors in maintaining the hygiene quality of stored grains.

The present work further highlighted that i) oats are prone to botanical impurities and earthy components, ii) maize are more physically damaged from processing and iii) barley are prone to pest contamination, especially after processing. Indeed, processing increases the grain's susceptibility to biotic and abiotic deterioration processes. Mechanically cracking of grains allows the secondary pests like moths, mites and psocids to feed on the broken grains (Stejskal et al., 2014). In the present study, the impurities and contaminants were detected already in the control, freshly opened bags taken from the manufacturers. This indicates their presence before the packaging, which may originate from the field as well as in the manufacturing facility. The impurities included foreign grains (e.g., rye, wheat, and wild oats), plant

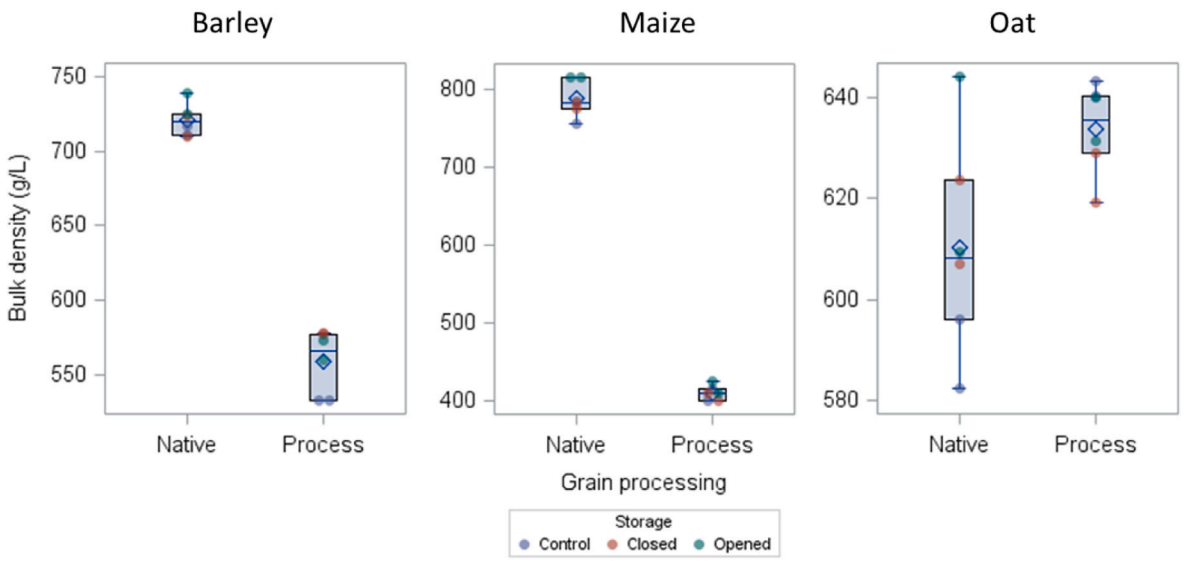

Fig. 5. Bulk density of native or processed (flaked) cereal samples intended for horse feeding. Note: Per group of grain, there were three different storage conditions (n = 2 per treatment), namely Control (freshly opened), Closed (closed bag stored for 42 d) and Opened (open-bag stored for 42 d). P values of grain sort, grain processing, sort × processing = P < 0.001, storage = 0.002.

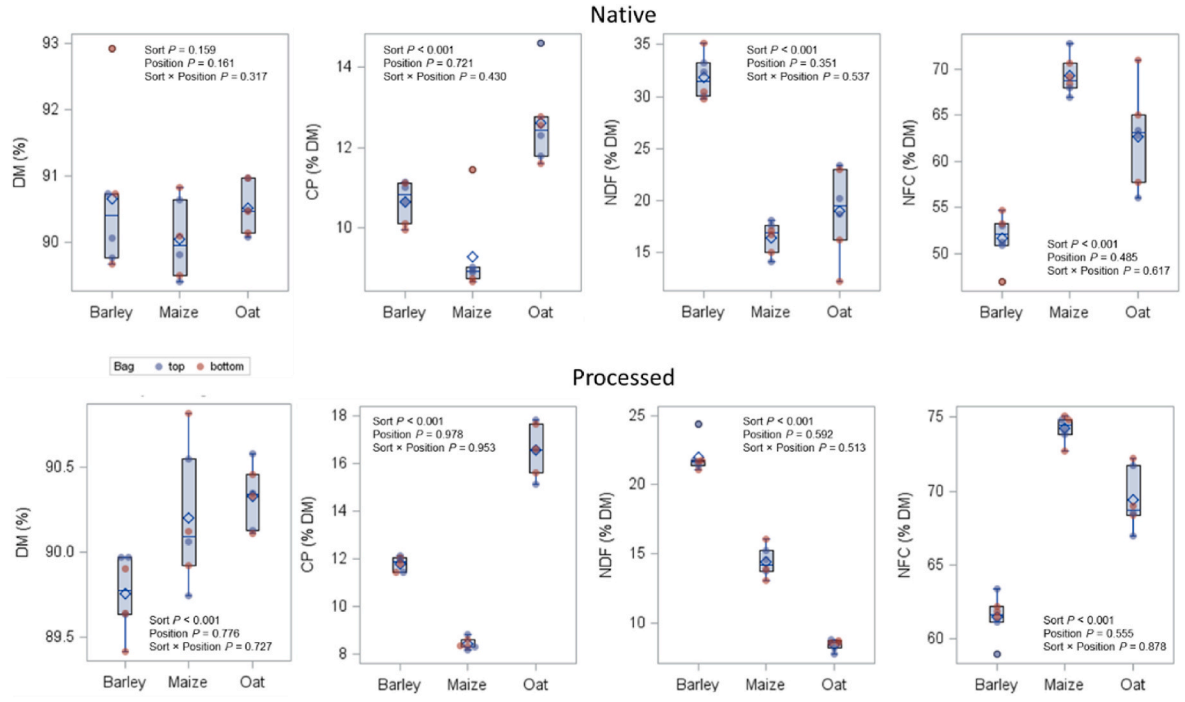

Fig. 6. Chemical composition of native or processed (flaked) cereal samples intended for horse feeding. Note: Per bag of grain, samples were taken from the top and bottom positions of the bag (n = 3 per treatment). DM = dry matter, CP = crude protein, NDF = neutral detergent fibre, NFC = non-fibre carbohydrates.

admixtures (e.g., chaff and husk) and earthy components (e.g., sand, earth, clumps of manure and sand), which would be related to impurity removal, cross-contamination prevention and the quality control of finished products. Counts of pest components were as high as 50 components per 250 g grain found in the processed barley samples. One native oat sample showed the highest count of 130 counts of impurities. The EU regulation on the catalogue of feed materials requires feed materials to have a minimum of 95% botanical purity (No 68/2013) and the maximum allowance of weed seeds is 3000 mg/kg (DIRECTIVE, 2002/32/EC). Based on this regulation, impurities detected in the samples did not seem to violate the standard of good production practice. However, the presence of still-living pest components was alarming as they can multiply rapidly when environmental conditions allow (warm and moist). Exposure to dust and other components such as fine feed particles, faeces, mites and microorganisms challenging to the respiratory tract can affect the health of horses (Kamphues, 2013). Even though our data did not cover the batch variation in manufacture, it offers evidence suggesting horse owners to be aware and inspect elevated impurities and pests especially when using commercial oats and barley for their horses.

Notably, the sensory evaluation failed to distinguish differences among samples showing different amounts of contaminants and

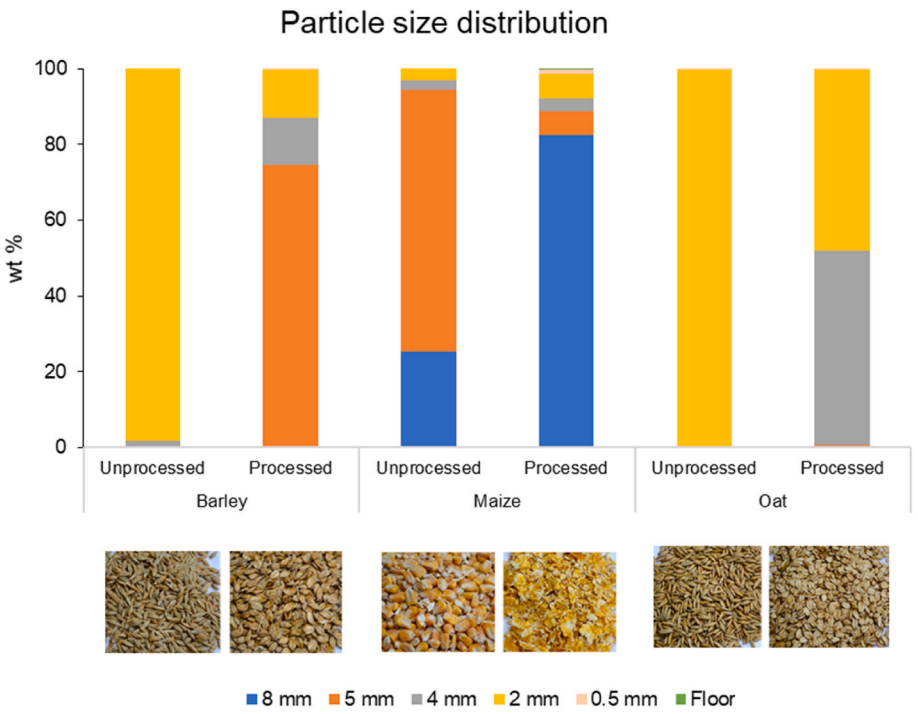

Fig. 7. Particle size distribution of native or processed (flaked) cereals intended for horse feeding. Note: The proportion (wt %) of sample retained on each sieve depicts the effects of grain sort, processing and sort × processing interaction (n = 6 per treatment). Significant interactions between grain sort and processing were detected for sieves 8, 5, 4 ($P < 0.001$) and 0.5 mm ($P < 0.05$) and a tendency for the floor sieve ($P = 0.10$).

impurities. This might be because the biotic and abiotic deterioration processes had not reached the level for detectable sensorial changes. For instance, all feeds are contaminated by microorganisms but the odour and the physical appearance of feed begin to change when spoilage-indicating microbes reach values up to 10^9–10^{10} cfu/g feed (Kamphues, 2013), which is triggered by high humidity and temperature and physical damages. Our work underlines the importance of microscopic evaluation for the assessment and early prevention of deterioration in feed quality and hygiene.

The processing (flaking and rolling) flattens grains and thus the particle size of processed cereal samples were larger than their native form as measured by the dry sieving method. Maize, as expected, had the largest particle sizes especially when flaked. Furthermore, our data underlined that maize is more easily damaged by processing as compared to oats and barley. This was likely related to the grain characteristics (husk vs. husk less) as well as the intense processing of maize that increased the brittleness of finished flakes. Steam flaking improves the starch digestibility of the maize in horses (Hymøller et al., 2012) and cattle (Malekkhahi et al., 2021; Theurer et al., 1999). The processing increases the surface area of the grain and results in the gelatinization process and therefore cooking of the starch, which in turn promotes physical damage and therefore would shorten the shelf life of processed grains.

The uniform quality of grains within the bag was another subject of interest. We expected the upper portion of a bag to be more exposed to pest infestation and environmental factors when stored openly for a prolonged period. With that in mind, we expected to see more fibrous and bulky particles in the upper location than in the bottom location. However, we did not detect the statistical differences, only higher variations in the NDF and NFC contents found with native oats. Oat samples also appeared to vary more in terms of bulk density as compared to maize and barley but multiple factors played a role. Apparently, the storage condition was proper, so did not result in major deterioration of the samples. This would explain the homogeneity, both physically and chemically, of the samples throughout the experiment.

Mycotoxins are secondary metabolites of fungi. They represent an important feed safety issue in horses. FB1 is the most studied in horses known to cause equine leukoencephalomalacia (ELEM), commonly called mouldy corn poisoning (EFSA, 2005). Our data are in good agreement with previous works showing that maize and maize products are risky feedstuffs concerning *Fusarium* mycotoxin contamination (Liesener et al., 2010; Magnoli et al., 2019). Fumonisin B_1 and B_2 are the only mycotoxins listed in the EU regulation (2006/576/EC) with the reference for horses. Accordingly, the legislation gave the guidance values for $FB_1 + FB_2$ of 60 mg/kg maize and maize products and 5 mg/kg for compound feed (with 88% DM) for horses. EFSA proposed the no observed adverse effect level (NOAEL) of 0.2 mg FB_1/kg BW/d (corresponding to 8.8 mg/kg feed) for developing ELEM for horses (Knutsen et al., 2018). Later risk assessment lowered it to 1 mg fumonisins/kg feed, which was referred to as the reference point for adverse health effects (Schrenk et al., 2022). In the present work, from the five selected samples, maize was the only grain sort that contained fumonisins and the d42 maize sample contained the highest level of fumonisins (0.27 mg FB1/kg, 0.3 mg FB_1+FB_2/kg). The detected levels of fumonisins were below the guidance value, therefore considerably safe. Other mycotoxins that could be tolerated in diets without adverse effects in horses (Kamphues, 2013) are aflatoxins (max. allowance of 0.02 mg/kg feed according to Directive, 2002/32/EC), zearalenone (2–3 mg/kg diet), deoxynivalenol (up to 10 mg/kg diet) and ergovaline (clinical symptoms at 0.3–0.5 mg/kg diet). Based on these limits, the studied samples also represented safe levels of these contaminants.

When feeding a single-source cereal to horses, horse owners should be aware of risky grain sources for specific mycotoxins. Using competitive enzyme immunoassays targeting five *Fusarium* mycotoxins and a group of ergot alkaloids in commercial horse feeds, Liesener et al. (2010) observed that single-grain cereals have higher concentrations of these fungal metabolites than in compound feeds (e.g. muesli, mash, pellets). They also showed that among the single-grain cereals, maize contained the highest concentrations of FB_1 and deoxynivalenol, while high concentrations of T2 and HT2 toxins were predominately in oat and ergot alkaloids in barley. A similar pattern for *Fusarium* mycotoxins was found in the present study. Accordingly, both maize samples consistently

	Concentrations (μg/kg) of the sum of contaminants assigned to the respective origin									
Sample	Fusarium	Ergot	Aspergillus	Penicillium	Alternaria	Other fungi	Bacteria	Plants	Pesticides	Others
Maize d1	1491	0	80	2	26	0	0	59	1	23
Maize d42	1746	0	0	25	646	0	0	0	0	48
Oat d42	960	0	0	60	576	60	0	21	1	46
Barley d42	16	0	0	6	47	0	0	391	303	655
Flaked barley d42	607	0	0	11	518	541	0	106	92	791

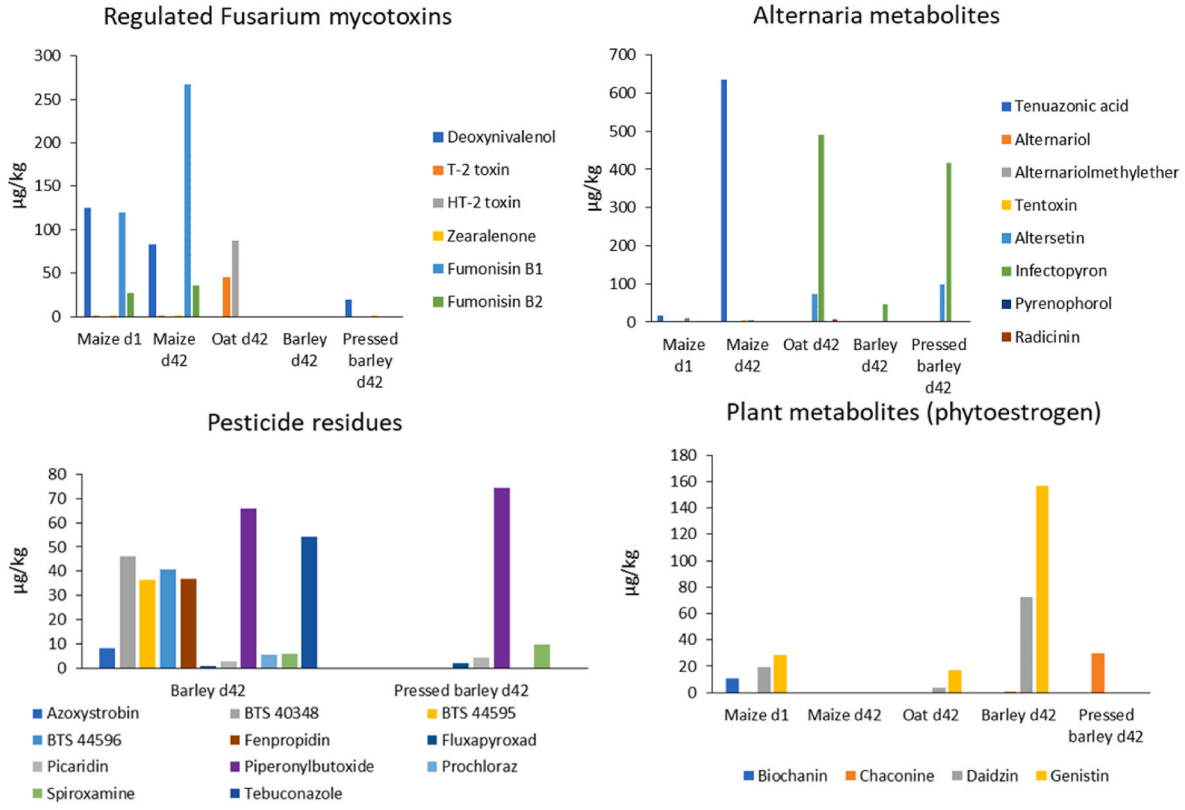

Fig. 8. Concentrations of secondary metabolites of fungi, phytoestrogens and pesticide residues in the native or processed cereal samples (μg/kg, on an as fed basis, the DM contents ranging from 89.4 to 92.9%). Note: The top panel depicts the total concentration of contaminants per origin (Others = contaminants on unspecific origins), whereas the chart panels depict concentrations of individual contaminants within the classification. Maize d1 was native grain in the control group (freshly opened), Maize d42 was native grain stored in a closed bag for 42 d, Oat d42 and Barley d42 were native grain stored in an opened bag for 42 days, and Flaked barley d42 was processed barley stored in an opened bag for 42 days. Pesticide residues (fluopicolide, fluopyram, fluxapyroxad, mandipropamid) with very low concentrations (<2 μg/kg) were detected in the Flaked barley d42 sample but are not included in the figure for scaling and visualizing purposes.

showed the highest concentrations of FB_1 and deoxynivalenol while the oat sample was the only significant source of T2 and HT2 toxins (0.13 mg/kg). Nevertheless, the concentration levels of deoxynivalenol in our maize samples were well below the EU regulation guidance value of 8 mg/kg for cereal products and 12 mg/kg for maize by-products (2006/576/EC).

None of the samples were positive for ergot alkaloids, which may suggest no sign of contamination of ergot sclerotia caused by an infection by *Claviceps* species. A Slovenian study (Babĭc et al., 2020) screened ergot alkaloids in cereals (wheat, rye, triticale, oat, spelt and barley) intended for animal feeding. They reported that their rye and oat were frequently contaminated with ergot alkaloids. Cross comparison of their findings with other EU countries as well as Canada and the USA confirmed the susceptibility of rye but it was varied for other cereals (Babĭc et al., 2020). Therefore, the prevalence of ergot alkaloid contaminations cannot be firmly associated with the grain sort besides rye. Occasionally, studies have reported ergot alkaloid concentrations exceeding 1000 μg/kg in various types of feed cereals (Liesener et al., 2010; Babĭc et al., 2020). So far, the feed regulation of the EU only limits the contamination of ergot sclerotia at 1000 mg/kg of unground cereals (Directive, 2002/32/EC), while for foods there are regulations for ergot alkaloid concentrations (20–500 μg/kg depending on cereal products) (EC No, 1881/2006). The unpredictable scenario of ergot alkaloid contamination in feed cereal signals the need for surveillance of these toxins in feedstuffs intended for animal feeding.

Using liquid chromatography-electrospray ionisation tandem mass spectrometry, we were able to reveal the common contamination of emerging *Fusarium* mycotoxins, such as enniatins, cumolin and siccanol. The emerging mycotoxins were not only present in all samples but also often shared the majority (>40%) of the pool of *Fusarium* metabolites detected. Furthermore, we detected random contamination with considerable levels (0.5–0.6 mg/kg) of *Alternaria* metabolites – the levels being higher than some other reports (Drakopoulos et al., 2021). The complexity of contaminants in feedstuffs consistently reported here and elsewhere (e.g., Drakopoulos et al., 2021; Santos Pereira et al., 2019) has pointed out the necessity to study the toxicity due to the interaction of fungal metabolites in animals. Indeed, mycotoxin research in horses has begun to emerge. For instance, mixtures of mycotoxins and metabolites from *Fusarium, Aspergillus* and *Penicillium* in forages, including emerging and modified mycotoxins from *Fusarium,* were associated with liver diseases in horses (Durham, 2022).

Another remark of the current work is that barley seems to prone to

contamination with pesticide residues. Accordingly, we detected pesticide residues, though at low levels, strictly in the barley products. These residues were mostly fungicides, one insecticide synergist (piperonylbutoxide), and one insect repellent (picaridin). However, we had a small number of samples and the data did not represent a survey. Thus, the detection of such contaminants could be a coincidence. Still, there is other evidence regarding pesticide contamination of barley, which could be due to the use of fungicides to control ramularia leaf spot and *Fusarium* head blight in barley production (Palladino et al., 2021). Moreover, other works have indicated the relevance of barley-derived products like brewery's spent grains as an important source of pesticide residues in dairy cow diets (Penagos Tabares et al., 2022, 2023).

The present study has the strength mentioned above, but also some limitations, in particular the limited replicates both in terms of batches of feeds and storage conditions. The test storage condition, though represents an actual farm condition, was relatively stable at the time of investigation. Moreover, temperate climate zones, such as our storage condition, are generally cooler and dryer than tropical conditions. In addition, there are endemic pest species of different geographical-climatic regions despite a number of introduced species being recognized (Ezcurra et al., 1978). Therefore, our findings may not be applicable to different climate conditions and further studies would be required. Furthermore, the method and analysis done in this study cannot reflect the role of endophytes in the hygienic quality of the samples. There is evidence that endophytic *Fusarium* species also produce mycotoxins in potato (Alijani Mamaghani et al., 2024). Storage conditions that promote proliferation of not only spoilage species but also endophytic species may contribute to higher contaminations of mycotoxins.

5. Conclusions

The present work emphasizes the importance of microscopic evaluation to assess the hygienic status of horse cereal grains and their products. Proper indoor storage conditions, i.e., low temperature (<25 °C) and low relative humidity (<75%) are essential for maintaining the quality of cereal grains and preventing the grains from deterioration even when cereal bags remain open for 42 days. The work further indicates specific impurities and damages as well as mycotoxins and pesticide residues associated with certain grain sorts. This implies that feeding a single-grain source can lead to a high intake of certain impurities and contaminants. The experimental assessment confirms the presence of various contaminants in commercial cereal products but the contamination levels, including after 42 d storage, were considered safe for feeding horses when compared to the legal limits and literature. The presence of living pests in the samples suggest that living biotic components could still prevail during storage when purchased horse cereals are stored under more severe and prolonged substandard storage conditions in the barn and stable. Thus, safe levels of contaminants may not be guaranteed under these circumstances. This marks a necessity for horse owners to examine the purchased products as well as the storage condition of their farm.

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CRediT authorship contribution statement

Ratchaneewan Khiaosa-ard: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Formal analysis. **Sophie Czermak:** Writing – review & editing, Investigation. **Manfred Hollmann:** Writing – review & editing, Investigation. **Felipe Penagos-Tabares:** Writing – review & editing, Investigation. **Michael Sulyok:** Writing – review & editing, Investigation. **Rudolf Krska:** Writing – review & editing. **Qendrim Zebeli:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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