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Assessment of the risk of mycotoxins and other related contaminants in dairy cattle diets in Austria with relevance for cow health and fertility as well as food safety

INAUGURAL-DISSERTATION

submitted for the fulfilment of the requirements for the degree of

DOCTOR MEDICINAE VETERINARIAE

of the University of Veterinary Medicine, Vienna

Submitted by

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Vienna, November 2022.

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I dedicate this dissertation to my family and friends,
*especially to my parents (**Julio** and **Maria**) as well as to my children (**Patrick** and **Sophie**)*

ACKNOWLEDGEMENTS

Initially, I want to express my gratitude to ***Prof. Dr. Qendrim Zebeli*** for the fantastic opportunity to accomplish my doctoral studies under his supervision. I thank him for his appropriate personal and professional guidance and mentorship during the fieldwork, lab work, and writing.

Especially, I want to thank the laboratory staff (***Anita, Sabine***, and ***Manfred***) for consistently providing excellent assistance and cooperation. I am also very thankful to the colleagues and cooperation partners of ***BIOMIN*** (***Johannes Faas***, ***Juan-Ignacio Artavia*** **and** ***Dr. Veronika Nagl***) and ***BOKU*** (***Dr. Michael Sulyok***, ***Prof. Dr. Krska*** and their team) for the excellent scientific cooperation as well as to ***Dr. Ratchaneewan Khiaosar-Ard*** for her assistance and valuable life experiences during the project.

Additionally, I want to thank my colleagues at our institute for the great moments. Mainly I want to thank ***Christoph, Mubarik, Arife, Marsel, Esther***, ***Catia, Anna, Julia, Ezequias, Raul, Sara, Parisa, Gregor, Annegret, Thomas, Frederike*** & ***Sisko*** for the excellent atmosphere, advice, and the shared moments during our scientific activities**.** I also recognize ***Marlene***, ***Eva-Maria***, and ***Johanna*** for their help during the research activities. Furthermore, I want to thank all my friends outside the institute who always supported me.

Especially, I want to thank my parents, ***Julio*** & ***Maria***, and my sister ***Laura*** for all the support during my life. ***Patrick*** and ***Sophie,*** you are my biggest motivation. A great thanks go to ***Katharina*** for her company and beautiful moments together. My immense thankfulness is also to ***Alexandra, Elfriede*** and ***Hans***.

My doctoral thesis was funded and performed within the framework of the sub-project entitled: "***Mycotoxin detection and implications for dairy performance - Screening mycotoxin contamination in feeds as causal agent of infertility and poor health in dairy cattle***", which is part of the project "***D4Dairy – Digitalization, Data Integration, Detection and Decision support in Dairying***". It was supported by BMK, BMWFJ, the federal state of Lower Austria, the city of Vienna and FFG.

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1. EXTENDED INTRODUCTION

1.1. Risk of mycotoxin contamination in dairy farming

The dairy industry is an essential economic sector that plays a major role in producing high nutritional value foods from fibrous matter, representing Austria's most important agricultural sector (Ledinek et al., 2018; BMLFUW, 2021). Feedstuffs are susceptible to mould infection/colonization with subsequent mycotoxin contamination during the complete feed-production chain (pre- and postharvest), affecting the rest of the productive chain, including animal health and performance as well as the quality/safety of the derived foods (FAO, 2014). A wide range of fungal (toxic and potentially toxic) metabolites are produced primarily by *Fusarium*, *Alternaria*, *Aspergillus* and *Penicillium* species (Jouany et al., 2009; Battilani et al., 2020). Additionally, other fungal genera such as *Cladosporidium*, *Phoma*, *Diplodia*, *Epichloë, Neotyphodium* (formerly *Acremonium*), *Pythomyces*, *Myrothecium*, *Stachybotrys*, *Mucor*, *Monascus*, *Eupenillium*, *Paecilomyces*, *Rhizopus*, *Trichoderma* and *Byssochlamys* have also been reported as toxigenic (Magan and Olsen, 2004; Jennessen et al., 2005; Storm et al., 2008; Bryden, 2012; Di Menna et al., 2012; Gallo et al., 2015b;). The characterization of the implicated mycotoxin mixtures requires to be assessed with an innovative and holistic approach based on multi-metabolite analyses for an optimal risk assessment (Battilani et al., 2020). Along with fungal toxins metabolites, other compounds like phytoestrogens and residues of pesticides and veterinary drugs can occur in whole diets of dairy cattle. They can also hazard feed and food safety (Kumar et al., 2018; Mostrom and Evans, 2018; Ortelli et al., 2018). While completing the current thesis project, some of these compounds (phytoestrogens, pesticide and veterinary drug residues) were also detected and reported. Some of the included publications mention these compounds; however, this thesis will be focused on mycotoxins and findings related to other kinds of feed contaminants and substances will be shortly discussed.

Mycotoxins and their mixtures can harm the herds' health, reproduction, and production. However, large-scale studies that characterized profiles of the most common fungal toxins and endocrine disruptors that naturally contaminated whole diets of dairy cattle are highly required to determine the impacts of these compounds (Fink-Gremmels, 2008b; Gallo et al., 2015b; Gallo et al., 2022). Dairy cattle diets vary widely through diverse production systems in different regions of the world, incorporating a wide range of components, including forages,

cereal grain, protein feeds and by-products of agro-industrial activities (FAO, 2014). The physiological nature of ruminants makes forages (including pastures and conserved forages) the most adequate and important feed source for dairy cattle (Webster, 2020). Like other crops/feedstuffs, forages are highly susceptible to mycotoxin contamination (Gallo et al., 2015b; Santos Pereira et al., 2019). However, research has been focused mainly on cereal grains (Gallo et al., 2015b). The production of these toxins can consequently be independent of the growth of the fungi, which is related to the primary metabolism (Jouany et al., 2009). These compounds may produce various unspecific disorders (called mycotoxicoses) through a natural route of exposure (commonly via ingestion of contaminated feed) (Bryden, 2012). Thus, the effects of mycotoxins on human and animal health have relevant public health and economic implications (Wild and Gong, 2010).

Mycotoxins have been a problem for humans since ancient times and are historically described. For instance, ergotism, caused by toxic metabolites derived from *Claviceps purpurea*, became an epidemic in the Middle Ages, the oldest identified type of human mycotoxicosis (Van Dongen and de Groot, 1995). However, the beginning of modern mycotoxicology started in the 1960s with the discovery of aflatoxins (AFs) after more than 100,000 young turkeys, ducklings, and other poultry animals in the UK deceased during a few months from unidentified diseases with high mortality, which was named "turkey x disease". A cautious assessment of the affected farms indicated that the disease was linked to the diet, specifically with peanut meal imported from Brazil. A clinical syndrome with the typical symptoms of turkey x disease was reproduced when animals were fed the same peanut meal. Rigorous investigations were then performed on the suspected ingredient to identify the nature of the toxin, which was soon found to be of fungal origin. The toxin-producing fungus was identified as *Aspergillus flavus* (Nesbitt et al., 1962). Subsequently, in the last decades, numerous mycotoxins have been discovered. So far, over 400 fungal toxins have been identified (Cinar and Onbaşı, 2019). However, the total number of mycotoxins that exist is not yet known, but there are probably thousands (Jouany et al., 2009; Klitgaard et al., 2014). The toxic potential of many of these fungal metabolites is unknown (van den Brand and Bulder, 2020). However, most studies have focused on AFs, ochratoxin A (OTA), fumonisins (FUMs), zearalenone (ZEN), trichothecenes (TCTs), like deoxynivalenol (DON), T-2 toxin and HT-2 toxin (Cinar and Onbaşı, 2019).

According to Mostrom and Jacobsen, 2020, the first documented reports of mycotoxicosis (stachybotryotoxicosis) in ruminants and horses date back to the 1930s and 1940s in Eastern Europe (Mostrom and Jacobsen, 2020). Posteriorly, during the 1950s and 1960s, additional reports of mycotoxicoses in cattle were described in the URSS and USA (Sarkisov, 1954; Stepanyuk et al., 1959; Crump et al., 1963). By then, the implicated toxins were not defined or characterized, but the causal relationship between mouldy feeds and toxic syndromes were corroborated. Initially, notable improvements in the clinical status of the affected animals were observed after contaminated feeds were not included in the diets (Stepanyuk et al., 1959). Additionally, the verification of causality was performed experimentally with bovines and rabbits, rats and Guinea pigs, observing similar clinical signs (Albright et al., 1964; Crump et al., 1963). The affected animals of here cited natural mycotoxicoses outbreaks presented diverse manifestations from an abrupt reduction of milk yield, profuse salivation, watery diarrhoea, salivation, and polyuria to sudden death (Albright et al., 1964; Crump et al., 1963; Izmailov et al., 1963; Izmailov and Moroshkin, 1962; Sarkisov, 1954; Stepanyuk et al., 1959), which evidenced the ambiguous and unspecific nature of the fungal toxicosis. The toxic effects of mycotoxins include cytotoxic, carcinogenic, immune-suppressive, nephrotoxic, hepatotoxic neurotoxic, mutagenic, estrogenic effects, among others (Kumar et al., 2020). Additionally, some mycotoxins, such as AFs can be carried over via milk is possible, which makes the mycotoxin issue in dairy animals a public health and economic concern (Fink-Gremmels, 2008a; Flores-Flores et al., 2015; Guerre et al., 2000).

Mycotoxicoses are diagnostic challenges due to their nonspecific signs. The ambiguous clinical/sub-clinical manifestations and lesions are due to many factors: (1) the co-occurrence of several mycotoxins, other toxicants or/and deficiency states (such as negative energy balance, heat stress and metabolic disturbances); (2) masking the toxic effects by secondary effects, e.g. infectious disease due to immunosuppression; (3) belated appearance of signs/lesions due to chronicity; (4) inter and intraspecies variations in response to the mycotoxin(s); (5) low awareness of the mycotoxins as a relevant causative factor for disease; (6) the non-homogeneous distribution of these compounds on feed charges and limited availability of commercial biomarkers for diagnostic. Some of the generic signs associated with substantial mycotoxin consumption are feed intake reduction, a decrease in nutrient absorption, presentation of metabolic disorders, endocrine alterations and a decline in reproductive as well

as productive performance (Fink-Gremmels, 2008b; Nešić et al., 2011; Richard and Thurston, 2012; Simion, 2018). An early and accurate diagnosis would permit veterinarians to recognise mycotoxicoses from other diseases and (ideally) determine the causal mycotoxin, contributing to minimising economic losses and preventing human exposure to mycotoxin residue levels in derived edible tissue or milk (Richard and Thurston, 2012; Fink-Gremmels and van der Merwe, 2019). Supported on diagnosis, it would be possible to determine how to deal whit the affected livestock (treating, not-treating, or euthanizing). If this were treated, an assertive diagnosis would help to define an appropriate clinical therapy that should be implemented. Additionally, the diagnosis is essential to deal with the contaminated feeds fed to the affected animals (removing contaminated feedstuffs of the animal feed chain, diluting with suitable feedstuffs, or treating whit biological, physical, and chemical methods, enabling to check the production (agricultural practices), transport, storage, and processing conditions of the same kind of feeds (Richard and Thurston, 2012; Gonçalves et al., 2015) (schematized in Figure 1).

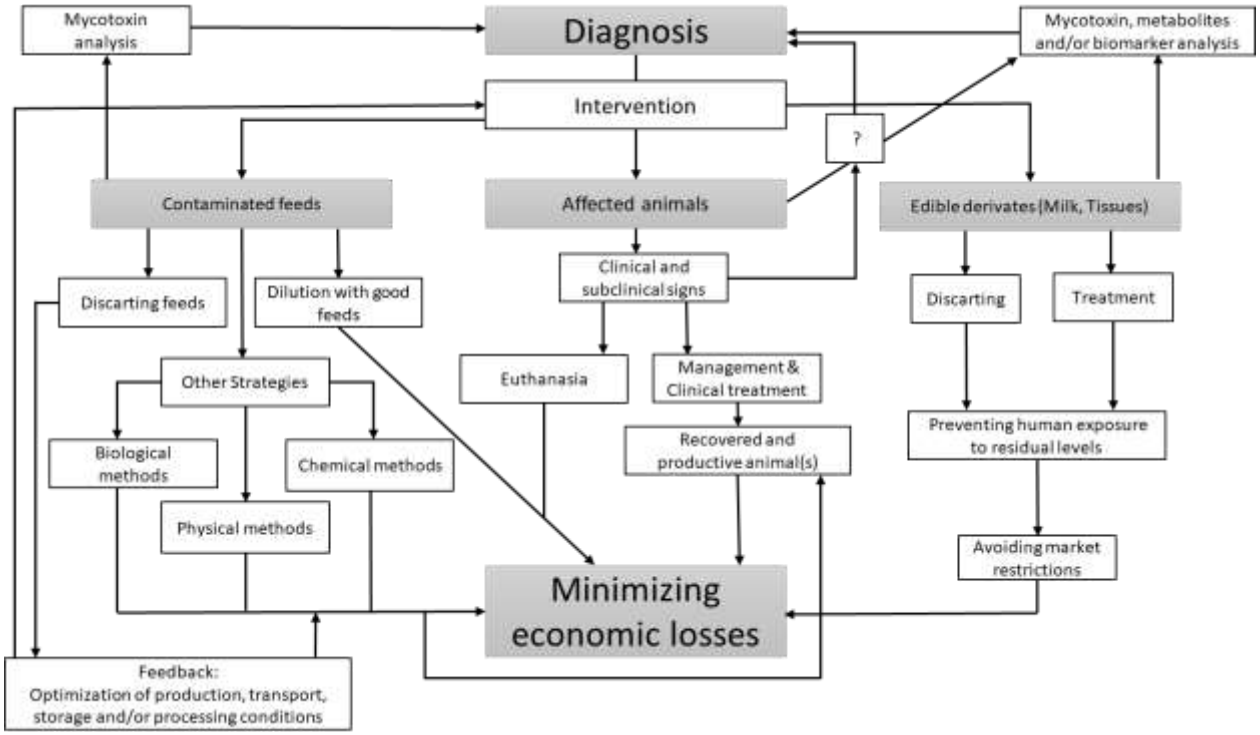

Figure 1 Scheme illustrating the mycotoxicoses as a diagnostic challenge. Accurate diagnosis is a basis for an adequate intervention. Diagnosis is based mainly on feed analysis and clinical signs (Modified from Richard and Thurston, 2012).

Compared to monogastric; adult ruminants (cattle, goats and sheep) are more resistant to mycotoxins as the ruminal microbiota (bacteria, protozoa and fungi) can partially degrade and inactivate some of these compounds, e.g., AFs and OTA (Engel and Hagemeister, 1978; Kurmanov, 1977; Kiessling et al., 1984; Westlake et al., 1989; Mobashar et al., 2010; Özpinar et al., 2002). Consequently, it was widely assumed that the resistance of ruminants to dietary mycotoxins as a fact and the negative consequences of the metabolites have been neglected and underestimated by dairy farmers worldwide (Rodrigues, 2014). However, other toxic fungal metabolites such as ergot alkaloids (EAs) and FUMs remain relatively stable in the rumen (Caloni et al., 2002; Fink-Gremmels, 2008b; Schumann et al., 2009). Moreover, other mycotoxins, such as the cyclic lactone patulin (PA), cannot only pass rumen unchanged; it also impairs the ruminal fermentative function by potent anti-bacterial and anti-protozoal properties (Escoula, 1992; Tapia et al., 2005). The relative resistance of ruminants could be suggested for the rare presentation of acute forms of mycotoxicoses. However, they are susceptible if unhealthy diets are fed over long periods (Gupta, 2019b). The metabolic and dietary particularities of high-producing dairy cows (e.g., ration with high energy density) seem to reduce the rumen's detoxifying ability, thereby increasing the risk of subclinical and clinical health disorders, impairing fertility, and affecting productivity (Fink-Gremmels, 2008b; Rodrigues, 2014).

Generally, dairy cattle are less tolerant than beef cattle and sheep to mycotoxins like the TCT DON. The higher grade of susceptibility could be related to the higher metabolic stress of high-producing dairy cows, which implies high dry matter intake, faster ruminal turnover, and reduced rumen microbial degradation time (Jouany and Diaz, 2005; Mostrom and Jacobsen, 2020). Figure 2 illustrates and explains the elevated risks of mycotoxicoses in high-yielding dairy cattle. On the one hand, under normal conditions, a well-functioning rumen maintains physiologically normal pH, metabolic activity, and passage rate, which provides the expected degradation of (myco)toxins by the rumen microbiota. On the other hand, feeding diets with high energy density impairs the microbiome (dysbiosis) and induces related rumen health disorders such as SARA (Sub-acute rumen acidosis). The rumen dysfunctionality impairs its detoxifying capacity and increases the passage rate out of the rumen and subsequent absorption of unmodified mycotoxins and other toxic compounds such as lipopolysaccharides (LPS). Thus, a higher amount of toxins reaches the systemic circulation, leading to inflammation, lowered

immunocompetence and liver damage, increasing the risk of mycotoxicosis in high-yielding cattle (Figure 2) (Fink-Gremmels, 2008b; Upadhaya et al., 2010; Debevere et al., 2020; Loh et al., 2020).

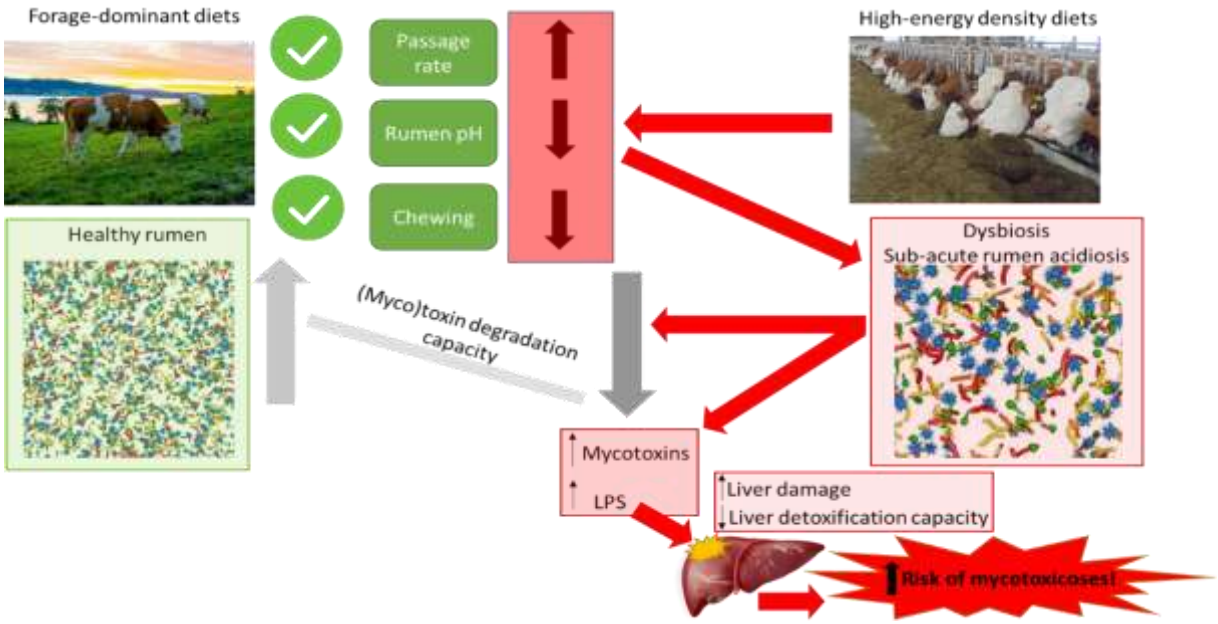

Figure 2 Summarized illustration explaining the higher risk of mycotoxicoses in high-yield dairy cows fed with high energy-dense diets (Based on personal communication, Prof. Dr. Qendrim Zebeli).

The state of the art of occurrence and adverse health effects of mycotoxins in dairy cattle feeds (especially in forages) have been focused on a few of these toxins and are still limited (Gallo et al., 2015b; Battilani et al., 2020). Their negative impacts vary considerably from acute to chronic syndromes, but acute mycotoxicoses are not usual in cattle. The chronic presentation forms seem to be the most recurrent, implying hidden disorders with reduced ingestion, productivity, and fertility (Fink-Gremmels, 2008a; Storm et al., 2008; Rodrigues, 2014). This depends on the kind and levels of mycotoxins exposed to, the time of exposure and animal particularities (species, age, immune status, gender) (Gashaw, 2016). Some of these toxic metabolites in high concentrations can cause acute toxicity with the evident sign of disease and causing even death. However, prolonged exposure to low toxin mixtures and intermittent rates is more likely to occur in standard diets (Gallo et al., 2015b). This constant exposure may lead to chronic mycotoxicosis (Fink-Gremmels, 2008b; Mostrom and Jacobsen, 2020).

Mycotoxins have diverse chemical structures and modes of action, making the classification extremely complex based on these characteristics (Fujimoto, 2011). In this doctoral thesis, mycotoxins are categorized into two major groups based on (CAST, 2003): Major classes and minor classes of mycotoxins.

1.2. Major classes of mycotoxins

This category of mycotoxins includes AFs, TCTs (DON, T-2 toxin and HT-2 toxins), FUMs, ZEN, OTA, and ergot alkaloids, which are the most studied kind of fungal toxins and include (at least partially) in the current European legislation (i.e., Directive and recommendations) (EC, 2002, 2006, 2013, 2016, 2021). The mycotoxins here included posing the most significant potential risk to human and animal health as food and feed contaminants (CAST, 2003). Tab. 1 summarizes the main associated adverse effects and proposed molecular mechanisms of action (taken from "Mycotoxins and nuclear receptors: A still underexplored issue" by Dall'Asta 2016). Tab. 2 European limit and guidance levels of major mycotoxins in diets and/or feedstuffs intended for dairy cow nutrition

Tab 1 Major mycotoxins, main associated adverse effects and proposed molecular mechanisms of action (taken from Dall'Asta 2016).

Mycotoxins	Effects	Cellular and molecular mechanisms of action
Aflatoxin B1 and M1	Hepatotoxicity Genotoxicity Carcinogenicity Immunomodulation	Formation of DNA adducts Lipid peroxidation Bioactivation by cytochromes P450 Conjugation to GS-transferases
Fumonisins	Central nervous system damage Hepatotoxicity Genotoxicity Immunomodulation	Inhibition of ceramide synthesis Adverse effect on the sphinganin/sphingosin ratio Adverse effects on the cell cycle.
Ochratoxin A	Nephrotoxicity Genotoxicity Immunomodulation	Effect on protein synthesis. Inhibition of ATP production Detoxification by peptidases
Trichothecenes (i.e.DON, T-2, HT-2)	Hematotoxicity Immunomodulation Skin toxicity	Induction of apoptosis in haemopoietic progenitor cells and immune cells. Effect on protein synthesis Abnormal changes to immunoglobulins
Zearalenone	Reproductive adverse effects	Binding to oestrogen receptors Bioactivation by reductases Conjugation to glucuronyltransferases

Tab 2 European maximum and guidance levels of major mycotoxins in diets and/or feedstuffs intended for dairy cow nutrition.

Mycotoxin	Maximum levels[a] / Guidance value[b,c,d] (µg/kg)[e]
Aflatoxin B1[a]	5
Deoxynivalenol[b]	5,000
Rye Ergot (*Claviceps purpurea*)[a]	1,000,000
Ergot alkaloids[c]	Recommendation for monitoring
Fumonisins B1 and B2[b]	50,000
Ochratoxin A[b]	250
Zearalenone[b]	500
T-2 + HT-2 toxins[d]	250

[a] European Commission - Directive 2002/32/EC
[b] European Commission Recommendation 2006/576/EC
[c] European Commission Recommendation - 2012/154/EU
[d] European Commission – Recommendation – 2013/165/EU
[e] Maximum levels in µg /kg (ppb) relative to a feedstuff with a moisture content of 12 %

1.2.1. Aflatoxins

These toxins are relevant contaminants of foods and feeds, produced primarily in warm, subtropical, and tropical climates, but can be found worldwide. *Aspergillus* spp. (like *A. flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii* and *A. niger*) are major producers (Gupta, 2019b; Yoko et al., 2001; Kurtzman et al., 1987). There are several kinds of AFs and respective methoxy, ethoxy and aceto-derivates (Bilgrami and Choudhary, 1998). The four major types are AFB_1, AFB_2, AFG_1, and AFG_2. Such designations are based on the fluorescence under ultraviolet light (B = blue and G = green), while the subscript is related to chromatographic mobility. Two additional metabolites, AFM_1 and AFM_2, are 4-hydroxylated metabolites of AFB_1 and AFB_2, were firstly isolated from the milk of lactating animals fed with diets contaminated with AFs (Mostrom, 2016). AFs are low-molecular-weight, lipophilic compounds passively absorbed from the gastrointestinal tract. Absorption of these toxins may take place in the mucosa of the oral cavity and/or oesophagus before entering the rumen, appearing rapidly (within 5 minutes) metabolized (as AFM_1) in the milk and clearing within three to four days after dosing cows (Frobish et al., 1986; EFSA, 2013; Mostrom and Jacobsen, 2020). Toxic effects of AFs include mutagenesis due to the alkylation of nuclear DNA, leading to cell death or its malign transformation (carcinogenesis), teratogenesis, reduced protein synthesis, and immunosuppression (CAST, 2003; Riley, 1998). Thus, reduced protein synthesis

results in impaired production of essential metabolic enzymes and structural proteins for growth (Gupta, 2019b). These compounds affect cell-mediated immunity, cytokine production, and nonspecific humoral factors, such as complement, interferon, and some bactericidal serum components, which can induce vaccine failure or poor antibiotic response (Mostrom and Jacobsen, 2020). AFs are also recognized as potent hepatotoxins, immunosuppressants, carcinogens (hepatocarcinoma) and mutagens in animals, but also in humans, representing a relevant public health concern (Gil-Serna, 2014; Gong et al., 2016).

Aflatoxicosis includes clinical signs such as poor weight gains, reduced feed conversion and milk production, inappetence, lethargy, ataxia, liver disease with elevated hepatic enzymes and bilirubin and prolonged clotting times (Diekman and Green, 1992). In a study carried out by Jones and Ewart (1979), cows fed with diets containing AFB1 at concentrations of 20 µg/kg presented a depletion in the feed intake and milk yield. Aspects started to improve three days after the AFB1-source was removed (Jones and Ewart, 1979). Similarly, another field study, which evaluated the effect of AF-contaminated corn on lactating dairy cattle, observed that with a decline in reproductive efficiency. After the inclusion of an AF-free diet, an increment of 25% of the milk yield was evidenced (Guthrie and Bedell, 1979) cited by (Jouany and Diaz, 2005). Several case reports of acute aflatoxicosis have been described. For example, a group of crossbred feeder steers fed with corn contaminated with 1,500 ng of AFs/g developed typical aflatoxicosis lesions, and residues of the mycotoxin were detected in kidney tissue (Colvin et al., 1984). In the same way, a small herd of cattle having access to mouldy and unharvested sweet corn was revealed via postmortem examinations, oedema of soft tissues and liver lesions consistent with aflatoxicosis. Weather conditions were favourable for the proliferation of *A. flavus* and *A. parasiticus* and the contamination levels of the corn samples taken from the field contained 2,365 ng of AFs/g. (Hall et al., 1989). Different studies revelated that feeding diets with AFB1 levels of 75 µg /kg DM to dairy cows can negatively impact animal performance. Queiroz et al., 2012 demonstrated that such dietary levels of AFB1 induced lower milk fat yield and milk protein concentration (Queiroz et al., 2012). Likewise, Ogunade et al. (2016) evidenced a reduced milk yield by 2.5 kg and a lowered 3.5% fat-corrected milk yield by 1.7 kg (Ogunade et al., 2016). However, some studies reported that AFs did not impact dairy cow productivity. For instance, dietary levels of 100 µg of AFB1/kg (Sulzberger et al., 2017) and a mixture of B1, B2, G1 and G2 at a concentration of 105 µg/kg (Rodrigues et al., 2019) did not

impact milk performance, intake, or efficiency. Aflatoxicosis diagnosis is based on typical clinical signs, lesions, and toxic (not trace) concentrations in the ration (Mostrom and Jacobsen, 2020).

AFB_1 is considered the most potent naturally occurring carcinogen, has been related to hepatocellular carcinoma in humans and has been classified in group 1 as a human carcinogen by the International Agency for Research on Cancer (IARC). Aflatoxin M1 is less toxic and classified by IARC as a human carcinogen in group 2B (IARC, 1993). Adult cattle, sheep, and goats are relatively resistant to the acute forms of aflatoxicosis but are more vulnerable if contaminated diets are fed over long periods (Gupta, 2019b). Chronic aflatoxicosis in cattle is associated with clinical signs of reduced appetite, feed efficiency, milk production, and icterus (Newberne, 1973). Hepatic enzymes are typically elevated, and prothrombin time can be prolonged. As with the other mycotoxicoses, aflatoxicosis decreases performance, the cause of which is multifactorial, involving nutritional interactions, anorexia, altered hepatic protein and lipid metabolism, and disruptions of hormonal metabolism (Raisbeck et al., 1991; Gil-Serna, 2014). AFB1 is the most strongly regulated and only mycotoxin with a maximum limit in feeds for dairy cows in the EU (5 µg/kg at a moisture content of 12 %) (Tab. 2) (EC, 2002).

1.2.2. Trichothecenes

TCTs are a group of sesquiterpene mycotoxins, produced mainly by *Fusarium* spp., but also by several genera of fungi, including *Stachybotrys*, *Myrothecium*, *Trichothecium*, *Trichoderma*, *Cephalosporium*, *Cylindrocarpon*, *Verticimonosporium*, and *Phomopsis* (Scott, 2017). Their production is increased under wet and cool conditions. TCTs are commonly found in cereals grains worldwide but also contaminate vegetative sections of the plant and can also be detected in high concentrations in forages (e.g., hay and straw) (Mostrom et al., 2005). Over 180 metabolites are considered as TCTs. These mycotoxins can be chemically classified into four types based on substitutions at five positions of the TCT skeleton, including Type A (with some of the most toxic TCTs like T-2 toxin, its deacetylated metabolite HT-2 toxin and diacetoxyscirpenol (DAS)); Type B (like nivalenol (NIV), fusarenon X (4-acetylnivalenol), DON and its derivatives); Type C (such as crotocin); and Type D (macrocyclics, such as satratoxin, roridin and verrucarin). The more common and problematic TCTs encountered in veterinary medicine are T-2 toxin and DON. However, all TCT should be considered toxic until

proven otherwise (Cope, 2018). These mycotoxins inhibit protein synthesis by binding to the peptidyl transferase (Feinberg and McLaughlin, 2017). Moreover, these compounds can induce apoptosis in the thymus, spleen and Peyer's patches (Poapolathep et al., 2002).

These mycotoxins are known for inducing the inhibition of DNA and RNA synthesis, which can be a secondary effect of the inhibition of protein synthesis or the apoptotic effect (Cope, 2018). The TCTs with the most potent immunosuppressive as well as protein synthesis inhibitors are T-2 toxin, DAS, DON, and fusarenon X (Corrier, 1991). Some studies of the metabolism of DON in adult cattle suggest that this toxin is transformed rapidly into metabolites with lower toxicity in the rumen before absorption. For example, the De-epoxidation of DON to de-epoxy DON (a much less toxic compound) is considered a ruminal deactivation step (Valenta et al., 2003). Rumen microbes (particularly bacterial and protozoal fractions) seem to be active in the deacetylation of the trichothecenes (Kiessling et al., 1984; Westlake et al., 1989; Guerre, 2020). Furthermore, no effect has been found of DON-contaminated diets on milk yield, feed intake or other parameters measured at levels used in the previous studies (Anderson et al., 1996; Charmley et al., 1993; Ingalls, 1996; Trenholm et al., 1985). The available data concerning the impact of feeds contaminated with trichothecenes in ruminant feed is still limited to allow a scientifically based risk assessment. For example, several trichothecenes like DAS, HT-2 and T-2 toxin, along with other mycotoxins like ZEN and FUMs, may co-occur, causing similar and intensified adverse effects (Battilani et al., 2020; Whitlow and Hagler, 2005). It has been suggested that TCTs are not likely to cause any harm to ruminants, and no guideline value is probably needed (Eriksen and Pettersson, 2004). However, the toxicokinetic could change in ruminants with acidosis or in young animals such as calves, for which the ruminal system is not fully functioning (EFSA, 2017b). For instance, nonruminating calves presented liver failure and higher bioavailability of DON (50.7%) compared to ruminating calves (4.1%). Both groups were fed with DON-contaminated concentrate (1.13 mg/kg) (Valgaeren et al., 2019). Concerning the type, A TCT, T-2 toxin, beef calves orally dosed with T-2 toxin at 0.3 mg/kg BW (approximately 10 mg T-2/kg diet) for six weeks presented a reduction in the feed intake. With higher contamination levels of T-2 toxins (0.6 mg/kg – approx. 20 mg T-2/kg diet), the calves developed marked anorexia, weight loss, rough hair coats, and intermittent diarrhea (Osweiler et al., 1981) reviewed by (Mostrom and Jacobsen, 2020). Additionally, mixed-breed beef calves, orally dosed with T-2 toxin at a level of 0.5

mg/kg BW, presented reduced serum concentrations of total protein, albumin, and globulin compared with the non-treated calves (control group) (Mann et al., 1983). Regarding residuality in milk and other animal-derived foods, is it stated that TCTs do not accumulate significatively in animal tissues due to the rapid excretion, and only traces can be found in animal-derived food products (Eriksen and Pettersson, 2004; Fink-Gremmels and van der Merwe, 2019). The guidance levels recommended by the European Commission for DON are 5,000 µg/kg (EC, 2006) and for the sum of T-2 toxin and HT-2 toxin 250 µg/kg, at a moisture content of 12 % (Tab. 2) (EC, 2013).

1.2.3. Ergot alkaloids

As mentioned previously, ergot alkaloids have a long history of affecting man and animals. (CAST, 2003; Matossian, 1989). These group of toxins consist of a large group of nitrogen-containing fungal compounds, which are classified into four major groups based on their chemical structures: (1) the clavines, (2) the lysergic acids, (3) the lysergic acid amides, and (4) the ergopeptines (Rehácek and Sajdl, 1990). Selected members of these groups of compounds (mostly of the ergopeptine class such as ergotamine, ergocristine, ergosine, ergocornine, ergocryptine, and ergovaline) are responsible for the majority of nervous or gangrenous syndromes in humans and animals, which consume grains, grain products or grasses contaminated with the sclerotia of the fungus (Gupta et al., 2018a). These toxins are mainly produced by several fungi in two different families - the Clavicipitaceae and the Trichocomaceae, being *Claviceps purpurea* and *Epichloë* spp. among the most relevant producers. The mentioned species parasitize a broad spectrum of monocotyledonous plants of different taxonomical families like *Poaceae*, which includes forage grasses and cereals (Schiff, 2006; Guerre, 2015; Robinson and Panaccione, 2015; Gupta, 2019a). According to the scientific opinion of EFSA, ergotism in ruminants is usually a chronic disease resulting from the continued ingestion of minor quantities of the fungus on grass (EFSA, 2012). The incidence of ergotism in Europe is undetermined, but in the United States, it is a severe problem in those areas where fescue grasses are the primary forage (Strickland et al., 2011). Ergovaline has been reported as the causal agent of severe intoxications in dairy farms when livestock consume pasture grasses with infected seed heads (Botha et al., 2004; Mostrom, 2016; Marczuk et al., 2019). Under pasture feeding conditions, frequent grazing or topping of grasslands susceptible

to ergot infestation during the summer months reduces flower-head production and helps control the disease (Gupta, 2019b).

The first clinical signs are usually diarrhoea, inappetence, lameness (hind limbs are affected before forelimbs), rigidity of the lower joints of the legs, and coldness and insensibility of the extremities. Posteriorly, vasoconstriction leads to necrosis of the extremities, ears, and tail due to thrombosis. A cold environmental temperature predisposes the extremities to gangrene. Other signs include hyperthermia, dyspnoea, agalactia and neurologic signs (Canty et al., 2014; Klotz, 2015; Gupta et al., 2018a; Gupta, 2019b; Malekinejad and Fink-Gremmels, 2020). Ergot alkaloids have also been linked with heat intolerance, similar to the "summer syndrome" induced by fescue toxicosis and may interfere with embryonic development. Treatment is constrained by economic limitations in the most severely affected animals, which must be euthanized (Gupta, 2019b). The rumen and small intestine are most likely the main sites of ergot alkaloid absorption (Strickland et al., 2011). The hyperthermia, uterine stimulation and vasoconstrictive effects induced by these compounds are explained by their chemical structures, which are like the biogenic amines norepinephrine, serotonin, and dopamine. These alkaloids can cause partial agonism or antagonism at adrenergic, dopaminergic, and serotonergic receptors (Mostrom, 2016). Some of these alkaloids, like ergovaline are antagonists to dopamine at D_1 vasodilatory receptors (Cross et al., 1995). The ergot compound can also induce agonist activity at D_2 receptors in the lactotroph cell in the adenohypophysis, reducing prolactin secretion (Goldstein et al., 1980; Poole and Poole, 2019). These disruptive endocrine effects have also been observed in multiple species exposed experimentally to ergopeptine alkaloids (Gupta et al., 2018a), which could also explain the presence of dysgalactia or agalactia observed in cases of ergot intoxications (Copetti et al., 2002; Poole and Poole, 2019). Research has shown that grazing endophyte-infected ergot alkaloid producing tall fescue impairs the cow-calf performance by depletion of reproductive rates, milk yield and calf weaning weights (Gay et al., 1988; Porter and Thompson Jr, 1992; Wilbanks et al., 2021). However, concentrations of ergovaline are exceptionally low in endophytes (parts per billion or low parts per million), so they are rarely detected in animal tissue or fluids. Their primary routes of elimination and excretion in cattle are mostly urine (96%) and at minor grade via bile, faeces and milk (Strickland et al., 2011; Gupta et al., 2018a). The content of ergot sclerotia in animal feeds is regulated (limit: 1,000,000 µg/kg or 1gr/kg at a moisture content of 12 %) (EC, 2002) and the

monitoring of ergot alkaloids in food and feed is recommended by the European Commission (EC, 2012). There is no guidance value for ergot alkaloids in animal feeds, but since January 2022, the commission regulation (EU) 2021/1399 has established a maximum level of ergot alkaloids in certain foodstuffs (like barley) (EC, 2021).

1.2.4. Fumonisins

These mycotoxins are primarily produced by *Fusarium* spp. (such as *F. verticillioides* [formerly *F. moniliforme*] and *F. proliferatum* (Gelderblom et al., 1988; Rheeder et al., 2002; Jouany et al., 2009). However, species of other genera such as *Aspergillus niger* and *Alternaria alternata* can also produce FUMs (Chen et al., 1992; Frisvad et al., 2007; Mogensen et al., 2010; Frisvad et al., 2011). Several analogues have been characterized, including the types B_1, B_2, B_3, B_4, A_1, A_2, C_1, C_4, P_1, P_2 and P_3 (Musser and Plattner, 1997). Such compounds are chemically characterized as an aliphatic hydrocarbon with a terminal amine group and tricarboxylic acid side chains, having structural similarity to sphingosine, the major long-chain base backbone of cellular sphingolipids. FUMs are competitive inhibitors of sphinganine and sphingosine N-acyltransferase, resulting in increased sphinganine and sphingosine, which can interfere with cellular growth, differentiation, and cell communication, resulting in toxicity and carcinogenicity (Wang et al., 1991; Smith, 2018).

It has been suggested that ruminants are relatively resistant to FUMs compared to other species like horses and pigs, which are affected by well-described mycotoxicoses: equine leukoencephalomalacia and porcine pulmonary oedema (PPE) (Gupta, 2019b; Mostrom and Jacobsen, 2020). In an experimental study, dairy cows consumed a diet naturally contaminated with FUMs at 100mg/kg DM for seven days prepartum and 70 days postpartum, evidenced by a reduction in feed intake and milk production (Diaz et al., 2000). Feeding a diet contaminated with FUMs with a concentration of 148 mg/kg for 31 days induced an intake depletion, elevated liver enzymes in crossbred feeder calves (Osweiler et al., 1993). In a study in Holstein steers (86–127 kg) fed with corn mixed with culture material of *F. moniliforme* with 328 mg/kg FUM B1 in the final corn mixture, presented feed refusal, changes in serum enzymes and biochemistry were observed in the calves. After lengthy exposition (> 230 days), the calves exhibited histopathologically: hepatocytes exhibited focal nuclear pyknosis and cellular shrinkage resembling apoptosis (Baker and Rottinghaus, 1999).

Fusarium moniliforme-contaminated corn resulted in feed refusal in cattle previously, but FUM B1 concentrations were not determined (Beasley et al., 1982). Not long ago, a short-term (two days) exposure experiment in Austria was performed with cows fed a basal diet with 40% grain (DM basis) and 20 mg of FUM per day. The outcome showed that FUM increased the number of observed features and significantly impacted β-diversity structure and metagenome predicted function. At the systemic level, FUM exposure induced a hepatotoxic effect (evidenced by an increment of liver enzyme concentrations), accompanied by altered heart and respiratory rates (Hartinger et al., 2022). The International Agency for Research on Cancer (IARC) classified FUM B1 as a possible carcinogen for humans (group 2B)(IARC, 2012). Dairy cows were provided with a naturally contaminated ration with levels of 100 mg /g of FUMs during seven days pre-parturition and 70 days post-parturition, presenting a reduction in feed intake and milk production (Diaz et al., 2000). EFSA identified a no-observed-adverse-effect level (NOAEL) for cattle of (31 mg /kg of FUM B1-3 in feed) considering endpoints the increase in serum enzymes, cholesterol, and bilirubin as well as the decrease in lymphocyte blastogenesis (EFSA et al., 2018). Tissue and milk residues are not considered to be a problem (Fink-Gremmels and van der Merwe, 2019). The guidance levels for diets of dairy recommended by the European Commission are 50,000 μg/kg of accumulate FUM B1 and FUM B2 at a moisture content of 12 % (Tab. 2) (EC, 2006).

1.2.5. Ochratoxins

Ochratoxins are structurally composed of a dihydroisocoumarin linked via a peptide bond to the amino acid phenylalanine and are usually produced during storage. Several species of genera, *Aspergillus* (e.g., *A. ochraceus, A. niger*) and *Penicilium* (such as *P. verrucosum*), can synthesize these toxins (Jouany et al., 2009; Mostrom and Jacobsen, 2020). The most relevant mycotoxin in this group is OTA, which is linked to nephrotoxicity and immunosuppressive effects in animals and humans (Krogh, 1976; Rodricks et al., 1977; Gupta, 2019b). These toxins cause oxidative stress and inhibit protein synthesis and deplete humoral factors, especially immunoglobulins, and decrease natural killer cell activity. The suggested action mechanism of the genotoxicity of OTA is by induction of oxidative DNA lesions coupled with direct DNA adducts via quinone formation (Gupta, 2019b). Additionally, OTA induces apoptosis by increasing Nicotinamide Adenine Dinucleotide Phosphate (NADPH) and the P450 enzyme,

which activates the caspase signalling pathway. Additionally, this toxin also induces apoptosis via oxidative stress by increasing the intracellular levels of reactive oxygen species by disrupting the mitochondria and endoplasmic reticulum, causing calcium release with subsequent inhibition of the cell cycle, mRNA splicing, DNA replication, lipid and nucleotide metabolism (Tao et al., 2018).

Ruminants are more resistant to ochratoxin than monogastric ones because rumen microbiota can inactivate as much as 60% of the dietary OTA to the less toxic compounds ochratoxin α and phenylalanine (Hult et al., 1976). Protozoa were considered the main microorganisms implicated in the ruminal degradation of OTA (Kiessling et al., 1984). The elimination half-life in ruminants is short, about 17 hours, contrasted with 100 hours in swine. As with other mycotoxins, the adverse effects of OTA and other OTs are more likely to occur in chronic low-level intoxication. The total amount necessary to generate acute toxicity in ruminants makes such occurrences improbable. For instance, adult cattle (Holstein) given a single oral dose of OTA at 13 mg/kg BW developed anorexia, reduced milk production, diarrhoea, and incoordination, with eventual recovery. The lethal level produced by repeated feeding to goats was 3 mg/kg BW. Ochratoxin A occurred in cow's milk and urine but only under the ingestion of massive doses. Abortion or foetal death, though occurring in rodents, is not likely to be caused in cows (Ribelin et al., 1978).

However, the panorama changes if the ochratoxins co-occur with other mycotoxins like citrinin, which are similarly nephrotoxic. *Penicillium* moulds produce citrinin under similar conditions to ochratoxins (Mostrom and Jacobsen, 2020), and both nephrotoxins have synergistic effects (Braunberg et al., 1994; Schulz et al., 2018). For instance, in three herds at the Iowa State University, 63 of 1190 animals died of uraemia, anorexia, depression, profuse diarrhoea, dehydration and hypothermia because of being fed diets based on maize silage, oats, sunflower hulls, or dry hay. Some of these ingredients were notably mouldy and contaminated with ochratoxin (up to 6 mg/kg) and citrinin (up to 4 mg/kg). The lesions were limited to occasional pneumonia and perirenal oedema at the macroscopic level. However, microscopic inspection showed nephrosis with hyaline casts, tubular dilatation, kidney fibrosis and fatty changes in the liver (Lloyd and Stahr, 1980). IARC has classified OTA as a possible human carcinogen (group 2B) (IARC, 1993). Concerns for ochratoxins in ruminants involve chronic exposure (Mostrom

and Jacobsen, 2020), and the likelihood of residues in edible tissues or milk of ruminants is low to negligible (Fink-Gremmels, 2008a; Fink-Gremmels and van der Merwe, 2019). Multiple source exposure assessment indicates that the overall contribution of animal products to human exposure does generally not exceed 3 – 10 % (EFSA, 2004). The European Commission has established guidance levels for OCA of 250 µg/kg relative to feeding stuffs with a moisture content of 12 % (EC, 2006) (Tab. 2).

1.2.6. Zearalenone

Previously known as F-2 or RAL/F-2 mycotoxin, ZEN poses a potent nonsteroidal estrogenic activity and is described chemically as 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-b-resorcyclic acid lactone (Mostrom, 2016; Gupta, 2019b). Its production is favoured by conditions of high humidity and low temperatures (CAST, 2003). This mycoestrogen and related molecules (such as α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalanone and the conjugates: ZEN-14-O-β-glucosides, ZEN-16-O-β-glucosides and ZEN-14-sulfate) are produced mainly by *Fusarium* spp. such as *F. culmorum*, *verticillioides* (*moniliforme*), *sporotrichioides*, *cerealis*, *semitectum*, *equiseti*, *oxysporum, F. incarnatum, F. crookwellense* (Fink-Gremmels and van der Merwe, 2019; Ropejko and Twarużek, 2021). The potency of ZEN is between two and four times less than 17β-estradiol. It is metabolized in the rumen to the also estrogenic α-zearalenol and β-zearalenol, which have respective potency factors of 60 and 0.2 compared to the parent compound (EFSA, 2017). Firstly was discovered that ZEN and its related metabolites can passively cross the cell membrane and bind directly with the cytoplasmic receptor for 17β-estradiol (E2), activating the estrogen pathways (Katzenellenbogen et al., 1979). More recently, it has been elucidated that ZEN and its derivates can exert disruptive endocrine effects via a membrane and nuclear E2 receptors (He et al., 2018), impacting the synthesis and secretion of sex hormones, including testosterone, oestrogens and progesterone (Zheng et al., 2019). The primary effect of ZEN is a reduction of reproductive efficiency, including decreasing embryonic survival rate, oedema, and hypertrophy of the genitalia in pre-pubertal females, decreasing the levels of LH and progesterone with subsequent morphological alteration of uterus and feminisation of males (Zheng et al., 2019). The most accentuated clinical signs in ruminants are vulvovaginitis, vaginal secretions, abortions, infertility, and mammary

hyperplasia in young heifers (Bloomquist et al., 1982; Roine et al., 1971; Jouany and Diaz, 2005).

Dairy cows exposed to varying concentrations of ZEN, ranging from 5 to 75 mg /kg feed, developed a drop in milk production, feed intake, and swelling of the vulva (Ványi et al., 1974) cited by (Gupta et al., 2018b). In an experimental study, 18 cycling heifers were dosed once daily without and with 250 mg of ZEN (purity of 99%) during one nonbreeding oestrous cycle and the next two consecutive oestrous cycles. The control group and treated heifers had respective conception rates of 87% and 62% (Weaver et al., 1986b). Another in vivo experiment carried out in dairy cattle with maximum daily doses of 500 mg per animal of 99% purified ZEN during two consecutive oestrous cycles evidenced no changes in serum progesterone concentration, erythrocyte and leukocyte blood counts, packed cell volume, oestrous cycle length, clinical health, or sexual behaviour (Weaver et al., 1986a). Recently, it was reported in an Austrian study that cows fed a basal diet with 40% grain (DM basis) and exposed to 5 mg of ZEN daily for two days presented a reduction of *Lachnospiraceae* and *Prevotellaceae* rumen populations, reduced ruminal pH and total short-chain fatty acid concentration, despite increased rumination activity. Additionally, ZEN also increased the body temperature up to a mild fever (Hartinger et al., 2022). Oral daily doses of 50-165 mg of ZEN for 21-days gave no presence of the mycoestrogen or related metabolites in either milk or plasma (detection limits: milk, 0.5 ng/ml, ZEN, α-ZEL; 1.5 ng/ml, β-ZEL; plasma, concentrations 2–3 times superior). The researchers concluded that milk would not usually represent a human health hazard because of feeding ZEN-contaminated diets to lactating dairy cows (Prelusky et al., 1990). However, ZEN is usually co-occurring with other mycoestrogens (i.e., *Alternaria*-derived toxins), mycotoxins (like DON and ergot alkaloids) and xenoestrogens (such as phytoestrogens), which undoubtedly have toxicological interactions of synergism, addition, and potentiation (Mostrom and Jacobsen, 2020; Reed and Moore, 2009; Vejdovszky et al., 2017a; Vejdovszky et al., 2017b). EFSA concluded that the contribution of ZEN residues in animal products is irrelevant to the total ZEN exposure of the consumers, which is higher in foodstuffs of plant origin (EFSA, 2017a). The guidance value of the European Commission for ZEN in feeds for dairy cows is 500 µg/kg at 88% of DM (Tab.2) (EC, 2006).

1.3. Minor classes of mycotoxins

Many other mycotoxins may affect ruminants, but they are less known, having lower occurrence and less potency (Whitlow and Hagler, 2005). Research on their (co-)occurrence in foods/feeds and their possible implications on animal health is still required (Battilani et al., 2020). Some of the mycotoxins included in this category have been a focus of interest by the scientific community. They have been denominated "emerging mycotoxins" and are described as non-contemplated in the legislation, and non-regularly examined, but occur commonly in agricultural commodities (Vaclavikova et al., 2013). Some of the mycotoxins considered emerging are produced mainly through species belonging to the genera *Fusarium* (enniatins (ENNs), beauvericin (BEA), moniliformin, fusaproliferin, fusaric acid and culmorin), *Alternaria* (alternariol, alternariol monomethyl ether and tenuazonic acid), *Aspergillus* (sterigmatocystin, emodin and cyclopiazonic) and *Penicillium* (mycophenolic acid) (Fraeyman et al., 2017; Gruber-Dorninger et al., 2017; Santini et al., 2012). Other mycotoxins which have risen interest are roquefortines (mainly the type C), gliotoxin, citrinin, patulin, among others. The reported general information (related to main producers, toxic effects, and probable action mechanisms) of the minor classes of mycotoxins are compilated in Tab. 3. Risks associated with some of these mycotoxins have been recognized but are not commonly tested for in animal feeds, and others are recently detected (Khoshal et al., 2019; Panasiuk et al., 2019; Mostrom and Jacobsen, 2020;). Knowledge of the occurrence in animal feed and acute and chronic toxicity of these compounds in animals, particularly ruminants, has been developed in the last years but is still very limited (Whitlow and Hagler, 2005; Mostrom and Jacobsen, 2020).

Tab 3. Minor mycotoxins: Main producers, general toxicological properties, and possibly implicated mechanisms of action.

Mycotoxin	Main producers	Toxic effects	Proposed action mechanisms	References
Alternariol Alternariol-Methyl-Ether	- *Alternaria* spp.	- Estrogenic - Genotoxic	-Induction of ROS production (Oxidative DNA damage) -Topoisomerase inhibitor (Disruption of DNA replication)	Tiessen et al., 2013; Dellafiora et al., 2018; Martins et al., 2020;
Averufin	- *Aspergillus* spp.	- Mutagenic?	- Interaction with ubiquinol-cytochrome c reductase complex - Inhibition of ATP synthesis	Fitzell et al., 1975; Wong et al., 1977; Kawai et al., 1984; Kawai et al., 1988; Wunch et al., 1992
Beauvericin	- *Fusarium* spp. - *Beauveria bassiana*	- Antibacterial - Cytotoxic	- Disrupting membrane potential (Ionophore) - Induction of apoptosis (via caspases)	Kouri et al., 2005; Santini et al., 2012; Wang and Xu, 2012; Mallebrera et al., 2018;Das et al., 2021
Citrinin	- *Penicillium* spp. - *Aspergillus spp.* - *Monascus* spp.	- Nephrotoxic - Hepatotoxic - Embryotoxic - Teratogenic	- ROS-mediated DNA damage	Yuliana et al., 2019; Bovdisova et al., 2021
Cyclopiazonic acid	- *Aspergillus* spp. - *Penicillium* spp.	- Hepatotoxic - Nephrotoxic - Cardiotoxic	- Specific inhibitor of Sarco(endo)plasmic reticulum Ca2+-ATPase.	Holzapfel, 1968; Luk et al., 1977; Pitt et al., 1986; Chang et al., 2009
Enniatins	- *Fusarium* spp.	- Antibiotic - Cytotoxic	- Disrupting membrane potential (Ionophore) - Induction of apoptosis (via caspases)	Hyun et al., 2009; Kamyar et al., 2004; Santini et al., 2012; Sy-Cordero et al., 2012; EFSA, 2014;
Fumigaclavines	- *Aspergillus* spp.	- Antibacterial - Neurotoxic - Immunosuppression	- NLRP3-caspase-1-IL-1β cascade in macrophages.	Cole et al., 1977; Pinheiro et al., 2013; Xu et al., 2020
Fusaproliferin	- *Fusarium* spp.	- Teratogenic	Unknown	Ritieni et al., 1997; Jestoi, 2008; Santini et al., 2012
Fusaric acid	- *Fusarium* spp.	- Cardiotoxic - Immunosuppression	- Increase levels of serotonin, 5-hydroxy indole acetic acid, tyrosine, and dopamine - Decline norepinephrine - Depletion of neuronal ATP levels	Wang and Ng, 1999; Dhani et al., 2020
Gliotoxin	- *Aspergillus* spp.	- Antimicrobial - Immunosuppression	- Selective binding to cytoplasmic membrane thiol groups	Pahl et al., 1996; Scharf et al., 2012; König et al., 2019; Esteban et al., 2021
Moniliformin	- *Fusarium* spp.	- Cardiotoxic	- Inactivation of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase - Inhibition of Krebs	Cole et al., 1973; Thiel, 1978; Zhang and Li, 1988; Hallas-Møller et al., 2016
Lolitrem B	- *Epichloë* spp. - *Neotyphodium* spp.-	- Neurotoxic	- Unknown - ABAA inhibition - Cholinergic activation? -BK channel inhibition?	Gallagher et al., 1981; Finch et al., 2018; Combs et al., 2019
Mycophenolic acid	- *Penicillium* spp.	- Antimicrobial - Immunosuppression	- Inhibition of the enzyme inosine monophosphate dehydrogenase - Highly expressed in proliferating cells such as T- and B-lymphocytes	Allison et al., 1993
Patulin	- *Penicillium* spp. - *Aspergillus* spp. - *Byssochlamys* spp.	- Antibacterial - Neurotoxic?	- Disruption of cell membrane - Inhibition of protein synthesis - Inhibition of Na+-coupled amino acid transport - Inhibition of DNA synthesis - Inhibition of interferon γ producing T-helper type 1 cells	Hatey and Gaye, 1978; Lee and Röschenthaler, 1987; Miura et al., 1993; Arafat and Musa, 1995; Mahfoud et al., 2002; Tapia et al., 2005; Pal et al., 2017
Roquefortines	- *Penicillium spp.*	- Antimicrobial - Neurotoxic	- Unknown	Kopp-Holtwiesche and Rehm, 1990; Ali et al., 2013; Malekinejad et al., 2015
Sterigmatocystin	- *Aspergillus* spp. - *Emericella* spp. - *Chaetomium* spp. - *Penicillium inflatum*	- Hepatotoxin - Nephrotoxic - Carcinogen	- Increase ROS production - Induction apoptosis (Via caspase 3) - Damage to DNA and impairment of cell cycle progression - Alteration of cellular signalling pathways	Rank et al., 2011; Kobayashi et al., 2018; Zingales et al., 2020
Tenuazoic acid	- *Alternaria* spp.	- Antibacterial - Carcinogenic?	- Inhibition of protein synthesis at the ribosomal level	Meronuck et al., 1972; Kumari and Tirkey, 2019

1.4. Modified and matrix-associated mycotoxins

Additional to the co-occurrence of emerging mycotoxins and the more studied parent ("free" or "unmodified") compounds, the presence of modified and matrix-associated forms of mycotoxins should be considered to clarify the total exposure and associated health risks (Freire and Sant'Ana, 2018; Suman, 2020; Zhang et al., 2020). Previously, several different terms such as "bound"," hidden" and "masked" were used to define mycotoxins with alterations in their chemical structure (Humpf et al., 2019). However, Rychlik et al., 2014 proposed a systematic definition of modified mycotoxins (Figure 3), used to describe fungal toxic compounds with any modification of the basic chemical structure of the parent fungal toxin. Such changes can be biological during phase-1 (functionalization) and phase-2 of metabolism (conjugation). Animals, microorganisms, and plants can conjugate mycotoxins. The term masked mycotoxin is referred only to the fungal toxins conjugated by plants. In addition, mycotoxins can also be modified chemically and can be classified as thermally and non-thermally formed. Besides the free and modified mycotoxins, another category proposed by Rychlik et al., 2014: is matrix-associated mycotoxins. It described the mycotoxins that form either complexes or are physically dissolved/trapped in matrix compounds and are covalently bound to matrix components or a combination of both effects. In difference to the parent compounds (e.g., DON, OTA, ZEN, FUMs), the data on the toxicokinetic and toxicodynamic of modified mycotoxins are still scarce, limiting the accuracy of the final assessment of *in vivo* toxicity for modified mycotoxins. Thus, research on their occurrence and toxicity should be addressed (Freire and Sant'Ana, 2018; Humpf et al., 2019; Lu et al., 2020).

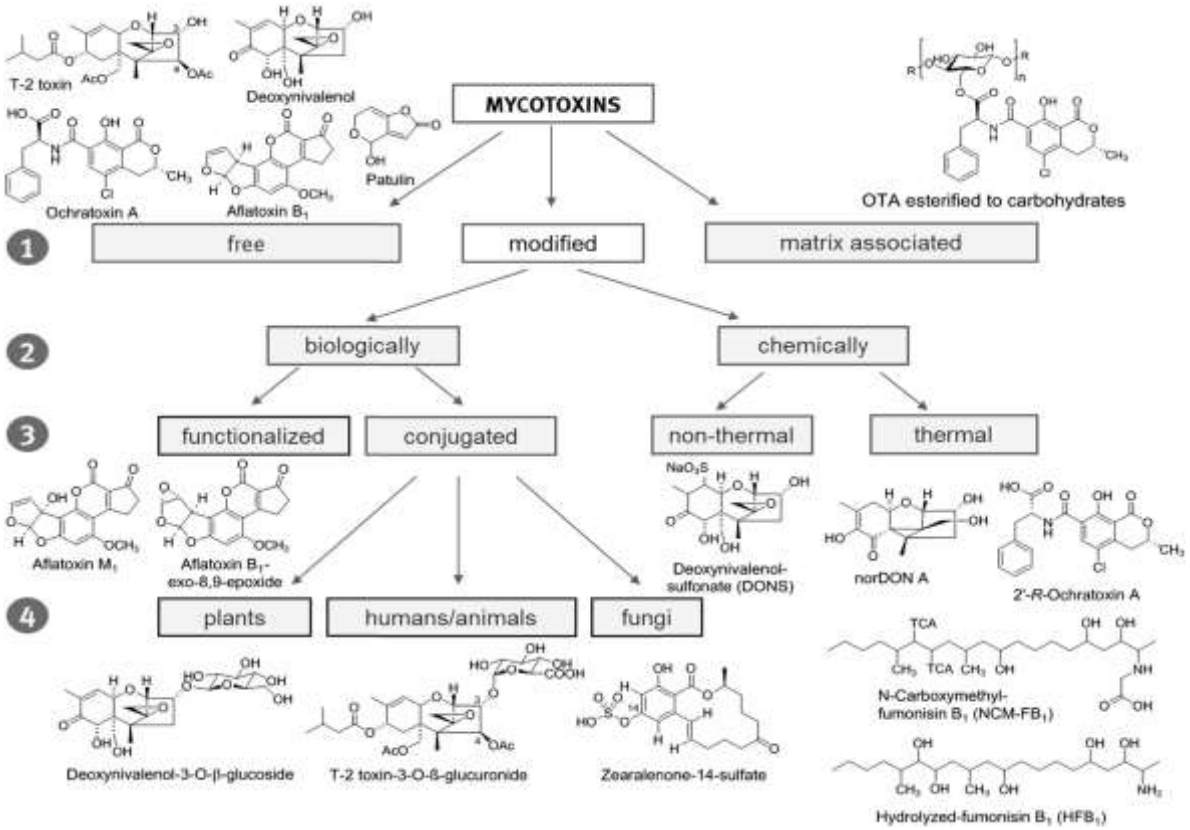

Figure 3. Systematic definition of modified mycotoxins (Schema taken from (Humpf et al., 2019) according to (Rychlik et al., 2014).

1.5. Mycotoxin mixtures and their toxicological interactions

Although most studies have focused on the occurrence and toxicology of single mycotoxins, feeds and foods are usually contaminated by numerous toxins. The combined effect of several co-occurring toxins can induce interactions like additivity, synergism, potentiation and antagonism, varying by mycotoxin type or/and concentration (Smith et al., 2016). Such biological effects of toxin mixtures on animal and human health have been growing notably in recent years, but related knowledge is still scarce (Gil-Serna, 2014; Smith et al., 2016; Weaver et al., 2020; Battilani et al., 2020). More data on mycotoxin mixtures in foods and feeds can help to prioritize research efforts (Gruber-Dorninger et al., 2019), making studies on the effects of mycotoxins mixtures a necessity but at the same time representing a challenge for scientists (Battilani et al., 2020). The research and monitoring of mycotoxin co-occurrence in feed and food would make accessible the characterization of the most prevalent mycotoxin mixtures.

The study on the occurrence and effects of single mycotoxins likely provides incomplete and biased information about the associated risks (Ogunade et al., 2018). For example, one extendedly reported synergic interaction occurs between OTA and citrinin, which increases the nephrotoxic activity (Braunberg et al., 1994; Das et al., 2014). Other frequently occurring combinations compilated by (Speijers and Speijers, 2004) are OTA/ZEN, OTA/AFB1, patulin/citrinin, FUM B1/Moniliformin, AFB1/FUM B1/ZEN/DON/NIV as well as diverse mixtures of different TCTs. Additionally, several studies suggest that naturally contaminated diets are more toxic than expected from the concentrations of tested mycotoxins, indicating the presence of unidentified toxins (Jouany and Diaz, 2005). For instance, it has been demonstrated that impure AF produced by culture reduced milk production, but equal amounts of pure AF did not (Applebaum et al., 1982). Recently, it has been proved that low doses of mycotoxins mixtures (below European regulatory limits) can negatively affect the performance of broiler chickens (Kolawole et al., 2020). Similar studies in other zootechnical species, including dairy cattle still missing. Under natural conditions, numerous mycotoxins habitually co-occur, making it crucial to evaluate the toxic effects of different combinations of mycotoxins. Based on the findings of various studies, such mycotoxin mixtures often exhibit different levels of (cyto)toxicity compared to the individual toxins, with a more substantial toxic effect *in vivo* and *in vitro* (Ficheux et al., 2012; Lu et al., 2013; Alassane-Kpembi et al., 2013; Alassane-Kpembi et al., 2015; Cheat et al., 2016; Demaegdt et al., 2016; Alassane-Kpembi et al., 2017; Skrzydlewski et al., 2022).

1.6. Relevance of proper sampling procedures

The distribution of mycotoxins in batches of agricultural commodities is a vital factor to be considered for establishing sampling criteria (Krska et al., 2008). Unlike nutritional compounds (proteins, lipids, carbohydrates, minerals and vitamins), the mycotoxin contaminated units derived from fungal growth and development are “spot processes” with highly inhomogeneous distribution, forming mycotoxin clusters throughout the feed lots (Richard, 2000; Miraglia et al., 2005; Maestroni and Cannavan, 2011). The mycotoxigenesis is influenced by several factors, for example, the implicated mould species, type/variety of crop, agronomic practices, weather conditions during growth and harvest, storage and processing conditions (Whitaker et al., 2005). *Fusarium* species are mainly associated with producing FUMs, ZEN and TCTs

during plant growth in wet and cold conditions. The distribution of *Fusarium* toxins is considered more homogeneous than storage-produced ones, like AFs and OTA, produced mainly by *Aspergillus* and *Penicillium*, respectively. These distribution phenomena between the field- and storage produced-mycotoxins could be attributable to mixing, manipulation processes at harvest, transport and storage, which explains the less heterogeneous spread of DON than OTA in truckloads of wheat (Maestroni and Cannavan, 2011). A "representative sampling" for AFs is assumed to be more complex than sampling for other known mycotoxins (Miraglia et al., 2005). This explains why several articles have been published on sampling schemes for AFs (Whitaker and Wiser, 1969; Whitaker et al., 1974; Whitaker et al., 1976; Schuller et al., 1976; Whitaker et al., 1979; Knutti et al., 1982; Whitaker et al., 1994; Whitaker et al., 1995; Whitaker et al., 2007; Brera et al., 2010; Bellio et al., 2016; Ozer et al., 2017a; Ozer et al., 2017b) and some for OTA (Biselli et al., 2008; Tittlemier et al., 2011). Thus, sampling procedures recommended for aflatoxins should be appropriate for other mycotoxins (Miraglia et al., 2005; Dickens and Whitaker, 1982).

Although sampling variability is unavoidable, a proper sampling plan should be implemented to overcome the problem caused by the heterogeneous distribution of mycotoxins. A total sample collected (denominated usually as aggregate or composite sample) is formed by the accumulation of many small portions, called incremental samples, which should be taken randomly (Dickens and Whitaker 1982; Maestroni and Cannavan, 2011). Collecting too small (inadequate mass) or few incremental samples are frequent errors that should be avoided not to compromise the sample representativeness (Maestroni and Cannavan, 2011). Since the high variability associated with each step of the mycotoxin testing procedure, a total and exact mycotoxin concentration of a bulk lot cannot be determined with 100% certainty (Whitaker, 2003). Sampling variation is often considered the most significant error in determining concentrations of mycotoxins in feed/food commodities (Whitaker, 2003). About 90 per cent of the error associated with mycotoxin assays can be attributed to how the original sample was collected (Carlson and Ensley, 2003). The worldwide safety evaluation of mycotoxins requires sampling plans that give acceptably accurate values for the level of contamination in specific batches of lots of a commodity. Although sampling variability is unavoidable, the precision of the sampling plan must be clearly defined and be considered acceptable by those responsible for interpreting and reporting the surveillance data. When sampling is undertaken, it is essential

that the following are clearly defined: the aim of the sampling exercise, the nature of the population being sampled, the sampling method, the efficiency of the sampling method and the sample preparation method (IACR, 2012).

1.7. Multi-mycotoxins analyses: An urgent necessity

Given the broad spectrum of fungal toxins (>400) described and the widely demonstrated co-occurrence of diverse mycotoxins, multi-metabolite analyses have been developed as powerful tools to take a more accurate picture of the realistic mixtures of these contaminants in the feed and food chain (see Figure 4) (Battilani et al., 2020; Steiner et al., 2020; Sulyok et al., 2020; Steiner et al., 2021;). Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is an instrumental reference technique utilized to quantitatively determine small molecules in foods, feeds, and other biological samples (Seger, 2012; Steiner et al., 2020). The method detects specific compounds directly based on their molecular characteristics as molecular mass and molecular disintegration patterns in mass spectrometric method (Kang et al., 2012). Mass spectrometry is a microanalytical technique, which can be employed selectively to detect and quantify the concentration of a given analyte. Due to its high sensibility and potent quantitative capacity, it has been called as “the smallest scale in the word”, not because of the size of the mass spectrometer but then because of the size of what it can weigh (molecules) (Siuzdak, 2004; Watson and Sparkman, 2007; Kang et al., 2012). According to Kang et al. (2012), the mass spectrometry apparatus generates a beam of gaseous ions from a sample, separates the subsequent mixture of ions corresponding to their mass-to-charge ratios, and produces signals which are a measure of relative abundance of each ionic species present. Mass spectrometry techniques are classified based on how the mass separation is achieved. Still, they all can be described as ion optical devices, which separate ions according to their mass-to-charge (m/z) ratios by employing electric and/or magnetic force fields (Kang et al., 2012). This method has high selectivity, sensitivity, robustness, and its multi-analyte capability facilitates the simultaneous determination of many analytes (Sulyok et al., 2020). These cutting-edge approaches not only screen mycotoxins but have also been adapted and improved to quantify other contaminant classes of pesticides, veterinary drugs, mycotoxins, other secondary metabolites, etc., with minimal or even without any clean-up (Sulyok et al., 2020). Such multi-

mycotoxin analysis techniques have been highly required to achieve a holistic risk assessment (Battilani et al., 2020).

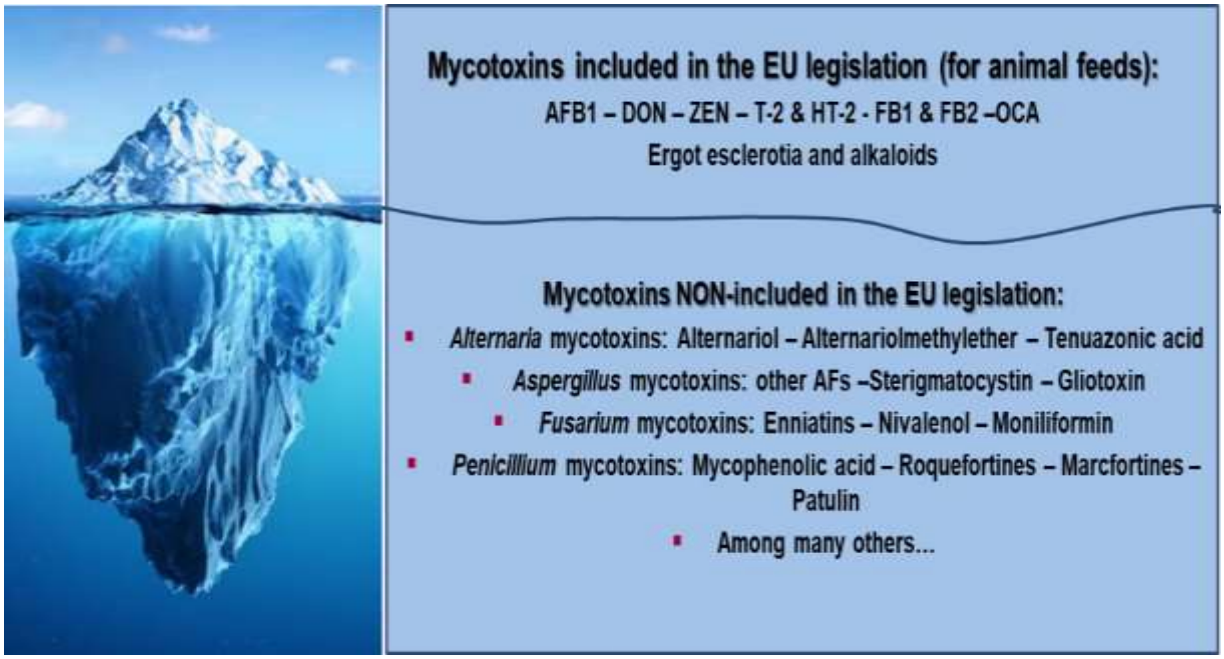

Figure 4 Mycotoxin research's "iceberg" status mainly focused on regulated metabolites. Less-known mycotoxins/metabolites are usually neglected and underestimated.

Regarding LC-MS/MS-based methods, the method employed during the investigations in the frame of this dissertation was developed at the Institute of Bioanalytics and Agro-Metabolomics, University of Natural Resources and Life Sciences, Vienna, Austria (BOKU). It can test animal feed samples for >700 fungal metabolites (including regulated mycotoxins, their modified forms, emerging mycotoxins and other less known secondary metabolites), several phytoestrogens, >300 pesticides and >150 veterinary drugs in one go (Krska et al., 2017). A broad spectrum of agro-contaminants and compounds makes this patented method a worldwide reference for commercial multianalyte screening for animal feeds. This method was employed during the development of the research project in this thesis, allowing us to generate new data on the exposome of Austrian dairy cows. This doctoral thesis focuses mainly on mycotoxins and other fungal secondary metabolites in some feeds and complete diets of Austrian dairy cattle and reported in some of the presented publications. Additionally, it was also aim to screen for other important contaminants and substances that can affect animal health and food safety like phytoestrogens, pesticides and veterinary drug residues.

2. AIMS AND HYPOTHESIS OF THE STUDY

During the last decade, several studies based on multi-mycotoxin analyses in diverse feeds and foods have been conducted, showing that a ubiquitous presence of multiple mycotoxins is a realistic scenario. However, to our best knowledge, multi-mycotoxin studies in Austria previously reported in dairy cattle feeds were extremely scarce. Thus, we hypothesized that feeds and complete diets of Austrian dairy cows are contaminated with a broad range of mycotoxins and fungal secondary metabolites, which could potentially affect the health, productivity, and reproductive performance, and potentially could be a threat of the food safety. We further hypothesized that several risk factors could contribute to the level of contamination such as geographical location, farm production system, weather conditions, and the feed used in the dairy cattle diets.

Based on the stated hypothesis, the primary research goals were:

1) To determine the co-occurrence and contamination levels of a broad spectrum of mycotoxin and fungal secondary metabolites in pastures of Austrian dairy farms, and to assess the risk factors associated with this contamination.

2) To determine the co-occurrence and contamination levels of a broad spectrum of mycotoxin and fungal secondary metabolites in mouldy spots of grass and maize silage of Austrian dairy farms.

3) To determine the co-occurrence and contamination levels of a broad spectrum of mycotoxin and fungal secondary metabolites in brewery's spent grains (BSG) intended for feeding cattle in Austrian dairy farms.

4) To determine the co-occurrence and contamination levels of a broad spectrum of mycotoxin and fungal secondary metabolites in complete dietary rations of Austrian dairy cows, as well as to assess the risk factors associated with this contamination.

The research performed in the frame of this thesis was conducted at the Institute of Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine, Vienna, Austria, in close cooperation with the Institute of Bioanalytics and Agro-Metabolomics, University of Natural Resources and Life Sciences, Vienna, Austria (BOKU) and Biomin (part of DSM Animal Nutrition & Health). One hundred dairy farms in Styria, Lower and Upper Austria were included in the investigations carried out as pilot farms.

3. PUBLICATIONS

3.1. Publication 1:

Mycotoxins, Phytoestrogens, and Other Secondary Metabolites in Austrian Pastures: Occurrences, Contamination Levels, and Implications of Geo-climatic Factors

Felipe Penagos-Tabares, Ratchaneewan Khiaosa-ard, Veronika Nagl, Johannes Faas, Timothy Jenkins, Michael Sulyok, and Qendrim Zebeli.

Toxins (2021) 13, 7:460. https://doi.org/10.3390/toxins13070460

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Mycotoxins, Phytoestrogens and Other Secondary Metabolites in Austrian Pastures: Occurrences, Contamination Levels and Implications of Geo-Climatic Factors

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Abstract: Pastures are key feed sources for dairy production and can be contaminated with several secondary metabolites from fungi and plants with toxic or endocrine-disrupting activities, which possess a risk for the health, reproduction and performance of cattle. This exploratory study aimed to determine the co-occurrences and concentrations of a wide range of mycotoxins, phytoestrogens and other secondary metabolites in grazing pastures. Representative samples of pastures were collected from 18 Austrian dairy farms (one sample per farm) between April to October 2019. After sample preparation (drying and milling) the pastures were subjected to multi-metabolite analysis using LC-MS/MS. In total, 68 metabolites were detected, including regulated zearalenone and deoxynivalenol (range: 2.16–138 and 107–505 µg/kg on a dry matter (DM) basis, respectively), modified (3-deoxynivalenol-glucoside, HT-2-glucoside) and emerging *Fusarium* mycotoxins (e.g., enniatins), ergot alkaloids and *Alternaria* metabolites along with phytoestrogens and other metabolites. Aflatoxins, fumonisins, T-2 toxin, HT-2 toxin and ochratoxins were not detected. Of the geo-climatic factors and botanical diversity investigated, the environment temperature (average of 2 pre-sampling months and the sampling month) was the most influential factor. The number of fungal metabolites linearly increased with increasing temperatures and temperatures exceeding 15 °C triggered an exponential increment in the concentrations of *Fusarium* and *Alternaria* metabolites and ergot alkaloids. In conclusion, even though the levels of regulated mycotoxins detected were below the EU guidance levels, the long-term exposure along with co-occurrence with modified and emerging mycotoxins might be an underestimated risk for grazing and forage-fed livestock. The one-year preliminary data points out a dominant effect of environmental temperature in the diversity and contamination level of fungal metabolites in pastures.

Keywords: pasture; mycotoxin; fungal metabolite; phytoestrogen; cyanogenic glucoside; ergot alkaloid; temperature; dairy cattle

Key Contribution: Mixtures of regulated, modified and emerging mycotoxins and phytoestrogens are frequently detected in pastures of Austrian dairy farms. Due to their incorporation into the feed chain, the unpredictable toxicological interactions and the transfer to animal products, these toxin mixtures may implicate a health risk for animals and humans.

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Citation: Penagos-Tabares, F.; Khiaosa-ard, R.; Nagl, V.; Faas, J.; Jenkins, T.; Sulyok, M.; Zebeli, Q. Mycotoxins, Phytoestrogens and Other Secondary Metabolites in Austrian Pastures: Occurrences, Contamination Levels and Implications of Geo-Climatic Factors. *Toxins* **2021**, *13*, 460. https://doi.org/10.3390/toxins13070460

Received: 26 May 2021
Accepted: 29 June 2021
Published: 30 June 2021

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1. Introduction

Grasses and grass-legume mixtures are essential sources of nutrients for herbivores, which can be consumed directly as fresh pastures and preserved as silage and hay. Pastures can be a source of toxic or endocrine-disrupting secondary metabolites originated from some plants, fungi, algae, bacteria and lichens residing in the pasture, which can induce a wide range of animal disorders [1–3]. Among these metabolites, mycotoxins, low molecular weight molecules produced by endophytic and epiphytic fungi, are one of the most relevant groups of metabolites due to their high incidence and their negative effects. The contamination of pastures marks an initial point of mycotoxins entering the feed chain. It has been shown that these fungal compounds can represent a risk for animals during grazing and stable periods, causing mycotoxicoses [1,4,5]. Even though ruminants are more resistant to mycotoxins than monogastrics, metabolic and dietary particularities of high producing animals seem to reduce the rumen's detoxifying ability, thereby increasing the risk of subclinical and clinical health disorders, impairing fertility and affecting productivity [6–8].

In general, less information is available regarding mycotoxin levels in pastures compared to the data in grains and conserved feeds [9,10]. Furthermore, although hundreds of compounds have been considered mycotoxins, most studies investigated a limited number of mycotoxins in pastures and other agricultural commodities [11,12]. The most investigated mycotoxins in pastures include the strictly regulated aflatoxin B1 (AFB1) and other mycotoxins with guidance levels (deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FBs), ochratoxin A as well as T-2 and HT-2 toxin) [13–17], which are addressed by the European legislation [18,19]. The ergot sclerotia are also regulated and monitoring of ergot alkaloids (EAs) in food and feed is recommended by the EU [20]. Other relevant but less studied groups of fungal toxins are the modified and emerging mycotoxins. Modified mycotoxins are structurally changed metabolites of the parent forms. These compounds result from biological or chemical modifications. [21]. The emerging mycotoxins have been described as those that are legislatively unregulated and non-regularly analysed, but which occur frequently in agricultural commodities [22]. In addition to single effects, there are toxicological interactions (addition, synergism, potentiation and antagonism) among mycotoxins and other fungal metabolites, which may have implications on animal's health and reproduction, and this necessitates more research and risk assessment from holistic and integrative approaches [12,23,24]. For instance, synergistic interactions of ZEN, trichothecenes, EAs and other mycotoxins contained in pastures have been discussed as a potential cause of infertility in grazing sheep and cattle [13].

Additionally, pastures are the source of plant secondary compounds such as phytoestrogens (PEs), pyrrolizidine alkaloids, cyanogenic glucosides (CGs), among others, which, at certain dietary levels, may induce detrimental effects on animal health and reproduction [1,25–28]. Negative effects of PEs on the reproduction of ruminants have been associated with pasture legumes such as clovers (*Trifolium* spp) and lucerne/alfalfa (*Medicago sativa*) [27]. In the context of the reproductive performance of livestock, it seems important that co-occurrences of fungal metabolites and PEs are taken into consideration [29,30].

The production of fungal and plant secondary metabolites is influenced by multiple biological (e.g., species, variety, plant age, parasitic and symbiotic interactions) as well as geo-climatic factors (temperature, relative humidity, rainfall, latitude and altitude) [31–34]. Some studies on pastures have shown that the geographic location, botanical species and sampling season affect the contamination levels of mycotoxins such as T2-toxin, ZEN and EAs [13,15,16]. Updated data and identification of the most influencing factors could assist in the prediction of contamination as well as the development of strategies for optimal management of forage grasses. The present exploratory study aimed to determine, via an LC-MS/MS-based multi-metabolite method, the presence, co-occurrence and concentrations of mycotoxins, PEs as well as other fungal, bacterial, lichenical and unspecific secondary metabolites in grazing pastures of Austrian dairy farms. Furthermore, potential correlations between the concentrations of the metabolites, and geo-climatic factors

of the farms (location, altitude, rainfall, humidity, temperature and time of sampling) were evaluated.

2. Results

2.1. Occurrence and Concentrations of the Detected Metabolites

2.1.1. Groups of Metabolites

The occurrence and concentrations (average, SD, median, minimum and maximum, expressed in µg/kg on a DM basis) of individual and grouped metabolites are shown in Table 1. The grouped metabolites were classified according to their main producers including *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, lichen-associated fungi, other (non-identified) fungi and unspecific (i.e., metabolites produced by fungi, bacterial and/or plants), or according to the kind of metabolites (EAs, PEs and CGs) based on previous reports [35,36]. In total 68 out of 481 targeted fungal, plant, lichenical and unspecific metabolites were detected in the studied pastures samples (Supplementary Table S1), consisting of 48 fungal compounds (over 30 known as mycotoxins), 11 plant and 9 unspecific metabolites (Table 1).

In total, 21 metabolites produced primarily by *Fusarium* spp. were present in the pasture samples and none of the samples was free from *Fusarium* metabolites (Table 1). The number of metabolites derived from *Alternaria* (4), *Aspergillus* (2) and other fungi (5) was considerably smaller with occurrences of 83, 44 and 44 %, respectively. The metabolite group derived from lichen-associated fungi and the EAs occurred in 44 and 39 % of the samples with a total of 2 and 13 metabolites of each respective group were detected. The group of fungal metabolites with the highest average, median and maximum concentrations were produced by *Fusarium*, followed by *Alternaria* and EAs (Figure 1). Only one metabolite produced by *Penicillium* was detected (pestalotin). Metabolites produced by lichen-associated fungi, and other fungal species showed low concentrations with values below 10 µg/kg and 60 µg/kg, respectively (Table 1, Figure 1).

Table 1. Occurrence and concentration of mycotoxins, fungal metabolites, phytoestrogens and other secondary metabolites detected in pastures collected from Austrian dairy farms.

Group	Metabolite	Positive Samples (%) [1]	Concentration (µg/kg DM) [2]		
			Average ± SD	Median	Range
Alternaria	Alternariol [3]	61	6.41 ± 7.43	2.81	1.00–23.7
	Alternariolmethylether [3]	56	7.30 ± 8.30	4.45	1.01–29.4
	Altersetin	83	220 ± 246	127	4.36–861
	Infectopyrone	33	76.5 ± 78.7	36.3	16.3–212
	Total [4]	83	260 ± 286	128	4.36–1010
Aspergillus	Averufin	6	-	-	1.15
	Sterigmatocystin [3]	44	2.94 ± 2.13	2.21	1.03–7.34
	Total [4]	44	3.08 ± 2.48	2.21	1.03–8.49
Ergot alkaloids [5]	Chanoclavine	17	152 ± 245	17.93	2.35–435
	Ergocornine	22	20.1 ± 26.1	7.83	5.57–59.2
	Ergocorninine	22	8.72 ± 8.83	4.86	3.27–21.9
	Ergocristine	17	38.0 ± 31.9	37.5	6.33–70.1
	Ergocristinine	17	8.21 ± 5.71	8.64	2.30–13.7
	Ergocryptine	28	24.8 ± 28.4	9.27	3.6–71.5
	Ergocryptinine	17	6.12 ± 6.30	3.01	1.97–13.4
	Ergometrine	22	8.76 ± 6.19	7.80	2.38–17.1
	Ergometrinine	11	1.92 ± 0.26	1.92	1.73–2.1
	Ergosine	22	15.9 ± 13.5	15.1	1.1–32.1
	Ergosinine	17	3.99 ± 2.39	3.24	2.06–6.66
	Ergotamine	11	75.7 ± 93.3	75.7	9.7–142
	Ergotaminine	11	11.6 ± 13.2	11.6	2.24–20.9
	Total [4]	39	163 ± 191	43.9	4.70–435

Table 1. *Cont.*

Group	Metabolite	Positive Samples (%) [1]	Concentration (µg/kg DM) [2]		
			Average ± SD	Median	Range
Fusarium	15-Hydroxyculmorin [3]	44	152 ± 243	39.2	13.0–721
	Antibiotic Y	67	254 ± 374	66.5	45.5–1290
	Apicidin [3]	39	31.3 ± 31.5	25.9	5.84–97.9
	Aurofusarin [3]	83	196 ± 213	133	7.89–835
	Beauvericin [3]	44	3.99 ± 3.03	2.6	1.02–9.34
	Chrysogine	61	13.6 ± 15.5	7.42	4.07–58.2
	Culmorin [3]	89	129 ± 216	51.1	9.53–882
	Deoxynivalenol [5]	11	306 ± 281	306	107–505
	DON-3-glucoside [6]	6	-	-	102
	Enniatin A [3]	6	-	-	2.01
	Enniatin A1 [3]	44	5.54 ± 6.03	2.92	1.22–19.1
	Enniatin B [3]	94	38.3 ± 63.9	11.8	1.30–241
	Enniatin B1 [3]	89	15.3 ± 24.8	5.49	1.19–93.3
	Enniatin B2 [3]	28	3.41 ± 2.74	2.27	1.19–7.90
	Epiequisetin [3]	56	9.27 ± 7.96	8.09	1.18–27.2
	Equisetin [3]	67	57.9 ± 60.4	37.6	2.72–179
	HT-2 Glucoside [6]	6	-	-	14.0
	Moniliformin [3]	100	5.70 ± 3.52	5.79	1.45–13.1
	Nivalenol	83	170 ± 182	78.6	38.1–574
	Siccanol [3]	61	716 ± 392	758	119.3–1480
	Zearalenone [5]	50	29.6 ± 44.3	9.93	2.61–138
	Sum of enniatins	94	57.4 ± 95.5	18.5	1.3–364
	Sum of type B Trichothecenes	83	218 ± 289	78.6	38.1–1070
	Total [4]	100	1280 ± 1430	983	40.2–5770
Penicillium	Pestalotin	11	3.79 ± 3.60	3.79	1.24–6.33
	Total [4]	11	3.79 ± 3.60	3.79	1.24–6.33
lichen-associated fungi	Lecanoric acid	39	2.31 ± 0.86	2.17	1.34–3.60
	Usnic acid	17	4.49 ± 0.53	4.19	4.18–5.10
	Total [4]	44	3.71 ± 2.18	3.44	1.34–7.13
other fungi	Ilicicolin A	22	1.92 ± 0.98	1.83	1.00–3.02
	Ilicicolin B	44	4.00 ± 3.33	2.85	1.23–11.7
	Ilicicolin E	11	1.44 ± 0.11	1.44	1.36–1.51
	Rubellin D	17	5.00 ± 5.00	2.7	1.56–10.7
	Monocerin	50	11.0 ± 11.8	2.97	1.32–33.4
	Total [4]	72	12.0 ± 15.4	5.73	1.23–56.9
	Sum of fungal metabolites	100	1570 ± 1580	1145	51.7–5880
Phytoestrogens	Biochanin	89	7060 ± 7560	3240	62.1–20,650
	Coumestrol	67	41.6 ± 34.4	32.9	7.88–130
	Daidzein	83	936 ± 1840	139	5.16–6110
	Daidzin	33	167 ± 200	88.7	15.8–543
	Genistein	83	2760 ± 4780	704	28.4–17,550
	Genistin	50	311 ± 513	139	14.6–1630
	Glycitein	83	7470 ± 10,700	1500	315–35,850
	Ononin	83	2230 ± 4210	186	47.1–15,130
	Sissotrine	78	4210 ± 9050	331	8.19–33,070
	Total [4]	89	23,570 ± 35,920	4850	78.8–130,530
Cyanogenic glucosides	Linamarin	83	50,620 ± 44,880	49,790	2030–147,500
	Lotaustralin	100	32,6200 ± 34,640	16,850	32.1–115,900
	Total [4]	100	74,800 ± 79,000	36,400	32.1–263,400
	Sum of plant metabolites	100	95,760 ± 81,560	85,700	32.1–265,3200

Table 1. *Cont.*

Group	Metabolite	Positive Samples (%) [1]	Concentration (µg/kg DM) [2]		
			Average ± SD	Median	Range
Unspecific	3-Nitropropionic acid	11	4.87 ± 1.91	4.87	3.52–6.22
	Brevianamid F	100	18.9 ± 13.7	14.1	6.50–62.4
	Citreorosein	50	18.1 ± 12.4	16.6	4.52–44.9
	cyclo(L-Pro-L-Tyr)	100	498 ± 347	361	172–1383
	cyclo(L-Pro-L-Val)	100	2190 ± 1000	1970	1080–4290
	Endocrocin	11	17.4 ± 6.77	17.4	12.6–22.1
	Iso-Rhodoptilometrin	22	2.25 ± 0.95	1.96	1.49–3.60
	Rugulusovine	100	13.7 ± 8.60	11.7	3.75–39.0
	Tryptophol	100	127 ± 118	74.0	53.1–485
	Sum of unspecific metabolites	100	2860 ± 1380	2460	1370–5910
	Sum of all detected metabolites	100	100,200 ± 80,900	92,100	4560–266,700

[1] n = 18 pastures, samples with values > limit of detection (LOD); [2] Excluding data < LOD. In case values > LOD and <limit of quantification (LOQ), LOQ/2 was used for calculation; [3] emerging mycotoxins [37–39], [4] accumulative values of occurrences and concentrations of all the metabolites belonging to the group, [5] regulated mycotoxins (European Commission, 2002, 2006, 2012) [18–20] and [6] modified mycotoxins [21].

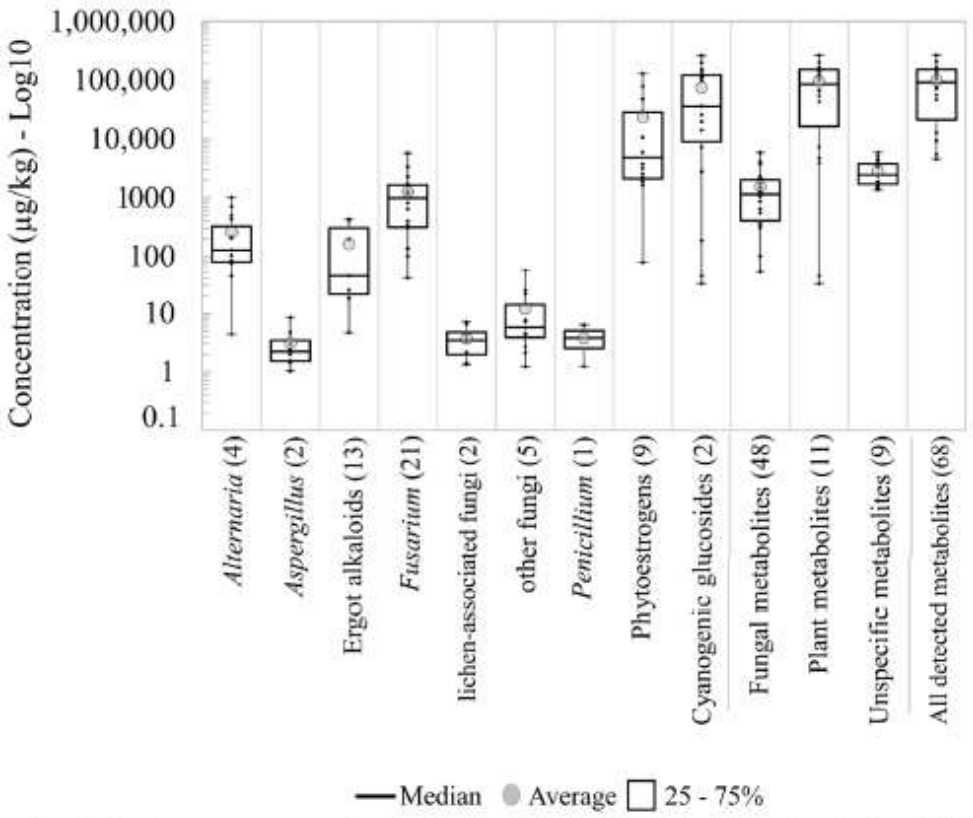

Figure 1. Boxplots for $\log_{10}$ concentrations of metabolite groups detected in the pasture samples taken from 18 Austrian dairy farms. The number in parentheses is the number of total detected metabolites per group.

As shown in Table 1, the groups of plant-derived metabolites, CGs (2 metabolites) and PEs (9 metabolites) were present at high frequencies and high concentrations, with total averages above 70,000 and 20,000 µg/kg, respectively. Nevertheless, the heterogeneity among the samples was evident and many of the samples showed values below the

average values (Figure 1). The presence of unspecific metabolites was ubiquitous and more homogenous among the pasture samples, with concentrations between 1370 and 5910 µg/kg. The total concentrations of all metabolites detected ranged from 4560 to 266,700 µg/kg with an average and median around 100,000 µg/kg.

2.1.2. Regulated Mycotoxins and Related Metabolites

The regulated AFB1, along with other AFs, FBs, T-2 toxin and OTA and structurally related forms were not detected in the pasture samples. Two regulated *Fusarium* mycotoxins were found: ZEN (50% positive samples; range: 2.61–138 µg/kg), and DON (11%, range: 107–505 µg/kg) (Table 1), being lower than EU guidance values: 500 and 5000 µg/kg (at 88% DM), respectively. Related to DON, nivalenol (NIV) occurred in more than 80% of the samples with concentrations ranged from 38.1 to 574 µg/kg of the tested pasture samples. The modified mycotoxins DON-3-glucoside (D3G) and HT-2-glucoside (HT-2G) co-occurred in the same sample with concentrations of 102 and 14.0 µg/kg, respectively (Table 1, Figure 2A).

The detected concentrations of individual EAs in the pasture samples ranged from 4.70 to 435 µg/kg (Table 1). In total, 13 different EAs were identified. Chanoclavine and ergotamine showed the superior mean concentrations of the group, 152 and 75.7 µg/kg, in that order. The rest of EAs contained average concentrations below 40 µg/kg. The presence of chanoclavine in the samples was highly heterogeneous, ranging from 2.35–435 µg/kg, but the median of ergotamine was higher than chanoclavine (Table 1, Figure 2B). Other targeted but not detected EAs were agroclavine, dihydroergosine, dihydrolysergol, elymoclavine, epoxyagroclavine, ergine and ergovaline (Supplementary Table S1).

2.1.3. Emerging Mycotoxins

The pasture samples contained 17 compounds considered emerging mycotoxins [37–39]. The majority of these emerging mycotoxins were derived from the genera *Fusarium* (in total 14 classified as emerging toxins) and, to a lesser extent, from *Alternaria* (2) and *Aspergillus* (1) (Table 1). Despite the high occurrence of fusarial emerging mycotoxins in the samples, the mean and median concentrations stayed below 150 µg/kg, except for siccanol (758 µg/kg) with noticeable variations among samples (Figure 2A). Concerning the frequency, all samples contained detectable levels of moniliformin. Other frequently found metabolites (over 80% of the pasture samples) were enniatin (ENN) B, ENN B1, culmorin and aurofusarin. Occurring in rates between 50 and 80% of the pasture samples were alternariol (AOH), alternariol methyl ether (AME), epiquisetin, equisetin and siccanol. Siccanol was the *Fusarium* metabolite with the highest average and median concentrations (Figure 2A). Lower occurrences (<50% occurrence) were detected for 15-Hydroxyculmorin, beauvericin (BEA), ENN A1, ENN A and ENN B2, as well as the *Aspergillus*-derived carcinogenic and aflatoxin precursor sterigmatocystin (STC) (Table 1). The concentration of STC showed a higher homogeneity among samples compared to other emerging mycotoxins from *Fusarium* and *Alternaria* (Figure 2A,C).

2.1.4. Other Mycotoxins and Metabolites from Fusarium, Alternaria, Aspergillus, Penicillium and Other Fungi

In addition to the known regulated and emerging mycotoxins, there were many other mycotoxins and metabolites associated with *Fusarium*, *Alternaria*, *Aspergillus* and *Penicillium* in the pasture samples (Table 1 and Figure 2A,C). Mycotoxin produced by *Fusarium*, including 15-hydroxyculmorin, apicidin, antibiotic Y, aurofusarin and chrysogine had occurrences over 55%, with exception of apicidin (39%) and 15-hydroxyculmorin (44%). Concerning compounds derived from *Alternaria*, altersetin (83%) was the most frequently found metabolite (Table 1). In terms of concentrations, altersetin and infectopyrone were the major detected metabolites produced by *Alternaria* (Figure 2C). The occurrence and concentrations of the *Penicillium* metabolite pestalotin (range: 1.24–6.33 µg/kg) were rather low (Figure 2C).

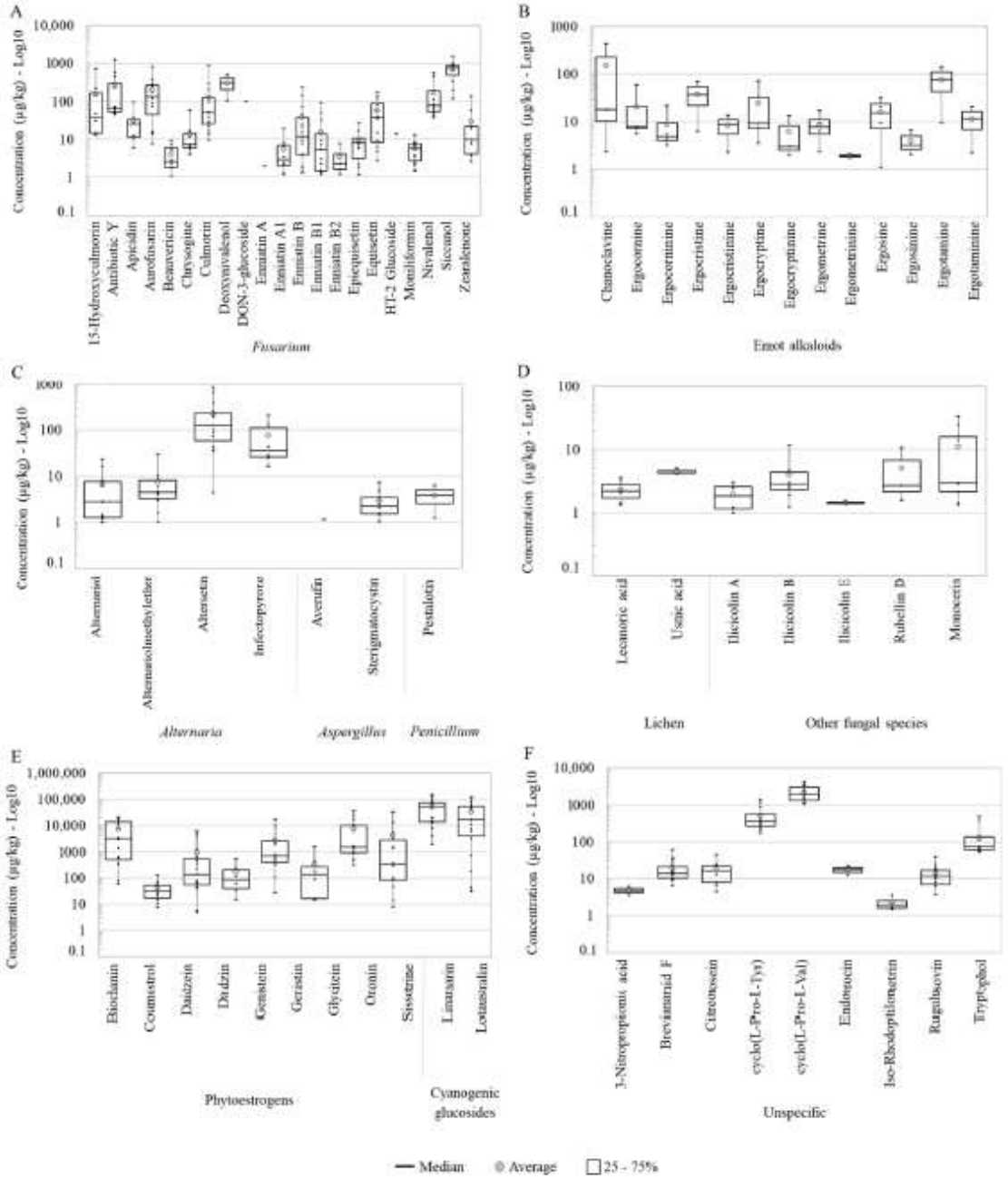

Figure 2. Boxplots for the $\log_{10}$ concentrations of individual metabolites in each category: (**A**–**D**) fungal, (**E**) plant and (**F**) unspecific metabolites (produced by fungi, plants and/or bacteria) detected in the pasture samples collected in Austrian dairy farms. The exact mean, SD, median, min and maximum values are shown in Table 1.

2.1.5. Metabolites from Lichen-Associated and Other Fungi Genera

The occurrence of metabolites produced by other fungi varied from 11–50% (Table 1). The most frequently found and most produced compound of this group was monocerin (50%; 1.32–33.4 µg/kg). The ilicicolins A, B and E occurred in concentrations below

12 µg/kg. Additionally, two lichen-derived metabolites, lecanoric acid (39%, range: 1.34–3.60 µg/kg) and usnic acid (17%, 4.18–5.10 µg/kg) were detected (Table 1, Figure 2D).

2.1.6. Plant Compounds (Phytoestrogens and Cyanogenic Glycosides) and Unspecific Metabolites

The identified PEs were biochanin, coumestrol, daidzein, genistein, genistin, glycitein, ononin and sissotrine, which occurred in ≥50% of the samples, and the less frequent daidzin (33.3%). Overall, for most PEs levels, the concentrations presented extremely variable, therewith maximum values achieved over 100 times more than the minimum values (Figure 2E). On average, glycitein and biochanin were the PEs that presented levels > 7000 µg/kg and those of genistein and ononin were about 3 times lower. Coumestrol, daidzein, daidzin and genistin had average concentrations below 1000 µg/kg. The CGs, linamarin were the metabolites with the highest concentrations (median, average and maximum) of the study (Table 2, Figure 2E).

Table 2. Effect of the sampling season on the number of detected metabolites per sample and concentrations of the groups of metabolites.

Variable	Early	Late	SEM [1]	*p*-Value
Number metabolites/sample				
All metabolites	24.4	39.6	3.51	0.008
Fungal metabolites	11.8	24.0	3.03	0.012
Concentration (µg/kg)				
from *Alternaria*	76	329	85.0	0.052
from *Aspergillus*	1.61	1.18	0.77	0.693
Ergot Alkaloids	5.32	110	44.6	0.120
from *Fusarium*	526	1890	431.8	0.041
from Lichen	1.76	1.56	0.81	0.865
from other fungi species	1.24	14.6	4.23	0.041
from *Penicillium*	0.00	0.76	0.50	0.303
Fungal Metabolites	611	2332	452	0.017
Phytoestrogens	7867	31,420	11,195	0.158
Cyanogenic glycosides	71,666	77,318	27,251	0.886
Plant metabolites	79,532	108,738	27,678	0.468
Unspecific metabolites	3144	4291	646	0.083
Total Metabolites	82,556	114,294	27,363	0.426

[1] Values are least-squares mean (LS means) and SEM is the standard error of the LS means; Sampling season: Early = samples in April–June; Late = samples in August–October.

Unspecific metabolites are analytes produced by different and unrelated species of fungi, bacteria and/or plants. In this group, five metabolites, namely brevianamide F, cyclo (L-Pro-L-Tyr), cyclo (L-Pro-L-Val), rugulusovine and tryptophol were present in all pasture samples and showed the highest levels of this category. The following unspecific compounds were detected less frequently: citreorosein (50%), iso-rhodoptilometrin (22%), 3-nitropropionic acid (11%) and endocrocin (11%) (Table 2, Figure 2F).

2.2. Co-Occurrence of Mycotoxins and Other Metabolites

The number of detected metabolites per sample are shown in Figure 3A. On average, 33 (range: 9–58) metabolites per sample were found and on average 7 PEs were detected per sample. On average 19 fungal metabolites (range: 3–40) were present in a sample. All pasture samples contained at least one CG.

The co-occurrence analyses of mycotoxins and metabolites are shown in Figure 3B. 94% of the pasture samples contained 20 or more metabolites. The most frequent combinations of mycotoxins detected in the pasture samples were MON and ENN B (94%), ENN B and ENN B1 (89%), CUL and ENN B (89%), aurofusarin and ENN B (83%) and aurofusarin and MON (83%), all of which are *Fusarium* metabolites. The combination of the other *Fusarium*

metabolites ZEN and NIV was found in 44% of the samples. Interestingly, most of the samples showed co-occurrences between *Fusarium* and *Alternaria* metabolites, especially for altersetin, which co-occurred with several *Fusarium* emerging mycotoxins (aurofusarin, CUL, ENN B and MON) in more than 70% of the samples and with ZEN in 50% of the samples. Two mycoestrogens from *Alternaria*, AOH and AME, had co-occurrences of 39% with ZEN. Up to 30% of the tested pastures showed co-contamination between detected EAs and *Fusarium* mycotoxins (Figure 3B).

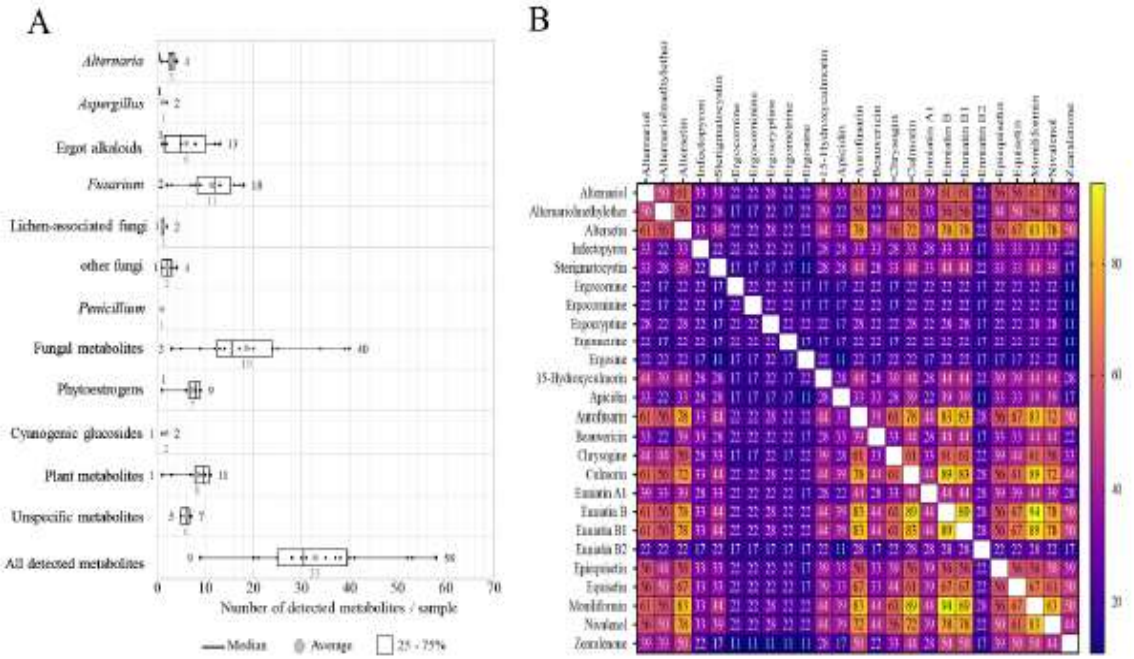

Figure 3. Co-occurrences of mycotoxins and other secondary metabolites detected in the pasture samples taken from 18 Austrian dairy farms. (**A**) Boxplots showing the number of metabolites per sample in each metabolite group. (**B**) Heatmap indicating the co-occurrence of the major mycotoxins (i.e., which occurred ≥20% of total samples) detected in the pastures.

2.3. Effect of Season, Locations and Pasture Diversity

Sampling was carried out once per farm during the grazing season of the year 2019. Subsequently, the sampling season was classified as early (April–June) and late (August–October). There was a significant difference in the co-contamination of metabolites (i.e., the number of metabolites/sample) and concentrations of several groups of mycotoxins and metabolites between the sampling seasons (Table 2). Samples collected late had higher levels of co-contamination of fungal metabolites ($p = 0.012$) and number of total metabolites increased ($p = 0.008$) compared to those of early sampling. A similar trend occurred with the concentrations of total metabolites from total fungi ($p = 0.005$), *Fusarium* ($p = 0.041$) and other fungal species ($p = 0.041$), which resulted in higher concentrations in the pastures during the late sampling season than in the early sampling. The location (classified by their federal state) and the pasture diversity did not affect the co-occurrence or the levels of metabolite groups in the tested pasture samples (data not shown).

We examined the influence of altitude and the climatic variables (temperature, humidity and rainfall at different time scales including whole-year average, 3-month average and sampling-month average). In line with the season effect, among the variables investigated, the 3-month average temperature (the mean of 2 months pre-sampling and the sampling month) was the only climatic variable that showed a significant correlation with the mycotoxin data (detailed data not shown). As shown in Figure 4A, the 3-month

average temperature showed a significant positive relationship ($p < 0.001$) with the co-occurrence of metabolites. Specifically, the number of metabolites per sample linearly increased with increasing temperature. Regression suggests an increase of 2.06 ± 0.5 fungal metabolites/sample per one degree Celsius of the 3-month average temperature ($p < 0.001$). Concentrations of total fungal metabolites along with *Fusarium* metabolites, EAs and *Alternaria* metabolites showed an exponential increment in response to the 3-month temperature. Accordingly, the concentrations of *Fusarium*, *Alternaria* and total fungal metabolites in the pasture samples remained comparably low when the temperature was below 15 °C and rapidly rose thereafter as underlined by a higher slope after this critical temperature (Figure 4B–D). Interestingly, the EAs concentrations were very low (<70 µg/kg) or absent at the temperature below 20 °C and rose strongly to concentrations over 400 µg/kg at 22 °C (Figure 4E).

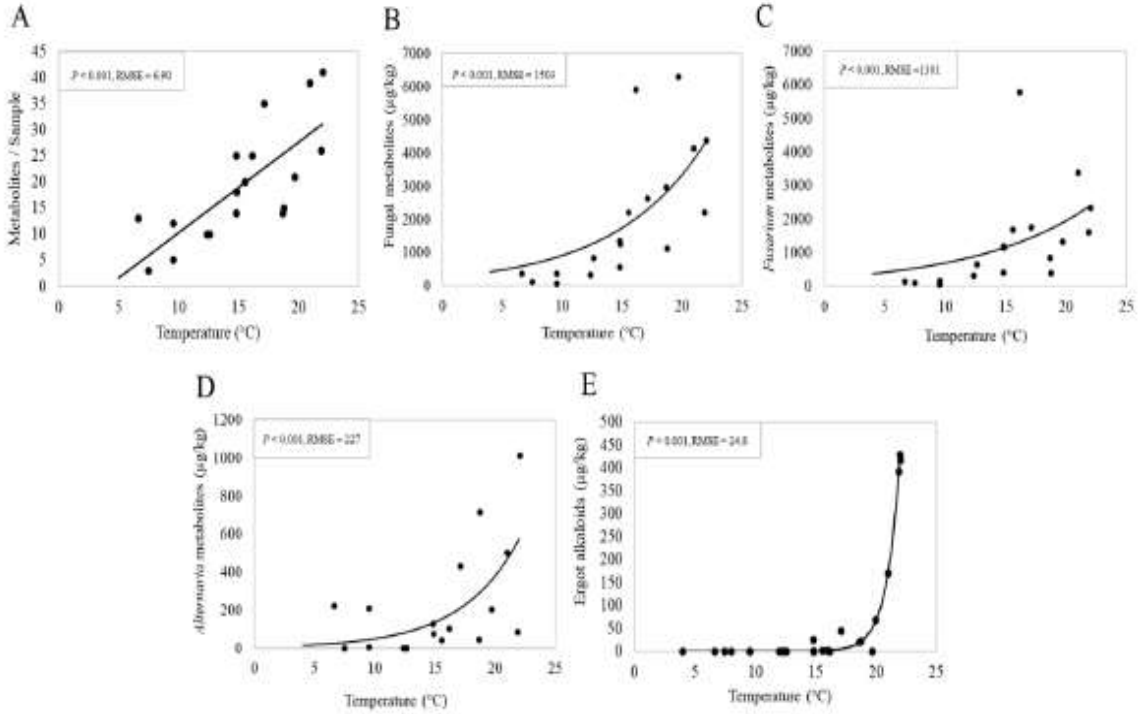

Figure 4. Linear regression showing a relationship between 3-month average temperature (the mean of 2 months pre-sampling and the sampling month) and the number of metabolites per sample (**A**) or concentrations of total and individual group of fungal metabolites (**B**–**E**). RMSE: Root mean square error.

3. Discussion

Mycotoxin contamination is an important feed safety issue that also attributes to the food safety issue due to the transfer of certain mycotoxins to animal products. Most of the previous studies have focused mostly on AFs, EAs, as well as *Fusarium* toxins DON, ZEN, T-2 toxin, HT-2 toxin and FBs [13–17]. There is a growing concern about the presence of modified and emerging mycotoxins in diets and associated risks for human and animal health according to the European Food Safety Authority (EPSA). Scientific opinions of EPSA and other authors have underlined the need for new information concerning the (co-) occurrence of those groups of fungal metabolites in foods and feed along with toxicity data [12,40–44]. To the best of our knowledge, the present study is the first study in Europe that documented the occurrences not only of mycotoxins but also of some relevant plant-derived compounds (phytoestrogens and cyanogenic glycosides) as well as unspecific

metabolites in pastures used for dairy production, which underlines pasture as a potential route of mycotoxins and other metabolites entering the feed chain.

The high occurrence of *Fusarium* metabolites found in the present study coincided with the findings of Nichea et al. in pastures collected in Argentina [45,46]. This corroborates once again the status of *Fusarium* as one of the most widespread fungal species in crops-growing areas of the planet and as a significant contributor to mycotoxin contamination in animal feeds [47–49]. Several *Fusarium* spp. capable of producing the mycoestrogen ZEN are common in pasture microflora [50], which explains the considerable incidence (50%) of this mycoestrogen in the Austrian pastures observed in the present study. Nevertheless, the detected levels of ZEN were below the guidance level (500 µg/kg DM) in feed intended for dairy cows recommended by the European Commission [19] and were low in comparison with previous studies from other geographic regions including New Zealand (max: ~4000 µg/kg) [50], Australia (36%, max: 3006 µg/kg DM) [13], Argentina (90% in 2011 and 81% in 2014, max: 2120 µg/kg), United States (61%, max: 1936 µg/kg) [51] and Russia (up to 5750 µg/kg) [16]. Studies on the effects of feeding ZEN contaminated oats at a concentration of 1.25 mg ZEN/kg feed DM were evaluated in heifers by EFSA (2004) revealing no related impacts on the oestrus cycle or histological structure of reproductive organs [52]. A study showed that ZEN intakes greater than 3 mg/ewe/day adversely affected reproduction, depressing ovulation rated and lambing percentages [53]. Based on these previous reports, by assuming an approximate 20 kg DM intake of pastures, found levels of ZEN in the Austrian pastures would not represent a high risk for ZEN-associated fertility problems in dairy cows. However, previous studies have not considered a synergistic effect related to co-occurrences of ZEN with other mycotoxins and xenoestrogens such PEs, which seems plausible [13].

Another important *Fusarium* mycotoxin is the type B trichothecene DON, which was found in a low frequency (11%) with a maximum concentration of 505 µg/kg being lower than the European guidance level (5000 µg/kg DM) [19]. Our findings stayed within the concentration range found in an Australian survey (129–682 µg/kg DM), although the authors reported DON at a higher frequency of 46% [13]. The maximum level of DON reported by Štýbnarová (2016) in Czech pastures was 715 µg/kg DM [54]. Remarkably, another type-B trichothecene NIV was detected at a much greater frequency (83%) with maximum concentrations of 574 µg/kg DM. Notably, an in vivo study using a mice model indicated that NIV has a higher oral toxic capacity (lower LD_{50}) than DON [55]. Due to its structural and toxicological similarities to DON, NIV has exhibited synergistic interactions in co-occurrence with DON and other types B trichothecenes in cell culture models [56–58]. Interestingly, another study found antagonistic effects [59]. The risks related to long-term exposure to low levels of NIV in animal feed are challenging to assess due to the limited information available in livestock species [40]. The emerging *Fusarium* mycotoxin ENN B was one of the most prevalent mycotoxins in the present study (94% occurrence), which was higher compared to a report in Argentinean grasses (70% occurrence) [45]. Metabolism of ENNs and BEA has been examined in monogastric animals, while data in ruminants are limited [60]. It is known that these compounds have antifungal, antibiotic and cytotoxic properties [61]. Our and other studies have underlined the significance of non-regulated (emerging) mycotoxins due to their high frequency. The impact of these emerging mycotoxins on dairy cattle as well as their influence on the rumen microbial ecology and digestive physiology have yet to be addressed [38].

Ergot alkaloids are produced mostly by *Claviceps* and *Epichloë* spp. These fungal species are known to parasitize a wide spectrum of monocotyledonous plants of different taxonomical families such as *Poaceae*, which includes forage grasses and cereals [62–64]. Ingestion of EAs by livestock can trigger a range of impacts from decreased performance and reduced fertility to acute clinical signs of ergotism including hyperthermia, convulsions, gangrene of the extremities and death [65–67]. Ergotism is primarily associated with *Claviceps* toxin ergotamine, which was detected in our samples with a greater mean concentration than most of the EAs detected. Fescue toxicosis is linked to ergovaline,

produced by *Neotyphodium coenophialum* in fescue grass (*Festuca arundinacea*) [65]. Ergovaline has been reported as the causal agent of severe intoxications in dairy farms [68,69]. These compounds can induce various cardiovascular, neurological as well as endocrinal effects [70–72]. Ergovaline was, however, not detected in the present study probably because only 2 pasture samples contained *Festuca pratensis* and it was a minor species in the pasture in both cases (Supplementary Table S2). Subclinical estrogenism has been proved as a significant disruptor of the reproductive performance of small ruminant herds in both Australia [73] and New Zealand [74]. It was proposed that feed contaminated with 250 µg/kg of EAs should not be fed to pregnant or lactating animals due to a higher risk of abortion and agalactia syndrome [75]. Two of the seven Austrian pastures contaminated with EAs contained a total concentration (418 and 434 µg/kg DM) above this recommendation, underlining a potential risk of pastures due to possibilities for high burdens of EAs. This emphasizes the need for close surveillance of EA contamination in pastures.

Concerning *Aspergillus* derived metabolites, although AFs were not detected, averufin and STC, two of their precursors were detected in our pasture samples [76,77]. Sterigmatocystin itself is known as a carcinogenic compound with high toxicological relevance. In general, the information available on exposure data of dairy cows to these precursors of AF is scarce [41]. Two detected emerging *Alternaria* mycotoxins, AOH and AME, belong to the chemical groups dibenzo-α-pyrones, are toxicologically relevant [78] and considered mycoestrogens, showing strong synergistic estrogenic effects in combination with the fusarial mycoestrogen ZEN even at very low concentrations [79]. However, EFSA declared that research data and information are scarce regarding toxic effects of *Alternaria* toxins on farm animals and companion animals and their occurrence in the feed, thus the health risk for different species associated with *Alternaria* toxins in feeds are not known [80]. The most occurrent toxin from *Alternaria* in this study was ALS with a mean concentration of 220 µg/kg DM but the maximum concentration reached 861 µg/kg DM. This toxin generated by species from the genus *Alternaria* has antimicrobial activity against several bacteria [81]. We also observed the co-occurrence of *Alternaria* mycotoxins with emerging *Fusarium* mycotoxins (such as ENNs and BEA, also with bactericidal properties) [60], thus ingestion of contaminated feed may have consequences for the ruminal bacterial community and functions that are important for the health and productivity of a ruminant.

Interestingly, we observed that the concentrations of both *Fusarium* and *Alternaria* metabolites responded to increasing temperature in a similar pattern with a critical temperature of 15 °C triggering the exponential increment of these metabolites. This matches with the fact that temperature is a primary determining factor implicated in the modulation of fungal growth and the subsequent mycotoxin production [82,83]. The effect on selective groups of fungal metabolites may suggest that the metabolism of these fungi driven by temperature may be interconnected. Fuchs et al., (2017) projected that the endophyte-mediated intoxications in livestock may increase on European grasslands with global warming [84]. The findings of the temperature effect reinforce the idea that global warming contributes to mycotoxin risk on crops [85–87]. Nevertheless, due to the small sample size, variations among the farms and short time of observation, the results presented in this exploratory study should be regarded as preliminary findings and thus must be interpreted with caution. Our results also suggest that the number of fungal metabolites was higher in pastures sampled later in the grazing season (July and October), which should be confirmed by future studies. Furthermore, the production of fungal secondary metabolites is mediated by several biotic and abiotic factors, [82], which cannot be entirely covered by the present study. Therefore, future studies with a larger sample size, more geographic locations and extended years of observation are pivotal to verify the current results regarding the critical temperature and its association with other geo-climatic and botanical factors for elevating mycotoxin contamination of pastures.

Phytoestrogens are produced, among other kinds of plants, by legumes such as *Trifolium prantense*, *T. repens* and *M. sativa*. [27]. The detected PEs in the present study belong to two different categories: isoflavones (biochanin A, daidzein, daidzin, glycitein,

genistein, genistin, onionine and sissotrine) and coumestans (coumestrol) [88,89]. The latter category seems to be more potent in inducing infertility problems [27], considering that coumestrol has a superior affinity to the 17β-estradiol than the isoflavone-derived equol [90]. Coumestrol can induce an acute or sub-acute decline of reproductive efficiency in sheep, cattle and horses [91–93]. The critical range of coumestrol in cattle feed was reported to be around 18–180 mg/kg [88]. In the current study, isoflavones were the predominant kind of PE and were detected in low quantities (7.9–129 µg/kg DM). Still, the impact of relatively low coumestan concentrations should not be ignored if the diet contains other xenoestrogens (e.g., isoflavones and mycoestrogens) [79], which were also present in the examined samples. Our results also underlined the co-occurrence of phytoestrogens and the mycoestrogen ZEN in pastures. Considering the estrogenic nature of both kind of compounds, an additive/synergistic interaction has been suggested [23]. Given the possibilities for synergistic effects of combinations of toxins, endocrine disruptors and other metabolites, these complex mixtures naturally occurring in pastures might be an underestimated risk for the health and productivity of dairy cattle, especially for high-producing cows with high feed intake.

4. Conclusions

The present study reveals that a broad range of mycotoxins, phytoestrogens and secondary metabolites are detected in pastures grown for dairy farming in Austria. Even though concentrations of individual fungal toxins and metabolites were generally low (often less than 200 µg/kg DM), the total fungal metabolite concentration could reach over 6000 µg/kg DM in pastures. Our data underline *Fusarium* as the major fungi in pastures. Still, the attention should also be paid to possibilities for high burdens of EAs and *Alternaria* mycotoxins in pastures. The preliminary data presented here suggests that an increment in the environmental temperature could drive the increased level of contamination from *Fusarium*, *Alternaria* and EAs in pastures. However, it should be further corroborated considering multifactorial influences from geo-climatic and botanical factors as well as year variations.

5. Materials and Methods

5.1. Sampling of Pastures

This study was part of a larger project surveying 100 dairy farms in the 3 states leading the country's dairy production (Lower and Upper Austria along with Styria) for detection of mycotoxins and implications for dairy performances. Of these 100 farms, 18 farms included partial grazing systems for the dairy cows and were selected for this study (Figure 5A). Under informed consent of the farmers, one representative sample of pasture was collected at a one-time point in each farm during the grazing season of 2019 (April–October). In this case, 8 farms were collected in April–June 2019 and 10 farms in August–October 2019. To obtain the representative sample of each farm 30 increment samples (Figure 5B) from a paddock being currently grazed were collected. Each incremental sample was taken from the area of 25 cm × 25 cm of pasture delimited by a metal frame. The pastures were cut 2–3 cm above the soil level using electric grass shears (Figure 5C). The 30 incremental samples were then composited, thoroughly mixed and approximately 1 kg of sample was taken, vacuum packed (−0.7 psi) and stored at −20 °C until sample preparation and analysis. The major botanical species of each sampled paddock were identified based on the morphological features of dissected specimens preserved in a herbarium by an expert. As identified, the sampled pastures contained mixtures of Gramineae (Family: Poaceae, including *Lolium perenne*, *Dactylis glomerata*, *Poa pratensis*, *Festuca pratensis*, *Alopecurus pratensis* and *Phleum pretense*) and Leguminosae (Family: Fabaceae; *Trifolium pretense*, *T. repens* and *Medicago sativa*). Visually, *Gramineae* were the dominating species of all pasture samples, but the exact proportions of individual species were not determined.

The climatic data (monthly averages of air temperature, air relative humidity and rainfall) of 2019 of the municipalities or districts were collected from the website of

the Austrian Agency of Meteorology and Geodynamics (Zentralanstalt für Meteorologie und Geodynamik-ZAMG, https://www.zamg.ac.at/cms/de/klima/klimauebersichten/jahrbuch). The pilot farms were in altitude ranges between 235–1340 m.a.s.l. The annual average temperature values in the areas of the farms ranged from 8.4 to 11.5 °C and the mean annual rainfall was between 502 to 954 mm, concentrated mostly during spring and summer. The average values of relative air humidity of the different locations during 2019 varied between 71.5 and 80%. Climatic data including temperature, humidity and rainfall (annual, monthly and 3-months averages) were checked and recorded for the correlation and regression analyses.

Figure 5. A representative sampling of pastures intended for multi-metabolite analysis. (**A**) Locations of the selected dairy farms (n = 18) in 3 Austrian federal states: Lower Austria, Upper Austria and Styria. (**B**) The sampling pattern (at least 30 incremental samples in a W shape) across a paddock that was being currently grazed at the time of sampling. Sample amount: ≥1–1.5 kg. (**C**) A quadrate (25 cm × 25 cm) used for sampling each incremental sample.

5.2. Mycotoxin Analysis

5.2.1. Chemicals and Reagents

Analytical grade reagents and chemicals were used for analysis. Glacial acetic acid (p.a.) and methanol (LC gradient grade) were acquired from Merck (Darmstadt, Germany); ammonium acetate (MS grade) from Sigma-Aldrich (Vienna, Austria) and acetonitrile (LC gradient grade) from VWR International (Leuven, Belgium). Standards of fungal, bacterial, plant and unspecific metabolites were acquired either via donation from various research institutions or purchased from commercial suppliers such as Romer Labs® Inc. (Tulln, Austria), Sigma-Aldrich (Vienna, Austria), Iris Biotech GmbH (Marktredwitz, Germany), Axxora Europe (Lausanne, Switzerland), LGC Promochem GmbH (Wesel, Germany), AnalytiCon Discovery (Potsdam, Germany), Enzo Life Sciences (Lausen, Switzerland), BioAustralis (Smithfield, Australia) and Toronto Research Chemicals (Toronto, Canada). Water was purified successively by reverse osmosis and an Elga Purelab ultra-analytic system from Veolia Water (High Wycombe, UK) to 18.2 MΩ. Stock solutions of each analyte were prepared by dissolving the solid substance, preferably at 250 µg/mL in acetonitrile, but depending on the respective solubility, a few compounds were dissolved in acetonitrile/water 1:1 (*v*/*v*), methanol or water instead as reported by Sulyok et al. [94]. Thirty-four combined working solutions were prepared to precede the spiking experiments by mixing the stock solutions of the corresponding analyte All solutions were stored at −20 °C and allowed to reach room temperature before the analysis.

5.2.2. Sample Preparation, Extraction and Estimation of Apparent Recoveries

The frozen pasture samples were thawed at room temperature for 24 h, then they were air-dried at 65 °C for 48 h. The average DM content of pasture samples was 22.3 ± 8.2% (range: 14.2–35.6%). The dried samples were sequentially milled to a final particle size of ≤0.5 mm. Firstly, the air-dried samples were processed using the cutting mill (SM 300, Retsch GmbH, Haan, Germany) at 1500 rpm for approximately 1 min. The remnant (mostly hard fragments of seeds) was subsequently milled using an ultra-centrifugal mill (ZM 200, Retsch GmbH, Haan, Germany) at 10,000 rpm for approximately 30 s. All milled fractions were combined and homogeneously mixed into one representative sample per farm.

Five grams (±0.01 g) of each homogenized sample were weighed into 50-mL polypropylene conical tubes (Sarstedt, Nümbrecht, Germany) and 20 mL of the extraction solvent (acetonitrile/water/acetic acid 79:20:1, *v*/*v*/*v*) was added. The samples were extracted on a GFL 3017 rotary shaker (GFL, Burgwedel, Germany) in a horizontal position at 180 rpm for 90 min. Then, the tubes were put in a vertical position for 10–15 min for sedimentation. A supernatant of 500 µL of the raw extract was diluted 1:1 with a dilution solvent (acetonitrile/water/acetic acid 20:79:1, *v*/*v*/*v*) in autosampler vials. The injection of 5 µL of the diluted raw extracts into the LC-MS/MS instrument was performed as described by Sulyok et al. 2020 [94]. Quantification was performed from external calibration by serial dilutions of a stock solution of multiple analytes. The results were corrected for apparent recoveries determined through spiking experiments [95].

5.2.3. LC-MS/MS Parameters

The chromatographic method and chromatographic and mass spectrometric parameters used in the current research were carried out at the Department of Agrobiotechnology (IFA-Tulln) at the University of Natural Resources and Life Sciences Vienna (BOKU) in Tulln, Austria and have been described detailed previously [94,95]. This fully validated method enables the accurate quantification of more than 500 fungal, bacterial, plant, lichenical and unspecific secondary metabolites, including all relevant mycotoxins. Analysis was performed with an Agilent 1290 Series HPLC System (Agilent, Waldbronn, Germany) coupled with a QTrap 5500 equipped with a TurboIonSpray electrospray ionization (ESI) source (Sciex, Foster City, CA, USA). Chromatographic separation was performed at 25 °C on a Gemini® C18-column, 150 × 4.6 mm inner diameter, 5 µm particle size, protected by a C18 security guard cartridge, 4 × 3 mm inner diameter (Phenomenex, Torrance, CA, USA). A methanol/water gradient containing 5 mmol/L ammonium acetate and 1% acetic acid was used at 1 mL/min.

Electrospray ionization-MS/MS was performed in the time-scheduled multiple reaction monitoring (MRM) mode both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. Qualitative and quantitative analyses were performed using Analyst, version 1.5 (AB Sciex, Framingham, MA, USA) and MultiQuant, version 2.0.2 (AB Sciex). The analyte identification was confirmed by the acquisition of two MRMs per analyte, yielding 4.0 identification points according to Commission Decision 2002/657/EC [18]. Furthermore, the LC retention time and the intensity ratio of the two MRM transitions agreed with the related values of an authentic standard within 0.1 min and 30% relative abundance, respectively. Quantification was based on external calibration (linear, 1/x weighted) using a serial dilution of a multi-analyte working solution. Results were corrected using apparent recoveries obtained through spiking experiments. The accuracy of the method is continuously validated by participation in a proficiency testing scheme organized by BIPEA (Gennevilliers, France) with a current rate of z-scores between −2 and 2 of >95% (>1500 results submitted).

5.3. Statistical Analysis

Descriptive statistics (occurrences and concentration values: average, median, minimum and maximum) were computed using only the positive values (x ≥ limit of detection (LOD)). Data below LOD were deemed not detectable. Metabolite concentrations below the respective limit of quantification (LOQ) were calculated as LOQ/2. The concentrations are presented on a DM basis in µg/kg–parts per billion (ppb) and on a logarithmic scale (Log10) where applicable. The co-occurrence analysis was performed constructing a matrix with the detection frequencies of the mycotoxins occurring ≥ 20% using Microsoft Excel and the heat map was elaborated by GraphPad Prism (Prism version 9.1, GraphPad Software, San Diego, CA, USA).

For correlations and climatic factors, the statistical analyses were performed using SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). A two-tailed Pearson correlation was accomplished (data not shown) to screen possible significant relationships between the

concentrations of the different groups of metabolites and climatic data, followed by the graphical evaluation. Subsequently, targeted pairs were evaluated in detail to quantify their responses. Linear regressions of the 3-month average temperature and the number of fungal metabolites per sample was performed using the Mixed procedure of SAS. The random effect of the farm was considered in the model. For the grouped fungal metabolites showing a non-linear relationship, then the NLIN procedure of SAS was used. An effect of sampling time, farm location or botanical diversity on the concentrations of grouped metabolites was evaluated using the MIXED procedure of SAS. The sampling time was grouped as early (sampled in April to June 2019, n = 8) or late (sampled in August–October 2019, n = 10). The farm location was designated to their federal state including Lower Austria (n = 5), Upper Austria (n = 5) and Styria (n = 8). Two groups of pasture diversity were defined including i) not diverse when one or two botanical species were identified in the samples (n = 11) and ii) diverse when three or more botanical species were detected (n = 7). The statistical model of each geo-climatic factor included a fixed effect of the test factor and a random effect of the farm. The resulting data reported are the least-squares means and standard error of the least-squares mean (SEM).

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/toxins13070460/s1, Table S1: List of 481 targeted metabolites via LC-MS/MS analysis. Compounds found in the pasture samples (values > the LOD) are located into grey cells, Table S2: Botanical composition of the sampled pastures.

Author Contributions: Conceptualization, F.P.-T., R.K.-a. and Q.Z.; methodology, F.P.-T., R.K.-a., T.J. and V.N.; software, F.P.-T. and R.K.-a.; formal analysis, F.P.-T., M.S. and R.K.-a.; investigation, F.P.-T.; data curation, F.P.-T. and M.S.; writing—original draft preparation, F.P.-T.; writing—review and editing, R.K.-a., J.F., M.S., T.J., V.N. and Q.Z.; visualization, F.P.-T.; supervision, R.K and Q.Z.; project administration J.F., V.N. and Q.Z.; funding acquisition, Q.Z. and V.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research was conducted as part of the Project "D4Dairy-Digitalization, Data Integration, Detection and Decision support in Dairying" supported by is supported by the Austrian Federal Ministry for Climate Action, Environment, Energy, Mobility, Innovation and Technology (BMK), Austrian Federal Ministry for Digital and Economic Affairs (BMDW) and the provinces of Lower Austria and Vienna within the framework of COMET-Competence Centers for Excellent Technologies., which is handled by the Austrian Research Promotion Agency (FFG).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Informed consent was obtained from all farmers involved in the study.

Data Availability Statement: Data available on request due to restrictions.

Acknowledgments: Open Access Funding by the University of Veterinary Medicine Vienna. We thank Remigius Chizzola (Institute of Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine) for the botanical identification of the specimens preserved in an herbarium and Annelies Müller (BIOMIN Research Center) for critically reading of the manuscript. The authors appreciate the excellent technical support provided by Manfred Hollmann, Anita Dockner, Arife Sener, Sabine Leiner, (Institute of Animal Nutrition and Functional Compounds, Vetmeduni, Vienna) and Thomas Erhäusl (BIOMIN Research Center). We express our gratitude to Marlene Suntinger, Franz Steininger and Christa Egger-Danner (ZuchtData EDV-Dienstleistungen GmbH) and the staff of LKV Austria Qualitätsmanagement GmbH-Federal Recording Association of Lower Austria, Upper Austria and Styria for the logistic support and the coordination with participating dairy farmers.

Conflicts of Interest: The authors declare no conflict of interest. N.V., J.F. and T.J. are employed by BIOMIN Holding GmbH, which operates the BIOMIN Research Center and is a producer of animal feed additives. This had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript or in the decision to publish the results.

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Supplementary Materials: Mycotoxins, Phytoestrogens, and Other Secondary Metabolites in Austrian Pastures: Occurrences, Contamination Levels, and Implications of Geo-climatic Factors

Felipe Penagos-Tabares, Ratchaneewan Khiaosa-ard, Veronika Nagl, Johannes Faas, Timothy Jenkins, Michael Sulyok and Qendrim Zebeli

Table S1. List of 481 targeted metabolites via LC-MS/MS analysis. Compounds found in the pasture samples (values > the LOD) are located into grey cells.

15-Acetyldeoxynivalenol	Aspyrone	Cladosporin	Emindole SA
15-Hydroxyculmorin	Asterric acid	Clonostachydiol	Emodin
15-Hydroxyculmoron	Atlantinon A	Colchicin	Endocrocin
2-Methylnitorubin	Atpenin A5	Communesin B	Enniatin A
3-Acetyldeoxynivalenol	Atropine	Cordycepin	Enniatin A1
3-Hydroxyterphenyllin	Atroventinmethylether	Coumestrol	Enniatin B
3-Nitropropionic acid	Aurantiamin A	Culmorin	Enniatin B1
4-Hydroxyalternariol	Aurantine	Curvularin	Enniatin B2
5-Hydroxyculmorin	Aurantioclavin	Curvulin	Enniatin B3
5-Methoxysterigmatocystin	Aurasperon B	cyclo(L-Leu-L-Pro)	Epiequisetin
5-Methylmellein	Aurasperon C	cyclo(L-Pro-L-Tyr)	Epoxyagroclavin
5-O-Methylsulochrin	Aurasperon G	cyclo(L-Pro-L-Val)	Epoxycytochalasin C
7-Hydroxykaurenolide	Aurofusarin	Cycloaspeptide A	Equisetin
7-Hydroxypestalotin	Austinol	Cycloheximide	Eremofortin A
Abscisic acid	Averantin	Cyclopenin	Eremofortin B
Acuminatum B	Averantinmethylether	Cyclopenol	Ergine
Acuminatum C	Averufanin	Cyclopeptine	Ergocornine
Aflatoxicol	Averufin	Cyclopiazonic acid	Ergocorninin
Aflatoxin B1	Bacitracin	Cylindrocarpon A4	Ergocristine
Aflatoxin B2	Bafilomycin A1	Cylindrol B	Ergocristinine
Aflatoxin G1	Barceloneic acid	Cytochalasin B	Ergocryptine
Aflatoxin G2	Bassianolide	Cytochalasin C	Ergocryptinine
Aflatoxin M1	Beauvericin	Cytochalasin D	Ergometrine
Aflatrem	Berkedrimane B	Cytochalasin E	Ergometrinine
Agroclavine	beta-Zearalenol	Cytochalasin H	Ergosin
Alamethicin	Bikaverin	Cytochalasin J	Ergosinin
alpha-Zearalenol	Biochanin	Daidzein	Ergotamine
Alteichin	Bis(methylthio)gliotoxin	Daidzin	Ergotaminine
Altenuene	BisdethioMTG	Deacetylneosolaniol	Ergovalin
Altenusin	Brefeldin A	Decalonectrin	Erucifolin
Alternariol	Brevianamid F	Dechlorogriseofulvin	Europin
Alternariolmethylether	Brevicomparine B	Dechlorononnidulin	Europin-N-Oxid
Altersetin	Butenolid	Dehydroaustinol	Fallacinol
Altersolanol	Butyrolacton III	Dehydrocurvularin	Fellutanine A
Altertoxin-I	Butyrolactone I	Dehydrocyclopeptine	Festuclavine
Amauromine	Calonectrin	Demethylsulochrin	Flavipucin
Aminodimethyloctadecanol	Calphostin	Deoxyfusapyron	Flavoglaucin
Amoxycillin	Calyxanthone	Deoxynivalenol	Fonsecin
Amygdalin	Carviolin	Deoxynortryptoquivalin	Formonetin
Anacin	Cephalochromin	Deoxytryptoquivaline A	Fulvic acid
Andrastin A	Cercosporamide	Desoxyverrucosidin	Fumagillin
Andrastin B	Cercosporin	Destruxin A	Fumagillol
Andrastin C	Cereulide	Destruxin B	Fumifungin
Andrastin D	Chaconin	Destruxin-Ed Derivat	Fumigaclavine
Andrastin Derivative	Chaetominine	Diacetoxyscirpenol	Fumigaclavine C

Anisodamine
Antibiotic F 1849 A
Antibiotic FF 1052
Antibiotic Y
Apicidin
Apicidin D2
Ascochlorin
Ascofuranone
Aspercolorin
Asperfuran
Asperglaucide
Asperlactone
Asperloxine A
Asperphenamate
Aspinolid B
Aspochracin A
Aspterric acid
Aspulvinone E
Fusarin C
Fusarinolic acid
Fuscofusarin
Geldanamycin
Genistein
Genistin
Gibberellic acid
Gibberellin A14
Gibepyron D
Gliotoxin
Glyantrypine
Glycitein
Glycitin
Griseofulvin
Griseophenone B
Griseophenone C
Harzianopyridine
Heliotrin
Heliotrin-N-Oxid
Helvolic acid
Helvolinic acid
Heptelidic acid
Herquline A
HT-2 Glucoside
HT-2 toxin
Hydroxycurvularin
Hydroxypaspaline
Hydroxysydonic acid
Hyoscin
Ilicicolin A
Ilicicolin B
Ilicicolin E
Ilicicolin F
Indicin/Intermedin/Lycopsamin
Indicin/Intermedin/Lycopsamin N-Oxid
Infectopyron
Integracin A
Integracin B
Isofusidienol
Iso-Rhodoptilometrin
Jacobin

Chaetoviridin A
Chanoclavin
Chevalone B
Chevalone C
Chlamydospordiol
Chlamydosporol
Chloramphenicol
Chlorocitreorosein
Chloronectrin
Chlortetracyclin
Chrodrimanin
Chrysogin
Chrysophanol
Citreohybridinol
Citreorosein
Citreoviridin
Citreoviridin C
Citrinin
Methylequisetin
Methylfuricone
Methylorsellinic acid
Methylsulochrin
Mevinolin
Mollicellin D
Monactin
Moniliformin
Monoacetoxyscirpenol
Monocerin
Monocrotalin
Mycophenolic acid
Mycophenolic acid IV
Myriocin
N-Benzoyl-Phenylalanine
Neocyclocitrinol
Neoechinulin D
Neosolaniol
Neoxaline
NG 012
Nidurufin
Nigericin
Nigragillin
Nivalenol
Nivalenol glucoside
Nonactin
Norlichexanthone
Norsolorinic acid
Norverrucosidin
Notoamide Derivative
Ochratoxin A
Ochratoxin alpha
Ochratoxin B
Ochratoxin C
O-Methylsterigmatocystin
O-Methylviridicatin
Ononin
Orsellinic acid
Oxaline
Oxyskyrin
Oxytetracyclin

Dichlordiaportin
Dihydrocitrinone
Dihydroergosine
Dihydrogriseofulvin
Dihydrolysergol
Dihydrosterigmatocystin
Dihydroxycalonectrin
Dihydroxymellein
DihydroxyZON Methylether
Dinactin
Diplodiatoxin
DON-3-glucoside
Doxycyclin
Echinulidin
Elymoclavine
Emericellamide A
Emericellamide C
Emericellamide E
Prelaptin
Prunasin
Pseurotin A
Puromycin
Purpactin A
Purpuride
Pyranonigrin
Pyrenocin A
Pyrenophorol
Pyripyropene A
Pyripyropene D
Pyrophen
Quadrone
Questiomycin A
Quinadoline A
Quinolactacin A
Quinolactacin B
Quinolone A
Radicicol
Rasfonin
Roquefortine C
Roquefortine D
Roridin A
Rubellin D
Rubrofusarin
Rugulosin
Rugulovasine A
Rugulusovin
Sambucinol
Scalusamid A
Sclerotinin A
Sclerotioramin
Sclerotiorin
Secalonic acid D
Secalonic acid F
seco-Sterigmatocystin
Senecionin_Senecivernin
Senkirkin
Siccanin
Siccanol
Sissotrine

Fumiquinazolin A
Fumiquinazolin D
Fumiquinazolin Derivat
Fumiquinazolin F
Fumitremorgin C
Fumonisin A1
Fumonisin A1 Vorstufe
Fumonisin A2
Fumonisin B1
Fumonisin B2
Fumonisin B3
Fumonisin B4
Fumonisin B6
Fungerin
Fusaproliferin
Fusapyron
Fusarenon-X
Fusaric acid
Ternatin
Terphenyllin
Terrein
Territrem B
Tetracycline
Thailandolide B
Tiamulin
Trichodermin
Trichotetronine
Trichothecolone
Trypacidin
Tryprostatin A
Tryprostatin B
Tryptophol
Tryptoquivaline A
Tryptoquivaline F
Tylosin
Unuigisin E
Usnic acid
Valinomycin
Vermistatin
Verrucofortine
Verrucosidin
Verruculotoxin
Versicolorin A
Versicolorin C
Violaceic acid
Violaceol I
Violaceol II
Viomellein
Viridicatin
Viridicatol
Viridicatum toxin
Xanthomegnin
Xanthotoxin
Zearalenone
Zearalenone-Sulfate
Zinndiol
Zinniamide
Zinniol

Josamycin	Papyracillic acid A	Skyrin
K-76 Derivative 4	Paraherquamide E	Solanin
Kojic acid	Paspalic acid	Sphingofungin B
Koninginin E	Paspalin	Sphingofungin D
Kotanin A	Paspalitrem A	Stachybotryamide
Lasiocarpin	Patulin	Stachybotrylactam
Lasiocarpin-N-Oxid	Paxillin	Staurosporin
Lecanoic acid	Penicillic acid	Stemphylperylenol
Linamarin	Penicillide	Sterigmatocystin
Lincomycin	Penicolinate	Sulochrin
LL-Z 1272e	Penigequinolone A	Surfactin A
Lolitrem B	Penitrem A	Surfactin B
Lotaustralin	Pestalotin	Sydonic acid
Macrosporin	Phenopyrrozin	Sydowinin A
Malformin A	Phomalactone	Sydowinin B
Malformin A2	Phomalone	T-2 Glucoside
Malformin C	Phomopsolide B	T-2 toxin
Marcfortine A	Physcion	T2-Tetraol
Marcfortine B	Pinselin	T2-Triol
Marcfortine C	Porritoxinol	Tensidol B
Meleagrin	Prehelminthosporol	Tentoxin
Methoxysterigmatocystin	Prehelminthosporollacton	Tenuazonic acid

Table S2. Botanical composition of the sampled pastures.

Sample	State	Botanical Composition (Dominant Species)[1]				
		1	2	3	4	5
1	LA	*Lolium perenne*	-	-	-	-
2	LA	*Lolium perenne*	*Trifolium repens*	*Dactylis glomerata*	-	-
3	LA	*Lolium perenne*	*Trifolium repens*	*Poa pratensis*	-	-
4	LA	*Phleum pratense*	*Trifolium repens*	-	-	-
5	LA	*Lolium perenne*	*Trifolium repens*	-	-	-
6	UA	*Lolium perenne*	*Trifolium repens*	*Medicago sativa*	-	-
7	UA	*Lolium perenne*	*Medicago sativa*	-	-	-
8	UA	*Lolium perenne*	*Trifolium pratense*	*Dactylis glomerata*	-	-
9	UA	*Lolium perenne*	*Trifolium repens*	-	-	-
10	UA	*Lolium perenne*	*Trifolium repens*	-	-	-
11	ST	*Lolium perenne*	*Alopecurus pratensis*	*Holcus lanatus*	*Poa pratensis*	-
12	ST	*Poa pratensis*	*Alopecurus pratensis*	*Festuca pratensis*	-	-
13	ST	*Dactylis glomerata*	*Phleum pratense*	*Trifolium repens*	*Cynosurus cristatus*	*Festuca pratensis*
14	ST	*Lolium perenne*	*Trifolium repens*	*Dactylis glomerata*	*Agrostis sp.*	-
15	ST	*Dactylis glomerata*	*Trifolium repens*	-	-	-
16	ST	*Lolium perenne*	*Medicago sativa*	*Buglossoides sp.*	*Cynosurus cristatus*	*Dactylis glomerata*
17	ST	*Lolium perenne*	*Trifolium repens*	-	-	-
18	ST	*Dactylis glomerata*	*Trifolium repens*	-	-	-

LA: Lower Austria; UP: Upper Austria; ST: Styria; [1] Taxonomical classification based on morphological keys.

3.2. Publication 2:

Fungal species and mycotoxins in mouldy spots of grass and maize silages in Austria.

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Mycotoxin Research (2022) 38:117–136. https://doi.org/10.1007/s12550-022-00453-3

Mycotoxin Research (2022) 38:117–136
https://doi.org/10.1007/s12550-022-00453-3

ORIGINAL ARTICLE

Fungal species and mycotoxins in mouldy spots of grass and maize silages in Austria

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Received: 20 September 2021 / Revised: 18 February 2022 / Accepted: 8 March 2022 / Published online: 26 March 2022

Abstract

Fungi and mycotoxins in silage can have detrimental consequences for both cattle and human health. This pilot study identified, via the routinary direct plating method, the dominant cultivable fungi in mouldy grass silages (GS) ($n = 19$) and maize silages (MS) ($n = 28$) from Austria. The profiles of regulated, modified, and emerging mycotoxins together with other fungal metabolites were analysed via LC-(ESI)MS/MS. *Penicillium roqueforti*, *Saccharomyces* spp., *Geotrichum candidum*, *Aspergillus fumigatus* and *Monascus ruber* were the most frequent fungal organisms identified. Other species including *Mucor circinelloides*, *Fusarium* spp. and *Paecilomyces niveus* were detected at lower frequencies. The presence of complex mixtures of toxic and potentially toxic compounds was evidenced by high levels and occurrences (≥ 50%) of *Penicillium*-produced compounds such as mycophenolic acid (MPA), roquefortines (ROCs), andrastins (ANDs) and marcfortine A. Mouldy silages contained toxins commonly produced by genus *Fusarium* (e.g. zearalenone (ZEN) and trichothecenes), *Alternaria* (like tenuazonic acid (TeA) and alternariol (AHO)) and *Aspergillus* (such as sterigmatocystin (STC)). Compared to those in GS, mouldy spots in MS presented significantly higher fungal counts and more diverse toxin profiles, in addition to superior levels of *Fusarium* spp., *Penicillium* spp. and total fungal metabolites. Generally, no correlation between mould counts and corresponding metabolites was detected, except for the counts of *P. roqueforti*, which were positively correlated with *Penicillium* spp. metabolites in mouldy MS. This study represents a first assessment of the fungal diversity in mouldy silage in Austria and highlights its potential role as a substantial contributor to contamination with complex mycotoxin mixtures in cattle diets.

Keywords Silage quality · Spoilage · Fungal contamination · Multi-mycotoxin analysis · Dairy farm

Introduction

Silage production is a widespread practice applied to preserve the nutritional value of forages for livestock feeding, using spontaneous lactic fermentation under anaerobic conditions (Muck et al. 2018). Grass silage (GS) and (whole plant) maize silage (MS) are the most frequently used dietary ingredients in modern dairy and beef farms in many countries, with GS being more widely used in Europe and MS in North America (Alonso et al. 2013; Wilkinson and Rinne 2018; Dänicke et al. 2020). Dairy farmers in several European countries store more than 90% of their forage production as silage (Alonso et al. 2013). These silages are produced by harvesting and chopping pastures and maize crops,

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which are subsequently stored under anaerobic conditions by compaction as well as airtight covers, mainly in trench/bunker silos and round bales (Resch et al. 2017).

In Austria, approx. 22% of the dairy farms feed cows a silage-free diet in order to match the haymilch (in German: "heumilch") standards, which does not allow the feeding with any kind of silage (BMLRT 2021). However, currently, most of the Austrian dairy farms feed their herds with silage year-round or seasonal green fodder plus silage. It has been estimated that Austrian dairy farms present an annual average intake of 3300 kg dry matter (DM)/cow/year of GS and 1200 kg DM/cow/year of MS (FAO, IDF, IFCN 2014). In 2019, 154,769 ha of grassland/pastures (primarily grasses, clovers and lucerne) and 85,684 ha of maize for silage were available for forage production in Austria. In practice, about 75% of the basic fodder is preserved by ensiling, which corresponded to approx. 2.55 million t DM of GS and 1.3 million t DM of MS in 2019 (Resch et al. 2021). Since the economic and dietary relevance of these silages for the cattle industry has been recognized, detailed information on safety concerning natural contaminants (such as mycotoxins) is required (Gallo et al. 2015a).

Despite its crucial role in livestock nutrition, silage quality assessment is often based only on chemical analysis (nutritional composition) without an additional evaluation of the occurrence of pathogenic/toxigenic microorganisms or toxins (Wambacq et al. 2016). Fungi and especially their toxic secondary metabolites—mycotoxins —have been shown to pose a health risk to ruminants, with silages as one of the main sources of exposure (Driehuis et al. 2008a; Ogunade et al. 2018). The fungal toxins produced on-field can persist during the ensiling process, endangering the feed safety (Storm et al. 2014). Even though the ensiling process inactivates most of the microorganisms involved in silage spoilage, some species of filamentous fungi such as *P. roqueforti*, *A. fumigatus*, *M. ruber* and *P. niveus* can tolerate the low pH, high levels of carbon dioxide and low availability of oxygen which occur during storage (Alonso et al. 2013; Wambacq et al. 2016). These moulds can therefore survive in the silos and proliferate when more oxygen is available leading to spoilage, thereby reducing the nutritional value, dry matter content and palatability of the silage. Ultimately, diverse fungi in silage can produce a wide spectrum of secondary metabolites (O'Brien et al. 2006) with different biological activities including immunosuppressive, hepatotoxic, nephrotoxic and neurotoxic effects in animals (Storm et al. 2008; Driehuis et al. 2018). When incorporated into the diets of dairy cows, mouldy silages may impair animal health and productivity (Fink-Gremmels 2008; Santos and Fink-Gremmels 2014). Some evidence suggests that sub-clinical disorders such as impaired rumen function or increased susceptibility to infections might be related to the impact of such complex mixtures of fungal secondary metabolites (Storm et al. 2008; Santos and Fink-Gremmels 2014). Exposure to mouldy feeds seems to induce a poorly characterized sub-clinical disorder described as mouldy silage syndrome (Santos and Fink-Gremmels 2014).

Recent research began to recognize possible synergistic interactions and consequences of long-term exposure to such mycotoxin mixtures and the importance of holistic and innovative approaches based on multi-mycotoxins analyses (Storm et al. 2014; Battilani et al. 2020). So far, research related to this topic has covered the study of fungal populations in silages (Alonso et al. 2013; Rodriguez-Blanco et al. 2020). Additionally, preharvest multi-mycotoxin surveys in maize (Hajnal et al. 2020; Kos et al. 2020) and grasses (Nichea et al. 2015; Penagos-Tabares et al. 2021) as well as postharvest in GS and MS have been carried out (Rasmussen et al. 2010; Shimshoni et al. 2013; Storm et al. 2014; Vandicke et al. 2019; Panasiuk et al. 2019; Reisinger et al. 2019; Rodríguez-Blanco 2019; Dänicke et al. 2020). However, research focused on a wide spectrum of storage-associated mycotoxins in mouldy silages is scarce and the risks of dietary contamination with mouldy spots of silage are not known. Furthermore, several studies suggested that MS represents a higher mycotoxicological risk compared to GS (Panasiuk et al. 2019; Reisinger et al. 2019; Dänicke et al. 2020). Therefore, this study aimed 1) to characterize the most recurrent spoiling fungal organisms (co-) occurring in GS and MS in Austrian dairy farms using the routinary fungal analysis and 2) to assess broad profiles of mycotoxins and other secondary fungal metabolites (> 400) presented in the mouldy portions of silages. The levels and diversity of mycotoxins and metabolites contained in mouldy spots of both silage types were statistically compared. Additionally, possible interrelationships between fungal counts and levels of mycotoxin/metabolites were investigated.

Materials and methods

Sampling procedure

With the consent of the farmers, samples were collected from a total of 35 dairy farms located in Lower Austria, Upper Austria, and Styria, corresponding to the three Austrian Federal states leading the country's milk production (Fig. 1a). The samples included in this pilot study were collected between May 2019 and August 2020, totalling 47 samples (19 samples of mouldy spots of GS and 28 of MS) from already opened and "ready to be fed" bunker/trench silos or round bales, which have been ensiled for at least 3 months. We aimed at sampling mouldy spots in silages, and thus, collecting a representative sampling of the complete silo as presented recommended by McElhinney et al. (2016) was not suitable for our goal. Samples from the available silos or bales fitting the aforementioned

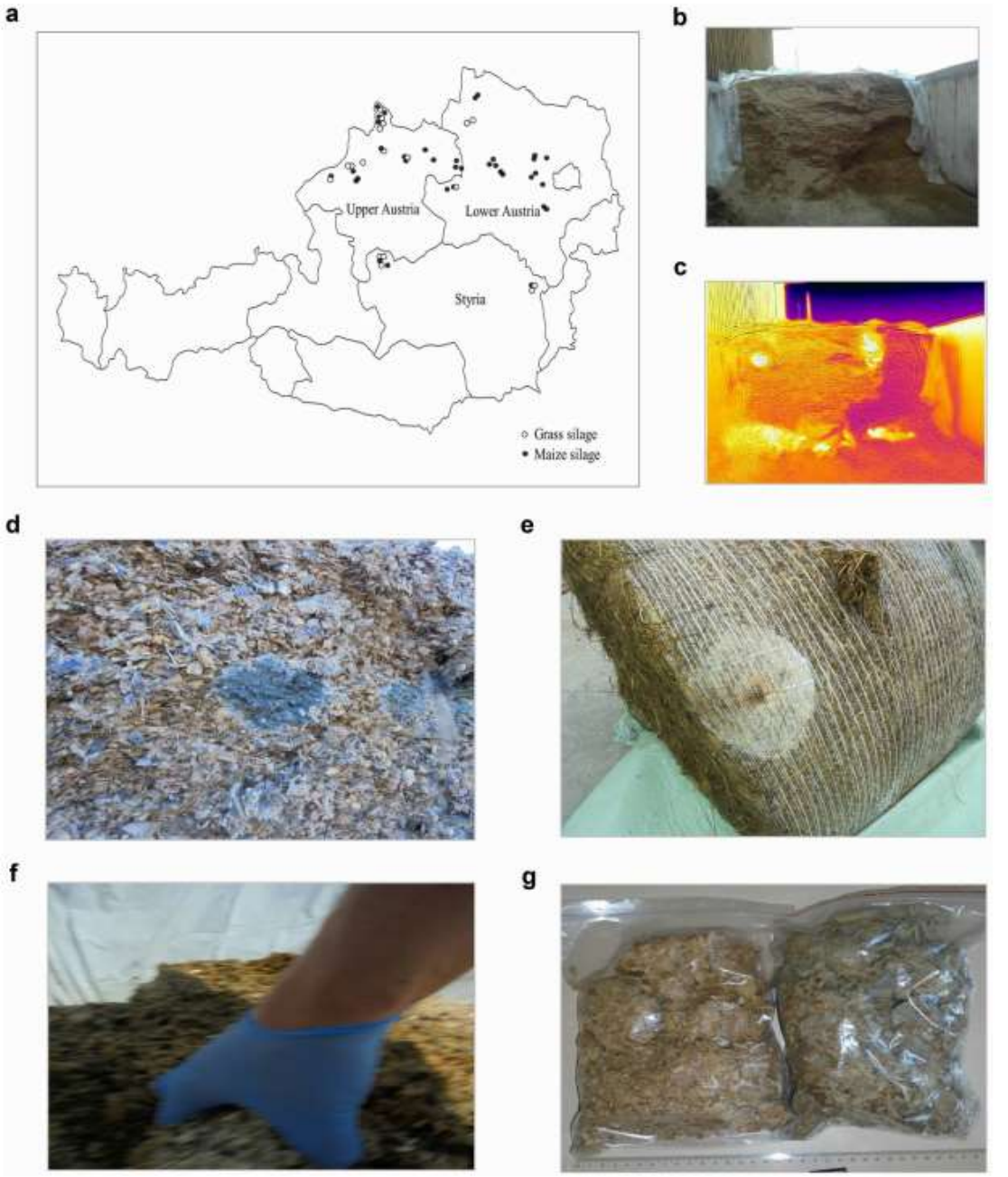


Fig. 1 Sampling of mouldy spots of grass and maize silages intended for feeding dairy cows **a** Map of Austria illustrating localization of surveyed samples. **b**, **c** Detection of mouldy spots via infra-red thermography in a ripped round bale of grass silage. **d** Visible mouldy spots of maize silage and **e** grass silage. **f** Sampling manually approx. 500 g of one hotspot with visible fungal growth per silo. Finally, **g** the samples were tightly sealed (the air was squeezed out) and stored at 4 °C in the dark until sample preparation

criteria across the pilot farms were collected and treated as individual samples independently as a means to account for the heterogeneity of the mouldy spots. Sections of silage with evidently dense fungal growth were detected via visual inspection (Fig. 1b) or by using thermal Imaging Camera FLIR ONE and FLIR Tools software (FLIR, Wilsonville, United States) (Fig. 1c). Per silo or bale, a subsample of a spot infested with apparent fungal growth (corroborated by observation of mycelial structures, Fig. 1e-d) was selected for sampling. Such mouldy hot spots were located in the superior and lateral sides of the trench/bunker silos and bales. The sampling consisted of the manual collection of one subsample of approx. 500 g on a wet weight basis of silage from one densely and compactly mould-colonized spot using nitrile gloves, superficially, not deeper than 20 cm (Fig. 1f). Each sample was subsequently stored in plastic bags, which were tightly sealed (the air was squeezed out) (Fig. 1g) and stored at 4 °C in the dark until arriving at the laboratory. Each sample of moulded silage was homogenized using a knife mill (Retsch GmbH, Haan, Germany; Type: GM200) at 10000 rpm for 10 s. Subsequently, 100 g was randomly selected for mycological evaluation and the remaining sample (approximately 400 g) was stored in the dark at -20 °C until further mycotoxin analysis.

Fungal identification (Plate Counting)

For mycological analysis, 20 g of the sample was mixed with180 ml of 0.1% peptone solution (achieving a 10–1 dilution) and further diluted until 10^{-4}. Dilution plating was carried out according to Samson et al. (2019), utilizing selective mycological media, namely, Malt Extract Agar (MEA; Merck, Darmstadt, Germany) supplemented with 100 µg/ml of chloramphenicol (Roth, Karlsruhe, Germany) and Dichloran Rose Bengal Chloramphenicol Agar (DRBC; Roth, Karlsruhe, Germany). These media have been used in studies of mycology of silages (O'Brien et al. 2005; O'Brien et al. 2007; Manfield and Kuldau 2007). For inoculation of the plates, 0.1 mL aliquots representing 10^{-2}, 10^{-3} and 10^{-4} dilutions were used, in triplicates. Plates were incubated at 25 °C for 5–7 days in the dark. Additional cultivation at 37 °C for 5 days was used for the isolation of opportunistic fungal pathogens. Each fungal colony isolated from a sample was considered as an individual isolate. Morphological identification of dominant fungal genera/species was performed by evaluation of macro- and microscopic morphological traits according to Samson et al. (2019) and de Hoog et al. (2020).

Multi-Mycotoxin analysis (LC–ESI–MS/MS)

For mycotoxin analysis, the frozen and previously milled sub-samples (approx. 400 g) were thawed for 12 h and subsequently dried at 65 °C in a ventilated oven for 48 h. Subsequently, the samples were milled through a 0.5-mm sieve using a cutting mill (SM 300, Retsch GmbH, Haan, Germany) at 1,500 rpm during approx.1 min. Five grams (± 0.01 g) of the homogenized samples were added to 50-ml polypropylene conical tubes (Sarstedt, Nümbrecht, Germany) and stored at – 20 °C until analysis. Glacial acetic acid (p.a.) and ammonium acetate (LC–MS grade) were purchased from Sigma-Aldrich (Vienna, Austria), HiPerSolv Chromanorm HPLC gradient grade acetonitrile was obtained from VWR Chemicals (Vienna, Austria), and LC–MS Chromasolv grade methanol was acquired from Honeywell (Seelze, Germany). Water was purified by reverse osmosis utilizing a Purelab Ultra system (ELGA LabWater, Celle, Germany). Standards of > 600 fungal and other secondary metabolites were acquired either via a donation from various research institutions or purchased from several commercial suppliers (Sulyok et al. 2020). Quantitative analysis of all relevant mycotoxins and other secondary metabolites was performed using a validated method based on liquid chromatography-electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) described by Sulyok et al. (2020). Briefly, 5 g of milled sample was deposited into a 250 ml Erlenmeyer flask along with 20 ml of extraction solvent. It was agitated for 90 min using a GFL 3017 rotary shaker (GFL, Burgwedel, Germany). Subsequently, the mixture was centrifuged for 2 min at 2,012 × g on a GS-6 centrifuge (Beckman Coulter Inc., Brea, CA, USA). The extract was transferred into glass vials and diluted 1:1 with dilution solvent. The injection volume of both raw extracts of the samples and the mycotoxin standard solutions was 5 µl. Identification and quantification of each mycotoxin were performed in the scheduled multiple reaction monitoring (sMRM) mode both in positive and negative polarity in two separate chromatographic runs using a QTrap 5500 LC–MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with a TurboV electrospray ionization (ESI) source was coupled to a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was accomplished by binary gradient elution. Quantification was based on external calibration using a serial dilution of a multi-analyte stock solution. Results were corrected for apparent recoveries determined during method validation according to Steiner et al. (2020). The accuracy of the method is verified by participation in a proficiency testing scheme with > 95% of the > 1600 results submitted so far exhibiting z-scores between -2 and 2. In particular,

15 out of 16 parameters submitted for a sample of whole-plant MS were in the satisfactory range with the exception being zearalenone (z = -2.04). The method used here has been employed to study multi-mycotoxin occurrence in diverse complex matrices of feedstuffs such as silage, pastures, concentrate feed and total mix rations (Shimshoni et al. 2013; Nichea et al. 2015; Kemboi et al. 2020; Penagos-Tabares et al. 2021; Awapak et al. 2021).

Statistical analysis

Occurrences and the descriptive statistics, i.e. minimum–maximum concentrations, median and mean values of the concentration of metabolites were calculated considering only the positive values (x ≥ limit of detection (LOD)). Concentrations of metabolites were presented on a dry matter basis in µg/kg. Values under the limit of quantification (LOQ) were computed as LOQ/2. To assess the significance of the differences between fungal counts and levels of mycotoxins and additional metabolites in mouldy GS and MS, a Mann–Whitney rank-sum test was performed, and statistical differences were considered significant at p-value < 0.05. A two-tailed Spearman's correlation test was conducted to explore possible relationships between fungal counts and levels of metabolites as well as relationships among metabolites within each kind of silage. For this, only data of metabolites with occurrence over 30% were considered. Spearman's correlation coefficients were considered significant at p-value < 0.05, and the interpretation was performed according to Schober et al. (2018). Accordingly, the correlation coefficients were considered significant at level p-value < 0.01 and the magnitude of the observed correlation was interpreted as "very strong" (0.90 up to 1.00), "strong" (0.70 up to 0.89) and "moderate" (0.40 up to 0.69) according to Schober et al. (2018). Linear regressions between fungal metabolites were performed to corroborate the promising relationships. The mentioned statistical analyses and graphs were performed using GraphPad Prism version 9.1 (GraphPad Software, San Diego, California, USA) and Microsoft® Excel®. Additionally, an effect of the occurrence of dominant mould species *P. roqueforti* on the concentration of *Penicillium* spp. metabolites was determined. For this purpose, the counts were classified into four groups: no (zero counts, $n = 13$), low (1×10^4 CFU/g – 5×10^5 CFU/g, $n = 19$), medium (1×10^6 CFU/g – 5×10^6 CFU/g, $n = 9$), and high (1×10^8 CFU/g, $n = 9$). Data were subsequently tested using a mixed model consisting of the fixed effect of the *P. roqueforti* group and the random effect of the kind of silage. The mixed model was analysed using PROC MIXED of SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). Pairwise comparisons of the resulting least-squares means were done using the PDIFF option, and significance was declared at p-value < 0.05.

Results

Occurrence and counts of fungal organisms

Seventeen distinct fungal organisms were detected in mouldy silages, consisting of 3 yeasts and 14 moulds identified at species or genus level (Fig. 2). Respectively, 12 different fungi in GS and 14 in MS were isolated. All samples were positive for moulds, whereas for yeasts only 68% and 75% of GS and MS were positive, respectively. The mould *P. roqueforti* was the most frequently isolated fungi in both types of mouldy silage, occurring specifically in 74% of GS and 71% of MS samples. For GS, the most common fungi were *Saccharomyces* spp. (47%), *M. ruber* (37%), *A. fumigatus* (26%), *G. candidum* (26%), *M. circinelloides* (16%), *Lichtheimia* (formerly *Absidia*) *corymbifera* (16%), *P. niveus* (formerly *Byssochlamys nivea*) (16%) and with lower incidence *Scopulariopsis brevicaulis* (11%) and *Hypopichia burtonii* (5%) as well as *Acremonium* sp. (5%). After *P. roqueforti,* MS samples were mostly contaminated with *G. candidum* (46%), *Saccharomyces* spp. (43%), *P. niveus* (36%), *A. fumigatus* (29%), *M. ruber* (29%), *M. circinelloides* (25%), *L. corymbifera* (14%) and *Pseudallescheria boydii* (14%). With occurrences under 10%, *Rhizomucor pusillus, F. verticillioides, Fusarium* spp., *Paecilomyces variotii* and *Verticillium* sp. were detected exclusively in MS. As shown in Supplementary Figure S1, in mouldy GS, *P. roqueforti* frequently co-occurred with *Saccharomyces* spp. (32%), *G. candidum* (21%), *M. ruber* (16%) and *A. fumigatus* (16%), while *Saccharomyces* spp. co-occurred with *A. fumigatus* (26%) and *M. ruber* (26%) as well as *M. ruber* with *A. fumigatus* (16%) and *L. corymbifera* (16%). In mouldy MS, *P. roqueforti* frequently co-occurred with *P. niveus* (32%), *G. candidum* (29%), *M. ruber* (29%) and *M. circinelloides* (18%), along with *M. ruber* and *P. niveus* (18%) (Supplementary Figure S1).

Mouldy spots of MS presented significantly superior total fungal counts, i.e. the sum of moulds and yeasts (p-value < 0.001) and total mould counts (p-value < 0.001) but not total yeast counts compared with the GS (Fig. 2). Total fungal count ranged from 1×10^4 CFU/g to 1.5×10^7 CFU/g in samples of GS and from 2.5×10^6 CFU/g to 2.2×10^7 CFU/g in MS (Fig. 2, Supplementary Table S1). No statistical differences between GS and MS were observed for the counts of other identified fungal organisms. The highest counts in mouldy GS were *M. circinelloides*, followed by *M. ruber*, *L. corymbifera*, *P. roqueforti* and *H. burtonii*, which presented average counts of over 1×10^6 CFU/g.

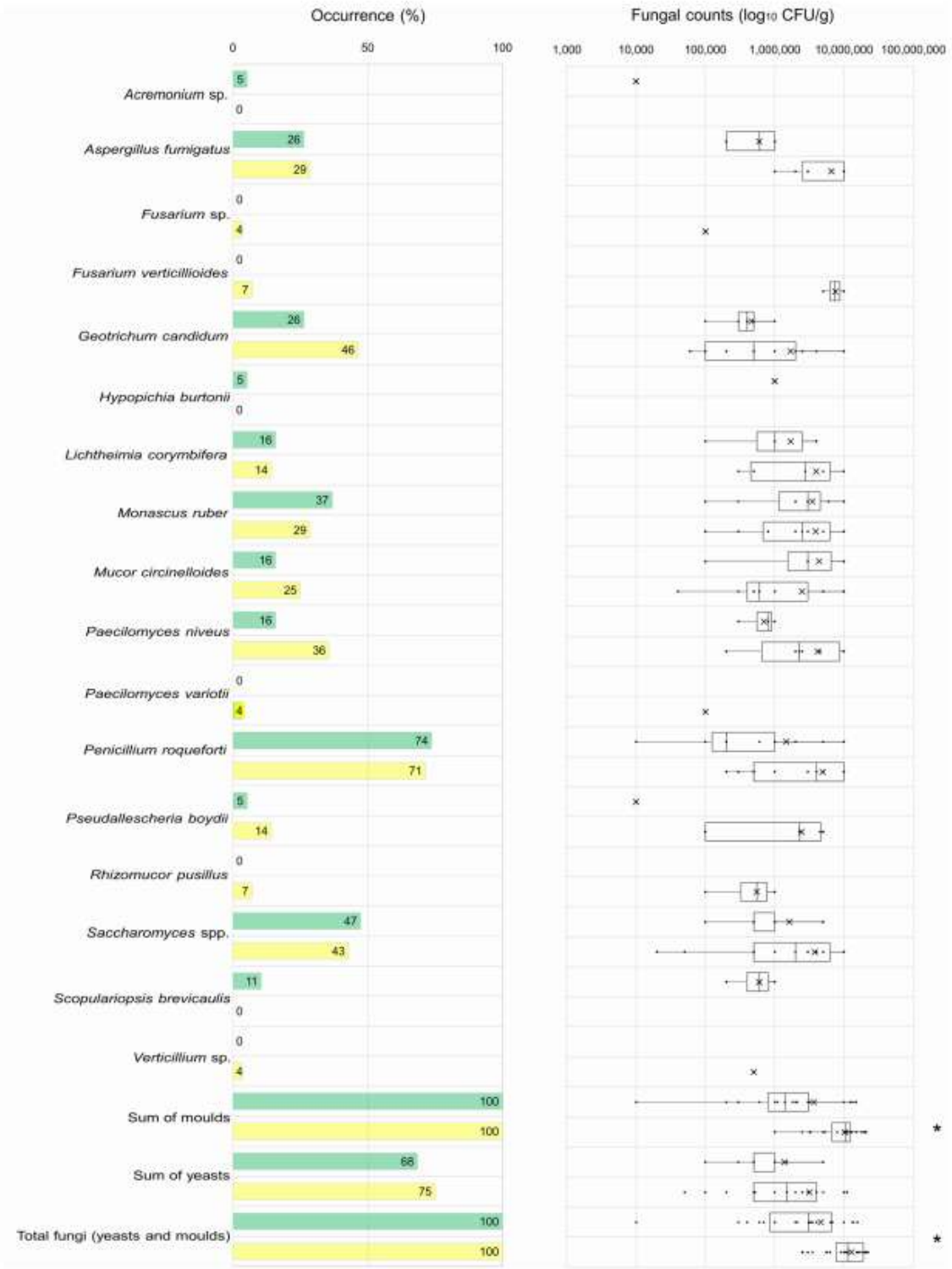


Fig. 2 Occurrences and counts (CFU/g) of fungal species isolated from mouldy grass (green) and maize silages (yellow)* Significantly different (p-value < 0.05)

Compared to mouldy GS, the analysed MS samples displayed superior average counts of *P. boydii*, *P. roqueforti*, *P. variotii*, *M. circinelloides*, *M. ruber*, *H. burtonii* and *G. candidum*, *F. verticillioides* and another *Fusarium* sp.

Occurrence and concentrations of mycotoxins and other secondary metabolites

General overview

A total of 106 and 83 secondary metabolites were detected across all MS and GS samples, respectively (Supplementary Table S1). To simplify the results' presentation along with their interpretation, the detected metabolites were classified by major producers based on previous reports with some modifications (Szulc et al. 2019; Hajnal et al. 2020; Penagos-Tabares et al. 2021) in the following categories: *Alternaria* spp. (5), *Aspergillus* spp. (23), *Fusarium* spp. (32), *Penicillium* spp. (16), other fungi (8), unspecific (19) and ergot alkaloids (EAs) (3). Figure 3 illustrates the occurrences and concentrations (mean, median, maximum and minimum) of the mentioned groups. Among the identified producers, metabolites mainly produced by *Penicillium* spp. were the most frequently detected and were found in all the samples of mouldy MS and 95% of GS. The highly diverse fusarial metabolites were positive in 100 and 89% of mouldy MS and GS, respectively. Diverse metabolites from *Aspergillus* spp. were also evident (Supplementary Table S1) but were detected in a lower frequency across the evaluated samples (82% in MS and 63% in GS, Fig. 3). Lower numbers of EAs metabolites, as well as metabolites derived from genus *Alternaria*, and other fungi (Supplementary Table S1) were detected in over 60% of the evaluated samples (Fig. 3). When comparing the two silages, MS samples presented significatively higher levels of total EAs (p-value = 0.045) as well as of total metabolites derived from *Fusarium* spp. (p-value < 0.001), *Penicillium* spp. (p-value = 0.017) and fungi (p-value < 0.001). All samples contained considerable amounts of unspecific metabolites, ranging from 602 µg/kg to13,400 µg/kg in GS and from 316 µg/kg to 17,500 µg/kg in MS (Fig. 3).

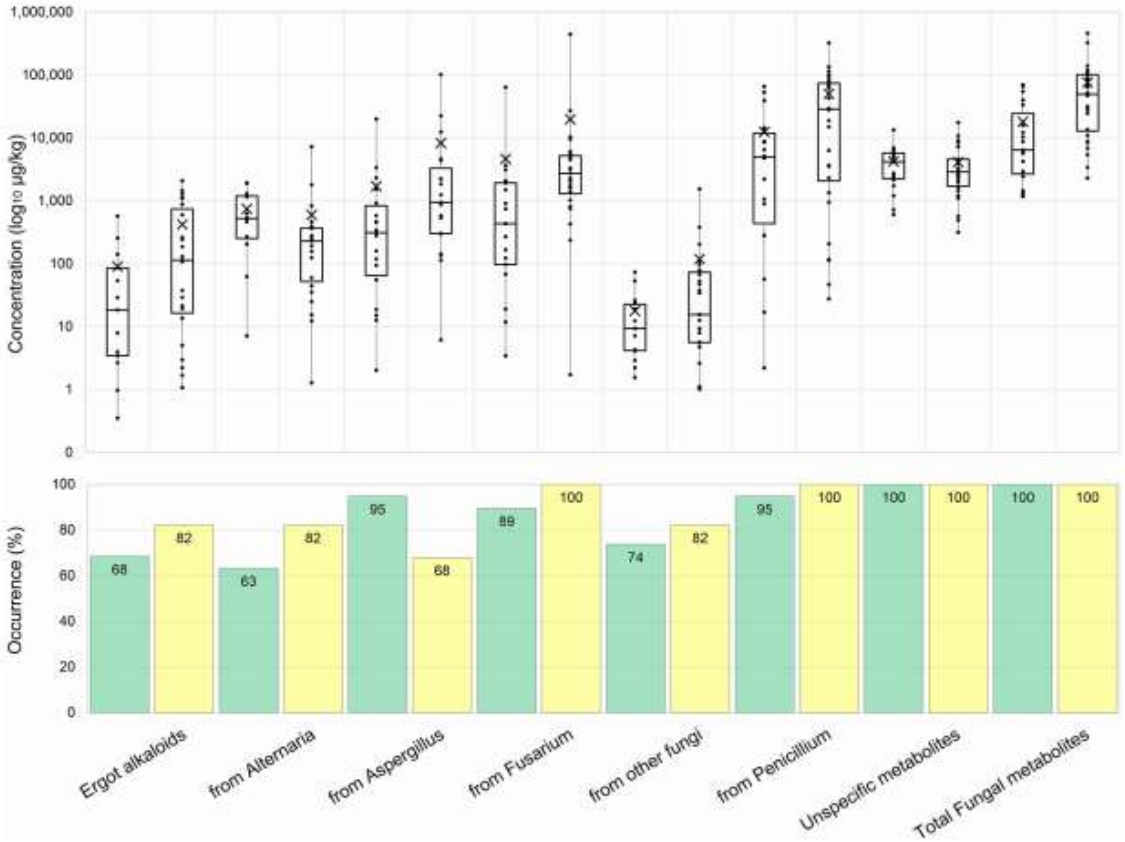


Fig. 3 Occurrences and concentration of grouped mycotoxins, other fungal and unspecific metabolites detected in mouldy spots of grass (green) and maize silages (yellow)* Significantly different (p-value < 0.05)

Table 1 Occurrences and levels of selected mycotoxins and metabolites detected in spots of mouldy grass and maize silages

Group	Metabolites	Grass silage ($n = 19$)							Maize silage ($n = 28$)						
		Positive Samples (%)[a]	Concentration[b]						Positive Samples (%)[a]	Concentration[b]					
			Average ± SD			Median	Range			Average ± SD			Median	Range	
Ergot alkaloids	**Agroclavine**	11	2.47	±	0.3	2.47	2.25	-	2.68	32	8.06	±	7.35	6.43	1.44	-	23.1
	Chanoclavine	58	36.5	±	73.6	5.78	0.16	-	225	54	160	±	445	18.7	0.17		1740
	Festuclavine*	63	63.7	±	123	14.9	0.35	-	435	82	313	±	444	86.9	1.07	-	1360
***Alternaria* spp.**	**Alternariol**	16	10.2	±	12.2	4.35	2.09	-	24.2	29	2.34	±	3.36	1.14	0.3	-	10.4
	Alternariolmethylether	26	4.13	±	2.3	3.5	1.6	-	7.32	29	1.78	±	1.62	1.31	0.13	-	4.71
	Altersetin	32	176	±	315	58.1	5.13	-	818	36	13.6	±	9.85	12.6	1.11	-	31.6
	Tenuazonic acid	53	781	±	552	569	195	-	1920	61	785	±	1720	275	57.2	-	7270
	Averufin	21	2.75	±	2.64	2.08	0.34	-	6.51	7	2.01	±	0.78	2.01	1.46	-	2.56
***Aspergillus* spp.**	**Bis(methylthio)gliotoxin**	11	133	±	184	133	2.19	-	263	32	152	±	242	63.8	6.53	-	756
	Fumigaclavine	26	276	±	557	5.34	1.56	-	1270	32	563	±	729	212	1.08	-	2040
	Fumigaclavine C	37	1800	±	4000	81.3	11.3	-	10,780	36	3950	±	7430	857	5.61	-	23,290
	Gliotoxin	5						79.3		14	47	±	46.6	46.8	5.09	-	89.2
	Helvolic acid	5						131		18	406	±	703	76.4	53.4	-	1660
	Kojic acid	21	63.2	±	44.5	43.5	36.2	-	129	43	97.7	±	103	54.3	16.1	-	353
	Sterigmatocystin*	37	6.89	±	9.79	1.3	0.09	-	26.6	7	2.49	±	3.2	2.49	0.23	-	4.75
***Fusarium* spp.**	**15-Hydroxyculmorin***	5						16.3		46	143	±	192	76.5	33.7	-	742
	alpha-Zearalenol									11	62.1	±	56.3	61.4	6.08	-	119
	Apicidin*	5						7.92		71	23.8	±	25.4	17.2	3.81	-	111
	Aurofusarin*	32	35.5	±	23.2	41.2	4.07	-	59.9	75	61.8	±	50.5	41	3.92	-	171
	Beauvericin*	47	19.7	±	40.4	1.83	0.2	-	125	86	30.1	±	36.7	17.7	3.93	-	153
	Bikaverin									46	9.7	±	5.68	7.04	3.53	-	22.7
	Chrysogine	53	34.4	±	31.8	23.1	4.61	-	102	29	6.64	±	5.07	4.44	2.35	-	15.8
	Culmorin	42	83.8	±	65.1	62.7	5.77	-	179	79	302	±	366	199	20.7	-	1360
	Deoxynivalenol	16	19.6	±	10.2	20	9.24	-	29.6	79	291	±	285	224	30	-	1220
	Enniatin A	37	1.36	±	1.75	0.81	0.02	-	4.9	43	0.85	±	0.81	0.67	0.01	-	2.17
	Enniatin A1	58	3.89	±	6.01	2.2	0.17	-	20.3	75	10.9	±	14.3	4.27	0.2	-	51.4
	Enniatin B	84	11.1	±	13.3	6.37	0.27	-	44.5	86	8.26	±	10.5	4.94	0.11	-	44.7
	Enniatin B1	68	12.6	±	21.1	7.19	0.64	-	80.7	68	19.4	±	26.8	7.16	0.05	-	95.3
	Enniatin B2	26	0.7	±	0.79	0.44	0.14	-	2.08	32	0.46	±	0.32	0.42	0.11	-	1.06

Table 1 (continued)

Group	Metabolites	Grass silage ($n = 19$)							Maize silage ($n = 28$)						
		Positive Samples (%)[a]	Concentration[b] Average ± SD		Median	Range			Positive Samples (%)[a]	Concentration[b] Average ± SD		Median	Range		
	Epiequisetin	37	7.15	± 8.45	2.65	1.01	-	22.3	46	6.42	± 6.95	3.45	0.3	-	23.5
	Equisetin	47	39.4	± 67.1	8.55	0.65	-	181	46	9.16	± 11.2	4.12	1.23	-	41.9
	Fumonisin B1								75	88.4	± 79.0	58.8	14	-	356
	Fumonisin B2								50	28.7	± 22.4	25.6	10.1	-	97.8
	HT-2 toxin								21	16.8	± 9.65	14.6	4.81	-	31
	Moniliformin	5					8.47		29	5.76	± 5.06	4.46	1.56	-	17
	Nivalenol	5					*36*		89	281	± 219	191.1	38.9	-	852
	Siccanol	47	8130	± 20,680	1400	200	-	63,200	82	3200	± 5380	1580	154	-	26,100
	Zearalenone	21	178	± 327	20.2	3.43	-	668	61	15	± 14.4	10.6	2.08	-	53.9
Penicillium spp.	**Andrastin A**	84	1030	± 1850	90.8	4.02	-	5840	86	3860	± 4160	2170	19.6	-	13,100
	Andrastin B	74	508	± 718	140	6.96	-	2270	79	3670	± 4300	1900	5.81	-	14,100
	Andrastin C	84	9580	± 14,800	723	71.3	-	36,720	79	45,200	± 58,100	32,800	21.5	-	252,100
	Marcfortine A	63	201	± 531	16.7	4.11	-	1880	68	2030	± 3060	777	1.01	-	12,900
	Mycophenolic acid	79	2530	± 2740	1960	18.1	-	7450	82	5570	± 9130	2000	2.59	-	30,900
	Mycophenolic acid IV	63	108	± 155	57.1	1.57	-	570	68	199	± 307	50.5	0.41	-	1050
	Questiomycin A	11	27.4	± 29.6	27.4	6.46	-	48.3	64	27.3	± 33.1	15.2	4.24	-	111
	Roquefortine C	79	2270	± 2940	1150	64.5	-	10,900	86	6360	± 6080	6530	6.36	-	20,000
	Roquefortine D	58	756	± 1340	160	32.7	-	4400	50	6220	± 9690	1970	129	-	31,200

*Significantly different (p-value < 0.05)

[a]Samples with values > limit of detection (LOD)

[b]Excluding data < LOD. In case values > LOD and < limit of quantification (LOQ), LOQ/2 was used for calculation

Selected mycotoxins and fungal metabolites

The occurrence, concentrations (mean, median and range) as well as the differences of selected mycotoxins levels between both kinds of silages are presented in Table 1. Other less known and lower recurrent mycotoxins and metabolites are given in Supplementary Table S3. Regarding mycotoxins contemplated in European legislation, GS samples presented relatively low frequencies (16% and 21%) of deoxynivalenol (DON) and zearalenone (ZEN) in comparison with the MS samples that were over 60% positive for both mycotoxins. Despite the low occurrence in GS, the maximum concentration of ZEN (668 µg/kg) exceeded the EU guidance level of 500 µg/kg (for ZEN in complementary and complete feedingstuffs for dairy cattle) (EC 2006), whereas ZEN ranged only from 2.08 µg/kg to 53.9 µg/kg in MS. All samples were negative for aflatoxin B1, ochratoxin A and T-2 toxin.

The fusarial mycoestrogen, alpha-zearalenol (α-ZEL) (11% occurrence) along with HT-2 toxin (21%), types B of fumonisins (FB) (1,2,3, and 4) (75%, 50%, 11% and 11%, respectively), nivalenol (NIV) (89%), fusaric acid (FA) (18%), butanolide (14%) and monoacetoxyscirpenol (MAS) (4%) were detected only in MS (Table 1). The most recurrent *Fusarium*-related mycotoxin in GS belonged to the enniatin (ENN) group: ENN B (84%), ENN B_1 (68%) and ENN A1 (58%). In MS, DON, NIV and FB_1, ENN A and B, beauvericin (BEA), siccanol, culmorin, aurofusarin and apicidin occurred in over 70% of the samples. The metabolites related to *Fusarium* spp. with the highest average concentrations were siccanol (8130 µg/kg) and fusaric acid (83300 µg/kg). In comparison with GS, MS samples showed significantly superior levels of DON (p-value < 0.001), NIV (p-value < 0.001), FB_1 (p-value < 0.001), FB2 (p-value < 0.001), ENN A_1 (p-value = 0.041), BEA (p-value < 0.001), aurofusarin (p-value = 0.004), bikaverin (p-value < 0.001), culmorin (p-value < 0.001), and apicidin (p-value < 0.001). Interestingly, the concentrations of siccanol (p-value = 0.015), ZEN (p-value = 0.0162) and chrysogine (p-value = 0.016) were significatively higher in GS samples.

Regarding *Penicillium*-derived metabolites, andrastins (AND) A, B, and C, marcfortine A, mycophenolic acid (MPA), MPA IV as well as roquefortines (ROQ) C and D were found in both silages in frequencies ≥ 50% (Table 1). Citrinin was detected only in one GS sample (99.7 µg/kg). The *Penicillium* mycotoxins with highest average concentrations in GS samples were AND C (9580 µg/kg), MPA (2530 µg/kg), ROQ C (2270 µg/kg) and AND A (1030 µg/kg). For MS samples, the metabolites with highest average concentrations were AND C (45,200 µg/kg), ROQ C (6360 µg/kg), ROQ D (6220 µg/kg), MPA (5570 µg/kg), AND A (3860 µg/kg), AND B (3670 µg/kg) and MAC A (2030 µg/kg). The samples of MS presented significantly higher concentrations of AND A (p-value = 0.003), questiomycin A (p-value < 0.001) and chevalone C (p-value < 0.001) compared to those in GS samples. The metabolite pestalotin was detected only in mouldy MS (Table 1, Supplementary Table S3).

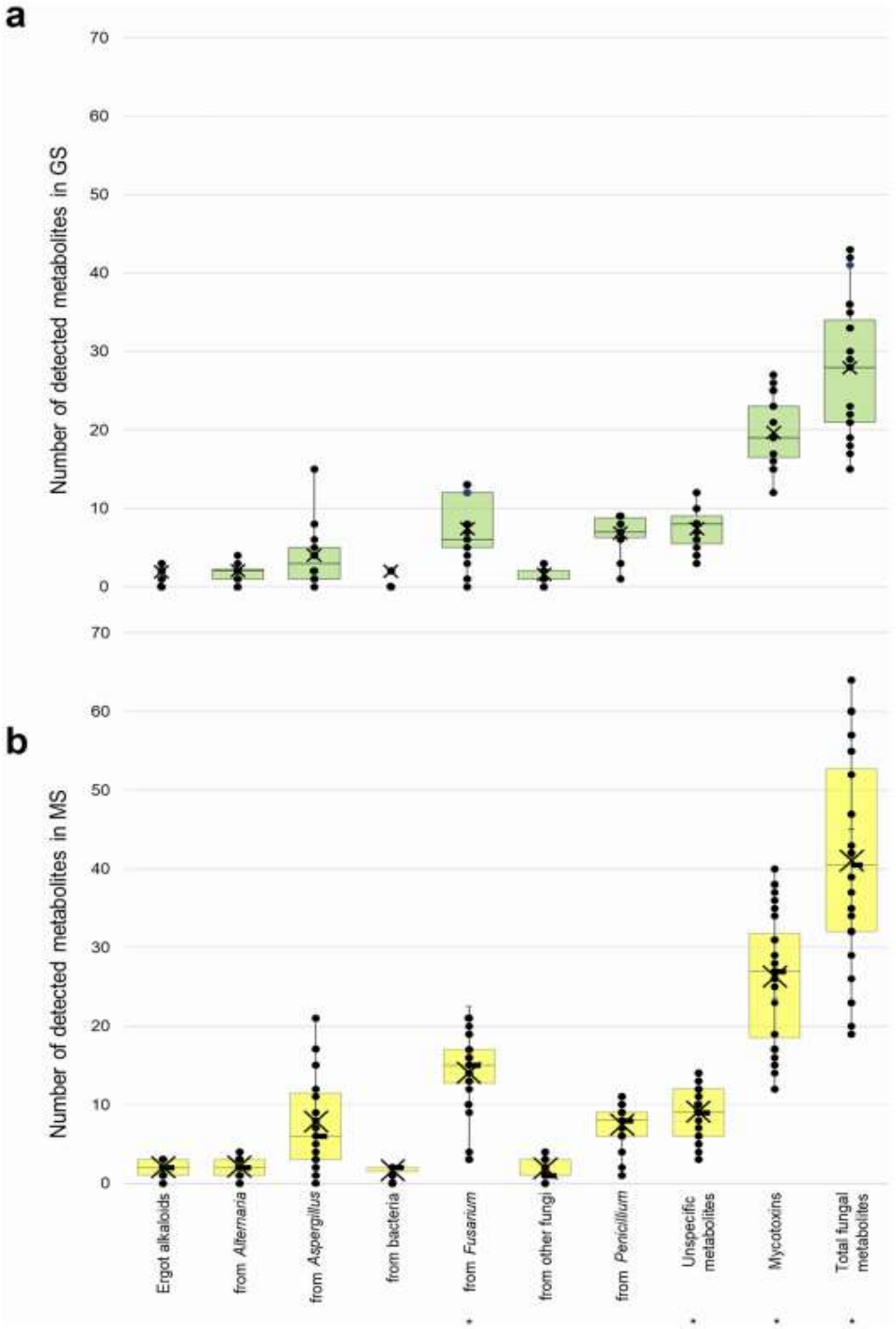
Fig. 4 Co-occurrence of grouped mycotoxins, other fungal and unspecific metabolites detected in mouldy spots of grass (green) and maize silages (yellow)* Significantly different (p-value < 0.05)

Three clavine alkaloids were found both silages: festuclavine (FES) (MS:82%, GS:63%), chanoclavine (MS:54%, GS:58%) and agroclavine (MS:32%, GS:11%) (Supplementary Table S2). The concentrations of these EAs were generally higher in MS compared to GS, but only FES (most produced EA in both groups of silages) reached significance (p-value = 0.026) (Supplementary Table S2). Tenuazonic acid (TeA) was the most frequent mycotoxin produced by *Alternaria* spp. detected in both GS and MS (53% and 61%, respectively) and with a lesser frequency alternariol (AHO) and alternariol-methyl-ether (AME) (< 40% and the concentrations under 1000 µg/kg) (Table 1). Both silage groups did not differ in the concentration of *Alternaria*-derived compounds.

Regarding *Aspergillus*-derived metabolites, the mycotoxins sterigmatocystin (STC), bis(methylthio)gliotoxin, gliotoxin, fumiquinazolines (FQ) A and D, fumigaclavine (FM) and fumigaclavine C (FMC) were detected. Their occurrences were under 40% for both GS and MS. FQA and FMC were the *Aspergillus*-derived mycotoxins with the highest average concentrations (over 3800 µg/kg) in MS. Despite having a higher average, sphingofungin B (7250 µg/kg) was found at a lower frequency (11%) (Supplementary Table S2). Likewise, GS samples also presented a predominant production of FMC and FQA, corresponding to average concentrations of 1800 µg/kg and 433 µg/kg. Interestingly, GS showed significantly higher contamination levels of SCT than MS (p-value = 0.0113) (Table 1). Other metabolites produced by other fungi and by organisms from other kingdoms (such as Bacteria and Plantae) are included in the Supplementary Table S1. Metabolites designated mycotoxins but also produced by plants, such as emodin (GS:95%, MS: 89%) and 3-Nitropropionic acid (GS:26%, MS: 54%) were also detected (Table 1). Differences between the mycotoxin content in mouldy MS and GS during the years 2019 and 2020 were analysed via Mann–Whitney Test. The metabolites with significant differences and the respective concentrations (average and median) are listed in the supplementary Table S3.

Co-occurrence analysis of mycotoxins and other fungal metabolites

All samples were co-contaminated with several mycotoxins and other fungal metabolites. Figure 4 shows the average, median and range of co-contamination (i.e. the number of metabolites detected per sample) of different groups of


a
Number of detected metabolites in GS
70
60
50
40
30
20
10
0
b
Number of detected metabolites in MS
70
60
50
40
30
20
10
0
Ergot alkaloids
from Alternaria
from Aspergillus
from bacteria
from Fusarium
from other fungi
from Penicillium
Unspecific metabolites
Mycotoxins
Total fungal metabolites

metabolites per silage type. GS had an average of 20 mycotoxins, with samples ranging from 12 to 27, whereas MS presented a mean of 26, varying from 19 to 64 mycotoxins. The number of *Fusarium* spp. metabolites (p-value < 0.001), total fungal metabolites (p-value < 0.001) and total mycotoxins (p-value < 0.003) was higher in MS than GS. Figure 5 illustrates the most common combinations of mycotoxins detected in GS and MS. Accordingly, the co-occurrence of several combinations of metabolites derived mostly from *Fusarium* spp. and *Penicillium* spp. in both mouldy silages was evident. Particularly in GS, over 50% of the samples presented a combination of ENN B and *Penicillium*-derived toxins AND A, AND B, AND C, ROQ C, MPA and MPA IV. MS also showed co-occurrence of ENNs, NIV, DON, FB1, ZEN ≥ 50%, and many of the previously mentioned toxins produced by *Penicillium* spp.

Relationship between fungal counts and concentrations of groups of metabolites

Spearman's correlations between total counts of fungi, moulds, and *P. roqueforti* and the groups of metabolites were mainly weak in GS. However, in MS, a positive moderate correlation ($\rho = 0.68$, p-value < 0.001) between the counts of *P. roqueforti* and the group of *Penicillium*-derived metabolites was found (p-value < 0.05). According to the mixed model analysis, as shown in Supplementary Figure S2a, a significant increase in the concentration of *Penicillium*-derived metabolites (60,000–65,000 µg/kg) was found with the groups with medium ($\times 10^6$ CFU/g) and high ($\times 10^8$ CFU/g) counts of *P. roqueforti* compared to the groups with non-detectable counts (0 CFU/g) and the low count group ($10^4 - 10^5$ CFU/g) of *P. roqueforti* (p-value < 0.05).

Relationship between concentrations and groups of mycotoxins and metabolites

In GS, a strong positive correlation ($\rho = 0.81$, p-value < 0.001) between *Penicillium* spp. metabolites and total fungal metabolites was evident (Supplementary Figure S2b and c). Specifically, the total of *Penicillium*-derived metabolites was strongly correlated with AND A ($\rho = 0.81$, p-value < 0.001), AND B ($\rho = 0.82$, p-value < 0.001), MPA ($\rho = 0.72$, p-value < 0.001), MPA IV ($\rho = 0.74$, p-value < 0.001) and ROQ C ($\rho = 0.81$, p-value < 0.001). However, only AND A, B and C in addition to ROQ C and D showed significance in the regression analysis. A strong relationship ($\rho = 0.80$, p-value < 0.001) between total *Penicillium*-produced and total fungal metabolites) was detected for both MS and GS (Supplementary Figures S2b, c and d). Additionally, metabolites associated with *Aspergillus* spp. presented a moderate relationship ($\rho = 0.73$, p-value < 0.001) with the unspecific metabolites (Supplementary Figure S2e). The mycotoxins DON was strongly correlated with ZEN ($\rho = 0.80$, p-value < 0.001) in MS (Supplementary Figure S2f). The correlation between FES and some of the *Penicillium* spp. toxins and metabolites (AND A, ROQ C and ROQ D) in samples of mouldy GS was confirmed by regression analyses (Supplementary Figure S2g).

Discussion

Mould contamination and associated mycotoxin production in silages are commonly occurring concerns in dairy farming and animal nutrition since mould growth deteriorates both nutritional and organoleptic properties of silage. Our results reveal the diversity of organisms co-occurring in individual samples of mouldy silages from Austria dairy farms as well as the presence of complex metabolites mixtures, which contain dozens of compounds with toxic or potentially toxic activity. Both toxigenic moulds, e.g. *A. fumigatus*, *P. niveus*, *M. ruber*, *M. circinelloides*, *F. verticilliodes*, and *Acremonium* sp. as well as silage-spoiling non-toxigenic fungi such as yeasts (*Saccharomyces* spp., *G. candidum* and *H. burtonii*) were detected in mouldy spots, which is in accordance with previous reports (Hollmann et al. 2008; Robledo et al. 2016; Wambacq et al. 2016; Rodríguez-Blanco et al. 2019). Silages comprise interesting microbial ecosystems, which can have diverse profiles of secondary metabolites (Alonso et al. 2013). Pre-harvest infestations of *Fusarium* spp., *Alternaria* spp., *Aspergillus* spp. as well as other endophytic symbionts in pastures or cereals, such as *Claviceps* spp. and *Neotyphodium* spp. can generate contamination and accumulation of the so-called field mycotoxins (Driehuis 2013; Driehuis et al. 2018). Our previous work indicated that the natural contamination of pastures with toxins derived mostly from *Fusarium* spp., but also *Alternaria* spp. and *Aspergillus* spp. in addition to EAs (Penagos-Tabares et al. 2021). During the harvesting and chopping processes, additional fungal contamination from the environment (air, soil, and dust) can take place. This newly established microbiota as well as existing field mycotoxins are ensiled together with the chopped raw plant materials (Mansfield and Kuldau 2007). Aerobic conditions and suboptimal silage management promote to the formation of fungi and during the ensiling process or the feeding out. Spots of dense fungal growth (mycelia) can be routinary found in silos intended for livestock feeding worldwide. Such mouldy spots are heterogeneous and not always visible, representing potential sources of mycotoxins and bioactive fungal metabolites that are associated with unspecific syndromes in dairy cattle (Santos

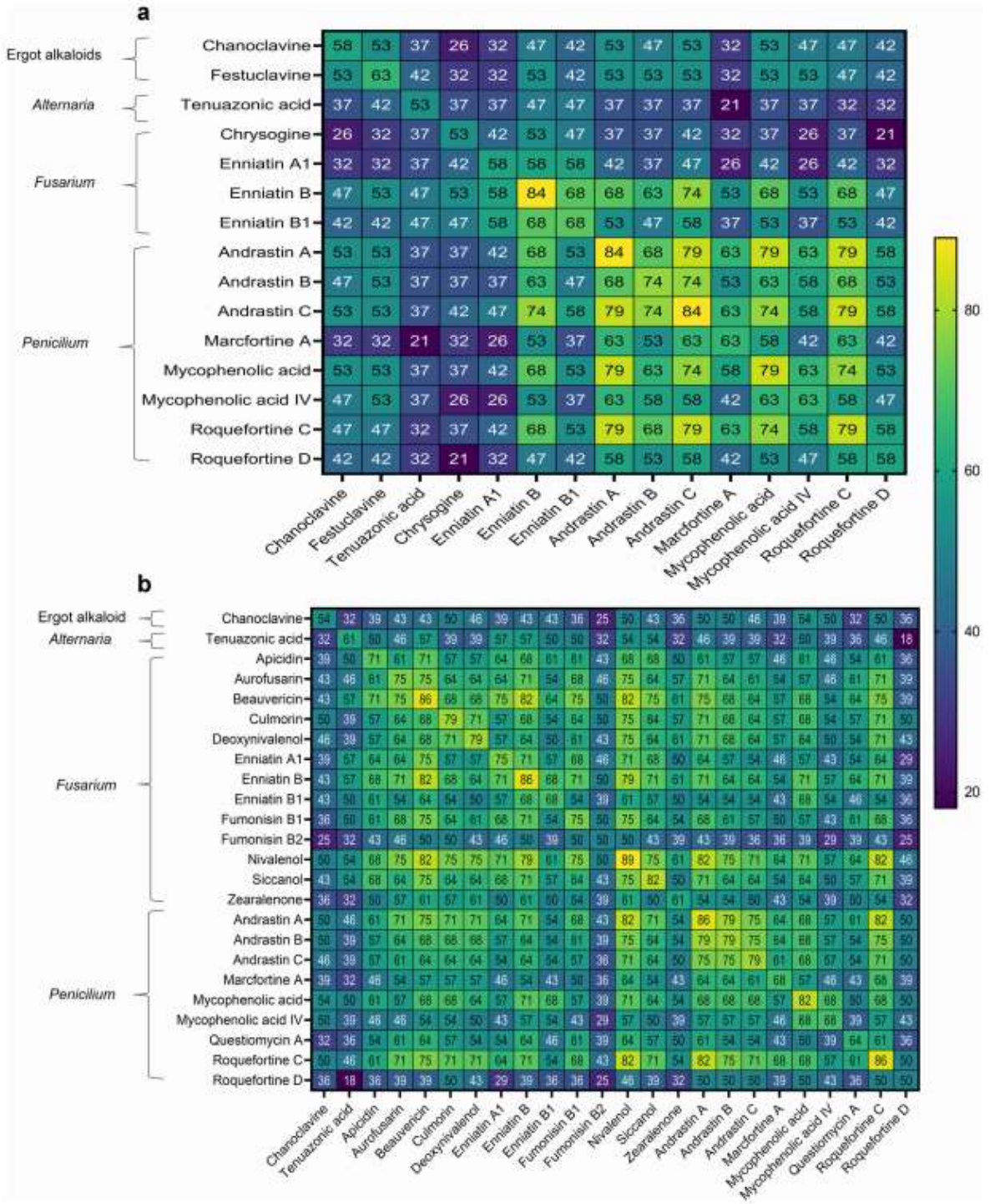


Fig. 5 Heatmap of the most frequent mycotoxins combinations (%) detected in mouldy spots of **a** grass and **b** maize silage. Mycotoxins include in this analysis occurred in ≥ 50% of the samples

and Fink-Gremmels 2014). This exploratory approach was planned to cover a general picture of the dominant cultivable fungi and an extremely wide toxin diversity associated with those visible or thermo-detectable common epicentres of postharvest deterioration. This study indicates that *Penicillium*-derived metabolites presented the highest mean concentrations within mouldy spots of silages, coinciding with the high frequency of *P. roqueforti*, the dominant species in mouldy spots. This supports the previous indication for the main role of *P. roqueforti* in the spoilage and toxin contamination of silages of different countries as reviewed previously (Alonso et al. 2013).

Moreover, we demonstrated that mouldy spots of MS presented a significantly higher diversity and concentration of *Penicillium*-derived toxins than those of GS. These fungal toxins are recognized as the most relevant postharvest toxins in conserved forages (Pahlow et al. 2003). Various *Penicillium*-derived compounds have been previously detected in silages, such as MPA, ROQs, AND A, agroclavine, marcfortine A (MAC A) and FES (Gallo et al. 2015b; O'Brien et al. 2006, 2008; Storm et al. 2014). The most studied *Penicillium*-derived compounds in ensiled products are MPA and ROQs (Gallo et al. 2015a). These compounds were shown to be more concentrated on the surface layer than in the core of silage (Dreihuis et al. 2008b), and high concentrations of MPA in GS were found in visible aerobic instability and mouldy spots (Santos and Fink-Gremmels 2014), likely due to the proliferation of aerobic fungi *Penicillium* spp. In terms of toxicity, ROQ C has been shown to cause neurotoxic effects. The clinical manifestations observed in a herd of cows after the ingestion of grain containing ROQ C (approx. 25,300 µg per kg DM) involved extensive paralysis that did not respond to treatment with calcium. The neurological signs disappeared as soon as the cows were no longer fed with mouldy grain (Häggblom 1990). In our study, the most common *Penicillium* spp. mycotoxins and metabolites co-occurring in both mouldy silages were AND C, followed by ROQ C, ROQ D, MPA, AND B, AND C and MAC A. Notably, the more diverse *Penicillium*-derived metabolites in MS compared to GS cannot be explained by the counts of *P. roqueforti*. Furthermore, the counts of *P. roqueforti* were positively correlated with the concentration of *Penicillium* spp. metabolites in MS but not in GS. The incidence of feed contamination with *Penicillium* spp. reported in the literature is variable (Auerbach et al. 1998; Gallo et al. 2015a; Mansfield et al. 2008; O'Brien et al. 2006), and there is not enough data to link *Penicillium* spp. and their produced metabolites. However, different profiles of metabolites could result from the same species depending on the high variability of strains and sometimes lack of adequate growing conditions. For example, previous studies showed that different strains of *P. roqueforti* isolated from mouldy GS and cultured in vitro presented remarkable differences in the profiles of mycotoxins produced (O'Brien et al. 2006, 2008). The agricultural and economic relevance of *Penicillium* spp. mycotoxins is considered underestimated since they are believed to be rapidly metabolized by gut microbiota and hepatic enzymes (Fuchs et al. 2008; Oh et al. 2013, 2015), but the detoxification process of mycotoxins can be disrupted by their antimicrobial and hepatotoxic properties (Noto et al. 1969; Kopp-Holtwiesche and Rehm 1990; Bentley 2000; Oh et al. 2015).

In the current study, many toxins were detected in the mouldy spots of silages, including regulated mycotoxins and related metabolites (such as DON, NIV, ZEN, α-ZEL, FBs, EAs) as well as emerging mycotoxins from *Fusarium* spp. (ENN, BEA, CUL), *Alternaria* spp. (TeA, AHO, AME) and *Aspergillus* (STC) along with *Penicillium* toxins (e.g. MA, ROQ C) and other less-studied metabolites. Specifically, the group of *Fusarium* spp. mycotoxins was the second most abundant, especially in MS having almost 8 times higher mean concentration compared to that of GS. One MS sample surpassed the maximum concentration of *Penicillium* spp. metabolites. *Fusarium*-derived mycotoxins such as DON, NIV, ZEN and ENN B are commonly found in whole-plant maize, pastures, and their silages (Gruber-Dorninger et al. 2017; Panasiuk et al. 2019; Reisinger et al. 2019; Vandicke et al. 2019). The levels of several fusarial mycotoxins (e.g. DON, NIV, ZEN, FB1, FB2, 15-hydroxyculmorin, culmorin, ENNs, equisetin, MAS and HT-2 toxin) found in mouldy MS in the current study in Austria were still below the maximum values reported in 158 MS samples (not specifically mouldy hot spots) from ten European countries (Reisinger et al. 2019). However, in our study, fusaric acid (FA) was found in high concentrations in the mouldy spots, especially in the two samples contaminated with *F. verticillioides* (precisely with 1.00×10^7 CFU/g and 5.00×10^6 CFU/g and respective concentrations of 408,000 µg/kg and 7,790 µg/kg), like previous reports (Brown et al. 2012; Merel et al. 2020). This could suggest that some fusarial potentially toxic metabolites such as FA could be produced during ensiling by *Fusarium* spp. (Wambacq et al. 2016). Interestingly, FA can enhance the activity of other *Fusarium* mycotoxins such as moniliformin, trichothecenes and fumonisins (Bacon et al. 1996; D'Mello et al. 1999). Additionally, its antimicrobial activity against *Ruminococcus albus* and *Methanobrevibacter ruminantium* has been described (May et al. 2000), possibly impacting the functionality of the rumen microbiome. AFs, OTA and T2 were not present in any sample, which was in accordance with previous European reports in non-mouldy silage (Driehuis et al. 2008a, b; Zachariasova et al. 2014; Panasiuk et al. 2019). Our study found a high occurrence of emerging fusarial mycotoxins

such as ENNs and BEA, in line with the results reported by McElhinney et al. (2016). One of the most studied mycotoxin combinations is DON-ZEN, which was detected in our study with a frequency of 61% in mouldy MS, similar to a previous European survey on non-mouldy MS (Reisinger et al. 2019). Considerably high occurrences of DON-ZEN co-contamination in MS and dairy diets have been reported by other authors (Kosicki et al. 2016; Panasiuk et al. 2019). Several studies proposed that MS is a major source of DON and ZEN in dairy feeds (Driehuis et al. 2008a, b; Panasiuk et al. 2019; Rodríguez-Blanco et al. 2019). Vandicke et al. (2021) proposed that, at the first phase of the ensiling process, the levels of mycotoxins such as parent forms could decline by elution, degradation, and absorption (caused by lactic acid bacteria). Subsequently, during the stable phase, under aerobic conditions (silos that are not properly sealed off) silage can be colonized by fungi again, producing additional mycotoxins, such as Afs, FBs, DON, ZEN and related metabolites. While the presence of field, fungi like *Fusarium and Alternaria* could become less significant in ensiled material as shown by Mansfield and Kuldau (2007) and the present study, our data further indicate that their metabolites may persist longer in the ensiled material. Still, available information about the effect of the ensiling on the fate of *Fusarium* spp. mycotoxins suggests a possible reduction in levels ZEN, DON and FBs after fermentation is contradictory (Richter et al. 2002; Boudra and Morgavi 2008; Vandicke et al. 2021), while other reports showed that the contamination levels remain unchanged (González Pereyra et al. 2014) or even increase (González Pereyra et al. 2008). Jensen et al. (2020) studied the fate of DON and ZEN as well as their modified forms using laboratory-scale silos. Comparing the concentration of mycotoxins before and after ensilage, they found that the levels of ZEN, α-ZOL, β-ZOL and ZEN-4-sulphate were constant, but the concentrations of DON increased significantly, whereas the levels of DON-3-glucoside and acetylated forms decreased proportionally. Additionally, to study the production of fungal secondary metabolites and their influencing/associated factors, controlled experimental approaches are needed. Studies under controlled environmental and ensiling conditions would reduce the external variation introduced by different locations, geo-climatic conditions, crop varieties, agricultural practices (e.g. use of fertilizers and fungicides) and other factors that influence the mycotoxins synthesis.

As found in previous studies, our results evidenced significantly higher levels of contamination with total fungal metabolites, specifically those produced mainly by Fusaria and Penicillia as well as EAs in MS compared to GS (Driehuis et al. 2008a, b; Panasiuk et al. 2019; Venslovas et al. 2021). In agreement with a recent study carried out in Germany (Dänicke et al. 2020), we also verified that mouldy spots of MS showed a broader spectrum of mycotoxins compared to GS. It has been described those high levels of water-soluble carbohydrates promote the growth of *P. roqueforti* (Pitt et al. 1991). Likewise, starch induces trichothecene production in *F. graminearum* (Oh et al. 2016). Thus, the higher content of water-soluble carbohydrates including starch found in maize plants in comparison with grasses, legumes and their mixtures could explain the higher levels of mycotoxins and other metabolites.

Regarding metabolites derived mainly from *Aspergillus* spp., although the strictly regulated aflatoxin B_1 and other AFs were not found, their precursors averufin and STC were detected in both mouldy silages. The latter, STC is a carcinogen compound and has been associated with immunotoxin and immunomodulatory activity, together with mutagenic effects, which justifies its toxicological interest (EFSA 2013; Viegas et al. 2020). The levels of STC found recently in pastures from Austria and in European MS presented a maximum concentration below 10 µg/kg (Reisinger et al. 2019), whereas the mouldy spots of GS and MS here studied here presented maximum levels of 26.6 and 4.75, respectively. It has been suggested that STC can be produced pre-and post-harvest (Mo et al. 2015). In general, the information available on exposure data of dairy cows to the mentioned precursors of AF is scarce (EFSA 2013; Gruber-Dorninger et al. 2017). Concerning detected emerging *Alternaria* mycotoxins, TeA, AOH and AME are considered to have toxicological relevance (Solfrizzo 2017). Regarding toxicity, the most important mycotoxin produced by *Alternaria* spp. is TeA (Kumari and Tirkey 2019), which targets protein synthesis inhibition at the ribosomal level, while the benzopyrene derivatives AOH and AME, known for their genotoxic effects (Gil-Serna et al. 2014), also showed strong synergistic estrogenic effects in combination with the fusarial mycoestrogen ZEN even at very low concentrations (Vejdovszky et al. 2017). In our study, levels of TeA in mouldy GS (range: 195 µg/kg -1920 µg/kg) and MS (range: 57.2 µg/kg -7,270 µg/kg) were considerably higher than levels found in ensiled maize from several European countries (maximum: 727 µg/kg) (Reisinger et al. 2019). *Alternaria*-derived toxins (AOH, AME and TeA) can be produced on-field and post-harvest. Contamination with *Alternaria* metabolites has been detected in pastures and maize (Nichea et al. 2015; Reisinger et al. 2019; Penagos-Tabares et al. 2021), and their production has been described during ensiling (Dacero et al. 1997). In our case, the relatively low levels of AOH and AME in mouldy silages (< 50 µg/kg) seem to indicate that these metabolites are not major products produced during ensiling or in mouldy spots, fitting with the findings of Storm et al. (2014). The current results emphasize the role of TeA as the most abundant mycotoxins produced by *Alternaria* spp. in mouldy spots of silages (median

concentration: 569 µg/kg in GS and 275 µg/kg in MS), while it was not detected in pastures (Penagos-Tabares et al. 2021). This may indicate that this mycoestrogen could be produced post-harvest in mouldy spots. Furthermore, the information is still scarce regarding the occurrence and toxic effects of *Alternaria*-derived toxins in animals, and therefore, health risks associated with these toxins in feeds have not yet been clarified (EFSA 2011).

Fungal biomass, DNA and colony counts are not directly associated with mycotoxin production, and there is not essentially a direct association between the presence of fungal species and the levels of mycotoxins in silage sampled at a certain point of time (Barug et al. 2006; Magan 2006; Storm et al. 2008). However, there is emerging evidence that they could be able to predict the presence of some mycotoxins (Cheli et al. 2013). Except for *P. roqueforti* and *Penicillium* metabolites in mouldy MS, our study found generally no correlation between mould counts and corresponding metabolites detected. The increased counts of *P. roqueforti* are closely related to superior levels of total *Penicillium*-derived metabolites (Supplementary Figure S2a), fitting with the results of Auerbach et al. (1998), which indicated that the *P. roqueforti* counts can be utilized as a criterion to predict the grade of contamination with toxins like ROQ C produced by this mould. In addition, these researchers emphasized that the feeding of silages with mouldy counts > 10^6 CFU/g should be stringently avoided of dietary rations of farm animals due to the possibility of contamination with *P. roqueforti*-toxins (Auerbach et al. 1998). Moreover, other studies seem to indicate that ROC C has a positive correlation with fungal growth because this secondary metabolite is produced by some fungi as a transportable extracellular nitrogen reserve (Boichenko et al. 2002; Wambacq 2017). However, a recent study analysed the presence of *Fusarium* mycotoxins in MS from seed to feed and found no correlations between fungal DNA and mycotoxin concentrations (Vandicke et al. 2021). Therefore, a simple investigation of microbial population is not always a good indicator of contamination with the most relevant regulated mycotoxins (AFs, OTA, ZEN, FBs, and DON) (Schmidt et al. 2015; Carvalho et al. 2016), which is in accordance with our results.

Additionally, it is important to remark that traditional and routinary techniques for the determination of mycobiota in feedstuffs by dilution and plating used in the present study, as well as in other studies (Baggerman 1981; Skaar and Stenwig 1996; O'Brien et al. 2005; Richard et al. 2007; Schenck et al. 2019). Although dominant and typical mycobiota responsible for the deterioration of silages such as *Penicillium spp.*, *Aspergillus* spp. and yeasts could be cultivated and identified (Mansfield and Kuldau 2007), selective media may not indicate with absolute certainty a complete profile of the mycobiota in the field or silage (Storm et al. 2008). The use of suitable and diversified culture conditions (different media and incubation in a modified atmosphere) may expand the picture of the silage's microbiota. Thus, the development of standardized methods has been strongly suggested (Storm et al. 2008). Furthermore, molecular approaches could provide a more complete picture of the microbial ecology of ensiling, aerobic deterioration, and subsequently a more accurate taxonomical identification (McAllister et al. 2018). For instance, Mansfield and Kuldau (2007) showed that a molecular approach using DNA sequences detected a greater number of fungal species than microbiological evaluation with selective media and morphological identification. For instance, *Alternaria* spp. were only detected with the molecular analysis. Also, considering the heterogeneity of mycotoxins in silages (McElhinney et al. 2016), interpretation and extrapolation of our findings may be limited to dominant mould species that colonize the superficial surfaces of certain kinds of silages.

The mouldy spots of silages investigated in this study were found to harbour several opportunist pathogens such as *A. fumigatus*, *M. circinelloides*, *Rhizomucor* spp., *Lichtheimia* spp. and *P. boydii*, pointing out an additional concern regarding the health risks for livestock and humans who are exposed to mouldy silages. These pathogenic moulds are relevant epidemiologically as causative agents of respiratory infectious diseases (mycosis), representing a higher health risk to animals and humans (farmworkers) (Alonso et al. 2013; de Hoog et al. 2020; Eucker et al. 2001; Pal et al. 2013). Mouldy silages could also contribute to a form of hypersensitivity pneumonitis denominated farmer's lung disease (Wuhrmann et al. 1965; Cano-Jiménez et al. 2016; Barnes et al. 2021) and possible cases of acute intoxications (mycotoxicosis) in workers handling high contaminated mouldy silage cannot be discarded (Emanuel et al. 1975; Gordon et al. 1993). Silages are economically relevant forage sources in dairy production, but they also represent sources of mycotoxin mixtures due to mould proliferation. Considering that spoilage of silage is heterogeneous and mouldy spots are not always visually detectable, the most important preventive measures thus consist of improving the storage conditions and sensibilization of farmworkers for the utilization of the respiratory protective equipment to avoid the inhalation of fungal organisms with pathogenic potential or their antigens (Cano-Jiménez et al. 2016).

This pilot study provides insight into the most occurrent fungal species spoiling GS and MS in Austria, confirming the previously called status of *P. roqueforti* as the "silage mould". The co-occurrence of other toxigenic along with non-toxigenic fungal organisms, some of them opportunistic pathogens of animals and humans was corroborated. Data on the profiles of mycotoxins and other metabolites

contained in mouldy silages demonstrated high concentrations of *Penicillium*-derived compounds and a considerable amount of wide spectrum regulated, emerging, modified and less known (potential) mycotoxins. The routinary fungal counts and the levels of (toxic) secondary metabolites in mouldy silages were not correlated, with exception of *P. roqueforti's* counts and some metabolites derived from *Penicillium* spp. in MS. Several pre-and post-harvest fungal toxins were detected in higher levels in MS compared to GS, suggesting that GS could be a better option as a source of animal feed in terms of lower mycotoxigenic risk. Further research focused on the occurrence, dietary levels and toxicity of mouldy silage-derived compounds, and their effects on the rumen microbiota, "mouldy silage syndrome" and carry-over via milk are needed. Diagnostics, prevention and remediation strategies for reducing at minimum the mould growth and mycotoxin production in ensiled feeds as well as the influencing environmental factors must be further investigated.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s12550-022-00453-3.

Acknowledgements We would like to Anneliese Müller (BIOMIN Research Center) for critically reading of the manuscript and to Marlene Suntinger, Franz Steininger and Christa Egger-Danner (ZuchtData EDV-Dienstleistungen GmbH) for the logistic support as well as the staff of LKV Austria Qualitätsmanagement GmbH – Federal Recording Association of Lower Austria, Upper Austria, and Styria.

Author contribution T.J., R.K., J.F., F.P.T. and Q.Z. conceived and designed the study. F.P.T. and Ma.S. collected the samples. R.L. performed the mycological analysis. Mi.S. completed the multi-metabolite (LC–ESI–MS/MS) analysis. F.P.T., R.K and C.P. analysed the data. F.P. T contributed to the original draft. R.K, C.P, T.J., V.N., C.P., Ma.S., R.L., J.F., M.S., and Q.Z. revised the manuscript. Q.Z. and V.N. acquired the research grant. All the authors read and approved the final paper.

Funding Open access funding provided by University of Veterinary Medicine Vienna. This research was conducted as part of the Project "D4Dairy-Digitalization, Data Integration, Detection and Decision support in Dairying" supported by the Austrian Federal Ministry for Climate Action, Environment, Energy, Mobility, Innovation and Technology (BMK), Austrian Federal Ministry for Digital and Economic Affairs (BMDW) and the provinces of Lower Austria and Vienna within the framework of COMET-Competence Centers for Excellent Technologies., which is handled by the Austrian Research Promotion Agency (FFG).

Availability of data and material Data transparency.

Code availability Software application or custom code.

Declarations

Conflicts of interest N.V., J.F. and T.J. are employed by BIOMIN Holding GmbH (now part of DSM), which operates the BIOMIN Research Center and is a producer of animal feed additives. This, however, did not influence sampling, analyses, or interpretation of data.

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Supplementary Table S1. Occurrences and counts (CFU/g) of fungal cultivated fungal species detected in spots of mouldy grass and maize silages

Fungal species	Grass silage (n=19)				Maize silage (n=28)				Mann-Whitney Test
		Count (CFU/g)				Counts (CFU/g)			
	Positive Samples [%]	Average ± SD	Median	Range	Positive Samples [%]	Average ± SD	Median	Range	*p*-value
Acremonium spp.	5			1.0×10^4	0				
Aspergillus fumigatus	26	$6.0 \times 10^5 \pm 4.6 \times 10^5$	6.0×10^5	2.0×10^5 - 1.0×10^6	29	$6.6 \times 10^6 \pm 4.3 \times 10^6$	1.00×10^7	1.0×10^6 - 1.0×10^7	0.688
Fusarium spp.	0				4			1.0×10^5	
Fusarium verticillioides	0				7	$7.5 \times 10^6 \pm 3.5 \times 10^6$	7.5×10^6	5.0×10^6 - 1.0×10^7	
Geotrichum candidum	26	$4.6 \times 10^5 \pm 3.4 \times 10^5$	4.0×10^5	1.0×10^5 - 1.0×10^6	46	$1.70 \times 10^6 \pm 2.8 \times 10^6$	5.5×10^5	6.0×10^5 - 1.0×10^7	0.142
Hypopichia burtonii	5			1.0×10^6	0				
Lichtheimia corymbifera	16	$1.7 \times 10^6 \pm 2.0 \times 10^6$	1.0×10^6	1.0×10^5 - 4.0×10^6	14	$3.95 \times 10^6 \pm 4.6 \times 10^6$	2.8×10^6	3.0×10^5 - 1.0×10^7	0.946
Monascus ruber	37	$3.5 \times 10^6 \pm 3.5 \times 10^6$	3.0×10^6	1.0×10^5 - 1.0×10^7	29	$3.9 \times 10^6 \pm 4.1 \times 10^6$	2.5×10^6	1.0×10^5 - 1.0×10^7	0.591
Mucor circinelloides	16	$4.4 \times 10^6 \pm 5.1 \times 10^6$	3.0×10^6	1.0×10^5 - 1.0×10^7	25	$2.5 \times 10^6 \pm 3.7 \times 10^6$	6.0×10^5	4.0×10^5 - 1.0×10^7	0.548
Paecilomyces niveus	16	$7.0 \times 10^5 \pm 3.6 \times 10^5$	8.0×10^5	3.0×10^5 - 1.0×10^6	36	$4.2 \times 10^6 \pm 4.2 \times 10^6$	2.3×10^6	2.0×10^5 - 1.0×10^7	0.103
Paecilomyces variotii	0				4			5.0×10^5	
Penicillium roqueforti	74	$1.5 \times 10^6 \pm 2.8 \times 10^6$	2.0×10^6	1.0×10^4 - 1.0×10^7	71	$4.9 \times 10^6 \pm 4.5 \times 10^6$	4.0×10^6	2.0×10^5 - 1.0×10^7	0.1097
Pseudallescheria boydii	5			1.0×10^4	14	$2.4 \times 10^6 \pm 2.7 \times 10^6$	2.3×10^6	1.0×10^5 - 5.0×10^6	
Rhizomucor pusillus	0				7	$5.5 \times 10^5 \pm 6.4 \times 10^5$	5.5×10^5	1.0×10^5 - 1.0×10^6	
Saccharomyces spp.	47	$1.6 \times 10^6 \pm 1.9 \times 10^6$	1.0×10^6	1.0×10^5 - 5.0×10^6	43	$3.8 \times 10^6 \pm 4.1 \times 10^6$	2.0×10^6	2.0×10^4 - 1.0×10^7	0.967
Scopulariopsis brevicaulis	11	$6.0 \times 10^5 \pm 5.7 \times 10^5$	6.0×10^5	2.0×10^5 - 1.0×10^6	0				
Verticillium spp.	0				4			5.00×10^5	
Sum of moulds	100	$3.7 \times 10^6 \pm 4.9 \times 10^5$	1.4×10^6	1.0×10^4 - 1.5×10^7	100	$1.0 \times 10^7 \pm 5.51 \times 10^6$	1.05×10^7	1.00×10^6 - 2.1×10^7	<0.001 *
Sum of yeasts	68	$1.4 \times 10^6 \pm 1.7 \times 10^5$	1.0×10^6	1.0×10^5 - 5.0×10^6	75	$3.2 \times 10^6 \pm 3.80 \times 10^6$	1.50×10^6	5.00×10^4 - 1.1×10^7	0.363
Total fungi (Yeasts and moulds)	100	$4.6 \times 10^6 \pm 5.0 \times 10^6$	3.1×10^6	1.0×10^4 - 1.5×10^7	100	$1.3 \times 10^7 \pm 6.50 \times 10^6$	1.14×10^7	2.50×10^6 - 2.2×10^7	<0.001 *

* Significantly different (p-value < 0.05)

Supplementary Table S2. Occurrences and levels of mycotoxins and other metabolites detected in spots of mouldy grass and maize silages

Group	Metabolites	Grass silage (n=19)				Maize silage (n=28)				Mann-Whitney Test	
		Positive Samples (%)[1]	Concentration[2]			Positive Samples (%)[1]	Concentration[2]			*p*-value	
			Average ± SD	Median	Range		Average ± SD	Median	Range		
Ergot alkaloid	Agroclavine	11	2.47 ± 0.3	2.47	2.25 - 2.68	32	8.06 ± 7.35	6.43	1.44 - 23.1	0.079	
	Chanoclavine	58	36.5 ± 73.6	5.78	0.16 - 225	54	160 ± 445	18.7	0.17 - 1,740	0.778	
	Festuclavine*	63	63.7 ± 123	14.9	0.35 - 435	82	313 ± 444	86.9	1.07 - 1,360	0.026	*
Alternaria spp.	Alternariol	16	10.2 ± 12.2	4.35	2.09 - 24.2	29	2.34 ± 3.36	1.14	0.3 - 10.4	0.482	
	Alternariolmethylether	26	4.13 ± 2.3	3.5	1.6 - 7.32	29	1.78 ± 1.62	1.31	0.13 - 4.71	0.857	
	Altersetin	32	176 ± 315	58.1	5.13 - 818	36	13.6 ± 9.85	12.6	1.11 - 31.6	0.792	
	Infectopyron	5			26.2	21	32.1 ± 21.7	22	11 - 66.1	0.183	
	Tenuazonic acid	53	781 ± 552	569	195 - 1,920	61	785 ± 1,720	275	57.2 - 7,270	0.651	
Aspergillus spp.	Averufin	21	2.75 ± 2.64	2.08	0.34 - 6.51	7	2.01 ± 0.78	2.01	1.46 - 2.56	0.15	
	Bis(methylthio)gliotoxin	11	133 ± 184	133	2.19 - 263	32	152 ± 242	63.8	6.53 - 756	0.088	
	Chaetominine*	5			439	32	468 ± 431	370	10.5 - 1,430	0.037	*
	Demethylsulochrin					14	129 ± 113	85.3	48.6 - 296	0.136	
	Fumagillin					14	2,190 ± 2,800	1,230	0.4 - 6,280	0.136	
	Fumigaclavine	26	276 ± 557	5.34	1.56 - 1,270	32	563 ± 729	212	1.08 - 2,040	0.544	
	Fumigaclavine C	37	1,800 ± 4,000	81.3	11.3 - 10,780	36	3,950 ± 7,430	857	5.61 - 23,300	0.948	
	Fumiquinazoline A	11	55.5 ± 52.9	55.5	18.1 - 92.9	25	520 ± 1,110	62.7	21 - 3,023	0.182	
	Fumiquinazoline D	26	433 ± 879	10.5	1.77 - 2,000	32	3,890 ± 9,230	1,080	1.66 - 28,400	0.514	
	Fumitremorgin C	5			33.9	21	169 ± 242	58.9	26.3 - 651	0.134	
	Gliotoxin	5			79.3	14	47 ± 46.6	46.8	5.09 - 89.2	0.39	
	Helvolic acid	5			131	18	406 ± 703	76.4	53.4 - 1,660	0.32	
	Kojic acid	21	63.2 ± 44.5	43.5	36.2 - 129	43	97.7 ± 103	54.3	16.1 - 353	0.136	
	Methylsulochrin	26	541 ± 1,060	10.9	1.98 - 2,420	43	797 ± 1,620	57.1	0.78 - 4,910	0.265	
	Mevinolin	53	516 ± 611	206	3.26 - 1,520	25	550 ± 740	185	4.03 - 2,050	0.065	
	Pinselin*	5			1.29	43	116 ± 173	48.5	1.91 - 594	0.003	*
	Pseurotin A	5			1,440	25	1,550 ± 2,560	247	20.6 - 7,100	0.101	
	Pyripyropene A	5			108	21	197 ± 315	53	17.6 - 824	0.183	
	Sphingofungin B					11	7,250 ± 9,670	3,280	206 - 18,300	0.262	
	Sphingofungin D	5			5.29	14	195 ± 313	58	4.39 - 658	0.292	
	Sterigmatocystin*	37	6.89 ± 9.79	1.3	0.09 - 26.6	7	2.49 ± 3.2	2.49	0.23 - 4.75	0.011	*
	Trypacidin	42	120 ± 290	2.88	0.91 - 833	21	451 ± 696	103	0.85 - 1,750	0.21	
	Versicolorin C	5			7	4			4.03	0.754	

[1]Samples with values > limit of detection (LOD); [2]Excluding data < LOD. In case values >LOD and < limit of quantification (LOQ), LOQ/2 was used for calculation.

* Significantly different (p-value < 0.05)

Supplementary Table S2. Cont. Occurrences and levels of mycotoxins and other metabolites detected in spots of mouldy grass and maize silages

Group	Metabolite	Grass silage (n=19)				Maize silage (n=28)				Mann-Whitney Test	
		Positive Samples (%)[1]	Concentration[2]			Positive Samples (%)[1]	Concentration[2]			p-value	
			Average ± SD	Median	Range		Average ± SD	Median	Range		
Fusarium spp.	15-Hydroxyzearalenone	5			16.3	46	143 ± 192	76.5	33.7 - 742	0.001	*
	alpha-Zearalenol	0				11	62.1 ± 56.3	61.4	6.08 - 119	0.262	
	Antibiotic Y	16	119 ± 198	9.54	3.58 - 345	7	295 ± 403	293	7.03 - 578	0.39	
	Apicidin	5	7.92			71	23.8 ± 25.4	17.2	3.81 - 111	<0.001	*
	Aurofusarin	32	35.5 ± 23.2	41.2	4.07 - 59.9	75	61.8 ± 50.5	43	5.92 - 171	0.004	*
	Beauvericin	47	19.7 ± 40.4	1.83	0.2 - 125	86	30.1 ± 36.7	17.7	3.93 - 153	<0.001	*
	Bikaverin	0				46	9.7 ± 5.68	7.04	3.55 - 22.7	<0.001	*
	Butenolide	0				14	15.2 ± 6.6	15.4	7.07 - 23.1	0.136	
	Chrysogine	53	34.4 ± 31.8	23.1	4.61 - 102	29	6.64 ± 5.07	4.44	2.35 - 15.8	0.018	*
	Culmorin	42	82.8 ± 65.1	62.7	5.77 - 179	79	162 ± 166	109	20.7 - 1,360	<0.001	*
	Deoxynivalenol	16	19.6 ± 10.2	20	9.24 - 29.6	79	291 ± 285	224	30 - 1,220	<0.001	*
	Enniatin A	37	1.36 ± 1.75	0.81	0.02 - 4.9	43	0.85 ± 0.81	0.67	0.01 - 2.17	0.74	
	Enniatin A1	58	1.89 ± 6.01	2.2	0.17 - 20.1	75	10.9 ± 14.3	4.27	0.2 - 51.4	0.041	*
	Enniatin B	84	11.1 ± 13.3	6.37	0.27 - 44.5	86	8.28 ± 10.5	4.94	0.11 - 44.7	0.572	
	Enniatin B1	68	12.6 ± 21.1	7.19	0.64 - 80.7	68	19.4 ± 26.8	7.16	0.05 - 95.3	0.887	
	Enniatin B2	26	0.7 ± 0.79	0.44	0.14 - 2.08	32	0.48 ± 0.32	0.42	0.11 - 1.06	0.719	
	Epiepoxydin	37	7.15 ± 8.45	2.85	1.01 - 22.3	46	6.42 ± 6.95	3.45	0.3 - 23.5	0.559	
	Equisetin	47	39.4 ± 67.1	8.55	0.65 - 181	46	9.16 ± 11.2	4.12	1.23 - 41.9	0.771	
	Fumonisin B1	0				75	88.4 ± 79.0	58.8	14 - 356	<0.001	*
	Fumonisin B2	0				50	28.7 ± 22.4	25.8	10.1 - 97.8	<0.001	*
	Fumonisin B3	0				11	20.9 ± 3.2	21.1	17.6 - 24	0.262	
	Fumonisin B4	0				11	10.7 ± 4.68	12.8	5.19 - 14.1	0.262	
	Fusaric acid	0				18	83,300 ± 182,000	217	108 - 408,000	0.1	
	Fusarin C	5			589	4			186	0.754	
	Fusarinolic acid	0				11	11,180 ± 19,210	85.5	84.4 - 53,400	0.282	
	Gibepyrone D	5			1,563	0				0.404	
	HT-2 toxin	0				21	16.8 ± 9.85	14.9	4.81 - 31	0.068	
	Moniliformin	5			0.47	29	5.78 ± 5.08	4.48	1.56 - 17	0.072	
	Monoacetoxyscirpenol	0				4			0.01	>0.999	
	Nivalenol	5	36			89	281 ± 219	191.1	38.9 - 852	<0.001	*
	Stecmol	47	8,130 ± 20,700	1,400	200 - 61,200	82	3,200 ± 5,380	1,580	154 - 28,100	0.015	*
	Zearalenone	21	178 ± 327	20.2	3.43 - 868	61	35 ± 34.4	20.6	2.08 - 53.9	0.016	*

[1]Samples with values > limit of detection (LOD); [2]Excluding data < LODin case values >LOD and < limit of quantification (LOQ), LOQ/2 was used for calculation.

* Significantly different (p-value < 0.05)

Supplementary. Table S2. Cont. Occurrences and levels of mycotoxins and other metabolites detected in spots of mouldy grass and maize silages

Group	Metabolite	Grass silage (n=19)				Maize silage (n=28)				Mann-Whitney Test	
		Positive Samples (%)[1]	Concentration[2]			Positive Samples (%)[1]	Concentration[2]			p-value	
			Average ± SD	Median	Range		Average ± SD	Median	Range		
Other fungi	Emindole SA					14	48.8 ± 51.9	27.9	8 - 123	0.136	
	Fellutamine A	11	38.3 ± 20.8	38.4	23.7 - 53.1	12	42.6 ± 37.1	37.8	5.65 - 129	0.108	
	Ilicicolin A	11	2.44 ± 1.75	2.44	1.2 - 3.68	4			2.11	0.429	
	Ilicicolin B	47	6.01 ± 5.7	4.17	0.64 - 19.2	46	11.5 ± 18.6	2.61	0.53 - 62.1	0.843	
	Isofistulinol					7	10.7 ± 9.68	10.7	3.89 - 17.6	0.508	
	Mollicellin D					14	75.5 ± 118	25.2	1.56 - 250	0.137	
	Monocerin	47	12.56 ± 23.3	3.81	0.91 - 73.4	36	14.3 ± 17.6	7.35	0.64 - 52.9	0.488	
	Trichoderxosine					4			1,210	>0.999	
Penicillium spp.	7-Hydroxyroridatin					21	6.78 ± 6.30	5.42	1.08 - 16.4	0.068	
	Andrastin A	84	1,030 ± 1,870	90.8	4.02 - 5,840	86	3,880 ± 4,160	2,170	19.6 - 13,100	0.03	*
	Andrastin B	74	508 ± 718	140	6.06 - 2,270	79	3,670 ± 4,300	1,900	5.81 - 14,100	0.05	
	Andrastin C	84	9,580 ± 14,800	723	71.3 - 36,720	79	45,200 ± 58,100	12,800	21.5 - 252,000	0.179	
	Asperin A5					11	17.6 ± 20.2	7.75	2.21 - 38.9	0.262	
	Chevalone C	47	11.2 ± 11.5	9.48	2.2 - 39.6	4			0.41	<0.001	*
	Citrinin	5			89.7					0.404	
	Eremofortin A					4			57	>0.999	
	Marcfortine A	63	201 ± 531	16.7	4.11 - 1,880	68	2,027 ± 3,060	777	1.01 - 12,900	0.196	
	Mycophenolic acid	79	2,530 ± 2,740	1,960	18.1 - 7,430	82	5,570 ± 9,130	2,000	2.59 - 50,900	0.771	
	Mycophenolic acid IV	63	108 ± 155	37.1	1.57 - 570	68	199 ± 307	50.7	0.41 - 1,050	0.771	
	Penitrem					43	7.13 ± 4.31	7.66	1.42 - 13.1	<0.001	*
	Quercitrin-cin A	11	27.4 ± 29.8	27.4	6.48 - 48.3	64	27.3 ± 33.1	13.2	4.24 - 111	<0.001	*
	Quinolactacin A					7	0.06 ± 0.04	0.06	0.03 - 0.08	0.508	
	Roquefortine C	79	2,270 ± 2,940	1,150	64.5 - 10,900	86	6,360 ± 8,080	6,530	8.56 - 30,000	0.148	
	Roquefortine D	58	756 ± 1,340	160	32.7 - 4,400	50	6,220 ± 9,600	1,870	129 - 31,200	0.159	

[1]Samples with values > limit of detection (LOD); [2]Excluding data < LODin case values >LOD and < limit of quantification (LOQ), LOQ/2 was used for calculation.

* Significantly different (p-value < 0.05)

Supplementary Table S2. Cont. Occurrences and levels of mycotoxins and other metabolites detected in spots of mouldy grass and maize silages

Group	Metabolite	Grass silage (n=19)				Maize silage (n=28)				Mann-Whitney Test	
		Positive Samples (%)[1]	Concentration[2]			Positive Samples (%)[1]	Concentration[2]				
			Average ± SD	Median	Range		Average ± SD	Median	Range	p-value	
Fusarium metabolites	5-Nitropropionic acid	26	109 ± 206	17.2	4.78 - 478	54	21 ± 40	7.23	1.7 - 159	0.181	
	Aspergillicidin	5			0.14	7	0.22 ± 0.04	0.22	0.19 - 0.25	0.705	
	Beauvamid F	74	80.97 ± 40.6	54.9	17.7 - 158	75	75.8 ± 123	39.5	8.02 - 593	0.791	
	Chlorocitreoviridin					21	49.2 ± 61.7	14.1	0.89 - 160	0.068	
	Chrysophanol	26	457 ± 721	117	75.8 - 1,740	36	1,410 ± 2,518	167	29 - 7,180	0.344	
	Citreoviridin	21	72.97 ± 64.5	73.7	12.6 - 132	50	212 ± 469	22.6	2.47 - 1,320	0.084	
	Cyclo(L-Pro-L-Tyr)	85	527 ± 310	470	59.9 - 1,240	100	259 ± 213	229	3.53 - 723	0.016	*
	Cyclo(L-Pro-L-Val)	100	1,950 ± 1,310	1,750	17.6 - 4,940	93	923 ± 911	488	6.98 - 3,380	0.007	*
	Emodin	85	187 ± 422	11.3	2.58 - 1,630	89	104 ± 167	47.7	0.48 - 712	0.802	
	Endocrocin	26	2,500 ± 3,000	897	159 - 7,250	71	1,940 ± 3,110	518	12.5 - 13,200	0.015	*
	Fellutamid	16	5.81 ± 5.47	4.92	0.64 - 11.9	18	24.1 ± 34	4.43	0.47 - 81.1	0.849	
	Iso-Rhodoptilometrin	32	3.9 ± 3.29	2.88	0.93 - 10.1	54	8.62 ± 7.93	5.1	0.41 - 28.2	0.073	
	Lecanoric acid	11	3.0 ± 2.77	3.01	1.03 - 4.97	7	4.7 ± 4.5	4.7	1.53 - 7.86	0.94	
	Mycotoxin					11	2,330 ± 1,370	1,940	1,210 - 3,860	0.242	
	N-Benzoyl-Phenylalanine	5			5.32	11	3.72 ± 2.37	3.13	1.71 - 6.34	0.73	
	Roquefortine	63	50.0 ± 32.6	41.55	8.78 - 135	68	44.4 ± 33.1	39.8	4.73 - 122	0.86	
	Skyrin	42	19.0 ± 34.8	4.04	0.61 - 100	39	45.4 ± 87.5	15.9	1.33 - 301	0.801	
	Terrestrol	89	773 ± 860	460	66.7 - 2,900	96	527 ± 491	370	38.1 - 1,800	0.66	
	Usnic acid	16	2.1 ± 0.64	2.07	1.47 - 2.75	14	1.66 ± 1.18	1.29	0.69 - 3.17	0.748	

[1]Samples with values > limit of detection (LOD). [2]Excluding data < LOD. In case values >LOD and < limit of quantification (LOQ), LOQ/2 was used for calculation.

* Significantly different (p-value < 0.05)

Supplementary Table S3. Significative differences in the levels of mycotoxins and other metabolites detected in spots of mouldy grass and maize silages during year 2019 and 2020

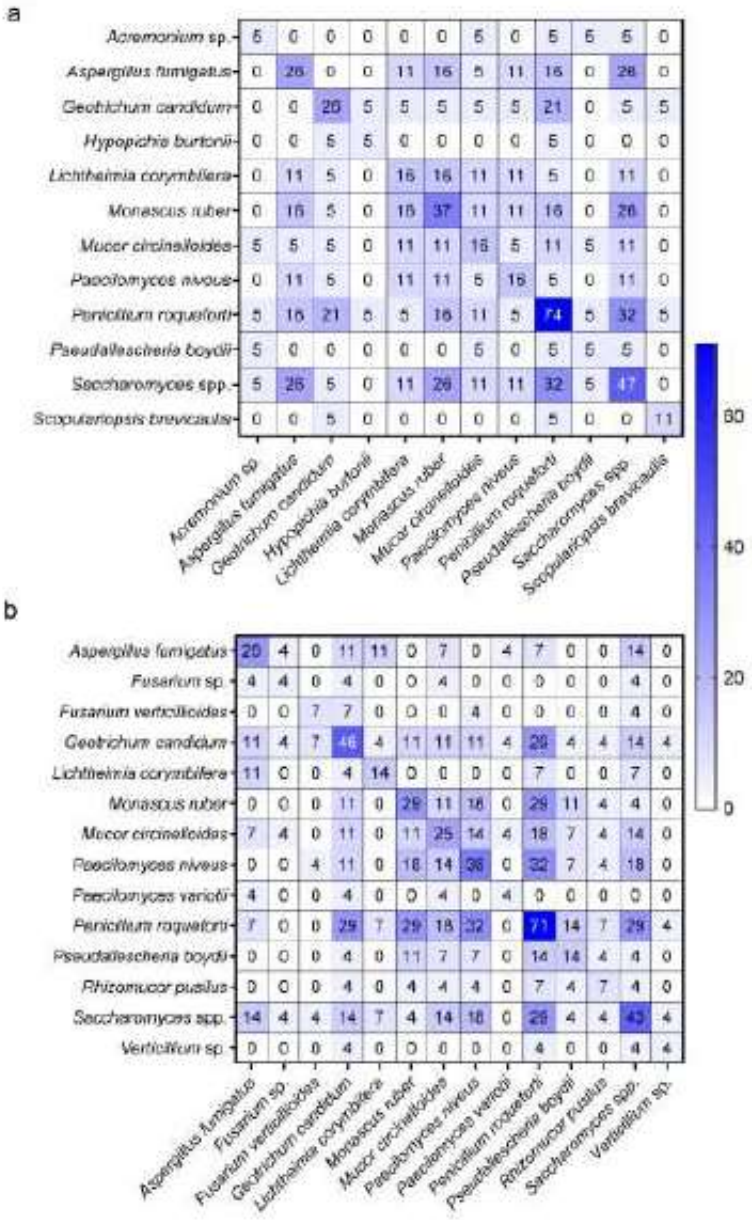
Metabolite / Group	Grass silage							Maize silage						
	2019 (n=9)			2020 (n=10)			Mann-Whitney Test	2019 (n=18)			2020 (n=10)			Mann-Whitney Test
	Concentration			Concentration				Concentration			Concentration			
	Positive samples (n)	Average	Median	Positive samples (n)	Average	Median	P-value	Positive samples (n)	Average	Median	Positive samples (n)	Average	Median	P-value
Andrastin C	7	384	398	9	12,600	4,270	0.006 *	14	31,928	38,052	8	61,810	28,930	0.161
Asperidin	0			1	7.92	7.92	>0.999 ns	15	24.3	20.0	5	22.2	9.88	0.039 *
Atpenin A5	0			0			>0.999 ns	2	20.5	20.5	1	5.75	5.75	0.787 ns
Beauvericin	7	24.9	3.77	2	1.61	1.61	0.016 *	17	38.2	23.8	7	10.5	7.74	0.007 *
Beauvamid F	9	47.7	47.8	5	84.9	106	0.273 ns	17	86.5	44.4	4	30.3	23.8	0.001 *
Chrysophan	7	40.0	19.8	3	22.4	26.5	0.041 *	3	7.49	4.30	5	6.13	4.57	0.078 ns
Citreoviridin	3	53.3	22.0	1	132	132	0.443 ns	13	228	17.8	1	27.0	27.0	0.003 *
Culmorin	4	89.2	98.1	4	98.4	102	>0.999 ns	12	231	118	10	388	241	0.001 *
cyclo(L-Pro-L-Tyr)	8	467	500	10	551	470	0.447 ns	18	327	206	10	139	85.3	0.024 *
Deoxynivalenol	1	9.24	9.24	2	24.8	24.8	0.598 ns	12	205	191	10	393	276	0.026 *
Emodin	8	178	11.3	10	159	11.3	0.720 ns	17	141	102	8	23.7	10.2	0.018 *
Endocrocin	7	2,500	897	0			0.011 *	16	2,099	518	4	1,300	1,099	0.014 *
Enniatin A	7	1.36	0.83	0			0.001 *	11	0.77	0.54	1	1.68	1.68	0.023 *
Enniatin A1	9	4.35	2.20	2	1.84	1.84	0.001 *	17	12.1	4.90	4	5.50	3.03	0.007 *
Enniatin B1	9	13.3	8.89	4	8.40	6.83	0.014 *	13	23.8	7.49	6	9.7	3.42	0.257 ns
Enniatin B2	7	0.70	0.44	0			0.011 *	8	0.38	0.34	1	1.06	1.06	0.139 ns
Ergosterin A	0			0			>0.999 ns	1	57.0	57.0	0			>0.999 ns
Ergic acid	4	63.2	63.7	0			0.033 *	12	87.7	34.3	0			0.001 *

[1]Samples with values > limit of detection (LOD). [2]Excluding data < LODIn case values >LOD and < limit of quantification (LOQ), LOQ/2 was used for calculation.

* Significantly different (p-value < 0.05)

27 **Supplementary Figure S1.** Co-occurrence (%) of fungal species isolated from mouldy spots of (a) grass and (b)
28 maize silage.

29



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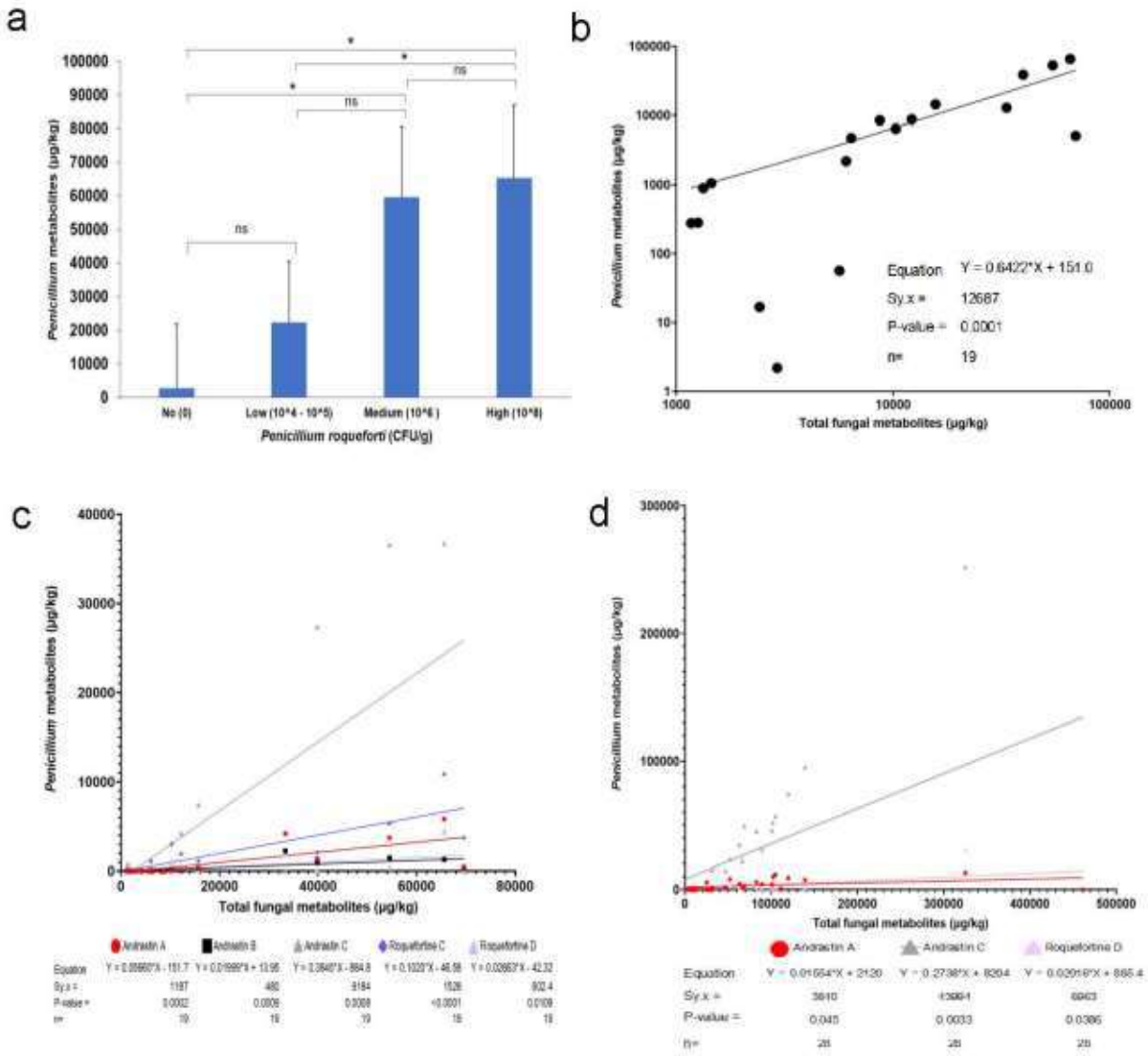
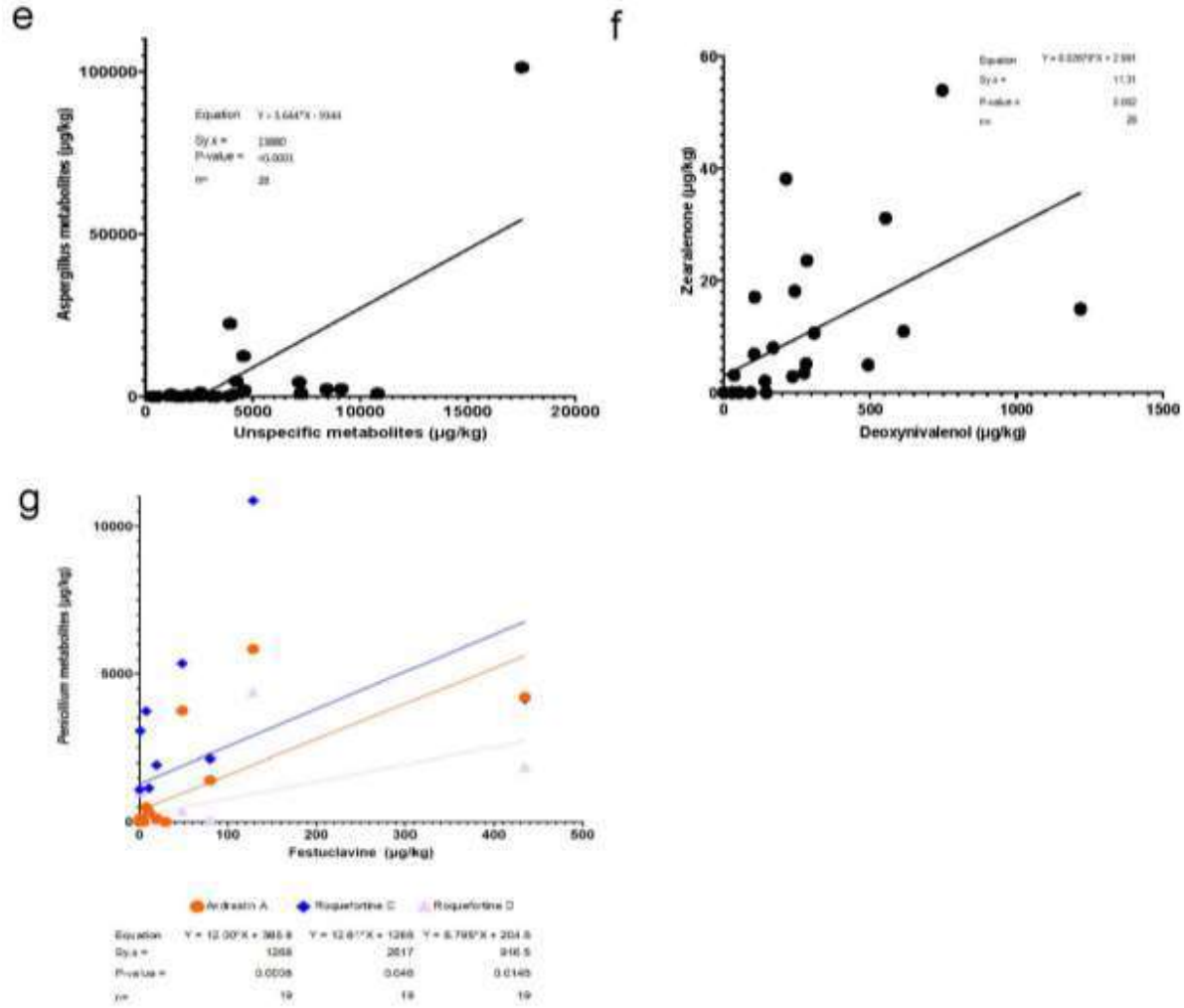

a
Penicillium metabolites (µg/kg)
Penicillium roqueforti (CFU/g)
No (0)
Low (10^4 - 10^5)
Medium (10^6)
High (10^8)
ns
b
Penicillium metabolites (µg/kg)
Total fungal metabolites (µg/kg)
Equation Y = 0.6422*X + 151.0
Sy.x = 12687
P-value = 0.0001
n= 19
c
Penicillium metabolites (µg/kg)
Total fungal metabolites (µg/kg)
Andrastin A
Andrastin B
Andrastin C
Roquefortine C
Roquefortine D
d
Penicillium metabolites (µg/kg)
Total fungal metabolites (µg/kg)
Andrastin A
Andrastin C
Roquefortine D
Equation Y = 0.0164*X + 2120 Y = 0.2738*X + 8264 Y = 0.0216*X + 885.4
Sy.x = 3610 6399 6963
P-value = 0.045 0.0033 0.0386
n= 28 28 28

Supplementary Figure S2. Cont.



3.3. Publication 3:

Cocktails of Mycotoxins, Phytoestrogens, and Other Secondary Metabolites in Diets of Dairy Cows in Austria: Inferences from Diet Composition and Geo-Climatic Factors.

Felipe Penagos-Tabares, Ratchaneewan Khiaosa-ard, Marlene Schmidt, Eva-Maria Bartl, Johanna Kehrer, Veronika Nagl, Johannes Faas, Michael Sulyok, Rudolf Krska, and Qendrim Zebeli.

Toxins (2022) 14, 493. https://doi.org/10.3390/toxins14070493

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Article

Cocktails of Mycotoxins, Phytoestrogens, and Other Secondary Metabolites in Diets of Dairy Cows in Austria: Inferences from Diet Composition and Geo-Climatic Factors

Felipe Penagos-Tabares [1], Ratchaneewan Khiaosa-ard [1,*], Marlene Schmidt [1], Eva-Maria Bartl [1], Johanna Kehrer [1], Veronika Nagl [2], Johannes Faas [2], Michael Sulyok [3], Rudolf Krska [3,4] and Qendrim Zebeli [1,5]

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Abstract: Dairy production is a pivotal economic sector of Austrian and European agriculture. Dietary toxins and endocrine disruptors of natural origin such as mycotoxins and phytoestrogens can affect animal health, reproduction, and productivity. This study characterized the profile of a wide spectrum of fungal, plant, and unspecific secondary metabolites, including regulated, emerging, and modified mycotoxins, phytoestrogens, and cyanogenic glucosides, in complete diets of lactating cows from 100 Austrian dairy farms. To achieve this, a validated multi-metabolite liquid chromatography/electrospray ionization–tandem mass spectrometric (LC/ESI–MS/MS) method was employed, detecting 155 of >800 tested metabolites. Additionally, the most influential dietary and geo-climatic factors related to the dietary mycotoxin contamination of Austrian dairy cattle were recognized. We evidenced that the diets of Austrian dairy cows presented ubiquitous contamination with mixtures of mycotoxins and phytoestrogens. Metabolites derived from *Fusarium* spp. presented the highest concentrations, were the most recurrent, and had the highest diversity among the detected fungal compounds. Zearalenone, deoxynivalenol, and fumonisin B1 were the most frequently occurring mycotoxins considered in the EU legislation, with detection frequencies >70%. Among the investigated dietary factors, inclusion of maize silage (MS) and straw in the diets was the most influential factor in contamination with *Fusarium*-derived and other fungal toxins and metabolites, and temperature was the most influential among the geo-climatic factors.

Keywords: mycotoxin; phytoestrogen; ergot alkaloid; co-exposure; dairy farming; feed safety

Key Contribution: The ubiquitous presence of complex mixtures of mycotoxins (considered and non-considered in the EU legislation), phytoestrogens, and other less-known secondary metabolites in the diets of lactating dairy cows is evident. Dietary rations with a high proportion of maize silage and straw tend to have higher mycotoxin contamination levels. Dietary exposure to the here-reported cocktails of toxic, potentially toxic, and endocrine-disrupting metabolites in the diets of food-producing animals can lead to unpredictable toxicological interactions and may involve health risks for animals and humans.

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Citation: Penagos-Tabares, F.; Khiaosa-ard, R.; Schmidt, M.; Bartl, E.-M.; Kehrer, J.; Nagl, V.; Faas, J.; Sulyok, M.; Krska, R.; Zebeli, Q. Cocktails of Mycotoxins, Phytoestrogens, and Other Secondary Metabolites in Diets of Dairy Cows in Austria: Inferences from Diet Composition and Geo-Climatic Factors. *Toxins* **2022**, *14*, 493. https://doi.org/10.3390/toxins14070493

Received: 9 June 2022
Accepted: 12 July 2022
Published: 15 July 2022

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1. Introduction

Dairy production is the most important agricultural sector in the Republic of Austria, representing 18% of the national agricultural production [1]. Animal feeding is a fundamental element of milk production, affecting the rest of the productive chain, including aspects such as animal health and performance as well the quality and safety of the derived foods [2]. The composition of dairy cattle diets varies widely among farms and production systems worldwide, incorporating a broad range of ingredients including roughage, cereal grains, and agroindustrial by-products [2]. The physiological nature of ruminants makes forages (including pastures and conserved forages: silages, hay, and straw) the most adequate and important feed sources for dairy cattle [3]. Additionally, the incorporation of high-density energy dietary sources (concentrate feeds) is essential to achieve the high milk yields demanded and expected in modern dairy farming [2,4,5]. Such diversity of ingredients contributes to the dietary exposure to a broad spectrum of toxic, potentially toxic, and endocrine-disrupting fungal and plant secondary metabolites [6–10].

Crops and feedstuffs are susceptible to mould infection and colonization with subsequent contamination with mycotoxins and other fungal secondary metabolites during the feed-production chain, both pre- and post-harvest, influenced by several biotic and abiotic factors [11]. Probably based on the paradigm that ruminants are less susceptible to the negative effects of fungal toxins [12], most studies concerning mycotoxins and animal feeds have focused on monogastric animals and their main dietary sources (cereal grains). However, a wide spectrum of fungal metabolites (several of them toxic and potentially toxic), primarily produced by *Fusarium*, *Alternaria*, *Aspergillus*, *Penicillium*, and other fungal species, has been found in cattle feed sources beyond cereal grains [6,13]. Some of the mycotoxins are included in the European legislation, which currently establishes a maximum limit for aflatoxin B1 (AFB1) and guidance values (GV) for zearalenone (ZEN), deoxynivalenol (DON), T-2 and HT-2 toxins, fumonisins B1 and B2 (FB1 and FB2), and ochratoxin A (OTA), and thus, their occurrences and levels in feeds and diets have received strong attention [14–16]. More recently, monitoring studies on contamination frequency and levels of ergot alkaloids (EAs) and emerging mycotoxins in animal feeds have been highly advocated [17–23]. Consequently, the characterization of the implicated mycotoxin mixtures needs to be performed with an innovative and holistic approach based on multi-metabolite analyses to achieve an optimal risk assessment [24]. Such multi-metabolite analytic approaches are relevant because, additionally to single negative effects, there are multiple toxicological interactions (such as addition, synergism, potentiation, and antagonism) among mixtures of mycotoxins and other metabolites, which could have implications for health and reproduction. These interactions require more investigation [25,26]. Beyond toxic fungal metabolites, the dairy cattle diet contains substantial levels of plant secondary metabolites, some of which may induce unfavourable impacts on the health and/or reproduction of livestock, such as pyrrolizidine alkaloids, cyanogenic glucosides (CGs), and phytoestrogens (PEs) [27]. Phytoestrogens can act as endocrine disruptors, impairing reproductive functions, generating temporal infertility, and potentially reducing the productive efficiency of dairy herds [9,28–31]. Interestingly, mycoestrogens (such as ZEN, alternariol (AOH), and their modified forms) and PEs (such as isoflavones) have synergistic effects [32–34], which must be considered in the context of a complete risk assessment on livestock reproductive performance [35–38]. Fungal and plant growth as well as concentrations of secondary metabolites in the dietary components and finally in the complete rations are influenced by multiple factors such as plant species/varieties, infecting/colonizing fungal species/varieties, climatic conditions, geography, parasitic/symbiotic interactions, use of pesticides, and other agricultural practices utilized [39–46]. The most influential factors favouring mycotoxin contamination and PE production of feedstuffs and diets of dairy cows should be studied. More data in this field would contribute to developing pre- and post-harvest preventive and management strategies to reduce exposure and optimize the health and productive performance of livestock farming [6,39,46].

Several studies have analysed the occurrence of some mycotoxins in different types of feed ingredients, including in pastures, cereals, and silages [6,7,22,42,47–49]. Research on the incidence of mycotoxins and other fungal secondary metabolites in complete diets (i.e., TMR) of cattle has been carried out during the last decade; however, it is still scarce [50–56]. Targeting the dietary levels of toxins and endocrine-disrupting metabolites is vital to assessing the risks for impacts on health, reproduction, and production [10,24]. Moreover, the whole-diet approach applied across many farms with different farm characteristics and feeding management could reveal true high-risk ingredients in dairy rations. Thus, the current study determined the frequency, levels, and co-occurrences of a wide spectrum of mycotoxins, PEs, and other secondary metabolites in representative samples of lactating cows' diets in 100 Austrian dairy farms, using a validated multi-metabolite liquid chromatography/electrospray ionization–tandem mass spectrometric (LC/ESI–MS/MS) method. Inclusion levels of the basal feed ingredients and their characteristics (chemical composition, particle size, hygienic status), dietary forage proportion, and geo-climatic factors (such as altitude, temperature, relative air humidity, and rainfall) were evaluated for their contribution to the dietary concentrations of mycotoxins, PEs, and other secondary metabolites.

2. Results

2.1. Characteristics of the Diets

2.1.1. Type of Rations and Main Dietary Components

The participating farms fed three kinds of dietary rations to cows: (i) partial mixed ration (87%) and (ii) exclusively forage-based mixed rations (11%), both with separately fed concentrate, as well as (iii) total mixed rations (2%). The frequency and rate of the inclusion levels of the main dietary ingredients in the rations of Austrian dairy cows are shown in Table 1. Grass silage (GS) and MS were the most common forages incorporated in the rations of the visited Austrian dairy farms, presenting frequencies of inclusion over 80% and representing maximums of around 87% and 59% of the rations, respectively. About 60% of the farms used straw in the rations, with maximal inclusion of 10% on a dry-matter basis. Hay was included in around 18% of the evaluated diets, representing from 0.6% to 30% of the ration. Wet brewery's spent grains (BSG) were included in 27% of the diets, with the maximal inclusion level of 13.5% of the total diet. Other silages (e.g., wheat, oats, barley, sunflower, and beep pulp) were included in 10% of the diets, with a maximal inclusion of 23.6% of the rations. The average forage-to-concentrate ratio was 66:34 (Table 1).

2.1.2. Chemical Composition and Particle Size Distribution of Basal Rations

Farms showed variation in the chemical (proximate) composition of the basal ration (Table 1). The dry matter of the basal rations ranged from 25.7% to 54.6%. The basal rations contained an average of around 50% neutral detergent fibre (NDF), ranging from 36.8% to 75.2%. Non-fibre carbohydrate (NFC) ranged from 0.4% to 41.3% (average: 23.3%), and crude protein ranged from 10% to 21.2% (average: 15.4%). Values of ash and crude fat also showed a wide range. Farms used rations with considerable variation in terms of the distribution of the particle sizes (Table 1). Large particles (>19 mm) represented the main particle size in the ration, accounting for 46.8 ± 18% (mean ± SD) of the ration (as-fed basis). Particles of 8–19 mm and 1.18–8 mm represented similar proportions in the ration, with averages of 22.7% and 25.6%, respectively. Finally, the proportion of fine particles (<1.18 mm) represented on average 4.6% of the ration. The value reached a maximum of 13.7% (Table 1).

Table 1. Potential factors influencing the levels of fungal (toxic) metabolites and phytoestrogens: Characteristics of the rations of lactating Austrian dairy cows, the hygienic status of the main ingredients, and geo-climatic parameters of farms' locations.

Dietary Related Factors				
Dietary Component	**Farm Frequency of Inclusion (%)**		**Average ± SD**	**Range**
Grass silage (%DM)	97.5		40.4 ± 16.3	10.4–86.7
Maize silage (%DM)	82.8		22.4 ± 14.3	1.7–59.1
Hay (%DM)	18.2		0.9 ± 3.2	0.6–29.8
Straw (%DM)	62.1		1.8 ± 2.1	0.01–10.0
BSG (%DM)	27.3		4.11 ± 2.4	0.34–13.5
Other silages (%DM)	10.1		6.29 ± 5.67	0.47–23.6
Forage (%DM)	100		65.9 ± 10.1	32.4–89
Chemical composition				
Dry matter (%)			37.1 ± 4.7	25.7–54.6
Crude protein (%DM)			15.4 ± 2.0	9.9–21.2
Ash (%DM)			8.2 ± 2.5	4.8–18.5
Crude fat (%DM)			2.7 ± 0.5	1.2–4.6
Neutral detergent fibre (% DM)			50.4 ± 7.0	36.8–75.2
Non-fibre carbohydrate (% DM)			23.3 ± 7.3	0.8–41.3
Particle size				
>19 mm (%)			46.8 ± 19.8	2.3–96.0
8–19 mm (%)			22.7 ± 11.2	2–53.6
1.18–8 mm (%)			25.6 ± 9.3	1.6–49.0
<1.18 mm (%)			4.6 ± 2.9	0.3–13.7
Hygienic status	**Proper**	**Minor deficiency**	**Significant deficiency**	**Vast deficiency**
Grass silage (%)	54.9	27.5	9.8	7.8
Maize silage (%)	45.7	43.9	3.7	6.7
Hay (%)	91.7	5.6	2.8	0
Straw (%)	80.5	17.1	1.6	0.8
BSG (%)	55.6	37	1.9	5.6
Concentrate (%)	97	1	1	1
Geo-climatic factors				
			Average ± SD	**Range**
Altitude (m a.s.l.)			480.3 ± 162.1	262–1300
Temperature (mean month of sampling) (°C) [a]			15.47 ± 6.19	−0.8–22.4
Temperature (maize's growing season) (°C) [b]			18.7 ± 1.1	13–22
Relative humidity (%) [c]			70.1 ± 3.3	60.3–78
Rainfall (mm) [d]			294.5 ± 60.3	179–594

[a] average temperature of the month of sampling; [b] average temperature of summer (June–September, maize's growing season); [c] average relative humidity of summer (June–September, maize's growing season); [d] rainfall during the summer (June–September, maize's growing season).

2.1.3. Hygienic Status of the Main Dietary Ingredients

The hygienic status of the main components of basal rations (GS, MS, straw, hay, BSG, and concentrate) was determined by sensory evaluation and scored as "proper", "minor deficiencies", "significant deficiencies", and "vast deficiencies" according to Kamphues et al., 2014 [57]. Most samples (>80%) of dried feedstuffs including straw, hay, and concentrates showed a proper hygiene score (Table 1). Wet conserved feeds presented major hygienic status concerns. MS was the feedstuff most often (over 50%) detected for hygiene deficiencies (minor to vast deficiencies). Ensiled grass presented minor deficiencies in hygienic status in 30% of the samples, significant deficiencies in 8%, and vast deficiencies in 3%. Around 44% of the BSG was not in proper hygienic conditions.

2.1.4. Geo-Climatic Factors

The climate conditions of the participating dairy farms were retrieved from the database of the Central Institution for Meteorology and Geodynamics of Austria and are shown in Table 1. Farms were in regions within altitudes ranging from 262 to 1300 m.a.s.l. The average temperature of the month of sampling (May 2019 to September 2020) ranged from −0.8 °C to 22.4 °C. The average temperature during maize's growing season (June–September) varied between 13 °C and 22 °C, with an average of 18.7 °C. The relative air humidity during the maize's growing season was on average 70.1%, fluctuating from 60.3% to 78%. The accumulated rainfall from June to September during the maize growing season was on average 294.5 mm, with minimum and maximum values of 178 mm to 594 mm, respectively (Table 1).

2.2. *Occurrence and Concentrations of the Detected Metabolites*

2.2.1. Groups of Metabolites

In total, 155 out of 863 targeted fungal, plant, and unspecific metabolites were detected in the analysed diets of lactating dairy cattle (Supplementary Table S1), consisting of 121 fungal compounds (including over 40 known mycotoxins), 17 plant metabolites, and 18 unspecific metabolites (Table 2). Their occurrences and respective average (with SD), median, and range of concentrations (expressed on a dry-matter basis in µg/kg) are indicated in Table 2. The detected metabolites were categorized in groups based on their main producers, consisting of *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, lichen-associated fungi, other fungal species, other plant metabolites, and unspecific (i.e., derived from fungi, bacterial and/or plants) metabolites, or corresponding to the kind of metabolites, such as EAs and PEs, according to previous reports [42,58,59]. Fusarial metabolites were detected in all samples and with the highest grade of diversity, with 35 different compounds identified (Table 2). Lower numbers of detected metabolites were derived from *Penicillium* (23), other fungal species (21), *Aspergillus* (16), *Alternaria* (11), and EAs (13). High occurrences (>90%) were detected for the groups of fungal metabolites (*Fusarium*, *Alternaria*, *Aspergillus*, and *Penicillium*), except for the total EAs (32.3% of total samples) and compounds produced by lichen-associated fungi (16.2%) (Table 2). Regarding the dietary contamination levels, the group of fungal metabolites with the highest average concentration was *Fusarium* (1380 µg/kg), followed by *Alternaria* (445 µg/kg), *Penicillium* (205 µg/kg), *Aspergillus* (177 µg/kg), other fungi (115 µg/kg), EAs (19.5 µg/kg), and minor grade lichen-associated fungi (4.57 µg/kg) (Table 2). As displayed in Figure 1, the distribution of the concentrations among groups of metabolites varied widely.

As presented in Table 2, ten different PEs and six additional plant metabolites were identified across all samples. Most of these plant metabolites occurred in high frequencies and high concentrations, with average concentrations of total PEs and other plant metabolites above 70,000 µg/kg and 3000 µg/kg, respectively. A high degree of variation among the samples was marked (Figure 1), with ranges from 1080 µg/kg to 411,000 µg/kg for total PEs and from 5.37 µg/kg to 24,500 µg/kg for the total of other secondary plant metabolites (Table 2). All diets were detected for unspecific metabolites (Table 2). The total concentrations of this category presented an average of 20,000 µg/kg and ranged from 3740 µg/kg to 52,400 µg/kg. The concentration heterogeneity was evident for this group of metabolites (Figure 1).

Table 2. Occurrence and concentration of mycotoxins, phytoestrogens, and other fungal, plant, and unspecific secondary metabolites detected in representative samples of whole diets of lactating cows (n = 198) from Austria.

Group	Metabolite	Positive Samples (%) [1]	Concentration (µg/kg DM) [2]						
			Average	±	SD	Median	Range		
Alternaria	Alternariol [3]	45.5	8.55	±	13.8	5.65	1.09	–	118
	Alternariolmethylether [3]	42.4	5.69	±	3.6	5.50	1.07	–	20.0
	Altenuisol	1.0	15.3	±	5.3	15.3	9.96	–	20.6
	Altersetin	47.0	34.3	±	26.4	26.4	4.16	–	143
	Infectopyrone	78.3	348	±	490	169	6.96	–	3810
	Pyrenophorol	2.5	8.31	±	9.6	4.05	1.90	–	27.5
	Radicinin	1.0	4.44	±	2.7	4.44	1.72	–	7.17
	Tentoxin	30.8	3.79	±	2.2	3.41	1.15	–	12.1
	Tenuazonic acid [3]	78.8	178	±	83.1	153	76.1	–	549
	Zinndiol	1.0	19.8	±	1.9	19.8	17.9	–	21.7
	Zinniol	2.5	42.0	±	23.7	36.4	22.4	–	87.6
	Total [4]	98.5	445	±	491	304	2.62	–	3930
Aspergillus	Aflatoxin B1 [5]	0		-		-		-	
	Averufin	7.6	2.69	±	1.6	2.95	1.07	–	8.03
	Bis(methylthio)gliotoxin	4.0	12.8	±	6.5	11.9	5.67	–	25.7
	Deoxygerfelin	2.0	9.37	±	11.5	3.84	0.75	–	29.0
	Deoxynortryptoquivalin	1.5	3.20	±	0.0	3.20	3.20	–	3.20
	Flavoglaucin	75.8	21.4	±	54.2	5.94	0.65	–	368
	Fumigaclavine	1.0	6.08	±	1.1	6.08	5.00	–	7.15
	Fumigaclavine C	2.0	28.4	±	20.9	24.2	6.52	–	58.6
	Fumiquinazolin D	2.0	26.7	±	11.5	25.8	14.3	–	40.9
	Integracin A	7.1	23.1	±	69.8	1.95	1.11	–	275
	Integracin B	11.6	50.7	±	219	2.87	1.05	–	1080
	Kojic acid	56.1	165	±	62.2	145	132	–	516
	Methylsulochrin	1.0	18.2	±	1.9	18.2	16.3	–	20.1
	Mevinolin	14.1	36.1	±	35.2	23.8	12.0	–	150
	Sterigmatocystin [3]	17.2	3.60	±	2.3	2.65	1.19	–	10.3
	Trypacidin	0.5		-		-		2.78	
	Versicolorin C	2.5	5.80	±	3.3	7.60	1.75	–	9.7
	Total [4]	88.4	141	±	159	150	1.03	–	1680
Ergot alkaloids	Chanoclavine	18.2	7.90	±	12.0	3.23	0.95	–	55.8
	Festuclavine	1.0	11.4	±	8.6	11.4	2.75	–	20.0
	Ergocornine	9.1	6.43	±	7.7	4.32	1.26	–	34.8
	Ergocorninine	5.6	5.53	±	5.7	3.38	1.60	–	22.1
	Ergocristine	4.5	7.86	±	3.4	6.90	1.90	–	13.5
	Ergocristinine	2.5	5.12	±	2.8	4.14	1.35	–	8.53
	Ergocryptine	8.6	10.6	±	11.0	7.21	0.95	–	43.2
	Ergocryptinine	2.0	8.94	±	4.0	9.63	3.49	–	13.0
	Ergometrine	0.5		-		-		7.18	
	Ergosine	9.1	6.76	±	4.8	5.43	0.30	–	17.2
	Ergosinine	8.6	5.01	±	6.1	2.90	0.30	–	24.5
	Ergotamine	6.6	9.64	±	15.4	4.94	1.61	–	62.3
	Ergotaminine	6.1	9.19	±	14.4	3.99	2.00	–	56.2
	Total [4]	32.3	19.5	±	37.3	8.01	0.95	–	219
	15-Hydroxyculmorin [3,6]	94.4	128	±	156	87.3	10.6	–	1600
	Acuminatum B	7.6	47.5	±	18.7	38.8	23.2	–	80.7
	Antibiotic Y	40.4	35.1	±	33.1	24.4	8.52	–	175
	Apicidin [3]	75.8	16.1	±	15.0	12.2	0.75	–	105
	Apicidin D2	8.1	14.7	±	13.1	6.95	6.95	–	57.2
	Aurofusarin [3]	96.0	59.3	±	42.3	46.9	6.79	–	349
	Beauvericin [3]	100	10.3	±	9.1	7.38	0.98	–	71.7
	Bikaverin [3]	66.2	25.6	±	24.6	18.4	3.83	–	161
	Chrysogine	8.6	32.0	±	33.6	23.9	1.68	–	136

Table 2. *Cont.*

Group	Metabolite	Positive Samples (%) [1]	Concentration (µg/kg DM) [2]						
			Average ± SD			Median	Range		
	Culmorin [3]	92.4	361	±	324	272	35.3	–	2952
	Deoxynivalenol (5000) [5]	92.4	153	±	230	104	14.8	–	2900
	DON-3-glucoside [6]	9.1	33.9	±	41.0	19.0	19.0	–	195
	Enniatin A [3]	65.2	1.79	±	3.2	1.07	0.20	–	31.1
	Enniatin A1 [3]	99.5	6.92	±	5.7	5.28	0.40	–	32.3
	Enniatin B [3]	100	40.2	±	28.1	31.4	4.34	–	175
	Enniatin B1 [3]	100	25.9	±	18.7	21.2	2.42	–	126
	Enniatin B2 [3]	69.2	1.34	±	0.9	1.07	0.22	–	6.81
	Epiequisetin [3]	53.5	5.08	±	8.2	3.07	1.07	–	63.4
	Equisetin[3]	97.0	13.4	±	22.3	7.73	1.60	–	224
	Fumonisin A1 (precussor)	1.0	3.97	±	0.4	3.97	3.62	–	4.32
	Fumonisin B1 [5]	70.7	120	±	118	93.5	26.5	–	1120
	Fumonisin B2 [5]	35.4	51.9	±	32.9	45.3	17.0	–	243
	Fumonisin B3	6.1	43.3	±	29.4	26.5	19.9	–	129
	Fumonisin B4	4.5	33.9	±	24.9	18.0	18.0	–	96.9
	Fusaproliferin	4.5	184	±	76.8	174	81.6	–	338
	Fusapyron [3]	2.0	10.9	±	9.6	6.42	3.49	–	27.5
	HT-2 glucoside [6]	1.0	14.4	±	8.4	14.4	6.00	–	22.7
	HT-2 toxin [5]	27.8	27.3	±	28.2	20.5	9.27	–	217
	Moniliformin [3]	40.4	23.4	±	22.4	16.1	4.61	–	148
	Monoacetoxyscirpenol	6.6	13.6	±	7.6	11.0	5.52	–	29.5
	Nivalenol	8.6	311	±	247	269	34.6	–	804
	Siccanol [3]	54.0	709	±	805	494	106	–	7220
	T-2 toxin [5]	12.1	4.97	±	2.5	4.25	2.13	–	14.6
	W493	65.7	21.7	±	69.9	5.64	1.00	–	671
	Zearalenone (500) [5]	77.8	25.2	±	36.9	14.7	1.90	–	378
	Sum of enniatins	100	75.0	±	50.4	61.1	7.36	–	324
	Sum of T-2 and HT-2 toxins (250) [5]	32.3	25.3	±	27.4	20.4	2.13	–	217
	Sum of fumonisins	71.2	150	±	169	106	26.5	–	1590
	Sum of fumonisins B1 and B2 (50,000) [5]	71.2	145	±	149	102	26.5	–	1370
	Sum of type A trichothecenes	36.9	25.0	±	29.8	19.0	2.13	–	246
	Sum of type B trichothecenes	92.9	184	±	266	113	14.8	–	3070
	Total [4]	100	1390	±	1510	1070	109	–	17,800
Penicillium	7-Hydroxypestalotin	3.0	4.39	±	2.6	2.60	2.60	–	9.07
	Andrastin A	16.7	25.8	±	33.7	12.0	1.80	–	140
	Andrastin B	4.0	68.8	±	66.6	48.4	16.5	–	238
	Andrastin C	3.5	270	±	170	247	43.4	–	603
	Barceloneic acid	18.2	36.4	±	29.9	24.7	7.84	–	133
	Citreohybridinol	1.0	3.77	±	1.6	3.77	2.16	–	5.38
	Citrinin	1.0	20.7	±	14.0	20.7	6.67	–	34.7
	Curvularin	6.1	49.7	±	64.3	14.9	2.54	–	182
	Dehydrocurvularin	1.5	54.5	±	35.2	32.8	26.5	–	104
	Fellutanine A	93.9	96.3	±	62.5	78.9	27.7	–	466
	Griseofulvin	0.5		-		-	1.83		
	Hydroxyandrastin C	3.0	10.8	±	7.0	9.41	3.10	–	20.8
	Marcfortine A	23.2	9.49	±	15.4	3.88	0.45	–	81.0
	Marcfortine C	6.1	3.08	±	3.2	1.57	0.45	–	12.1
	Mycophenolic acid [3]	21.2	47.5	±	104	15.8	1.52	–	661
	Ochratoxin A (250) [5]	1.0	7.50	±	0.3	7.50	7.16	–	7.84
	Pestalotin	14.1	5.59	±	2.8	3.30	1.88	–	11.3
	Phenopyrrozin	96.5	52.8	±	36.8	42.7	10.8	–	352

Table 2. *Cont.*

Group	Metabolite	Positive Samples (%) [1]	Concentration (µg/kg DM) [2]							
			Average ± SD			Median	Range		
	Questiomycin A	5.1	27.1	±	14.1	20.8	11.1	–	59.5
	Questiomycin Derivat	18.7	58.4	±	153	32.6	9.82	–	973
	Questiomycine	36.4	8.17	±	9.3	5.23	1.50	–	49.2
	Roquefortine C	18.7	30.3	±	64.7	14.5	3.56	–	387
	Roquefortine D	1.5	9.69	±	7.7	4.25	4.25	–	20.6
	Total [4]	99.5	205	±	176	166	2.71	–	1680
Lichen-associated fungi	Lecanoric acid	6.1	4.71	±	6.0	1.45	1.45	–	18.1
	Usnic acid	11.6	3.83	±	3.2	2.53	0.50	–	12.7
	Total [4]	16.2	4.57	±	4.9	2.47	0.50	–	18.9
Other fungi	Alamethicine	1.5	65.5	±	40.1	61.2	18.8	–	117
	Ascochlorin	9.6	3.35	±	3.3	2.07	1.15	–	13.6
	Ascofuranone	0.5		-		-	3.57		
	Bassianolide	2.0	8.25	±	9.4	3.90	0.80	–	24.4
	Calphostin C	3.0	2.89	±	2.5	1.98	1.09	–	8.34
	Cytochalasin B	13.1	48.3	±	51.2	34.6	8.87	–	234
	Cytochalasin C	1.0	8.77	±	0.9	8.77	7.90	–	9.6
	Destruxin B	27.3	5.66	±	7.5	3.26	0.20	–	44.1
	Emestrin	3.5	16.2	±	11.3	22.3	3.50	–	31.0
	Epoxycytochalsin C	7.6	3.59	±	3.7	0.60	0.60	–	12.2
	Ilicicolin A	13.1	2.53	±	2.8	1.42	0.50	–	10.1
	Ilicicolin B	38.9	4.79	±	6.5	1.89	1.02	–	36.5
	Ilicicolin E	5.6	4.53	±	3.0	3.93	0.50	–	10.2
	Ilicicolin H	22.2	16.1	±	20.8	10.5	0.50	–	123
	LL-Z 1272e	1.5	10.4	±	8.3	8.89	1.03	–	21.3
	Monocerin	33.3	68.1	±	162	11.9	0.65	–	893
	Myriocin	1.0	41.4	±	24.0	41.4	17.4	–	65.3
	Rubellin D	57.1	34.8	±	54.2	15.5	0.85	–	301
	Neoechinulin A	35.9	27.6	±	54.2	17.7	2.00	–	429
	Sporidesmolide II	51.5	65.8	±	114	23.6	0.25	–	617
	Ternatin	1.5	7.59	±	6.5	6.39	0.25	–	16.1
	Total [4]	89.9	115	±	177	45.1	1.15	–	1060
Sum of fungal metabolites		100	2260	±	1690	1993	302	–	19,100
Phytoestrogens	Biochanin	100	21,900	±	15,800	23,000	226	–	52,050
	Coumestrol	80.8	524	±	1140	111	2.50	–	8290
	Daidzein	99.5	5780	±	6670	3110	25.0	–	45,900
	Daidzin	89.9	4527	±	4580	3300	3.38	–	23,900
	Formonetin	21.2	78,700	±	67,900	58,400	13,800	–	289,000
	Genistein	100	9460	±	8950	6730	179	–	52,600
	Genistin	93.4	6000	±	6130	3980	33.0	–	36,500
	Glycitein	53.0	9430	±	10,200	4530	138	–	48,100
	Glycitin	80.8	1205	±	1160	930	12.5	–	7540
	Ononin	73.7	435	±	1050	160	14.0	–	11,540
	Total [4]	100	70,200	±	67,100	50,800	1080	–	411,000
Other plant metabolites	Abscisic acid	89.4	785	±	552	627	136	–	4315
	Chaconin	11.6	31.4	±	41.3	7.50	5.60	–	161
	Colchicine	3.5	71.2	±	87.9	31.6	13.5	–	282
	Linamarin	47.0	2850	±	2860	1520	82.5	–	14,200
	Lotaustralin	74.2	1300	±	2160	558	18.1	–	13,700
	Xanthotoxin	62.6	37.4	±	74.2	10.9	0.90	–	450
	Total [4]	98.0	3090	±	4260	1522	5.37	–	24,400
Sum of plant metabolites		100	73,500	±	67,300	54,500	1204	–	413,000

Table 2. *Cont.*

Group	Metabolite	Positive Samples (%) [1]	Concentration (µg/kg DM) [2]						
			Average ± SD			Median	Range		
Unspecific	3-Nitropropionic acid	8.1	43.4	±	41.2	20.8	10.7	–	158
	Asperglaucide	72.7	5.82	±	12.5	3.23	0.60	–	136
	Asperphenamate	69.2	8.41	±	24.1	2.64	0.50	–	216
	Brevianamid F	100	264	±	147	256	17.0	–	899
	Chrysophanol	53.5	576	±	1390	276	19.0	–	12,500
	Citreorosein	18.2	178	±	197	108	28.3	–	954
	cyclo(L-Pro-L-Tyr)	100	4100	±	2320	3720	569	–	15,400
	cyclo(L-Pro-L-Val)	100	13,700	±	6390	12,780	2720	–	36,900
	Emodin	97.0	249	±	355	92.4	4.26	–	1957
	Endocrocin	9.6	292	±	255	215	40.5	–	1090
	Iso-Rhodoptilometrin	45.5	2.07	±	2.1	1.22	0.40	–	9.01
	N-Benzoyl-Phenylalanine	5.6	28.9	±	37.8	14.2	1.00	–	111
	Norlichexanthone	25.8	20.2	±	103	0.55	0.55	–	745
	Oxyskyrin	0.5		-		-	6.36		
	Physcion	20.2	844	±	683	655	49.7	–	2560
	Rugulusovine	100	271	±	132	257	19.6	–	817
	Skyrin	49.0	4.2	±	4.1	2.92	0.15	–	28.8
	Tryptophol	77.8	1030	±	1200	564	49.2	–	6380
Sum of unspecific metabolites		100	20,000	±	8870	18,600	3740	–	52,400
Sum of all detected metabolites		100	95,400	±	68,900	78,300	15,100	–	432,000

[1] with values > limit of detection (LOD); [2] computations performed without data < LOD. In case values > LOD and < limit of quantification (LOQ), LOQ/2 was used for calculation; [3] classified as emerging mycotoxins [60–62]; [4] accumulative values of occurrences and concentrations of all the metabolites belonging to the group, [5] classified as regulated mycotoxins and their respective maximum level (for AFB1) and guidance levels (for the other mycotoxins) expressed in µg/kg for a dairy cattle feedstuff with a moisture content of 12% (European Commission, 2002, 2006, 2012) [14–17], and [6] modified mycotoxins [63].

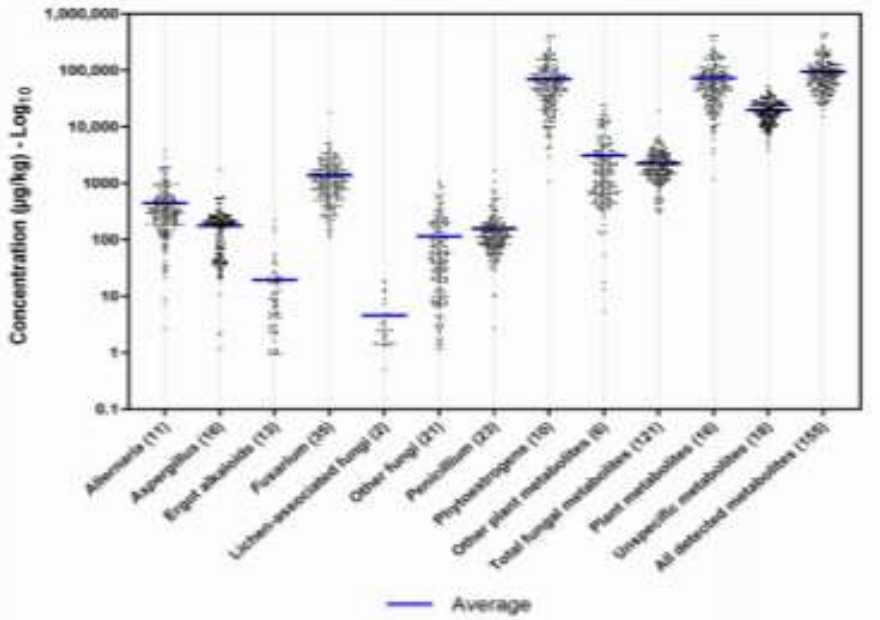

Figure 1. Scatter plot for concentrations (log_{10}) of metabolite groups detected in whole diets of lactating cows (n = 198) from Austrian dairy farms. The total number of metabolites detected per group is shown in parentheses.

2.2.2. Mycotoxins Included in the EU Legislation and Related Compounds

The mycotoxins with GV in the European legislation but not the strongly regulated AFB1 were found in the dietary rations tested in the present study (Table 2). The level of occurrences and heterogeneity in concentrations across samples differed among these mycotoxins (Figure 2A). Accordingly, DON, ZEN, and FB1 were the most abundant and frequently found regulated mycotoxins (Table 2). Type A trichothecenes, T-2 toxin, and HT-2 toxin were detected in frequencies <30%. Metabolites structurally and toxicologically related to the regulated fusarial metabolites, including DON-3-glucoside, nivalenol (NIV), monoacetoxyscirpenol, HT-2 glucoside, FA1, FB3, and FB4, occurred in the studied diets but at lower frequency compared to their parental form (Table 2, Figure 2A). Of these, NIV showed the highest concentration (range: 34.6–804; mean 311 µg/kg). The mycotoxin OTA (produced mainly by *Penicillium* spp. but also by *Aspergillus* spp.) was detected only in 1% of the samples and in low concentrations (<8 µg/kg). In total, 13 different EAs were identified. The individual levels of EAs detected in the evaluated samples of diets' averages were below 12 µg/kg and presented maximum concentrations less than 65 µg/kg, and their occurrences were lower than 20% (Table 2). The concentration distribution across samples was similar among the EAs (Figure 2B).

2.2.3. Emerging Mycotoxins

This study detected 20 compounds classified as emerging toxins [60–62] (Table 2). Emerging mycotoxins were derived mainly from the genera *Fusarium* (15) and, to a lower degree, from *Alternaria* (3), *Aspergillus* (1), and *Penicillium* (1) (Table 2). In total, five forms of enniatins (ENNs) were detected, including ENN A, ENN A1, ENN B, ENN B1, and ENN B2. All of them occurred in at least 65% of the total samples. ENN B, ENN B1, and ENN A1 presented the most frequent detection. The average levels of the individual ENNs were ≤40.2 µg/kg, and the maximum levels were not superior to 180 µg/kg. The sum of ENNs presented an average of 75 µg/kg, ranging from 7.36 µg/kg to 324 µg/kg. Other frequently found metabolites (presented in more than 80% of analysed diets) were aurofusarin (AUR), beauvericin (BEA), bikaverin, culmorin, 15-hydroxyculomorin, epiequisetin, equisetin, and siccanol. Despite the high frequency of contamination with *Fusarium*-produced emerging mycotoxins in the samples, the mean and median concentrations remained below 400 µg/kg, except for siccanol (mean: 709 µg/kg; median: 494 µg/kg; range: 106 µg/kg–7220 µg/kg). All fusarial emerging mycotoxins showed noticeable variations among samples (Figure 2C). The emerging toxins and mycoestrogens derived from *Alternaria* were detected, consisting of AOH, alternariol methyl ether (AME), and tenuazonic acid (TeA). These metabolites were detected at rates between 40% and 80% of the samples, with average concentrations below 180 µg/kg. Among the *Alternaria* metabolites, TeA presented the highest frequency (78.8%) and contamination levels (range: 76.1 µg/kg–549 µg/kg) (Table 1), but its concentrations across samples were more homogenous than infectopyrone (Figure 2D). For *Aspergillus*-derived emerging mycotoxins, the carcinogenic and aflatoxin precursor sterigmatocystin (STC) was detected in 17.2% of the samples, with an average concentration of 3.6 µg/kg, ranging from 1.19 µg/kg to 10.3 µg/kg (Table 2). Mycophenolic acid (MPA) and roquefortine (ROQ) C were detected with frequencies around 20%, showing concentrations varying from 1.52 µg/kg to 661 µg/kg and from 3.56 µg/kg to 387 µg/kg, respectively.

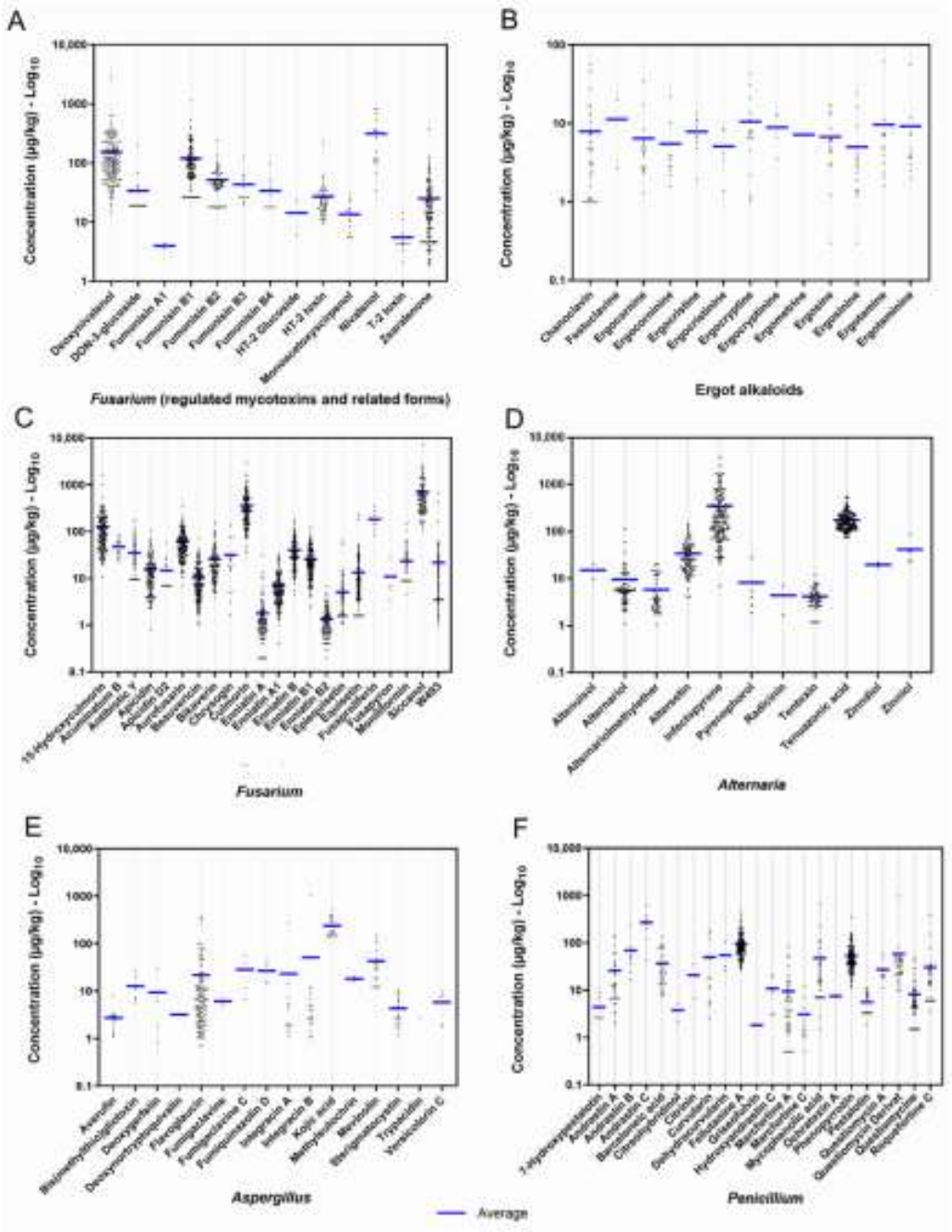

Figure 2. Scatter plots illustrating the distribution of individual concentrations (log_{10}) of mycotoxins and fungal metabolites presented in complete diets of Austrian dairy cows. (**A**) *Fusarium* mycotoxins considered in the legislation, as are related compounds, (**B**) ergot alkaloids, (**C**) other mycotoxins and metabolites from *Fusarium*, and (**D**) mycotoxins and metabolites derived from *Alternaria*, (**E**) from *Aspergillus*, and (**F**) from *Penicillium*. The mean, SD, median, minimum, and maximum values are presented in Table 2.

2.2.4. Other Mycotoxins and Metabolites from *Fusarium*, *Alternaria*, *Aspergillus*, and *Penicillium*

Additionally, many other less-known mycotoxins and metabolites associated with *Fusarium*, *Alternaria*, *Aspergillus*, and *Penicillium* were found in the diets of Austrian dairy cows (Table 2, Figure 2). Metabolites produced by *Fusarium*, including 15-hydroxyculmorin, antibiotic metabolites, and W493, were found in more than 40% of the samples, whereas acuminatum B, apicidin D2, chrysogine, fusaproliferin, and fusapyrone had lower occurrences at below 10%. Concerning other compounds derived from the genus *Alternaria*, infectopyrone (78.3%) and altersetin (47%) were the most frequently found metabolites, after the previously mentioned TeA (Table 2). In terms of concentrations, infectopyrone was the major contaminant produced by *Alternaria* (Figure 2D). Multiple compounds produced by members of the genus *Aspergillus* and *Penicillium* were detected in diverse frequencies of occurrence and contamination levels. Most of the *Aspergillus* and *Penicillium* secondary metabolites were detected in rates lower than 10% of the samples and presented average and median concentrations below 100 µg/kg. For *Aspergillus*-derived metabolites, while kojic acid showed the highest mean concentration (165 µg/kg), flavogluacin was the most frequently found metabolite and presented high concentration heterogeneity across samples (Table 2, Figure 2E). Fellutanine A and phenopyrrozin were the most frequently found *Penicillium* metabolites and had relatively high mean concentrations as compared to other *Penicillium* metabolites (Table 2, Figure 2F).

2.2.5. Metabolites from Lichen-Associated Fungi and Other Fungi Genera

The occurrence of the individual metabolites produced by other fungal species was under 40%, with the exception of rubellin D (57.1%) and sporidesmolide II (51.5%) (Table 2). Monocerin was the most abundant compound in this group (average: 68.1 µg/kg; range: 0.65–893 µg/kg). The ilicicolins A, B, E, and H occurred in concentrations below 125 µg/kg. The two lichen-derived metabolites detected were usnic acid (11.6%, 0.50–12.7 µg/kg) and lecanoric acid (6%, range: 1.45–18.1 µg/kg). Despite relatively low concentrations, the concentrations of other fungi- and lichen-derived metabolites varied considerably among samples (Figure 3A).

2.2.6. Plant Secondary Metabolites (Phytoestrogens and Other Plant Metabolites)

The detected PEs in the rations consisted of nine isoflavones, namely biochanin, daidzein, daidzin, formonetin (synonym: formononetin), genistein, genistin, glycitein, glycitin, and ononin, and a coumestan (coumestrol). With the exception of formonetin (21.2%), all of the phytoestrogens occurred in ≥70% of the samples (Table 2). The contamination levels of isoflavones biochanin, daidzein, daidzin, genistein, genistin, and glycitein were higher than 4500 µg/kg. The metabolites with the highest contamination levels found in this study were formonentin (average: 78,700 µg/kg; range: 13,800–289,000 µg/kg) and biochanin (average: 21,900 µg/kg; range: 226–52,100 µg/kg). Regarding other plant metabolites, abscisic acid, lotaustralin, and xanthotoxin occurred in more than 60% of the evaluated dairy cattle diets, whereas linamarin, chaconin, and colchicine presented lower occurrences (47%, 11%, 6%, and 4%, respectively). The cyanogenic glycosides linamarin (average: 2850 µg/kg; range: 82.5–14,200 µg/kg) and loustralin (average: 1300 µg/kg; range: 18.1–13,700 µg/kg) presented the highest levels within the category of other plant metabolites (Table 1, Figure 3B).

2.2.7. Unspecific Metabolites (Derived from Multi-Kingdom Producers)

Unspecific metabolites can be produced by different and unrelated organisms belonging to diverse kingdoms (Plantae, Fungi, and/or Eubacteria). In this category, four metabolites, namely brevianamide F, cyclo (L-Pro-L-Tyr), cyclo (L-Pro-L-Val), and rugulusovine, were evidenced in all the assessed diets. The compounds asperglaucide, asperphenamate, chrysophanol, emodin, and tryptophol occurred at a rate superior to 50%. Skyrin, iso-rhodoptilometrin, citreorosein, norlichexanthone, and physcion were detected in frequencies between 15% and 50%. Low rates (<10%) of 3-nitropropionic acid, endocrocin,

and N-benzoyl-phenylalanine were detected in the samples. The superior concentrations in the category of unspecific metabolites corresponded to the bioactive cyclic dipeptides cyclo (L-Pro-L-Val) (average: 13,700 µg/kg; max.: 36,900 µg/kg), and cyclo (L-Pro-L-Tyr) (average: 4100 µg/kg; max.: 15,400 µg/kg), as well as the alcohol tryptophol (average: 1030 µg/kg; max.: 6380 µg/kg). The other metabolites of this group presented average concentrations lower than 850 µg/kg (Table 1, Figure 3C).

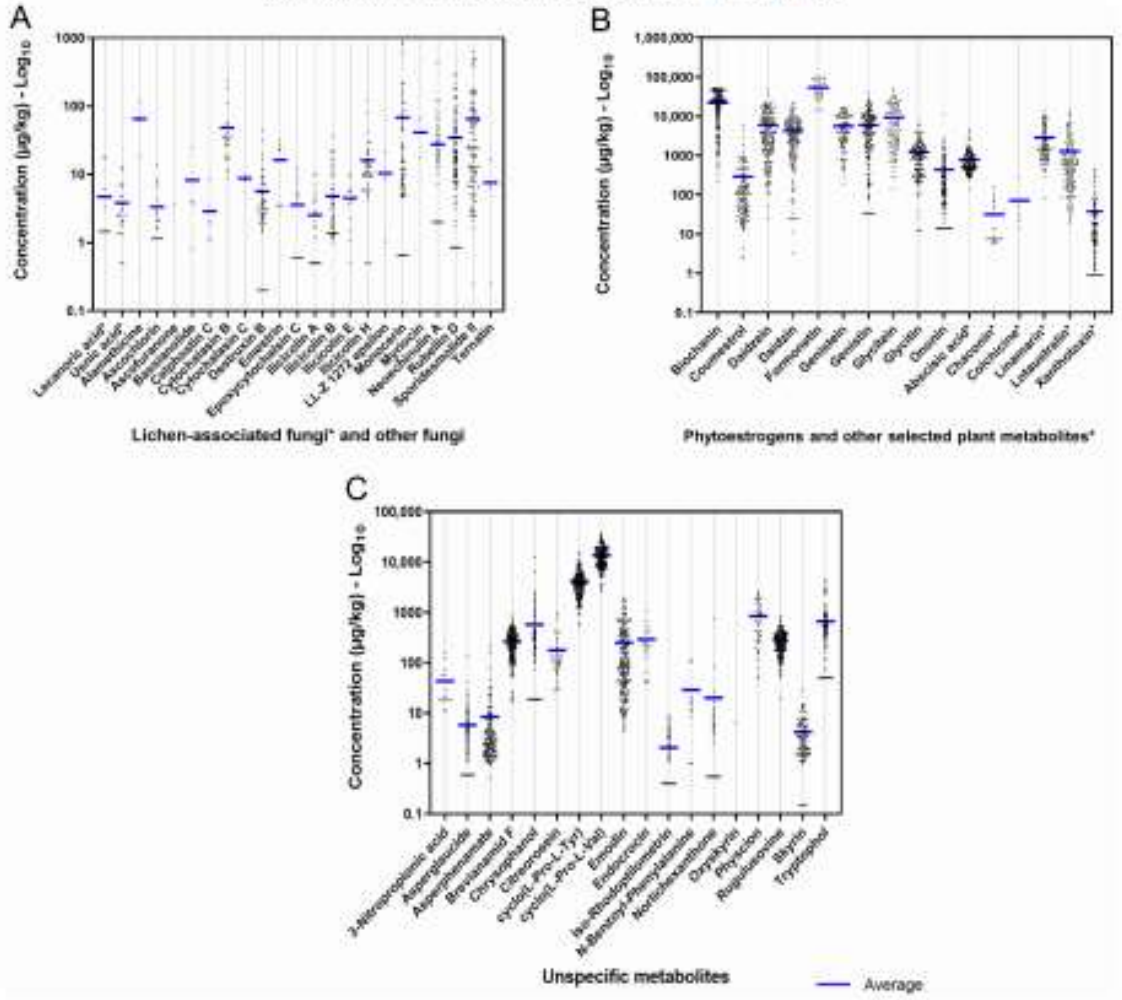

Figure 3. Scatter plots illustrating the distribution of individual concentrations (log_{10}) of metabolites produced by (**A**) lichen-associate fungi and other fungal species, (**B**) phytoestrogens and other plant-derived metabolites, and (**C**) unspecific metabolites (produced by fungi, plants, and/or bacteria) presented in complete diets of Austrian dairy cows. The exact average, SD, median, minimum, and maximum values are presented in Table 2.

2.3. Co-Occurrence of Mycotoxins, Phytoestrogens, and Other Secondary Metabolites

Apparent differences in the number of detected metabolites per sample were observed (Figure 4). Samples were co-contaminated with 29 to 81 metabolites, with an average of 51 co-contaminating metabolites per sample. Considering metabolites derived from fungi, the number per sample ranged from 12 to 58, with an average of 31 compounds. On average, each sample presented a mixture of 8 PEs. The samples contained a mean

of 11 plant-derived and 10 unspecific metabolites, ranging from 3 to 14 and from 5 to 16 metabolites per sample, respectively (Figure 4).

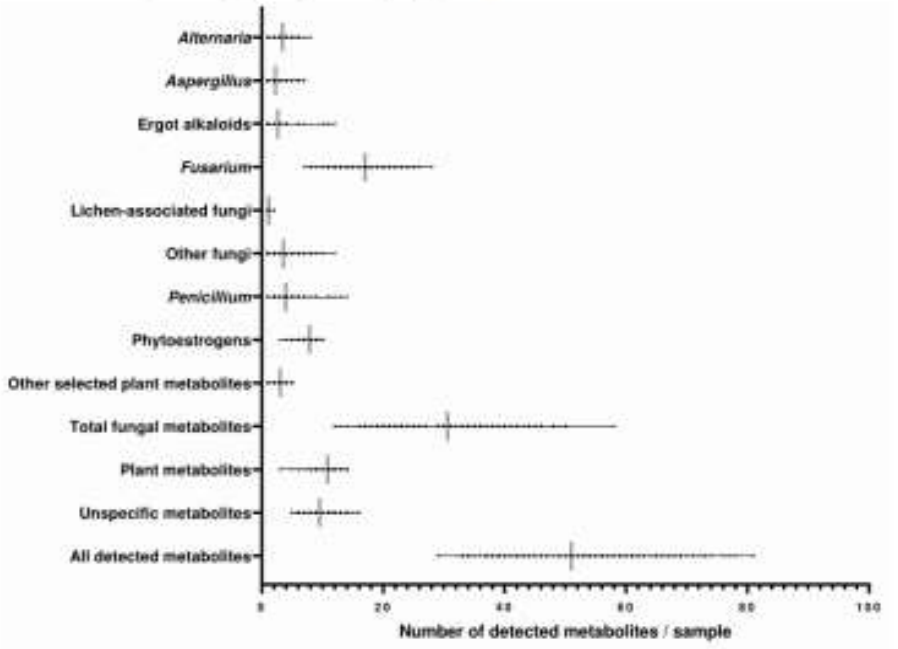

Figure 4. Scatter plots showing the number of metabolites per sample in each metabolite group detected in whole diets of lactating dairy cows in Austria. The grey lines indicate the average numbers of detected metabolites per sample.

The frequencies of co-occurrence analyses between mycotoxins are presented in Figure 5. The most recurrent combinations of mycotoxins detected in the complete rations of dairy cows were between fusarial emerging mycotoxins (ENN A1, ENN B, ENN B1, 15-hidroxy-culmorin, AUR, and equisetin) (100%), which presented co-occurrences over 90%. ENN A1 and ENN B (94%), ENN A1 and ENN B (94%), and ENN A1 and ENN B1 were widespread combinations. The combinations of the other *Fusarium* regulated mycotoxins ZEN and DON (75%), DON and FB1 (68%), and ZEN and FB1 (59%) were considerably frequent. *Aspergillus*-derived metabolites such as flavoglaucin and kojic acid presented co-occurrence with fusarial metabolites up to 79%. Remarkably, more than one-third of the samples showed co-contamination between several emerging *Fusarium* (ENNs, BEA, AUR) and *Alternaria* (AOH, AME, and TeA) mycotoxins.

The co-occurrence rates of PEs, other plant-derived metabolites, and mycoestrogens (AOH, AME, TeA, and ZEN) are illustrated in Figure 6. All tested samples presented co-contamination between biochanin and genistein. Samples often presented with mixtures of PEs with high occurrences (>70%), including the metabolites coumestrol, daidzein, daidzin, genistein, and genistin. Many of the PEs co-occurred with the mycoestrogens in more than 30% of the samples. Particularly, ZEN and TeA showed relatively higher co-occurrences of PEs compared with the mycoestrogens from *Alternaria*.

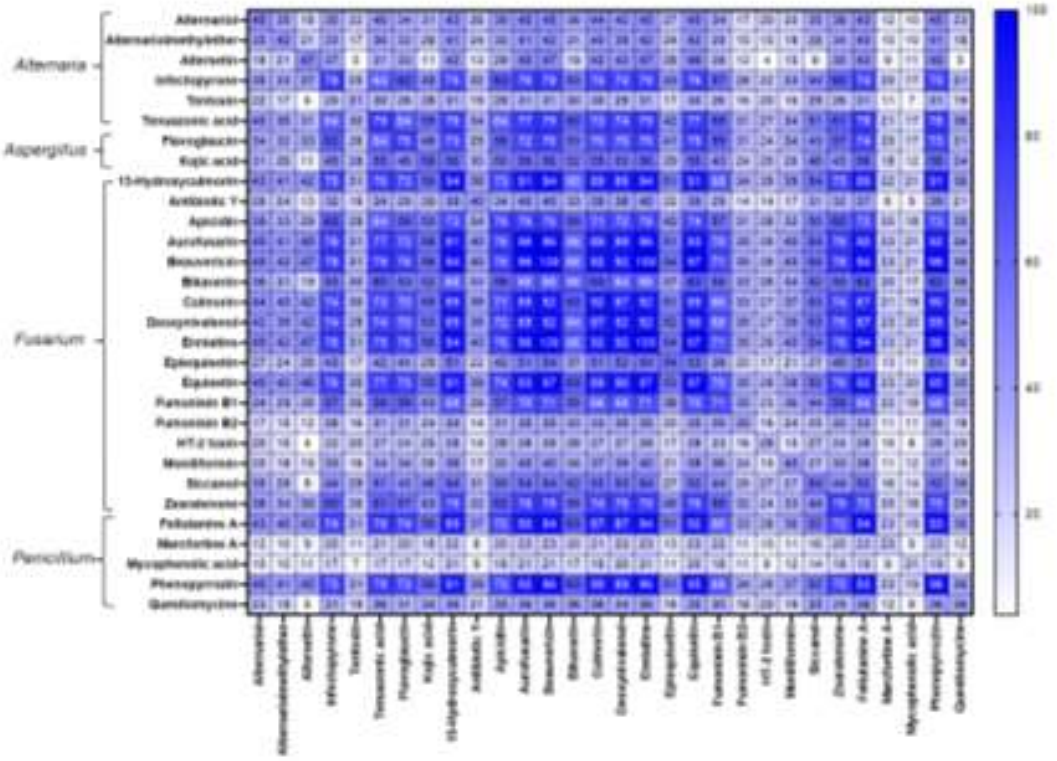

Figure 5. Heatmap indicating the co-occurrence (%) of the selected mycotoxins, which occurred in ≥20% of total samples, detected in the diets of Austrian dairy cows.

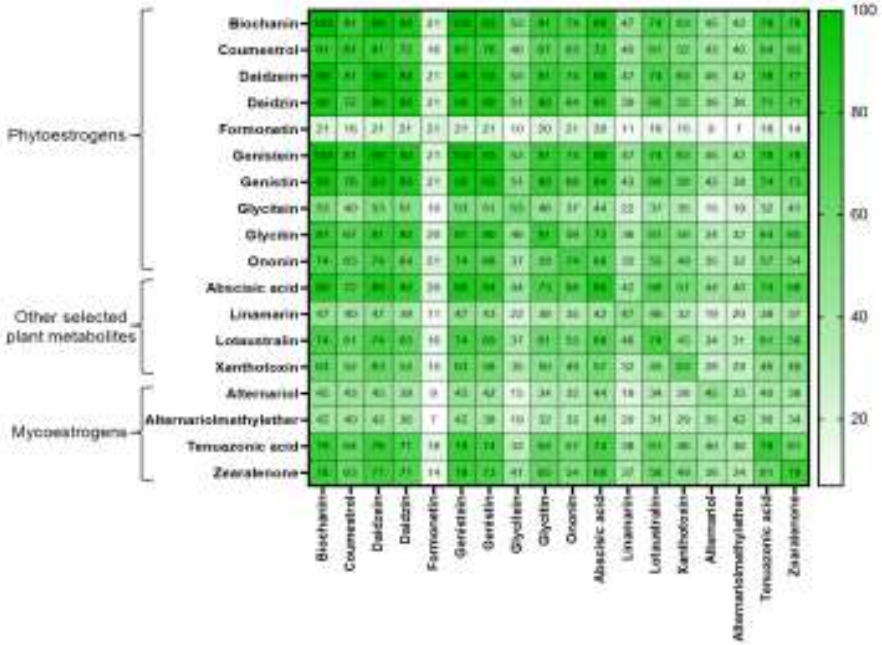

Figure 6. Heatmap indicating the co-occurrence (%) of phytoestrogens, other plant-derived metabolites, and mycoestrogens detected in the whole diets of Austrian dairy cows.

2.4. Dietary Composition and Geo-Climatic Factors in Relation to the Concentration of Mycotoxins, Phytoestrogens, and Other Secondary Metabolites

Correlations between recorded dietary and geo-climatic factors (see Table 1) with the contamination with fungal (toxic) metabolites of interest were screened using Spearman correlation analysis. Based on this approach, we observed some potential factors (i.e., Spearman rank correlation coefficient (ρ) $\geq$ 0.3). Among dietary ingredients, we found that MS showed the highest correlations with the concentration of *Fusarium* mycotoxins such as DON (ρ = 0.40, $p < 0.001$), sum of type-B trichothecenes (ρ = 0.38, $p < 0.001$), ZEN (ρ = 0.30, $p < 0.001$), CUL (ρ = 0.32, $p < 0.001$), BEA (ρ = 0.42, $p < 0.001$), and total *Fusarium* metabolites (ρ = 0.36, $p < 0.001$). The content of straw in the ration showed a significant positive correlation with infectopyrone (ρ = 0.62, $p < 0.001$), total *Alternaria*-derived metabolites (τ = 0.52, $p < 0.001$), and total fungal metabolites (ρ = 0.33, $p = < 0.001$). The dietary proportion of BSG presented a significant positive correlation with the contamination levels of many *Fusarium*-derived mycotoxins such as ENN A1 (ρ = 0.47, $p < 0.001$), ENN B1 (ρ = 0.38, $p < 0.001$), and total ENNs (ρ = 0.35, $p< 0.001$). The proportion of feed particles with size between 1.18 and 8 mm presented a low positive correlation with the presence of fusarial metabolites (ρ = 0.33, $p < 0.001$), whereas the proportion of ration with a size longer than 19 mm correlated negatively (ρ = −0.33, $p < 0.001$). Of the geo-climatic conditions studied (see Table 1), the temperature during the maize's growing season showed a positive correlation with type B trichothecenes (ρ = 0.36, $p < 0.001$), AUR (ρ = 0.33, $p < 0.001$), BEA (ρ = 0.37, $p < 0.001$), and total fusarial metabolites (ρ = 0.30, $p < 0.001$).

Multiple regression models of the log-transformed concentration values of compounds derived from species of *Alternaria*, *Fusarium*, and *Penicillium*, total fungal and some individual mycotoxins such as ZEN, DON, ENNs, BEA, CUL, as well as the sum of FB1 and FB2 are presented in Table 3. Influences of some dietary factors based on the simple correlation method were confirmed by a multiple regression approach. Importantly, the multiple regression approach revealed a joint effect of multiple factors attributed to the dietary concentration of mycotoxins. Inclusion levels of MS and straw, the proportion of particles >19 mm, and dietary NFC content affected total concentrations of *Fusarium* metabolites. Together, these factors explained 52% of the variance, which is the highest value observed in this present study. Specifically, the proportion of MS and its combination with straw positively influenced the contamination levels of *Fusarium*-derived metabolites (slope = 0.004, p = 0.042, Table 3 and Figure S1). As shown in Figure 7, at the same level of MS, farms using more straw showed higher *Fusarium* contamination and vice versa. Interestingly, a quadratic effect of the proportion of MS was observed, and the total *Fusarium* metabolite peaked at an MS level around 30–35% of the basal diet DM before dropping to a higher MS level (Figure 7). A similar outcome was observed via logistic regression analysis that estimated an odds ratio of 1.05 (95% confidence limits: 1.01–1.08) and predicted a close to 75% chance for high loads of *Fusarium* metabolites at MS inclusion level of 30% of the diet DM (Supplementary Data Table S2 and Figure S1). For individual fusarial mycotoxins, the inclusion of MS positively influenced the contamination level of DON and emerging mycotoxins BEA, CUL, and SIC, while the proportion of particles >19 mm negatively influenced the contamination of ZEN, CUL, SIC, and total *Fusarium* metabolites. However, this depended on the inclusion level of MS. As shown in Figure 7, when no MS was used, the contamination of *Fusarium* metabolites increased with an increment in the proportion of particles >19 mm. With the inclusion of MS, the effect of MS dominated the effect of the proportion of particles >19 mm. Only ZEN was related to the level of ether extract (i.e., crude fat) of basal diet and hygiene score of GS. In agreement with simple correlation analysis, the inclusion level of straw affected the contamination level from *Alternaria* but with a significant quadratic effect. The influence of the temperature during the maize's growing season was only confirmed for the BEA contamination. The influence of the dietary proportion of BSG on the total ENN commination was confirmed by the multiple regression approach; however, it explained only 19% of the variance. In addition to the factors listed above, the ash content in basal diets (with a positive quadratic response)

contributed to the concentration of total fungal metabolites. None of the factors studied substantially explained the concentration of phytoestrogens and plant metabolites (data not shown).

Table 3. Influences of the dietary parameters and geo-climatic factor on the concentration of mycotoxins, fungal metabolites, and phytoestrogens.

Concentration (Log-µg/kg)	*n*	Intercept	SE	*p* Value	Influencing Factors	Coefficients	SE	*p* Value	R^2	RMSE
Alternaria metabolites	190	5.2607	0.0821	<0.001	Straw	+0.3851	0.0616	<0.001	0.26	0.757
					Straw × Straw	−0.0282	0.0082	<0.001		
Fusarium metabolites	198	6.2526	0.5082	<0.001	MS	+0.0695	0.0147	<0.001	0.52	0.579
					Sieve > 19 mm	−0.0158	0.0072	0.030		
					Straw	−0.5902	0.1714	<0.001		
					NFC	−0.0413	0.0174	0.019		
					MS × MS	−0.00082	0.0002	<0.001		
					MS × Straw	+0.00398	0.0019	0.042		
					MS × Sieve > 19 mm	−0.00041	0.0002	0.016		
					Straw × NFC	+0.02352	0.0069	<0.001		
					Straw × Sieve > 19 mm	+0.01151	0.0031	<0.001		
					NFC × Sieve > 19 mm	+0.00064	0.0003	0.047		
					Straw × NFC × Sieve > 19 mm	−0.00047	0.0001	<0.001		
Deoxynivalenol	182	5.7616	0.8443	<0.001	MS	+0.09058	0.0247	<0.001	0.22	0.677
					Rainfall	−0.01240	0.0049	0.013		
					MS × Rainfall	−0.00017	0.0001	0.031		
					MS × MS	−0.00057	0.0002	0.010		
					Rainfall × Rainfall	+0.000022	0.0000	0.009		
Zearalenone	154	0.9462	0.5023	0.057	EE	+0.3897	0.1421	0.007	0.22	0.918
					Sieve > 19 mm	−0.0124	0.0041	0.003		
					Hygiene GS	+0.2147	0.0745	0.004		
Fumonisins B1 and B2	125	4.6964	0.110	<0.001	Straw	−0.07163	0.0278	0.011	0.09	0.606
					Hygiene MS	+0.1470	0.0585	0.013		
Beauvericin	198	−1.3010	0.6717	0.054	MS	+0.0152	0.0037	<0.001	0.32	0.654
					Sieve 1.18–8 mm	+0.0198	0.0055	<0.001		
					Crop temperature	+0.1439	0.0374	<0.001		
Culmorin	183	4.4483	0.3138	<0.001	MS	+0.06254	0.0157	<0.001	0.34	0.611
					Sieve > 19 mm	−0.00234	0.0046	0.611		
					MS × MS	−0.00072	0.0002	0.001		
					MS × Sieve > 19 mm	−0.00039	0.0002	0.025		
Enniatins	198	3.5175	0.1333	<0.001	Brewery's spent grains	+0.1111	0.0192	<0.001	0.19	0.600
Siccanol	107	7.0348	0.4132	<0.001	MS	+0.0016	0.0067	0.808	0.30	0.627
					Sieve > 19 mm	−0.0098	0.0033	0.003		
					Straw	−0.0487	0.0524	0.353		
					MS × Straw	+0.0052	0.0021	0.016		
Penicillium metabolites	187	3.9964	0.2731	<0.001	Temp sampling	+0.0726	0.0233	0.002	0.12	0.483
					Forage	+0.1135	0.0035	0.002		
					Temp sampling × Temp sampling	−0.0024	0.0097	0.013		
Total fungal metabolites	190	9.0404	0.7690	<0.001	MS	−0.0118	0.0012	0.345	0.44	0.408
					Straw	+0.0864	0.0509	0.091		
					Ash	−0.3912	0.1364	0.005		
					NFC	−0.0148	0.0055	0.008		
					Sieve > 19 mm	+0.0003	0.0035	0.932		
					Hygiene GS	−0.0633	0.0314	0.045		
					Ash × Ash	+0.01395	0.0056	0.014		
					Straw × Straw	−0.01337	0.0046	0.004		
					Straw × Sieve > 19 mm	+0.00185	0.0008	0.019		
					MS × Sieve > 19 mm	−0.00031	0.0001	0.011		
					MS × Ash	+0.00421	0.0017	0.015		

SE = standard error; RMSE = root mean square error; MS = proportion of maize silage in the diets; Straw = proportion of straw in the diets; Ash = proportion of ash in the mixed rations; NFC = proportion of non-fibre carbohydrates in the mixed rations; EE = proportion of etheric extract in the mixed rations; Sieve > 19 mm = proportion of feed particles with diameter longer than 19 mm in the diets; Temp sampling = temperature at the sampling month; Crop temperature = average temperature of summer (June–September, maize's growing season); Rainfall = accumulated rainfall (mm) during the summer (June–September, maize's growing season); Humidity crop = average relative humidity (%) of summer (June–September, maize's growing season); Hygiene MS = hygienic score of maize silage; Hygiene GS = hygienic score of grass silage.

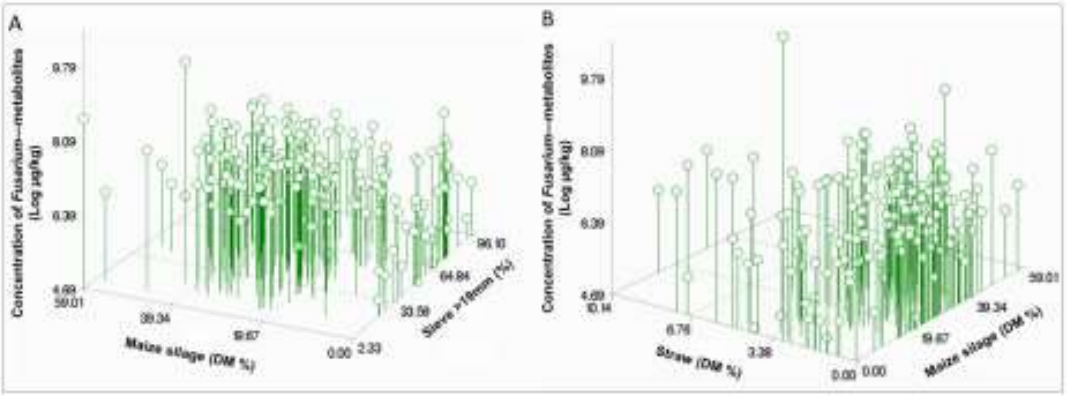

Figure 7. Scatter plots in 3D of the combined influence of dietary factors on the levels of *Fusarium* metabolites (Log-µg/kg) in diets of dairy cows in Austria. (**A**) Influence of content (% DM) of maize silage and particle size > 19 mm (%). (**B**) Influence of the content (% DM) of maize silage and straw.

3. Discussion

Fusarium metabolites were the most relevant fungal contaminants in the rations of dairy cattle surveyed in the present study, corroborating again the importance of *Fusarium* as one of the most widespread mycotoxigenic species in crops and the main contributor to mycotoxin contamination in animal feeds [7,64,65]. Among the EU-regulated mycotoxins, the type B trichothecene DON (occurrence: 92%) was predominant, followed by the mycoestrogen ZEN (77%) and FB1 (71%). The type A trichothecenes, T-2 and HT-2 toxin, which are more cytotoxic than the type B trichothecenes [66], were detected in low frequencies (12% and 18%, respectively) and concentrations (on average < 30 µg/kg). The contamination levels of these regulated mycotoxins were not over the guidance values of the European Union for feeds of dairy cattle. However, it has been proven that even dietary contamination under the EU values can negatively affect the performance, digestion, and immunity of dairy as well as beef cattle [67]. We showed that numerous non-regulated emerging and modified toxins produced by *Fusarium* spp. were even more recurrent and presented higher contamination levels. Although the tangible implications resulting from exposure to modified and emerging mycotoxins are not properly characterized, it is known that these compounds interact with other well-recognised fungal toxins, increasing their toxicological activity [24,25]. The high occurrences, concentrations, and diversity of metabolites derived from *Fusarium* spp. confirm the omnipresence and relevance of this genus in the mycotoxin contamination of crops and animal feeds [64,65]. Emerging *Fusarium* mycotoxins ENNs and BEA have antibacterial and cytotoxic properties; however, their implications for health and performance in ruminants are underexplored [21,68]. Research on the impact of such kinds of compounds on rumen ecology and functionality is crucial [12,61,69]. Maize silage and straw were the main forage components that drove the increasing concentration of *Fusarium* metabolites, which lined up with previous studies in the Netherlands [70,71] and Spain [50]. Viable *Fusarium* spp. is rarely isolated in ensiled maize, suggesting that *Fusarium* species do not grow properly during the ensiling process [72]. However, it has been widely proposed that mycotoxins of *Fusarium* spp. are mainly produced during crop growing [73,74] and, therefore, field conditions such as temperature influence mould proliferation and mycotoxin synthesis, supporting the notion that global warming promotes mycotoxin contamination in crops and feeds [46,75–77]. Various studies mark the key effects of temperature and humidity on mycotoxin contamination [39,42,46]. Based on our correlation analysis, environmental temperature increments during the crop's late growing season (June to September) and the sampling month were associated with a higher accumulated concentration of some *Fusarium* mycotoxins (type B trichothecens, AUR, and BEA) and *Penicillium* metabolites, respectively. However, its significance was not confirmed by

the multiple regression approach, except for BEA. We also did not observe significance for humidity. This might be explained by the accuracy of available climatic data when studying dietary contaminations coming from multiple sources (self-produced and purchased feed as well as different time of storage). This means that spot or average climatic data do not match the concentration at sampling as precisely as studies of single feed sources such as pasture [42].

In general, we did not observe dietary concentrations of regulated mycotoxins exceeding the EU maximum limit and GVs. Compared to the earlier study in Spain by Rodriguez-Blanco et al. (2020), we observed higher occurrences of regulated *Fusarium* mycotoxins. The researchers studied a similar number of total mixed rations (n = 193) from different areas of Spain during the period from February 2016 to January 2018 and found that DON (16.6%), ZEN (16.0%), and the sum of FB1 and FB2 (34.2%) presented lower occurrences and slightly higher average concentrations than those found in our study. However, all the samples showed values under the EU recommendations [78]. Other mycotoxin surveys performed in several European countries have also evidenced high occurrences and contamination levels of *Fusarium* mycotoxin in MS [61,79]. Dreihuis et al. (2008) estimated the dietary intake of four mycotoxins (DON, ZEN, ROQC, and MPA) of high-producing dairy cows in different regions of the Netherlands. The detected mean concentrations of DON, ZEN, ROQC, and MPA in complete diets were 273 µg/kg, 28 µg/kg, 114 µg/kg, and 54 µg/kg, respectively. Consistent with our findings, they reported that MS was the major feed source of these mycotoxins in the diet [70]. Similarly, other studies underline MS as the potential feed source of *Fusarium* mycotoxins [61,78]. Matching our results, *Fusarium*-derived mycotoxins were the most recurrent fungal contaminants with the highest concentrations detected in total mixed rations of Brazilian feedlots [54]. Europe-based studies, including the present research, rarely report the detection of AFB1. Nevertheless, this was the case in a recent study on Lithuanian dairy farms [56]. In that study, the analysis of total mixed rations (n = 51) collected in 2019–2020 showed that 60.8% of the rations were positive for AFB1, 54.9% for DON, 49% for ZEN, and 29.4% for T-2 toxin, and AFB1 exceeded the maximum concentration limits in haylage samples [56]. Moreover, the maximum average concentrations of AFB1 and T-2 toxin were found in the GS samples, while some samples of ensiled maize had ZEN and DON concentrations exceeding the EU GVs. Relating to toxic compounds produced by Aspergilli, the absence of strongly regulated AFB1 and other AFs was expected, because the occurrence of these mycotoxins in central Europe has been considered rare [46]. However, we detected precursors of AFs, such as averufin, STC, and versicolorin C [79,80], albeit at low frequencies (<20%) and concentrations (<11 µg/kg). Regarding STC, it has been suggested that this mycotoxin can be produced pre-and post-harvest [81]. Like AFs, STC is a known carcinogenic with immunotoxic and immunomodulatory activity. In general, the information available on exposure data of dairy cows to these precursors of AF is still very limited [18,60]. Fungi of the genus *Bipolaris*, *Chaetomium*, and *Emiricella* are able to synthesize STC [82]. OTA, considered in the European regulation, is produced by *Penicillium* and *Aspergillus* spp. and presented very low occurrence and contamination levels in the present survey, which suggests that this mycotoxin presents a minor risk for Austrian dairy herds. Additionally, kojic acid, produced primarily by *Aspergillus* spp. but also by *Penicillium* and *Acetobacter* fungi [83], has been shown to have low toxicity for human macrophages, along with antibacterial and immunomodulatory properties [84–86]. In the present study, due to low frequencies as well as high heterogeneity of the metabolite composition among farms, we did not identify factors associated with the contamination of *Aspergillus* metabolites.

Other potentially harmful contaminants occurring in dairy cows' diets were compounds derived from the genus *Alternaria*, some of which are considered emerging mycotoxins, such as AOH, AME, and TeA. Our study indicates that they are commonly presented in the diets of Austrian dairy cows. *Alternaria* spp. can grow and produce toxins in various crops in the field and post-harvest stage, causing considerable losses due to decomposition [87,88]. Our analysis further indicates that straw contributes to contamination from

Alternaria. Data and information regarding occurrence in the feeds and toxicological implications of *Alternaria* toxins for livestock systems are still missing [88–90]. Our survey suggests that the occurrence of metabolites of *Alternaria* should not be ignored. For instance, TeA was the most frequently detected *Alternaria* metabolite in the diets of Austrian dairy cows. This mycoestrogen targets protein synthesis inhibition at the ribosomal level and is considered, concerning toxicity, the most important metabolite produced by *Alternaria* spp. [91]. The benzopyrene derivatives AOH and AME are not related to acute toxicity but are known for their genotoxic effects [92–94]. Moreover, AME, AOH, and TeA are also classified as mycoestrogens, showing strong synergistic estrogenic effects in combination with mycoestrogen ZEN even at very low concentrations [32,33,95]. Our co-occurrence analysis showed that 30% to 60% of the samples displayed co-contamination between ZEN and *Alternaria*-derived AOH, AME, and TeA.

The analysed diets presented several *Penicillium*-derived toxins, which are considered the most relevant post-harvest mycotoxins contained in silages [6,96–100]. However, the production of such toxins is also possible in the field [72,101]. MPA and ROQs are considered the most investigated *Penicillium* metabolites occurring in silage [6]. A common feature of many *Penicillium*-derived exometabolites such as MPA, ROQs, CIT, and OTA is their immunotoxic properties [102,103], which could interfere with the activity of innate and adaptative immune responses, predisposing the animals to secondary infectious diseases [104]. *Penicillium* toxins have been linked with appetite reduction, affecting nutrient efficiency, and increasing the incidence of abomasal ulcers, laminitis, gastroenteritis, abortion, and paralysis [105]. Additionally, toxins produced by *Penicillium* spp. Such as ROQ C have neurotoxic activity [106]. Despite their abundance in feeds and their potential harmful properties, the economic relevance of *Penicillium* mycotoxins in livestock farming is considered underestimated, because even though mycotoxins are believed to be rapidly metabolized by gut microbiota and hepatic enzymes [104,107–109], the detoxification process of mycotoxins can still be disrupted by their antimicrobial and hepatotoxic properties [104,107,110–114]. *Penicillium*-derived mycotoxins are mostly associated with storage, being detected frequently in mouldy spots of silages [100,115,116]. Although the temperature of the samplings' month presented a negligible correlation ($\rho = 0.20$, $p = 0.004$) in our study, several studies performed under controlled conditions have proven that *Penicillium* growth and toxin production were strongly increased by higher temperatures [117–120]. *Penicillium roqueforti* has been described as the most predominant fungi in mouldy sections of silages in Austrian dairy farms [100]. Contamination with storage mycotoxins (mainly associated with *Penicillium*) can occur even in good-quality silages, since aerobic spoilage is practically unavoidable during feed-out [121]. Our findings did not reveal relationships between the hygienic status of the main feedstuffs (GS, MS, straw, hay, BSG, and concentrate) and the contamination levels, which has been reported previously in forages [122]. This can be explained by the fact that toxin production by a fungus does not correlate directly with its growth [123]. Over 30% of the evaluated diets contained EAs, toxic compounds associated with diverse endocrine, vascular, and neurological effects [124]. These can be commonly detected in cereal grains as well as in pastures [42,125,126]. Dietary exposure to EAs in dairy cattle can produce unspecific effects such as reduced productive and reproductive performance and acute clinical signs of ergotism including hyperthermia, convulsions, gangrene in distal portions of the body, and fatalities [127–129]. It was stated that feeds exceeding 250 µg/kg of EAs should not be fed to pregnant or lactating animals, because it could increase the risk of abortion and agalactia syndrome [126]. Additionally, further less-known metabolites are produced by other fungi detected in the diets of dairy cows. Some of them have antibacterial activity, for example, the anthraquinone rubellin D [130,131], illicicolins [132], monocerin [133,134], and cytochalasins [135,136].

Interestingly, the recent analysis indicates that as compared with contamination from other fungal groups, contamination of *Fusarium* metabolites can be explained to a greater extent by dietary factors that are mainly related to forage components. We demonstrated a complex relationship between MS, straw, and proportions of NFC and large particles

(>19 mm) that drives the contamination of *Fusarium* metabolites in dairy cow diets. With our multiple regression approach, the independent factors can explain 50% of the variance, substantially higher than a previous study that used a simple correlation analysis [137]. As explained before, other studies have underlined MS as a potential feed source of *Fusarium* mycotoxins. This could be explained by the fact that starch induces mycotoxin production (e.g., trichothecenes) in *F. graminearum* [137]. Thus, the superior content of non-fibre carbohydrates (such as starch) in maize and cereal plants compared to other forages such as GS and hay could explain the elevated levels of mycotoxins and other secondary metabolites. Furthermore, we found that, in addition to MS, straw was likewise an influential forage component. Straw is often added to dairy cow diets containing high grains and high MS to compensate for physical characteristics (long fibre) of the diet. As minor dietary components, the hygienic as well as chemical characteristics of straw likely receive less attention as compared to main forage sources such as MS, GS, and hay. Mould infection could be present in straw but might not be screened out before feeding. We found that, in addition to *Fusarium* metabolites, straw was also a determinant for contamination with *Alternaria* metabolites. The black mould genus *Alternaria* includes various saprophytic, endophytic, and pathogenic species, which occur worldwide in different habitats such as soil, as well as on dead or dying plant tissues such as straw [138]. A recent Swiss survey targeting a broad spectrum of mycotoxins in barley products found higher concentrations of total fungal metabolites in straw than in grains [139]. Interaction of dietary large particle size with MS and with straw partly represented shifts in the physical characteristics of the diet based on the combination of forage choices. Dietary ash content did not influence concentrations of metabolites from *Fusarium*, *Alternaria*, or *Penicillium*, but it did influence total fungal metabolites. Its positive quadratic effect indicates that high fungal metabolite loads are associated with high dietary ash content. High dietary ash contents are an indicator of contamination with soil, which affects the hygienic quality of the feedstuffs. All in all, although the current data could prove partial roles of the main dietary factors, the outcome underlines that there is no single factor that dominantly influences the dietary contamination. Rather, the dominant influence comes through the combination of forage choice, management (particle length), and the hygienic status of feed sources.

Another novel outcome of the present study was related to PEs, which constitute the extensively recurrent class of metabolites contained in dairy rations. PEs are of concern in veterinary medicine and public health due to their endocrine-disrupting activity. These substances especially affect the reproductive organs and process, inducing infertility in livestock [140,141]. These metabolites are found primarily in *Leguminosae* plants, such as soy, but also in clovers (*Trifolium* spp.) and alfalfa/lucerne (*Medicago sativa*) [9,28,142]. Coumetrans such as coumestrol seem to be more potent in estrogenic activity [9,31]. The levels of coumestrol detected in diets of Austrian dairy herds in the present study were below the reported critical range (18–180 mg/kg) [141]. Their interaction with other estrogenic substances (such as mycoestrogens) is currently the focus of interest [38]. Other plant-derived compounds such as the cyanogenic glucosides linamarin and lotaustralin observed in the present study did not exceed the maximum limit (50 mg/kg) of total cyanogenic compounds established by the European Union [143]. Both compounds (linamarin and lotaustralin) have a relatively broad distribution in the plant kingdom, being found in high concentrations in cassava, soy, cereal, clovers, and other plant species [53,144]. In general, levels of these compounds in clover are not high enough to cause acute toxicity. Some clinical manifestations include dyspnoea, muscular contractions, and oedemas in mucous membranes [145]. Nevertheless, reports of cyanide poisoning of livestock are rare, suggesting that levels of cyanide- or HCN-producing compounds in the feed are generally low [143], as is also the case for the present study. The inclusion of hay showed a major correlation with both linamarin and lotastratin in this study. Among the unspecific metabolites detected were molecules of some biologically active toxins, which increase the toxicological complexity of the cocktails of secondary metabolites evidenced. These include, for instance, emodin (antibacterial and immunosuppressive) [146,147], 3-nitropropionic acid

(neurotoxic) [148,149], skyrin [147], brevianamide F (cyclo-L-Trp-L-Pro) (antifungal and antibacterial) [150], cyclo (L-Pro-L-Tyr), and cyclo (L-Pro-L-Val) (antibacterial) [151,152]. The complex profiles of co-contamination with different mycotoxins, PEs, CGs, and other metabolites occurring in the diets of high-yielding dairy cows suggest unexplored and unpredictable synergistic as well as antagonistic toxic effects. Most of the detected metabolites represent unregulated compounds with a high diversity of biological and toxic activity, indicating that the characterization of the regulated contaminant in dairy feeds is only the tip of the iceberg of fungal and other environmental toxins.

4. Conclusions

This study underlined the omnipresence of a broad number of mycotoxins (most of them unregulated), Pes, and other metabolites occurring in diets of dairy cows in Austria. Overall, the Austrian dairy rations are safe when considering that the detected contamination levels were below the guidance values of the EU commission. Nevertheless, a vast majority of mycotoxins and metabolites are emerging ones, as well as less-known and less-studied fungal metabolites. Overall, *Fusarium*-produced metabolites and mycotoxins were the dominant fungal contaminants. Additionally, we found that dietary factors related to the use of forages, rather than concentrating sources, contribute to increased contamination of mycotoxins in Austrian dairy rations. Among typical forage sources, the content of MS and straw were the most influential factors linked to the concentration of *Fusarium* metabolites in the complete rations. The analysis further addressed the influences of characteristics of diets and hygienic substandard of forages. Individually, the detected mycotoxins represented a relatively low or safe level based on EU regulation and literature. However, the co-exposure to mycotoxins and other (fungal and plant) secondary metabolites has unpredictable effects. Our findings make clear that the evaluation of contamination with only regulated mycotoxins offers a limited picture of the possible toxicological risks to animal health, reproduction, and productivity. Therefore, it is crucial to elaborate surveillance and monitoring programs for a broad spectrum of metabolites in the dairy feed chain and to understand their toxicological effects. Furthermore, there is a need to increase awareness of the importance of feed management and nutrition as reduction and prevention measures for mycotoxin contamination in dairy production. Monitoring and further research based on multi-metabolite approaches in the dairy industry in other geographic regions are still necessary.

5. Materials and Methods

5.1. Sampling and Sample Preparation

Under the agreement of written informed consent with the farmers, 100 dairy farms located in Lower Austria (n = 33), Upper Austria (n = 51), and Styria (n = 16), representing the 3 provinces leading the country's dairy production, were involved in the survey, lasting from 2019–2020 (Figure 8A). The herd sizes (number of lactating cows) during both visits were on average 59 ± 15 SD lactating cows per farm, varying from 32 to 140 lactating cows per farm. Each representative sample of complete diets (n = 198) consisted of at least 30 incremental samples of mixed rations from the feeding table (feed bunk), and at least 30 subsamples of concentrate feed on the automatic feeders were collected. The final sample amount was 1–1.5 kg of each kind of sample (basal feed ration and additional concentrate) (Figure 8B). An additional sample of basal ration (approx. 1 kg) was collected for particle size determination. The samples were immediately vacuum-packed (−0.7 psi) and stored in the dark at −20 °C to avoid subsequent microbial spoilation until sampling preparation (Figure 8C). Sampling was performed during the period April 2019 to September 2020, at two time points with a divergence of at least six months between the first (n = 100) and the second sampling (n = 98; two farms did not continue in the study). Since the formulations, feed components, and batches of the different feedstuffs varied between the two visits, both visits within each farm were treated independently (n = 198). The frozen basal feed samples were thawed at room temperature for 24 h and air-dried at

65 °C for 48 h. The average dry-matter content of basal feed samples was 37.06% ± 4.72% (mean ± SD, range: 25.73–54.72%). The dried samples were sequentially milled to a final particle size of ≤0.5 mm. Firstly, they were milled in the cutting mill (SM 300, Retsch GmbH, Haan, Germany) at 1500 rpm for approximately 1 min. The non-milled residues (mostly hard fragments of seeds) were subsequently milled using an ultra-centrifugal mill (ZM 200, Retsch GmbH, Haan, Germany) at 10,000 rpm for approximately 30 s. All milled fractions of each kind of sample were combined, homogeneously mixed, and packed in plastic bags (Figure 8D). Twenty grams (±0.01 g) of the whole diet representative samples was obtained by mixing proportionally milled basal and the additional concentrated feeds (supplemented based on the daily milk production) according to the average intake of each farm provided by the farmers (see Section 5.2). Then, five grams (±0.01 g) of each homogenized representative sample of the diets intended for multi-analysis was weighed in 50-mL polypropylene conical tubes (Sarstedt, Nümbrecht, Germany), and 100 g of basal feed was utilized for the chemical (proximate) analysis and stored at −20 °C until analysis.

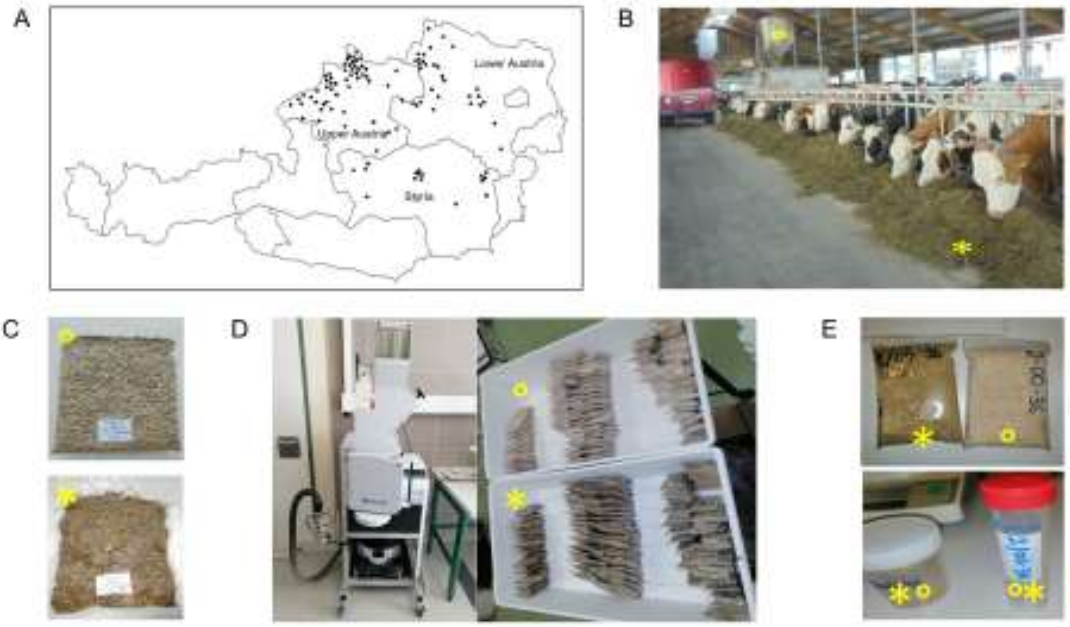

Figure 8. (**A**) representative sampling and sample preparation of whole diets of lactating dairy cows intended for multi-metabolite analysis via LC-MS/MS. (**A**) Map of locations of the selected dairy farms (n = 100) involved in this survey. (**B**) The representative sampling consisted of basal feed (total, partial, or forage mixed ration) collected from the feeding table (*) as well as samples of concentrated feeds (°). (**C**) Vacuum packing and preservation at −20 °C until sample preparation and subsequent analysis. Sampling preparation consisted of drying, (**D**) milling (to a particle size of ≤ 0.5 mm), and subsequent (**E**) pooling and homogenization according to the reported average intakes of basal feed and additional concentrate.

5.2. Data Collection

Information regarding the kind of farming system (organic or conventional), the composition of the basal feed (major ingredients and their proportions), and total intakes of basal feeds (forage, partial, or total mixed rations), as well as the amount of additional concentrate and feed supplemented (based on the daily milk production) were obtained from those responsible for feeding management via personal interview guided by questionnaire. Per farm, the hygienic status of conserved forages and concentrates included in the rations of the lactating cows were evaluated. For the hygienic status assessment, representative

samples (of at least 10 subsamples) were composited and immediately assessed. The sensory evaluation was performed considering characteristics of the appearance (colour was considered along with the presence of impurities), odour, and texture based on the methodological approaches described by Kamphues et al., 2014 [57]. The geo-climatic data, including altitude, average air temperature of the month of sampling, the average air temperature, relative humidity, and the accumulated rainfall during the growing season of maize (June–September of the previous year); average relative humidity of summer (June–September, maize's growing season); rainfall during the summer (June–September, maize's growing season); and averages of the air temperature of the municipalities/districts of the farms, were retrieved from the website of the Central Institution for Meteorology and Geodynamics (in German: Zentralanstalt fur Meteorologie und Geodynamik—ZAMG) (available at https://www.zamg.ac.at/cms/de/klima/klimauebersichten/jahrbuch) (accessed on 1 June 2021). Summarized data are illustrated in Table 1.

5.3. Chemical Proximate Analysis and Particle Size Distribution of the Rations

The chemical proximate (nutrient) analysis of the samples of basal feed rations was conducted according to the protocols of the Association of German Agricultural Analytic and Research Institutes (VDLUFA, Darmstadt, Germany, 2012) [153]. The dry-matter content was determined by oven-drying the samples at 103 °C for at least 4 h (method 3.1). Ash was analysed by combustion in a muffle furnace at 550 °C overnight (method 8.1). Crude protein was determined using the Kjeldahl method (method 4.1.1) and ether extract using the Soxhlet extraction system (method 5.1.2). Analyses of NDF and the estimation of NFC were performed following the methods described by Van Soest et al. (1991) [154]. Particle size distribution of the basal rations was determined using a manually operated Penn State Particle Separator (PSPS) (model C24682N, Nasco, Fort Atkinson, WI, USA) with three sieves with aperture diameters of 19 mm, 8 mm, and 1.18 mm in diameter, according to Lammers et al. (1996) [155] and Kononoff et al. (2003) [156]. For each visit, the test was performed in duplicate, and the sieve fraction values (%) were averaged.

5.4. Sample Extraction and Multi-Metabolite Analysis (LC-ESI-MS/MS)

For simultaneous multi-metabolite quantification, five grams (±0.01 g) of each homogenized sample was extracted in 20 mL of the extraction solvent (acetonitrile/water/acetic acid 79:20:1, *v*/*v*/*v*) and following the procedures reported by Sulyok et al. [157]. Glacial acetic acid (p.a.) and methanol (LC gradient grade) were acquired from Merck, Darmstadt, Germany), and the water was reverse-osmosis-purified using an Elga Purelab ultra-analytic system (Veolia Water, High Wycombe, UK). Then, for sedimentation, the samples were put in a vertical position for 10–15 min. A supernatant of 500 µL of the raw extract was diluted 1:1 with a dilution solvent (acetonitrile/water/acetic acid 20:79:1, *v*/*v*/*v*) in vials. The injection volume of both raw extracts of the samples and standard solutions of the analytes was 5 µL. These volumes were put into the QTrap 5500 LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with a TurboV electrospray ionization (ESI) source, which was coupled to a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany) as described by Sulyok et al., 2020 [157]. A subsequent quantification from external calibration by serial dilutions of a stock solution of analysed compounds was completed. Finally, the results were adjusted for apparent recoveries defined through spiking experiments according to Steiner [158]. Standards of fungal, plant, and unspecific secondary metabolites were purchased from several commercial suppliers or obtained via a donation from different research institutions [157,158]. This analytical methodology has been validated [157,158] and has been employed to study multi-mycotoxin occurrence in complex feedstuff matrices such as silage, pastures, concentrate feed, and total mix rations [53,61,159,160]. The accuracy of the method is verified on a routine basis by participation in proficiency testing organized by BIPEA (Gennevilliers, France). Satisfactory *z*-scores between −2 and 2 have been obtained for >95% of the >1700 results submitted so

far. In particular, 17 out of 18 results submitted for a sample of MS were in this range, the exception being zearalenone exhibiting z = −2.05.

5.5. Statistical Analysis

Frequencies of contamination (occurrences) and the descriptive statistics of the concentrations of metabolites (average, SD, median, and range values) were calculated considering values over the limit of detection (LOD). Values lower than the limit of quantification (LOQ) were processed as LOQ/2. Concentrations of metabolites are expressed in µg/kg parts per billion (ppb) on a dry-matter basis and plotted on a logarithmic scale (Log_{10}) where applicable. The co-occurrence analyses of mycotoxins and plant metabolites were performed separately using Microsoft Excel, constructing matrices that included metabolites with detection frequencies over 20%. Spearman's correlation coefficients were computed, and heatmaps were plotted using GraphPad Prism (Prism version 9.1, GraphPad Software, San Diego, CA, USA). The correlation analysis was interpreted considering only significative correlations with $\rho \geq 0.3$, based on Hinkle et al. (2003) [161]. Multiple regression analysis was performed using SAS (version 9.4; SAS Institute Inc., Cary, NC, USA) to investigate the influences of dietary and geoclimatic factors on dependent variables of interest: concentrations of total metabolites produced by fungi, plants, *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, EAs, DON, ZEN, FUM (the sum of FB1 and FB2), BEA, ENNs, CUL, siccanol, and phytoestrogens. Data were log-transformed to normalize the data. For some variables (DON, FUM, siccanol, total *Alternaria* metabolites, total *Penicillium* metabolites, and total fungal metabolites), extreme data that still led to screwed data were manually excluded. The normality of data based on the Shapiro–Wilk test ($p > 0.05$) and Q-Q-plot were ensured before further data analysis. For each dependent variable, a set of independent variables including dietary proportions of MS, straw, hay, BSG, feed particle size > 19 mm and between 8–1.18 mm, the content of crude protein, ash, ether extract, ash, and non-fibre carbohydrate, the hygienic status of MS and GS, altitude, temperature, relative humidity, and rainfall were tested, and the candidate independent variables were selected based on a step-wise selection using the procedure SELECT of SAS. All candidate independent variables passed the collinearity test, having a variance inflation factor less than 10. Next, the effects of candidate variables, including their squared terms and interactions, were investigated using the mixed procedure of SAS. The model also included the random effect of two rounds of visits. Backward elimination was performed to obtain the final model using the protocol described previously [162]. Additionally, R^2 and RMSE of the final model were calculated. In addition, the odds ratio and predicted probabilities for high contamination of *Fusarium* metabolites due to the inclusion levels of forage sources were determined using PROC LOGISTIC (SAS version 9.4; SAS Institute Inc., Cary, NC, USA). For this analysis, data classified as low (25 percentile, $n = 49$) and high *Fusarium* metabolite concentrations (75 percentile, $n = 60$) were used. The model included dietary levels of MS, GS, straw, hay, BGS, other silages, and quadratic terms of MS, because they were found to show significance in multiple regression analysis.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/toxins14070493/s1. Table S1. List of 863 targeted metabolites via a validated multi-metabolite liquid chromatography/electrospray ionization–tandem mass spectrometric (LC/ESI–MS/MS) method. Table S2. Odds ratio estimates and profile-likelihood confidence intervals of forage inclusion levels as dietary risk factors for high *Fusarium* mycotoxin loads (above 75th percentile concentrations). Figure S1. Predicted probabilities for *Fusarium* mycotoxin loads (above 75th percentile concentrations) related to the proportion of (a) maize silage and (b) straw in the dietary rations of Austrian dairy cows.

Author Contributions: Conceptualization, F.P.-T., R.K.-a. and Q.Z.; methodology, F.P.-T., R.K.-a., J.F., V.N., M.S. (Michael Sulyok), R.K. and Q.Z.; formal analysis, F.P.-T. and R.K.-a.; investigation, F.P.-T., M.S. (Marlene Schmidt), E.-M.B., J.K., R.K. and M.S. (Michael Sulyok); data curation, F.P.-T. and M.S. (Michael Sulyok); writing—original draft preparation, F.P.-T.; writing—review and editing, R.K.-a., M.S. (Marlene Schmidt), E.-M.B., J.K., J.F., M.S. (Michael Sulyok), V.N., R.K. and Q.Z.; visualization, F.P.-T. and R.K.-a.; supervision, R.K.-a. and Q.Z.; project administration J.F., V.N. and Q.Z.; funding acquisition, Q.Z. and V.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research was conducted as part of the Project "D4Dairy-Digitalization, Data Integration, Detection and Decision support in Dairying" supported by the Austrian Federal Ministry for Climate Action, Environment, Energy, Mobility, Innovation and Technology (BMK), Austrian Federal Ministry for Digital and Economic Affairs (BMDW), and the provinces of Lower Austria and Vienna within the framework of COMET-Competence Centers for Excellent Technologies, which is handled by the Austrian Research Promotion Agency (FFG).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Informed consent was obtained from the owners of the involved farms.

Data Availability Statement: Data available on request due to restrictions (Data protection agreement).

Acknowledgments: Open access funding by the University of Veterinary Medicine Vienna. The authors are grateful for the exceptional assistance and recommendations provided by Cátia Pacífico, Anita Dockner, Arife Sener, Sabine Leiner, and Manfred Hollmann (Institute of Animal Nutrition and Functional Compounds, Vetmeduni, Vienna) and Ing. Thomas Erhäusl (DSM—BIOMIN Research Center). We also express gratitude to Annelies Müller (DSM—BIOMIN Research Center) for critical reading of the manuscript as well as to Marlene Suntinger, Franz Steininger, and Christa Egger-Danner (ZuchtData EDV-Dienstleistungen GmbH) and the LKV staff for the logistic support.

Conflicts of Interest: J.F. and N.V. are employed by BIOMIN Holding GmbH (part of DMS), which operates the BIOMIN Research Center and is a manufacturer of feed additives. This, however, did not influence sampling, analyses, or data interpretation.

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Cocktails of Mycotoxins, Phytoestrogens, and Other Secondary Metabolites in Diets of Dairy Cows in Austria: Inferences from Diet Composition and Geo-climatic Factors

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Table S1. List of 863 targeted metabolites via a validated multi-metabolite liquid chromatography/electrospray ionization-tandem mass spectrometric (LC/ESI–MS/MS). * Compounds found in diets of Austrian dairy cows (values > the LOD)

10-Norparvulenone	Aflatoxin M2	Apicidin	Aurantine
15-Acetyldeoxynivalenol	Aflatoxin P1	Apidicin C	Aurantioclavin
15-Desoxyoxalicine B	Aflatoxin Q1	Apidicin D2*	Aurantiogliocladin
15-Hydroxyculmorin*	Aflatrem	Aristolochic acid A	Aurasperon B
15-Hydroxyculmoron	Aflavarin	Ascochlorin*	Aurasperon C
16-Ketoaspergillimide	Agistatin B	Ascofuranone*	Aurasperon G
1-Deoxypebrolide	Agistatin D	Ascolactone	Aureobasidin
2-Chlorunguinol	Agistatin E	Ascomycin	Aurofusarin*
2-Methylmitorubin	Agroclavine	Asparason A	Austalide A
3,4,15Triacetylnivalenol	Aigualomycin D	Aspercolorin	Austalide B
3,4-Diacetylnivalenol	AJ 296	Asperflavine	Austalide Derivative
3-Acetyldeoxynivalenol	Alamethicin*	Asperfuran	Austalide F
3-Acetylneosolaniol	alpha-Zearalenol	Aspergamid A	Austamide
3-Acetyl-T-2 Toxin	alpha-Zearalenol Glucoside	Aspergillicin Derivat	Austdiol
3-Hydroxy-3-acetyl-T-2 Toxin	Alteichin	Aspergillimide	Austinol
3-Hydroxy-HT-2 Toxin	Altenuene	Asperglaucide*	Austocystin A
3-Hydroxyterphenyllin	Altenuisol*	Asperlactone	Austocystin B
3-Nitropropionic acid*	Altenusin	Asperloxine A	Austocystin D
4,7,15Triacetylnivalenol	Alternarian acid	Aspermytin A	Austocystin I
4-Hydroxyalternariol	Alternarienoic acid	Aspernigrin A	Australide D
4-Methoxycyclopeptin	Alternariol*	Asperphenamate*	Australide F
4-Monoacetoxyscirpenol	Alternariol-3-Glucoside	Asperthecin	Averantin
5-Hydroxyculmorin	Alternariol-9-Glucoside	Aspinolid B	Averantinmethylether
5-Methylmellein	Alternariolmethylether*	Aspinonene	Averufanin
7-Hydroxykaurenolide	Alternariolmethylether-Glucoside	Aspochalasin C	Averufin Derivat
7-Hydroxypestalotin*	Altersetin*	Aspochalasin D	Averufin*
8-Acetylneosolaniol	Altersolanol	Aspochalasin H	Bacitracin
8-O-Methylaverufin	Altertoxin II	Aspochalasin I	Bafilomycin A1
A 23187	Altertoxin-I	Aspochalasin J	Banksialactone A
A 26771 B	Amauromine	Aspochracin	Barceloneic acid*
AAL TA-Toxin	Amidepsin B	Aspterric acid	Bassianolide*
AAL TB Toxin	Aminodimethyloctadecanol	Aspulvinone E	Beauvericin*
AAL TD Toxin	Amoxycillin	Aspulvinone O	Benzomalvin A
AAL TE Toxin	Amphotericin	Aspyrone	Benzomalvin B
Abscisic acid*	Anacin	Asteltoxin	Benzomalvin C
Acetylchaetoglobosin D	Andrastin A*	Asterric acid	Berkedrimane B
Achaetolide Derivat	Andrastin B*	Asterriquinonedimethylether	Berkeleyacetal B
Acuminatum B*	Andrastin C*	Azonapyrone A	Berkeleylctone E
Acuminatum C	Andrastin D	Atlantinon A	Berkeleylctone F
Aflatoxicol	Andrastin Derivative	Atpenin A5	beta-Zearalenol
Aflatoxin B1	Anisomycin	Atropine	beta-Zearalenol-Glucoside
Aflatoxin B2	Antbiotic L 696474	Atroventinmethylether	Bikaverin*
Aflatoxin G1	Antibiotic F 1849 A	Aurantiamin A	Biochanin*
Aflatoxin G2	Antibiotic PF 1052	Auranticin A	Bis(methylthio)gliotoxin*
Aflatoxin M1	Antibiotic Y*	Auranticin A	Bongkrekic acid

Botryodiplodin	cyclo(L-Pro-L-Val)*	Dihydrolysergol	Fonsecin
Brasiliamide A	Cycloaspeptide A	Dihydrosterigmatocystin	Formonetin*
Brefeldin A	Cycloechinulin	Dihydrotrichotetronine	FS-4
Brevianamid F*	Cycloheximide	Dihydroxycalonectrin	Fulvic acid
Brevicompanine B	Cyclopenin	Dihydroxymellein	Fumagillin
Butenolid	Cyclopenol	DihydroxyZONMethylether	Fumarprotocetaric acid
Butyrolacton III	Cyclopeptine	Dinactin	Fumifungin
Butyrolactone I	Cyclopiazonsäure	Diplodiatoxin	Fumigaclavine C*
Butyrolactone II	Cyclosporin A	DON-3-glucoside*	Fumigaclavine*
ButyrolactonIImethylether	Cyclosporin B*	Doxorubicin	Fumiquinazolin A
Byssochlamic acid	Cyclosporin C*	Doxycyclin	Fumiquinazolin D*
Calonectrin	Cyclosporin D	Drimane 6	Fumiquinazolin Derivat
Calphostin	Cyclosporin H	Drimane 8	Fumiquinazolin F
Calphostin C*	Cylindrocarpon A4	Duclauxin	Fumitremorgin A
Calyxanthone	Cylindrol B	Echimidin	Fumitremorgin B
Carnequinazolin A	Cytochalasin A	Elymoclavine	Fumitremorgin C
Cephalochromin	Cytochalasin B	Elymoclavine-Fructoside	Fumonisin A1
Cercosporamide	Cytochalasin C	Emericellamide A	Fumonisin A1 (precussor)*
Cercosporin	Cytochalasin D	Emericellamide C	Fumonisin A2
Cereulide	Cytochalasin E	Emericellamide E	Fumonisin AK2
Cerulenin	Cytochalasin J	Emestrin*	Fumonisin B1*
Chaconin*	Daidzein*	Emindole SA	Fumonisin B2*
Chaetocin	Daidzin*	Emodin*	Fumonisin B3*
Chaetoglobosin A	Daunorubicin	Endocrocin*	Fumonisin B4*
Chaetoglobosin C	Deacetylneosolaniol	Enniatin A*	Fumonisin B6
Chaetoglobosin D	Decalonectrin	Enniatin A1*	Fungerin
Chaetoglobosin F	Dechlorogriseofulvin	Enniatin B*	Fusaproliferin*
Chaetominine	Dechlorogriseofulvin	Enniatin B1*	Fusapyron*
Chaetoviridin A	Dechloroisochromophilon IV	Enniatin B2*	Fusarenon-X
Chanoclavin*	Dechlorononnidulin	Enniatin B3	Fusaric acid
Chetomin	Deepoxy-deoxynivalenol	Epiequisetin*	Fusarielin A
Chetoseminudin A	Deepoxy-T-2 toxin	Epoxyagroclavin	Fusarin C
Chevalone B	Deepoxy-T-2tetraol	Epoxycytochalsin C*	Fusarinolic acid
Chevalone C	Dehydroaustinol	Equisetin*	Fusarisetin A
Chevalone E	Dehydrocurvularin*	Eremofortin A	Fuscofusarin
Chlamydospordiol	Dehydrocyclopeptine	Eremofortin B	Galbinic acid
Chlamydosporol	Dehydrogriseofulvin	Ergine	Geldanamycin
Chloramphenicol	Demethoxyviridol	Ergocornine*	Genistein*
Chlorocitreorosein	Demethylasteltoxin	Ergocorninin*	Genistin*
Chloronectrin	Demethylsulochrin	Ergocristine*	Geodin
Chlortetracyclin	DeoxyAltersolanol	Ergocristinine*	Geodin hydrate
Chrodrimanin	Deoxybrevianamid E	Ergocryptine*	Gibberellic acid
Chromomycin A3	Deoxyfusapyron	Ergocryptinine*	Gibberellin A12
Chrysogin*	Deoxygerfelin*	Ergometrine	Gibberellin A14
Chrysophanol*	Deoxynivalenol*	Ergometrinine*	Gibberellin A4
Cinereanin	Deoxynortryptoquivalin*	Ergosin*	Gibberrellin A7
Citreohybriddione	Deoxytryptoquialanine	Ergosinin*	Gibepyron D
Citreohybridinol*	Deoxytryptoquivaline A	Ergotamine*	Gigantenone
Citreoindole	Desoxypaxillin	Ergotaminine*	Gliocladic acid
Citreorosein*	Desoxyverrucosidin	Ergovalin	Gliotoxin
Citreoviridin	Destruxin A	Erucifolin	Glisoprenin D
Citreoviridin C	Destruxin B*	Erucifolin-N-Oxid	Glyantrypine
Citreoviridinol	Destruxin CHL	Erythromycin	Glycitein*
Citrinin*	Destruxin D	Ethylorsellinic acid	Glycitin*
Citromycetin	Destruxin-Ed Derivat	Europin	Grayanotoxin I
Cladosporin	Dethiosecoemestrin	Europin-N-Oxid	Griseofulvin acid
Cladosporone Derivat	Diacetoxyscirpenol	Expansolid	Griseofulvin*
Clonostachydiol	Diacetylcercosporin	F01 1358-A	Griseophenone A
CNM 115443	Diacetylnivalenol	Fallacinol	Griseophenone B
Cochlioquinone A	Dichlordiaportin	FB1 Methylester	Griseophenone C
Colchicine*	Dichlormethylasterric acid	Fellutanine A*	Harzianopyridine
Communesin B	Diffractaic acid	Fellutannine B	Harzianum A
Cordycepin	Dihydroaspyrone	Festuclavine*	HC Toxin
Coumestrol*	Dihydrochlamydocin	Filipin	Heliotrin
Culmorin*	Dihydrocitrinone	FK 506	Heliotrin-N-Oxid
Curvularin*	Dihydrocompactin	FK 9775 A	Helvolic acid
Curvulin	Dihydroergosine	FK 9775 B	Helvolinic acid
Cyclo(l-Ala-L-Pro)	Dihydroergotamine	Flavipucin	Heptaibin
cyclo(L-Leu-L-Pro)	Dihydrogriseofulvin	Flavoglaucin*	Heptelidic acid
cyclo(L-Pro-L-Tyr)*	Dihydroinfectopyron	Folipastin	Herquline A

Botryodiplodin	cyclo(L-Pro-L-Val)*	Dihydrolysergol	Fonsecin
Brasiliamide A	Cycloaspeptide A	Dihydrosterigmatocystin	Formonetin*
Brefeldin A	Cycloechinulin	Dihydrotrichotetronine	FS-4
Brevianamid F*	Cycloheximide	Dihydroxycalonectrin	Fulvic acid
Brevicompanine B	Cyclopenin	Dihydroxymellein	Fumagillin
Butenolid	Cyclopenol	DihydroxyZONMethylether	Fumarprotocetaric acid
Butyrolacton III	Cyclopeptine	Dinactin	Fumifungin
Butyrolactone I	Cyclopiazonsäure	Diplodiatoxin	Fumigaclavine C*
Butyrolactone II	Cyclosporin A	DON-3-glucoside*	Fumigaclavine*
ButyrolactonIImethylether	Cyclosporin B*	Doxorubicin	Fumiquinazolin A
Byssochlamic acid	Cyclosporin C*	Doxycyclin	Fumiquinazolin D*
Calonectrin	Cyclosporin D	Drimane 6	Fumiquinazolin Derivat
Calphostin	Cyclosporin H	Drimane 8	Fumiquinazolin F
Calphostin C*	Cylindrocarpon A4	Duclauxin	Fumitremorgin A
Calyxanthone	Cylindrol B	Echimidin	Fumitremorgin B
Carnequinazolin A	Cytochalasin A	Elymoclavine	Fumitremorgin C
Cephalochromin	Cytochalasin B	Elymoclavine-Fructoside	Fumonisin A1
Cercosporamide	Cytochalasin C	Emericellamide A	Fumonisin A1 (precussor)*
Cercosporin	Cytochalasin D	Emericellamide C	Fumonisin A2
Cereulide	Cytochalasin E	Emericellamide E	Fumonisin AK2
Cerulenin	Cytochalasin J	Emestrin*	Fumonisin B1*
Chaconin*	Daidzein*	Emindole SA	Fumonisin B2*
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Chrysogin*	Deoxygerfelin*	Ergometrine	Gibberellin A14
Chrysophanol*	Deoxynivalenol*	Ergometrinine*	Gibberellin A4
Cinereanin	Deoxynortryptoquivalin*	Ergosin*	Gibberrellin A7
Citreohybriddione	Deoxytryptoquialanine	Ergosinin*	Gibepyron D
Citreohybridinol*	Deoxytryptoquivaline A	Ergotamine*	Gigantenone
Citreoindole	Desoxypaxillin	Ergotaminine*	Gliocladic acid
Citreorosein*	Desoxyverrucosidin	Ergovalin	Gliotoxin
Citreoviridin	Destruxin A	Erucifolin	Glisoprenin D
Citreoviridin C	Destruxin B*	Erucifolin-N-Oxid	Glyantrypine
Citreoviridinol	Destruxin CHL	Erythromycin	Glycitein*
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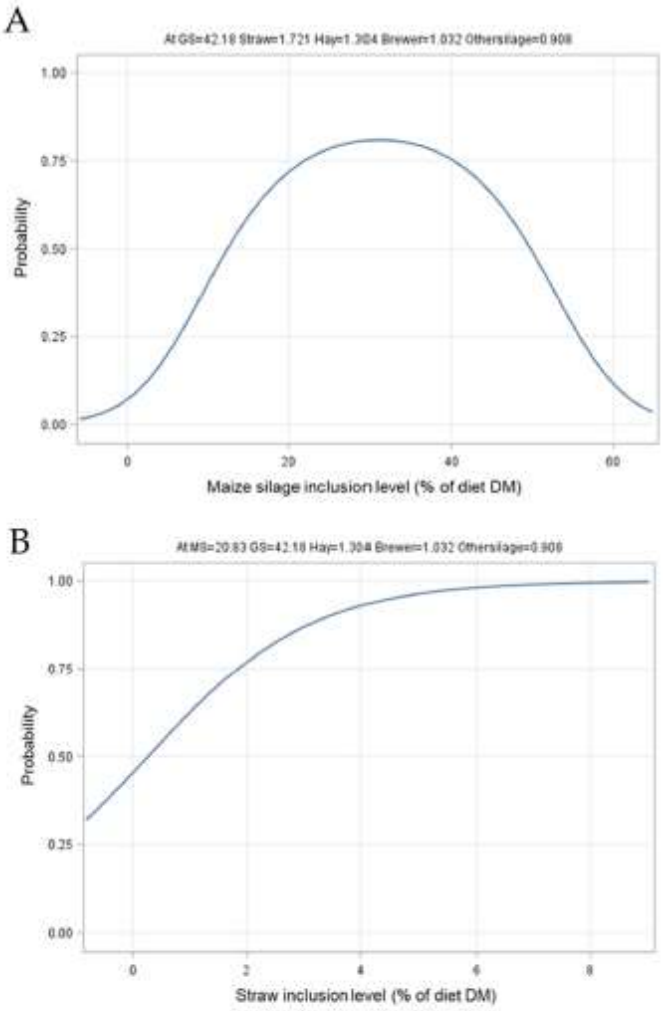
cyclo(L-Pro-L-Tyr)*	Dihydroinfectopyron	Folipastin	Herquline A

Sphingofungin B	Tenuazonic acid*	Trichoverrin A	Versiconol
Sphingofungin D	Ternatin*	Trypacidin*	Verticillin A
Spiculisporic acid	Terpendole C	Tryprostatin A	Violaceic acid
Spiramycin	Terpendole E	Tryprostatin B	Violaceol I
Spirodihydrobenzofuranlactam IV	Terpendole I	Tryptophol*	Violaceol II
Sporidesmolide II*	Terphenyllin	Tryptoquialanine	Viomellein
Sporogen AO I	Terragine E	Tryptoquialanine Derivat	Vioxanthin
Stachybotryamide	Terrecyclic acid	Tryptoquivaline A	Viridicatin
Stachybotrylactam	Terrein	Tryptoquivaline F	Viridicatol
Stachybotrysin B	Terretonin	Tryptoquivaline G	Viridicatum toxin
Staurosporin	Terretonin F Derivat	Tylosin	Viriditoxin
Stemphylperylenol	Territrem A	Ulocladol	Viridol
Sterigmatocystin*	Territrem B	Unguinol	Vulpinic acid
Sticitc acid	Tetraacetlnivalenol	Unugisin E	W493*
Strobilactone A	Tetraacetyl-T-2 Tetraol	Usnic acid*	WIN 68577
Sulochrin	Tetracycline	Ustiloxin A	WIN-64821
Surfactin A	Tetrahydrobostrycin	Ustiloxin B	Wortmannin
Surfactin B	Thailandolide B	Ustiloxin D	Xanthomegnin
Sydonic acid	Thaxtomin A	Ustusol A	Xanthotoxin*
Sydonol	Thielavin B	Valinomycin	Xantocillin X1
Sydowinin A	Toxoflavin	Vancomycin	Yaequinolone J2
Sydowinin B	Triacetoxyscirpenol	Vermistatin	Zearalenone*
sydowinol	Triacetyl-Deoxynivalenol	Verrucarin A	Zearalenone-14-glucoside
T-2 Glucoside	Trichalasin B	Verrucarin J	Zeralenone-16-Glucoside
T-2 toxin*	Trichodermamide C	Verrucarol	Zinndiol*
T2-Tetraol	Trichodermin	Verrucofortine	Zinniamide
T2-Triol	Trichodesmin	Verrucosidin	Zinniol*
Tanzawaic acid B	Trichodimerol	Verruculogen	
Taxol	Trichostatin A	Verruculotoxin	
Tenellin	Trichotetronine	Versicolorin A	
Tensidol B	Trichothecin	Versicolorin C*	
Tentoxin*	Trichothecolone	Versiconal Acetat (Hemiacetal)	

Table S2. Odds ratio estimates and profile-likelihood confidence intervals of forage inclusion levels as dietary risk factors for high *Fusarium* mycotoxin loads (above 75th percentile concentrations)

Forage sources	Estimate	95% confidential limits	
Maize silage	1.085	1.007	1.168
Grass silage	0.966	0.908	1.023
Straw	2.004	1.372	3.193
Hay	0.997	0.850	1.215
Brewer spent grain silage	1.056	0.792	1.446
Other silage	1.624	1.086	2.689

Figure S1. Predicted probabilities for high *Fusarium* mycotoxin loads (above 75th percentile concentrations) related to the proportion of (**A**) maize silage and (**B**) straw in the dietary rations of Austrian dairy cows.



3.4. Publication 4:

Mixtures of mycotoxins, phytoestrogens, and pesticides cooccurring in wet spent brewery grains (BSG) intended for dairy cattle feeding in Austria.

Felipe Penagos-Tabares, Michael Sulyok, Veronika Nagl, Johannes Faas, Rudolf Krska, Ratchaneewan Khiaosa-ard, Qendrim Zebeli

Food Additives and Contaminants: Part A (2022) 39:11, 1855-1877. https://doi.org/10.1080/19440049.2022.2121430

FOOD ADDITIVES & CONTAMINANTS: PART A
2022, VOL. 39, NO. 11, 1855–1877
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Mixtures of mycotoxins, phytoestrogens and pesticides co-occurring in wet spent brewery grains (BSG) intended for dairy cattle feeding in Austria

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ABSTRACT

Spent brewery grains (BSG) are the main by-product of beer production and are incorporated in rations of food-delivering animals, mainly dairy cows. Like other agricultural commodities, BSG can be contaminated by a broad spectrum of natural and synthetic undesirable substances, which can be hazardous to animal and human health as well as to the environment. The co-occurrence of mycotoxins, phytoestrogens, other fungal and plant secondary metabolites, along with pesticides, was investigated in 21 BSG samples collected in dairy farms in Austria. For this purpose, a validated multi-metabolite liquid chromatography/electrospray ionisation tandem mass spectrometry (LC/ESI–MS/MS) was employed. Metabolites derived from *Fusarium*, *Aspergillus*, *Alternaria* and pesticide residues, were ubiquitous in the samples. Zearalenone (ZEN), T-2 and HT-2 toxins were the only regulated mycotoxin detected, albeit at concentrations below the European guidance values for animal feeds. Ergot alkaloids, *Penicillium*-derived metabolites, and phytoestrogens had occurrence rates of 90, 48 and 29%, respectively. *Penicillium* metabolites presented the highest levels among the fungal compounds, indicating contamination during storage. Aflatoxins (AFs), ochratoxins and deoxynivalenol (DON) were not detected. Out of the 16 detected pesticides, two fungicides, ametoctradin (9.5%) and mandipropamid (14.3%) revealed concentrations exceeding their respective maximum residue level (MRL) (0.01 mg kg^{-1}) for barley in two samples. Although based on European guidance and MRL values the levels of the detected compounds probably do not pose acute risks for cattle, the impact of the long-time exposure to such mixtures of natural and synthetic toxicants on animal health and food safety are unknown and must be elucidated.

Abbreviations: BSG: Spent brewery grains; ZEN: Zearalenone; DM: Dry matter; OTA: Ochratoxin A; AFs: Aflatoxins; DON: Deoxynivalenol; EDCs: Endocrine-disrupting chemicals; ZAMG: Agency of Meteorology and Geodynamics, Zentralanstalt für Meteorologie und Geodynamik; LOD: Limit of detection; LOQ: Limit of quantification

ARTICLE HISTORY
Received 15 June 2022
Accepted 28 August 2022

KEYWORDS
Dietary exposure; feed safety; mixtures toxicology; mycotoxins; pesticide residues; phytoestrogens; spent brewery grains

Introduction

The use of agro-industrial by-products as valuable feeds in dairy farming is a strategy that can reduce the direct dependence on whole cereals grains and oilseeds, which are essential in human nutrition. Due to the low cost of by-products, their incorporation into livestock rations improves the economics, also contributing to increasing the edible feed conversion ratio (Bocquier and González-García 2010; Ertl et al. 2015). Beer is the most consumed alcoholic beverage in Europe and worldwide (Violino et al. 2020; Ambra et al. 2021). Spent brewery grains (BSG) are the main by-product of beer production, accounting for around 85% of its agricultural waste and allowing the availability of this by-product throughout the year (Mussatto 2014;

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/19440049.2022.2121430

Petit et al. 2020). Most of the generated BSG are utilised in animal nutrition; however, this product has also been used for biogas production or, in a minor proportion, simply disposed of in landfill (Bianco et al. 2020). The vast potential of this by-product as a valuable feed/foodstuff in animal and human nutrition is because of a high content of digestible fibre (usually fluctuating from 30 to 50% on a dry matter (DM) basis), good quality protein (varying from 19 to 30% on DM basis), lipids and several minerals (José et al. 2013; Mussatto 2014; Lynch et al. 2016). Additionally, the BSG contain arabinoxylans and β-glucans, which can be used as prebiotics, promoting the activity of beneficial bacteria, such as *Bifidobacterium*, *Enterococcus*, and *Lactobacillus* species in monogastric livestock (Lao et al. 2020). In the feeding of ruminants, wet BSG are ideal for mixing with forage rations, offering high-quality protein feed, which is inexpensive and can reduce the dependency on commercial concentrate feeds (Gonzalez Pereyra et al. 2011). Although BSG have been fed to beef cattle, horses, pigs, sheep and poultry, the primary market for wet BSG is as a dairy cattle feedstuff (Westendorf and Wohlt 2002; Mussatto 2014; Kamboh 2017; Pack et al. 2021).

Like other agricultural products, the presence of contaminants and residues in BSG is associated with feed safety issues. Potential hazards of feedstuffs can be of natural (e.g. mycotoxins and plant toxins) as well as synthetic origin (like pesticides) (FAO and WHO 2019). Safety of wet BSG can be jeopardised by hundreds of fungal toxic compounds, which can be produced pre- and postharvest (especially during storage on the farms). However, most studies have investigated only a limited number of them in feedstuffs (including BSG) and other agricultural commodities. Specifically, research on mycotoxin contamination has focused mostly on a limited range of mycotoxins such as aflatoxins (AFs), fumonisins (FBs), trichothecenes, ochratoxin A (OTA) and zearalenone (ZEN) (Lynch et al. 2016; Cinar and Onbaşı 2019; Battilani et al. 2020; Pack et al. 2021), which are regulated by European legislation (EC 2002, 2006, 2012, 2013). Barley is the main cereal utilised for beer production (Palmer 2018). Like other cereal grains, barley is susceptible to mould infection with subsequent mycotoxin contamination and other secondary metabolites during the complete feed-production chain, pre-and postharvest (Pascari et al. 2018). Thus, research concerning other fungal toxins such as emerging and modified mycotoxins from *Fusarium*, *Alternaria*, *Aspergillus* and *Penicillium* is still limited but often advocated (Battilani et al. 2020; Lao et al. 2020). Additionally, other compounds of natural origin like phytoestrogens can occur in feeds, affecting animal farming. For instance, these plant secondary metabolites are known endocrine disruptors that, at certain levels, can impair reproductive functions and reducing the productive efficiency of dairy herds (Wocławek-Potocka et al. 2008, 2013).

The pre- and post-harvest use of pesticides under conventional farming systems protects crops, like barley, from insects, pests, weeds and plant pathogens, improving the production yields. Residues of such pesticides can be accumulated in crops and the environment, with potentially toxic effects on human and animal (including wildlife) health as well as on the soil microorganisms (Igbedioh 1991; Damalas and Eleftherohorinos 2011; Cozma et al. 2017). Additionally, pesticide applications can generate the presence of residues, which are usually at lower levels than the mycotoxins and phytoestrogens, but which should be analysed in order to address the exposure to entire mixtures of toxicants and endocrine-disrupting chemicals (EDCs), which can have public health and environmental implications (Igbedioh 1991; Connolly 2009; Rivera-Becerril et al. 2017; Guo et al. 2020; Geissen et al. 2021; Pires et al. 2021). Like some mycotoxins, pesticides are also regulated by European Union legislation (specifically by Regulation (EC) No 396/2005) (EC 2005). Thus, this study aimed to analyse a broad-spectrum profile of mycotoxins, phytoestrogens, other secondary metabolites, and pesticide residues in wet BSG intended to feed Austrian dairy cattle. This was achieved using a validated multi-analyte method based on liquid chromatography-electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS).

Materials and methods

Sample collection and preparation

Representative samples of wet BSG intended for dairy cattle feeding were collected from batches acquired by farmers from regional breweries

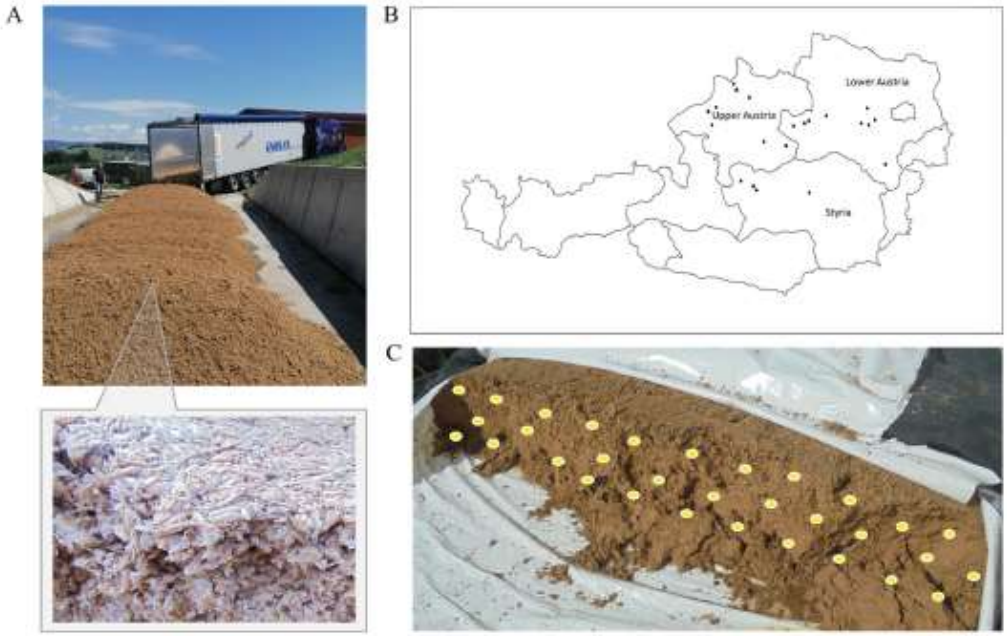

Figure 1. Representative sampling of wet barley brewery's spent grains (BSG) intended for feed dairy cattle in Austria. A) The samples were collected from piles or silo bags, which were stored for a maximum of 2 weeks (Picture gently provided by Mr. Alexander Kopper©). B) The farms ($n = 21$) were in Lower Austria ($n = 9$), Upper Austria ($n = 8$) and Styria ($n = 4$). C) Subsamples were collected manually (around 20 – 30 handfuls) from the next-to-be-fed section of the BSG charge of each farm, for a final sample of 1 – 1.5 kg.

(Figure 1(A)). The sampling was conducted between June and September 2020 from 21 conventional dairy farms located in Lower Austria ($n = 9$), Upper Austria ($n = 8$) and Styria ($n = 4$) (Figure 1(B)). Upon sample collection, BSG batches had been stored for less than two weeks at the respective farms. Each representative sample was collected manually, consisting of 20–30 incremental subsamples (randomly selected handfuls) using nitrile gloves, collected superficially (not deeper than 20 cm) from the next-to-be-fed section of the wet BSG (Figure 1(C)). The incremental samples were composited, giving a final sample amount of 1.5 kg, vacuum-packed in plastic bags and stored at −20 °C in the dark until sample preparation. Subsequently, the samples were thawed for 12 h, freeze-dried in a SCANVAC CoolSafe™ freeze (Labogene, Lillerød, Denmark) for 24 h, and milled through a 0.5 mm sieve using a cutting mill (SM 300, Retsch GmbH, Haan, Germany) at 1500 rpm for approximately 1 min. Five grams (± 0.01 g) of the homogenised samples were added to 50 ml polypropylene conical tubes (Sarstedt, Nümbrecht, Germany) and stored at −20 °C until analysis. The DM content of the sampled wet BSG was, on average, 25.3%, fluctuating from 20.9 to 30.6%. The farms were in altitude ranges between 266 and 814 m. The temperature during the months of sampling of participating farms fluctuated from 14.8 to 21.5 °C, being on average 18.4 °C (According to the Agency of Meteorology and Geodynamics, Zentralanstalt für Meteorologie und Geodynamik (ZAMG), https://www.zamg.ac.at/cms/de/klima/klimauebersichten/jahrbuch).

Analysis multiple of metabolites and pesticides

The previously dried and milled sample (5 ± 0.01 g) was placed into a 250 ml Erlenmeyer flask with 20 ml of extraction solvent according to the protocol described by Steiner et al. (2020). After agitation with a GFL 3017 rotary shaker (GFL, Burgwedel, Germany) for 90 min, the solvent

solution-sample mixture was centrifuged for 2 min at 2012 × *g* on a GS-6 centrifuge (Beckman Coulter Inc., Brea, CA). The extract was diluted 1:1 with dilution solvent. The injection volume of both diluted extracts of the samples and the standard analyte solutions was 5 µl. Identification and quantification of each analyte were performed in the mode of multiple reaction monitoring with positive and negative polarity in two separate chromatographic runs using a QTrap 5500 LC-MS/MS system (Applied Biosystems, Foster City, CA) equipped with a TurboV electrospray ionisation (ESI) source coupled to a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Quantitative analysis of all the analytes was performed using a validated method based on LC-ESI-MS/MS described by Sulyok et al. (2020). Quantification was based on external calibration using a serial dilution of a multi-analyte stock solution. Results were corrected for apparent recoveries determined during method validation according to Steiner et al. (2020). The values of the method performance (apparent recovery, limit of detection [LOD] and limit of quantification [LOQ]) of each analyte are presented in Table 1. The apparent recovery for each pesticide was calculated using the equation proposed by Awapak et al. (2021). The method's accuracy is verified on a routine basis by participating in a proficiency testing scheme organised by BIPEA (Gennevilliers, France) with current z-scores between −2 and 2 indicating > 95% confidence. All values submitted for a sample of wheat chaff were within this satisfactory range.

Data analysis

Concentrations of all detected contaminants, residues, and other non-regulated metabolites were presented on a DM basis in $\mu g\ kg^{-1}$. Descriptive statistics, i.e. frequencies, mean, median and ranges of the concentration of analytes, were calculated considering only the positive results ($x \geq$ limit of LOD). Results below the LOQ were computed as LOQ/2. All statistical evaluations, tables, and graphs were performed using Microsoft Excel® and GraphPad Prism® version 9.1 (GraphPad Software, San Diego, CA).

Table 1. Performance values of LC–MS/MS analysis targeting fungal and other contaminants as well as pesticide residues detected in wet brewery's spent grains intended for dairy cattle nutrition.

	Method performance		
Analyte	Apparent recovery (%)	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
15-Hydroxyculmorin	100	0.3	0.9
Alternariol	45	3.4	11.3
Alternariolmethylether	52	3.3	11
Altersetin	75	0.7	2.3
Altertoxin-I	26	22	73
Ametoctradin	57	2.7	8.9
Andrastin A	88	4.3	14
Andrastin B	84	0.4	1.2
Andrastin C	41	3.5	12
Antibiotic Y	80	0.1	0.4
Apicidin	93	0.2	0.8
Apicidin D2	71	0.9	2.8
Asperphenamate	75	0.1	0.4
Aurofusarin	51	0.6	1.9
Azoxystrobin	64	1.7	5.6
Beauvericin	103	0.05	0.2
Benzovindiflupyr	73	0.8	2.8
Bikaverin	72	10	30
Bixafen	56	2.8	9.3
Boscalid	41	2.7	8.9
Brevianamid F	49	2.4	8.1
BTS 44595	67	1.3	4.3
Butenolid	93	7	23.4
Chanoclavin	44	2.6	8.5
Chrysogine	95	5.7	19
Citreorosein	33	2	6.6
Culmorin	65	0.5	1.5
Cyclo (L-Pro-L-Tyr)	31	15	52
Cyclo (L-Pro-L-Val)	31	15	52
Cyclosporin A	56	4.9	16.4
Daidzein	42	50	180
Deoxynortryptoquivalin	52	1.9	6.4
Deoxytryptoquivaline A	52	0.9	3.1
Emodin	71	2.1	7
Enniatin A	37	4.2	13.9
Enniatin A1	52	0.5	1.7
Enniatin B	75	2	6
Enniatin B1	51	17	58
Enniatin B2	92	0.7	2.4
Epiequisetin	138	1	3.2
Equisetin	138	1	3.2
Ergocornine	96	0.3	0.9
Ergocristine	63	1.1	3.8
Ergocristinine	76	0.8	2.7
Ergocryptine	55	0.1	0.4
Ergometrine	61	0.06	0.2
Ergometrinine	77	0.3	1
Ergosin	58	0.2	0.6
Ergosinin	65	0.2	0.6
Ergotamine	65	0.1	0.4
Ergotaminin	65	0.1	0.4
F01 1358-A	100	1	3
Festuclavine	50	0.5	1.5
Flavoglaucin	47	0.4	1.3
Fluopyram	6.4	2.3	7.5
Fluxapyroxad	57	2.2	5.3
Fumiquinazolin D	53	1	3.2
Fungerin	72	0.4	1.3
Fusaproliferin	100	10	30
Fusaric acid	80	10	30
Genistein	62	28	92
Gibberellin A12	57	1.2	4.1
Glycitein	59	31	105
HT-2 toxin	74	2.6	8.5
Hydroxyandrastin A	52	2.5	8.5

(*continued*)

Table 1. Continued.

Analyte	Method performance		
	Apparent recovery (%)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
Hydroxysindrastin C	85	2.1	7.1
Infectopyrone	210	0.7	2.5
Isopyrazam	75	1.1	3.7
Kotanin A	55	1.5	5
Macrosporin	79	2	6.7
Mandipropamid	51	2.4	7.8
Marcfortine A	56	1.9	6.2
Marcfortine C	56	1.9	6.2
Metrafenone	64	0.5	1.8
Monocerin	67	1.3	4.2
Mycophenolic acid	52	0.6	1.9
Mycophenolic acid IV	57	0.6	1.9
Neoechinulin A	38	4	14
Patulin	89	0.6	2.1
Phenopyrrozin	75	0.3	0.9
Physcion	45	11	37
Pinselin	57	1.3	4.2
Piperonyl butoxide	100	5	15
Pirimiphos-methyl	97	1	3.3
Pseurotin A	56	7.4	28
Pyraclostrobin	62	2.3	7.7
Pyrenophorol	74	2.4	8.1
Questiomycin	74	2.8	9
Quinadoline A	112	0.8	2.6
Roquefortine C	58	0.9	3.1
Roquefortine D	66	1.3	4.4
Rubellin D	49	1.7	5.6
Rugulusovin	56	1	3.2
Siccanol	72	2.3	7.6
Sporidesmolide II	100	0.02	0.05
T-2 toxin	111	7	23
Tebuconazole	68	1.1	3.7
Tentoxin	58	2.2	7.5
Tenuazonic acid	150	10	30
Trifloxystrobin	67	1.2	4
Tryptophol	30	100	300
Tryptoquialanine derivate	35	1	3.3
Tryptoquivaline A	58	1.8	6
Verrucofortine	57	1.5	5
Viridiotoxin	100	2.5	7.5
W493	195	2.1	7
Zearalenone	70	2.8	9.2

Results and discussion

General overview of detected groups of analytes

Analytes of natural origin were categorised into groups: *Alternaria*, *Aspergillus*, ergot alkaloids, *Fusarium* and *Penicillium* mycotoxins, other fungal species, phytoestrogens and unspecific metabolites, as in previous reports (Szulc et al. 2019; Hajnal et al. 2020; Penagos-Tabares 2021). Table 2 shows the occurrences and respective average, median, and range concentrations (μg kg^{-1} DM) of observed secondary metabolites. A total of 107 out of more than 1400 targeted secondary metabolites and pesticides were detected: 78 fungal compounds, three phytoestrogens, 16 pesticides and 10 unspecific metabolites (Figure 2). The categories of fungal metabolites with the highest number of detected metabolites in this exploratory study were *Fusarium* spp. (26 metabolites), *Penicillium* (18), *Aspergillus* (11), ergot alkaloids (10), *Alternaria* (9), with fewer metabolites from other fungal genera (4). All the samples contained metabolites derived from *Aspergillus*, *Alternaria*, *Fusarium* and other fungal species, while ergot alkaloids and *Penicillium*-derived metabolites occurred in 90 and 48% of BSG, respectively. Phytoestrogens were found in 29% of the analysed BSG, and the detection of pesticide residues was ubiquitous (100%). In this study, *Fusarium* metabolites showed the highest level of diversity, which has been previously observed in other naturally-contaminated samples from Austria and Europe (Reisinger et al. 2019; Penagos-Tabares et al. 2021, 2022). This again suggests the status of *Fusarium* as the most widespread fungal genus in cereal growing areas and as a significant contributor to mycotoxin contamination in animal feeds (Nesic et al. 2014). Additionally, it also corroborates the widespread occurrence of other mycotoxigenic genera (*Aspergillus*, *Alternaria* and *Penicillium*) (Grenier and Oswald 2011). Regarding the detected levels, the group of unspecific metabolites showed the highest average concentration (7010 μg kg^{-1}), followed by fungal metabolites (5140 μg kg^{-1}), phytoestrogens (957 μg kg^{-1}) and pesticides (208 μg kg^{-1}). Specifically for the fungal metabolites, the highest average levels were for the category of *Penicillium* (5600 μg kg^{-1}), followed by *Fusarium* (2200 μg kg^{-1}), *Alternaria* (107 μg kg^{-1}), ergot alkaloids (69.5 μg kg^{-1}), *Aspergillus* (79.6 μg kg^{-1}) and metabolites from other fungi (15.5 μg kg^{-1}) (Table 2, Figure 2(A)).

Regulated mycotoxins and related forms

Among the mycotoxins included in the European legislation were detected ZEN, T-2 toxin, and HT-2 toxin, which have recommended GVs (EC 2006, 2013). ZEN was the regulated mycotoxins that occurred the most, detected in 57% of the samples with a maximum concentration of 32.3 μg kg^{-1}, whereas T-2 and HT-2 toxins were detected in only one sample (5%), showing concentrations of 3.8 and 4.25 μg kg^{-1}, respectively.

Table 2. Occurrences and levels of fungal and other natural contaminants detected in wet brewery's spent grains intended for dairy cattle nutrition.

Group	Metabolite	Occurrence (%)[a]	Concentration (µg kg^{-1})[b]		
			Mean ± SD	Median	Range (GV)[c]
Alternaria spp.	Alternariol	81	5.99 ± 1.41	5.65	5.65–11.5
	Alternariolmethylether	86	5.50 ± 0	5.50	5.50–.50
	Altersetin	14	15.8 ± 14	8.70	6.85–31.9
	Altertoxin-I	5		–	36.5
	Infectopyrone	100	76.2 ± 30.1	74.2	25.9–141
	Macrosporin	52	3.64 ± 0.37	3.75	2.51–3.75
	Pyrenophorol	5			14.9
	Tentoxin	5			1.15
	Tenuazonic acid	48	29.6 ± 16.8	22.8	15.0–56.7
	Total	100	107 ± 52.3	95.1	30.9–229
Aspergillus spp.	Deoxynortryptoquivalin	33	3.20 ± 0	3.20	3.2–3.20
	Deoxytryptoquivaline A	90	26.5 ± 15.1	22.9	1.55–53.2
	Flavoglaucin	71	1.82 ± 1.42	1.63	0.65–5.19
	Fumiquinazolin D	10	4.92 ± 4.7	4.92	1.60–8.25
	Kotanin A	33	2.50 ± 0	2.50	2.50–2.50
	Pinselin	43	4.40 ± 3.33	1.55	1.55–10.3
	Pseurotin A	5			31.7
	Quinadoline A	71	13.1 ± 5.9	11.0	5.2–24.8
	Tryptoquialanine derivate	76	11.6 ± 5.09	11.7	5.07–20.9
	Tryptoquivaline A	14	3.00 ± 0	3.00	3.00
	Viriditoxin	48	65.3 ± 25.9	60.5	28.9–116
	Total	100	79.6 ± 46.8	72.9	22.2–228
Ergot alkaloids	Ergocornine	52	9.77 ± 4.95	8.67	3.72–22.7
	Ergocristine	86	26.8 ± 22.8	22.6	7.66–104
	Ergocristinine	29	2.23 ± 1.42	1.35	1.35–4.70
	Ergocryptine	90	13.9 ± 8.3	12.4	3.37–33.0
	Ergometrine	43	0.11 ± 0.04	0.1	0.10–3.75
	Ergometrinine	57	1.62 ± 1.02	1.53	0.37–3.75
	Ergosin	90	7.8 ± 6.24	7.02	0.30–23.3
	Ergosinin	57	1.92 ± 1.56	1.24	0.30–5.62
	Ergotamine	76	12.9 ± 7.06	10.2	4.38–27.9
	Ergotaminin	71	3.7 ± 3.29	2.41	0.94–12.3
	Total	90	69.5 ± 52.7	57.4	14.0–210
Fusarium spp.	15-Hydroxyculmorin	14	11.7 ± 0	11.7	11.7–11.7
	Antibiotic Y	5			9.50
	Apicidin	57	15.1 ± 13.3	9.64	2.74–43.0
	Apicidin D2	5			6.95
	Aurofusarin	100	137 ± 107	116	15.4–364
	Beauvericin	100	6.37 ± 6.25	4.37	1.40–32.4
	Bikaverin	100	13.6 ± 5.93	11.4	4.61–26.4
	Butenolid	5			237
	Chrysogin	19	3.46 ± 1.65	3.74	1.20–5.17
	Culmorin	100	390 ± 210	348	118–802
	Enniatin A	100	5.56 ± 1.83	5.12	2.06–8.73
	Enniatin A1	100	35.0 ± 12.8	30.0	12.2–60. 9
	Enniatin B	100	201 ± 77.3	173	84.4–380
	Enniatin B1	100	171 ± 61.5	151	65.2–321
	Enniatin B2	100	4.40 ± 1.67	3.91	1.98–7.71
	Epiequisetin	14	1.60 ± 0	1.60	1.60–1.60
	Equisetin	95	3.91 ± 6.18	1.60	1.60–29.0
	Fungerin	43	2.38 ± 2.28	1.41	0.65–7.27
	Fusaprolifetin	24	36.2 ± 24.9	32.5	15.0–75.1
	Fusaric acid	10	481 ± 617	481	45.1–917
	Gibberellin A12	95	189 ± 89.6	168	43.1–389
	HT-2 toxin	5			3.80 (250)
	Siccanol	100	966 ± 297	989	275–1503
	T-2 toxin	5			4.25 (250)
	W493	14	18.4 ± 16.3	15.8	3.50–35.7
	Zearalenone	57	13.2 ± 8.92	11.5	4.60–32.3 (500)
	Total	100	2200 ± 579	2343	1049–3100
Penicillium spp.	Andrastin A	19	2910 ± 4540	1020	14.7–9570
	Andrastin B	10	5990 ± 5420	5990	2160–9830
	Andrastin C	10	6400 ± 3630	6400	3840–8970
	Chanoclavin	5			0.95
	F01 1358-A	5			19.2
	Festuclavine	5			74
	Hydroxyandrastin A	5			46.9
	Hydroxyandrastin C	10	15.4 ± 12.5	15.4	6.60–24.2

(*continued*)

Table 2. Continued.

Group	Metabolite	Occurrence (%)[a]	Concentration (µg kg^{-1})[b] Mean ± SD	Median	Range (GV)[c]
	Mardfortine A	14	292 ± 498	848	0.99–867
	Mardfortine C	5			7.25
	Mycophenolic acid	14	261 ± 399	33.0	28.6–722
	Mycophenolic acid IV	5			1094
	Patulin	5			9201
	Phenopyrrozin	24	533 ± 558	130	1.29–12.5
	Questiomycin	19	286 ± 167	251	1.5–492
	Roquefortine C	10	480 ± 450	480	162–798
	Roquefortine D	10	60.7 ± 64.8	60.7	14.9–106
	Verrucofortine	29	250 ± 0	250	2.50–2.50
	Total	48	5600 ± 10,400	140	1.3–30,300
Other fungi	Cyclosporin A	5			222
	Monocerin	5			1.86
	Rubellin D	90	5.12 ± 3.57	280	2.8–12.8
	Sporidesmolide II	90	0.20 ± 0.10	0.18	0.09–0.42
	Total	100	15.5 ± 48.2	3.01	0.10–225
Sum of total fungal metabolites		100	5140 ± 7750	2750	1280–34,000
Phytoestrogens	Daidzein	24	501 ± 682	90.0	90.0–1660
	Genistein	29	508 ± 707	149	46.0–850
	Glycitein	10	93.6 ± 58.2	93.6	52.5–135
	Total	29	957 ± 1400	213	136–3650
Unspecific metabolites	Asperphenamate	62	0.25 ± 0.12	0.20	0.20–0.58
	Brevianamid F	100	307 ± 150	268	138–738
	Citreorosein	19	9.90 ± 4.26	8.46	6.62–16.0
	Cyclo(L-Pro-L-Tyr)	100	2304 ± 1387	2080	580–6370
	Cyclo(L-Pro-L-Val)	100	1937 ± 1118	1690	527–4770
	Emodin	52	3.99 ± 1.64	3.50	3.50–8.93
	Neoechinulin A	24	10.9 ± 8.83	7.00	7.00–26.7
	Physcion	5			535
	Rugulusovin	100	107 ± 61.3	99.4	24.2–280
	Tryptophol	100	2330 ± 2480	,090	150–8240
	Total	100	7010 ± 3400	6380	2620–16,160

[a]$n = 21$ representative samples of wet barley-derived brewery spent grains from Austria, considered as positive values > limit of detection (LOD); [b]calculations without data < LOD. In case values > LOD and < limit of quantification (LOQ), LOQ/2 was used for the calculation; [c]GV: Guidance value according to European Commission (EC 2006, 2013)

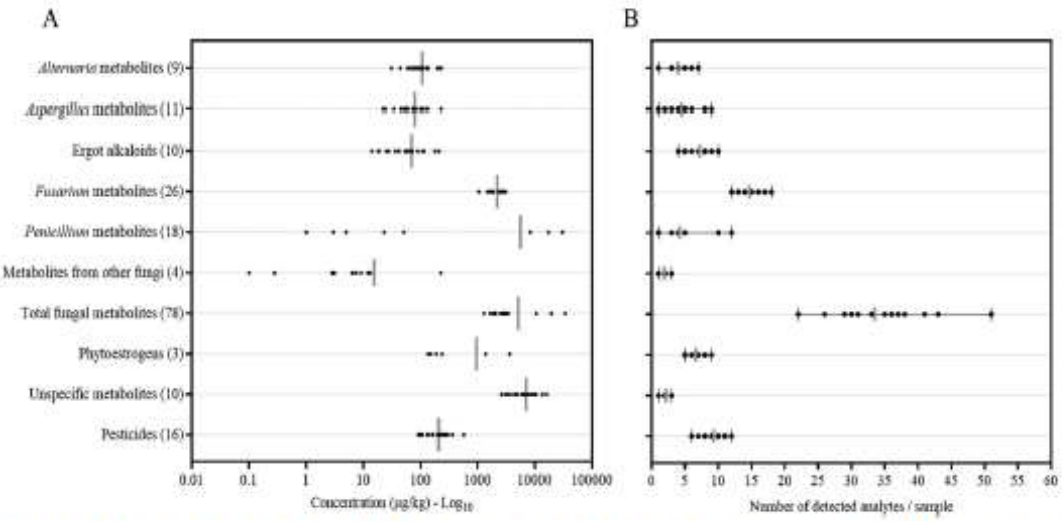

Figure 2. (A) Distribution of concentrations and (B) co-contamination grade (detected analytes per sample) of major categories of analytes detected in wet brewery's spent grains intended for the nutrition of dairy cattle in Austria.

The observed concentrations were still under the GVs of the European Union, which are 500 μg kg^{-1} for ZEN and 250 μg kg^{-1} for the sum of T-2 toxin and HT-2 toxin relative to a feed with moisture of 12% (EC 2002, 2006, 2013). The findings align with a previous report, which detected ZEN contamination in beer (Bauer et al. 2016). Other regulated mycotoxins such as AFB1, deoxynivalenol (DON), OTA and FB1 and FB2, which are regulated in European legislation for dairy cattle, were not detected. Regulated mycotoxins are known to cause adverse health effects. For example, ZEN is linked with hyper-estrogenism, reduced milk production, early abortion and other reproductive abnormalities (Hussein and Brasel 2001; Marczuk et al. 2012). Trichothecenes, like T-2 toxin and HT-2 toxin, are known for inducing inhibition of DNA and RNA synthesis, which can be a secondary effect of the inhibition of protein synthesis or due to apoptosis (Cope 2018). *Fusarium* head blight is a common disease in barley associated with ZEN, type A trichothecenes and other mycotoxins. This disease is a significant threat to the brewing industry, with *Fusarium graminearum* considered the predominant causal species worldwide (Bai and Shaner 2004; Starkey et al. 2007; Schwarz 2017). Other species like *Fusarium culmorum*, *Fusarium poae* and *Fusarium avenaceum* have also been described as some of the most widely occurring in Europe (Becher et al. 2013). *F. graminearum* and *F. culmorum* are important producers of ZEN, DON and nivalenol (Bottalico and Perrone 2002). *F. langsethiae* and *F. sporotrichioides* are producers of HT-2 and T-2 toxins (Thrane et al. 2004). It has been suggested that low levels of fusarial mycotoxins like ZEN and type-B trichothecenes (like DON, 15-acetyldeoxynivalenol and 3-acetyldeoxynivalenol) are retained in BSG (Pack et al. 2021). Previous studies found that the residual concentrations of several fungal toxins including OTA, AFB2, FB2, AFG1, AFB1, ZEN and patulin decreased to less than 20% during the brewing process (Inoue et al. 2011; Piacentini et al. 2019). Although T-2 toxin has received special attention in the malting barley chain, due to its occurrence in this cereal crop and its toxic potency, the contamination levels of this toxin generally decrease during the brewing process (Edwards et al. 2009; Malachova et al. 2010). A study of mycotoxins in BSG in Argentina evidenced FB1 (100%; range: 104–145 μg kg^{-1}) and AFB1 (18%; 19–44.5 μg kg^{-1}), whereas AFB2, AFG1, AFG2 and ZEN were not detected (Pereyra et al. 2011).

Regarding ergot alkaloids, which were detected in high occurrence (90%), the European Union recommends the monitoring of these metabolites (Recommendation 2012/154/EC) (EC 2012). There is no specific guidance value for ergot alkaloids in animal feeds. Still, the limit of 1000 mg kg^{-1} rye ergot (*Claviceps purpurea*) represents a maximum value relative to a feedstuff with a moisture of 12% described in the directive 2002/32/EC (EC 2012). Stricter regulation is in place for foodstuffs. Since January 2022, the Commission Regulation (EU) 2021/1399 established a maximum level of ergot alkaloids in certain foodstuffs. For instance, for milling barley products intended for human consumption (with an ash content lower than 9000 mg kg^{-1}), a maximum level of 100 μg kg^{-1} (50 μg kg^{-1}) will be implemented from 1.7.2024). For milling barley products (with an ash content equal to or higher than 9000 mg kg^{-1}) or barley grains placed on the market for the final consumer, the maximum level set is 150 μg kg^{-1} (EU 2021).

Moreover, our results show that members of the ergopeptine class alone, including ergocryptine, ergosin and ergocristine, presented with occurrences of over 85%. The average concentrations of individual ergot alkaloids were under 30 μg kg^{-1}, and the maximal accumulated concentration of total ergot alkaloids was 210 μg kg^{-1}, which should not be ignored. Feed contaminated with 250 μg kg^{-1} of ergot alkaloids should not be fed to pregnant or lactating animals due to a higher risk of abortion and agalactia syndrome; even low concentrations of alkaloids in the diet (<100 kg^{-1} total) can reduce the growth efficiency of livestock (Coufal-Majewski et al. 2016). Some members of the ergot alkaloids such as ergotamine, ergocristine, ergosine, ergocornine, ergocryptine and ergovaline are responsible for the majority of nervous or gangrenous syndromes in humans and animals, which consume grains, grain products or grasses contaminated with the sclerotia of the

fungus (Gupta et al. 2018). Ingestion of this kind of alkaloids by livestock can trigger a range of impacts from decreased performance and reduced fertility to acute clinical signs of ergotism, including nervous or gangrenous syndromes, hyperthermia, convulsions, necrosis of the extremities and death (Evans 2011). According to the scientific opinion of EFSA, ergotism in ruminants is usually a chronic disease and the result of continued ingestion of minor quantities of the fungus on grass (EFSA 2012). A large proportion of the original peptide alkaloids can be removed during brewing which is believed to result from thermal degradation (Schwarz et al. 2007). However, our data confirm that the reduction is not absolute.

Other fungal toxins and metabolites

Emerging mycotoxins are the focus of high scientific interest. They are defined as commonly occurring in feed and foods (agricultural commodities) and are legislatively unregulated and non-regularly tested (Vaclavikova et al. 2013). We showed that several emerging and non-regulated mycotoxins and metabolites were detected in samples of BSG intended for cattle feeding on Austrian farms. Several *Fusarium*-derived emerging mycotoxins include culmorin, siccanol, aurofusarin, beauvericin, bikaverin and enniatins (A, A1, B, B1 and B2), were detected in all the samples. The enniatins B and B1 presented the highest concentrations among the fusarial mycotoxins, with averages exceeding 170 μg kg^{-1}. Enniatins and beauvericin have haematotoxic, immunotoxic and antibiotic activities (Sy-Cordero et al. 2012; EFSA 2014; Juan et al. 2019; Křížová et al. 2021). Research on the impact of such fungal antimicrobial compounds on rumen ecology and functionality is essential (Fink-Gremmels 2005, 2008; Reisinger et al. 2019). Siccanol (also called terpestacin) (Chan and Jamison 2003) was the fusarial compound with the highest concentration (average: 966 μg kg^{-1}) in BSG. Another fusarial metabolite, fusaric acid occurred with a frequency of 19%. This compound can increase the toxicity of other *Fusarium* mycotoxins such as moniliformin, trichothecenes and FBs (Bacon et al. 1996; D'Mello et al. 1999).

Emerging *Alternaria* mycotoxins, such as alternariol (81%), alternariol methyl ether (86%) and tenuazonic acid (48%) were detected in relatively low concentrations. It is known that these compounds have estrogenic activity and genotoxic effects (Escrivá et al. 2017; Aichinger et al. 2019, 2021). The genus *Alternaria* is widely distributed in the environment and is one of the leading causes of disease in cereal crops like wheat, barley and sorghum (Deshpande 2002). However, information is still missing regarding *Alternaria* mycotoxins in the feeds and their toxicological repercussions on animal health (EFSA 2011). Infectopyrone was found in all the BSG samples and the compound with the highest average and maximum concentration among the *Alternaria*-derived metabolites. Is a potential mycotoxin whose biological activities are unknown and should be further explored (Andersen et al. 2002; Larsen et al. 2003). *Alternaria*-derived compounds like altersetin, altertoxin-I, pyrenophorol and tentoxin were also found. Although at this time, there are no global regulations establishing limits for these toxins in food and feed, the European Food Safety Authority (EFSA) has raised concern about *Alternaria* mycotoxins in relation to public health (EFSA 2011; Escrivá et al. 2017). *Aspergillus*-derived compounds were omnipresent in Austrian BSG evaluated in this study. The most frequently-occurring metabolites from *Aspergillus* were deoxytryptoquivaline A, tryptoquialanine derivate, flavoglaucin and quinadoline A, detected in frequencies above 70%. Viriditoxin and deoxytryptoquivaline A presented the highest average, median and maximum concentration of *Aspergillus*-derived metabolites. Other molecules produced by this genus including deoxynortryptoquivalin (33%), fumiquinazolin D (10%), kotanin A (33%), pinselin (43%), pseurotin (5%) and tryptoquivaline A (14%) were also found in BGS (Table 2). Viriditoxin, fumiquinazolin D, quinadoline A exhibit antibacterial properties (Qian et al. 2019; Urquhart et al. 2019; Almeida et al. 2021). Tryptoquialanines belong to the group of tremorgenic mycotoxins that can be produced by species of *Aspergillus* and *Penicillium* (Clardy et al. 1975; Ariza et al. 2002).

Strongly linked to postharvest contamination, *Penicillium*-derived metabolites presented an occurrence of 48%. This category of fungal compounds showed the highest average and maximal

concentration (average: 5604 µg kg^{-1}; max: 30,300 µg kg^{-1}). The metabolites verrucofortine (29%) phenopyrrozin (24%) were the more recurrent compounds from penicillia, but their mean concentrations were very low (<10 µg kg^{-1}). The *Penicillium*-derived metabolites detected in highest concentrations were andrastin C (average: 6,400 µg kg^{-1}, occurrence: 10%), andrastin B (5990 µg kg^{-1}, 10%) and andrastin A (2910 µg kg^{-1}, 19%). These compounds, among other *Penicillium*-derived substances, are commonly found in silage, with higher concentrations in mouldy hot spots (Reisinger et al. 2019; Gallo et al. 2021; Penagos-Tabares et al. 2022; Manni et al. 2022). The complete spectrum of the biological activities and toxicological effects of the andrastins has not been elucidated. It is known that the andrastins are protein farnesyltransferase inhibitors, which can inhibit the efflux of anticancer drugs from multidrug-resistant cancer cells and are devoid of antimicrobial activity. They are commonly found in European blue (mould) cheeses (Uchida et al. 1996). Other compounds detected in this study were mycophenolic acid, and roquefortines, the most investigated *Penicillium* metabolites in silages (Gallo et al. 2015). A common feature of many detected metabolites like mycophenolic acid, roquefortines and patulin, is their immunotoxic properties (Oh et al. 2012; Brennan et al. 2017), which could interfere with the activity of innate and adaptive immune responses, predisposing to secondary infectious diseases (Oh et al. 2015). Phenopyrrozin, along with marcfortines A and C, were also detected in the analysed BSG samples. Several researchers have proposed that *Penicillium* toxins can induce unspecific clinical signs like appetite reduction, affecting nutrient efficiency and increasing the incidence of abomasal ulcers, laminitis, gastroenteritis, abortion and paralysis (Dzidic et al. 2006; Nielsen et al. 2006; Fink-Gremmels 2008; Alonso et al. 2013; Gallo et al. 2015). Additionally, further less-known metabolites produced mainly by other fungal species were detected in this study. Rubellin D and sporidesmolide occurred at a frequency of 90%, whereas cyclosporin A and monocerin were detected in only one sample (5%). The quantified levels of these metabolites were below 15 µg kg^{-1}, except for cyclosporin A (222 µg kg^{-1}) (Table 2). Rubellin D is an anthraquinone derived from *Ramularia collo-cygni* with antibacterial activity (Walters et al. 2008; Miethbauer et al. 2009). Cyclosporin A has a potent immunotoxic activity, which has even been used commercially in human and veterinary medicine as an immunosuppressant (Laupacis et al. 1982; Shevach 1985; Stähelin 1996).

Postharvest infestations with moulds proliferate under aerobic conditions, producing potent toxins, disruptive endocrine substances and antimicrobial compounds. Moreover, fungal growth leads to spoilage, thereby reducing the nutritional value, DM content, intake and palatability (O'Brien et al. 2006). The high proportion of moisture in wet BSG makes this product especially susceptible to microbial growth and spoilage in a short period (7–10 d) (Lilly et al. 1980; Stojceska and Ainsworth 2008; Chanie and FieVez 2017). Strategies suggested for preserving wet BSG include drying with solar radiation and ensiling. Drying by solar radiation is found to be challenging due to costs (Conrad and Rogers 1977). Alternatively, the ensiling of wet brewery grain alone or mixed with dry fodders is the proposed practice for dairy farmers, especially in developing countries (Kindbom 2012; Souza et al. 2012). The preservation of wet BSG by lowering the water activity of the material using beet molasses (30%) and further stabilising the mixture by incorporating an anti-mycotic agent (0.3% of potassium sorbate) has been achieved at both laboratory-scale and pilot-scale. For practical preservation, the stabilised grains should be stored under anaerobic conditions in plastic bags, squeezing the air out and sealing tightly (Lilly et al. 1980). However, more applied research on preservation strategies for BGS is still required.

Contamination of phytoestrogens and other secondary metabolites

In the present research, three isoflavones were detected in medium-low frequencies: Daidzein (24%), genistein (29%) and glycitein (10%) (Table 2). The predominant daidzein and genistein presented average concentrations above 500 µg kg^{-1} and maximum above 1600 µg kg^{-1}. These

metabolites are found primarily in *Leguminosae* plants, such as soy (*Glycine max*) but also occur in clovers (*Trifolium* spp.) and alfalfa/lucerne (*Medicago sativa*) (Reed 2016). Glycitein presented an average concentration of 93.6 μg kg^{-1}, ranging from 52.5 to 135 μg kg^{-1}. Liggins et al. (2002) reported daidzein and genistein in cereal-derived products for human consumption. In pearl barley, only genistein was detected with an average concentration of 86 μg kg^{-1}. The concentration of the two isoflavones in the remaining foods ranged from 33 to 11,873 µg kg^{-1} (Liggins et al. 2002). Coumetrans like coumestrol, which were not found. seem to have a more potent estrogenic activity than the detected isoflavones here (Romero et al. 1997). The detected concentration of phytoestrogens (isoflavones) found in our study apparently does not represent a potential risk for cattle (Grgic et al. 2021).

In addition, several unspecific secondary metabolites were detected. These analytes can be produced by different and unrelated living systems belonging to diverse kingdoms (Plantae, Fungi, Animalia and/or Eubacteria). Several of the unspecific secondary metabolites detected in our study are biologically active molecules. These compounds could influence the toxicological complexity of the complete cocktails of secondary metabolites evidenced in this investigation. They included, for example, emodin (antibacterial and immunosuppressive) (Kiyoshi et al. 1984; Dong et al. 2016) as well as the diketopiperazines cyclo-(L-Pro-L-Tyr) (synonym: maculosin) and cyclo-(L-Pro-L-Val) (antibacterial) (Park et al. 1993; Rahman et al. 2020; Zin et al. 2020; Paudel et al. 2021). Other detected unspecific metabolites were neoechinulin A, physcion, rugulusovine and tryptophol. The most predominant unspecific metabolites (detected in all the samples) were tryptophol, cyclo-(L-Pro-L-Tyr) and cyclo-(L-Pro-L-Val), which also showed the highest average concentrations of this group (>1900 µg kg^{-1}) (Table 2).

Pesticide residues

All the samples presented residues of pesticides, varying from six to twelve different biocides per sample (Figure 2(B)). No illegal compounds (EU-MRL-Database 2022) were detected. In total, 16 pesticides were found: 14 fungicides, one insecticide (pirimiphos-methyl) and an insecticide synergist (piperonyl butoxide) as classified in previous studies (Table 3) (Huang and Subramanyam 2005; Opalski et al. 2006; Lamberth et al. 2008; Sooväli and Koppel 2010; Harp et al. 2011; Lazzari et al. 2012; Rodrigues et al. 2013; Kanungo and Joshi 2014; Rumbos et al. 2016; EFSA 2017, 2018; McLean and Hollaway 2019; Xu et al. 2020; Basak et al. 2021; Yao et al. 2021; EU-MRL-Database 2022; Rathod et al. 2022). Occurrences along with the corresponding average, median and range concentrations (expressed in μg kg^{-1} DM) of the detected pesticide residues, their respective uses, the maximum residue levels (MRLs) in barley, and the proportion of samples above the respective MRL are presented in Table 3. The most frequently detected pesticides in the analysed BSG samples were fluopyram, piperonyl butoxide, fluxapyroxad, bixafen, mandipropamid and tebocunazol, which were seen in ≥85% of the samples. The fungicides azoxystrobin, benzovindiflupyr and boscalid as well as the insecticide pirimiphos-methyl showed occurrences between 43 and 62%. Residues of ametoctradin, isopyrazam, pyraclostrobin and trifloxystrobin were found in less than 40% of the evaluated BGS samples. The pesticides with the highest average levels of residues were piperonyl butoxide (116µg kg^{-1}), metrafenone (30.4 µg kg^{-1}) and fluopyram (24.7 µg kg^{-1}) (Table 3). Notably, 9.5% and 14.3% of the samples exceed the respective current MRLs (0.01 mg kg^{-1}) of ametoctradin and mandipropamid for barley. The other pesticides were detected in amounts lower than the MRLs (EU-MRL-Database 2022). Piperonyl butoxide occurred frequently and presented the highest levels among the groups of pesticides. Piperonyl butoxide enhances the potency of certain pesticides such as carbamates, pyrethrins and pyrethroids but has no pesticide activity of its own (Basak et al. 2021). It has been demonstrated that this insecticide synergist can induce the formation of liver tumours in mice *via* the constitutive androstane receptor, which is qualitatively not plausible for humans due to the lack of effect on replicative DNA synthesis in human hepatocytes (Lake et al.

Table 3. Occurrences and levels of pesticide residues detected in wet brewery's spent grains intended for dairy cattle nutrition.

	Occurrence (%)[a]		Concentration (µg kg^{-1})[b]						
Analyte	Positive samples	≥ MRL[c]	Mean ± SD	Median	Range	WHO classification by hazard[c]/ listed as highly hazardous pesticides by PAN[d] (+)	MRL[e] (µg kg^{-1})	Use	References
Ametoctradin	23.8	9.5	12.0 ± 12.7	4.45	4.45–33.8	III	10	Fungicide	Dreinert et al. (2018)
Azoxystrobin	61.9	0.0	7.17 ± 5.15	2.8	6.20–17.3	U	1500	Fungicide	Rodrigues et al. (2013)
Benzovindiflupyr	47.6	0.0	1.40 ± 0.00	1.4	1.4–1.40	II	1500	Fungicide	Yao et al. (2021)
Bixafen	95.2	0.0	7.51 ± 3.96	4.65	4.65–18.1	–	400	Fungicide	Lazzari et al. (2012)
Boscalid	42.9	0.0	9.75 ± 16	4.45	4.45–52.1	U	4000	Fungicide	Xu et al. (2020)
BTS 44595 (Metabolite of prochloraz)	38.1	0.0	3.65 ± 4.26	2.15	2.15–14.2	II	30	Fungicide	EFSA (2018)
Fluopyram	100	0.0	24.7 ± 15.8	19.8	12.5–75.6	III	200	Fungicide	Rathod et al. (2022)
Fluxapyroxad	95.2	0.0	8.45 ± 6.22	6.84	2.65–24.5	III	3,000	Fungicide	McLean and Hollaway (2019)
Isopyrazam	19.0	0.0	1.85 ± 0.00	1.85	1.85–1.85	II/+	600	Fungicide	Harp et al. (2011)
Mandipropamid	90.5	14.3	11.8 ± 22.1	3.9	3.90–99.1	U	10	Fungicide	Lamberth et al. (2008)
Metrafenone	71.4	0.0	30.4 ± 45.6	15.7	2.48–191	U	600	Fungicide	Opalski et al. (2006)
Piperonyl butoxide	95.2	N/A	116 ± 58.8	80.1	15.5–254	U	N/A	Insecticide synergist	Basak et al. (2021)
Pirimiphos-methyl	47.6	0.0	3.57 ± 2.61	2.54	1.65–9.29	II/+	5000	Insecticide	Huang and Subramanyam (2005)
Pyraclostrobin	4.8	0.0	3.85 ± 0	–	3.85	–	1000	Fungicide	Kanungo and Joshi (2014)
Tebuconazole	85.7	0.0	10.1 ± 3.35	9.41	1.24–16.7	II/+	2000	Fungicide	Soovälli and Koppel (2010)
Trifloxystrobin	14.3	0.0	7.04 ± 8.73	2	2–17.1	U	500	Fungicide	EFSA (2017)
Total	100	N/A	208 ± 113	201	92.6–572	N/A	N/A		

[a]$n = 21$ representative samples of wet barley-derived brewery spent grains from Austria, considered as positive values > limit of detection (LOD); [b]calculations without data < LOD. In case values > LOD and < limit of quantification (LOQ), LOQ/2 was used for the calculation; [c]WHO classification of pesticides by hazard: Ia (Extremely hazardous, Ib (highly hazardous), II (moderately hazardous), III (slightly hazardous) and U (unlikely to present acute hazard) (WHO 2020); [d]according to pesticide action network international (PAN 2021); [e]maximal residue level for barley according to the European Union guidelines expressed at 88% DM (EU-MRL-Database, 2022). N/A: Not available/not apply.

2020). This insecticide synergist is not a cholinesterase inhibitor and has low toxicity; it is also employed for other purposes than crop protection. It may also be used in conjunction with flea or tick dips, collars and oral medications in farm animals (Keane 1999). A widespread use of fluopyram, a pyridinyl ethylbenzamide, applied as a broad-spectrum fungicide with nematicide activity (Becker et al. 2020; Rathod et al. 2022) was recorded in this study, because it was detected in all the samples. The literature suggests that the high persistence of fluopyram in the environment (soil and water/sediment) can present risks for human, animal and soil health. The fate of this fungicide in diverse soil environments is still to be studied (Rathod et al. 2022). Fluopyram is authorised for use on crops that might be fed to livestock (EFSA 2020). A feeding study (Schoening and Wolters 2008 Ref: MR-07/367 (unpublished) cited by Lunn (2010) investigated the residue depuration of fluopyram in lactating dairy cattle, finding fluopyram and its metabolites in milk and different tissues. Barley has frequently been found contaminated with traces of fungicides (Palladino et al. 2021). In terms of risk, according to the WHO, five of the detected biocides here (benzovindiflupyr, prochloraz [confirmed by its metabolite of BTS 44595], isopyrazam, pirimiphos-methyl and tebuconazole) were classified as moderately hazardous (II) (WHO 2020). The last three are included in the list of highly hazardous pesticides (HHP) of the Pesticide Action Network (PAN 2021). Isopyrazam was added to the HHP list in 2011, it is considered likely carcinogenic for humans, very persistent in water, soil or sediment and very toxic for aquatic organisms (<50 ng/L) (US EPA 2017; Yao et al. 2018). Pirimiphos methyl was added to the HHP list in 2009, which is considered highly toxic for bees (Barnett et al. 2007; PAN 2021). Also, in the HHP list, tebuconazole can induce acute toxicity and long-term effects (PAN 2021) and can have ecotoxicological effects on an aquatic decomposer-detritivore system (Zubrod et al. 2011). Other detected pesticides like ametoctradin, fluopyram and fluxapyroxad are classified as slightly hazardous (III). In contrast, azoxystrobin, boscalid, mandipropamid, metrafenone, piperonyl butoxide and trifloxystrobin are grouped as unlikely to present acute hazards (WHO 2020). It has been suggested that reduction in pesticide levels during the malting and brewing processes reduced considerably the risks of contaminating beer with pesticides and only a few pesticides remained without being removed or resolved (Navarro et al. 2007; Kong et al. 2016). Several pesticides found during beer production are adsorbed onto the spent grain after mashing. Moreover, some pesticides are degraded or transformed during boiling and fermentation, indicating that such reduction was caused primarily by adsorption, pyrolysis, and hydrolysis (Inoue et al. 2011; Xi et al. 2014). In the European Union, pesticide residue levels in particular plant and animal-derived foods and feeds, have been set by the Commission (EC) No 396/2005 (EC 2005). Information concerning MRLs and toxicity is available in the EU Pesticide database (EU-MRL-Database 2022).

Co-occurrence of fungal toxins, phytoestrogens and pesticide residues

Bioaccumulation rates and effects of long-term exposure to contaminant mixtures are unpredictable and should be investigated through the feed and food production chain. The co-contamination of several natural and synthetic contaminants was found (Figure 2(B)). All the samples presented co-contamination with several fungal secondary metabolites, fluctuating from 22 to 51 fungal metabolites per sample; 34 on average. Similarly, a broad spectrum of co-contamination with fungal and other metabolites has been observed in different complex matrices of feedstuffs such as silage, pastures, concentrate feed, and total mix rations (Shimshoni et al. 2013; Nichea et al. 2015; Kemboi et al. 2020; Awapak et al. 2021). Interestingly, we generated data concerning pesticide residues, which occurred with an average of nine compounds per sample, ranging from six to twelve (Figure 2(B)). Additionally, Figure 3 illustrates co-occurrence matrices of mycotoxins (with recurrence over 25%) and phytoestrogens (Figure 3(A)) as well as pesticides (Figure 3(B)). All the samples evidenced that three fusarial emerging mycotoxins, aurofusarin, beauvericin and enniatins, were

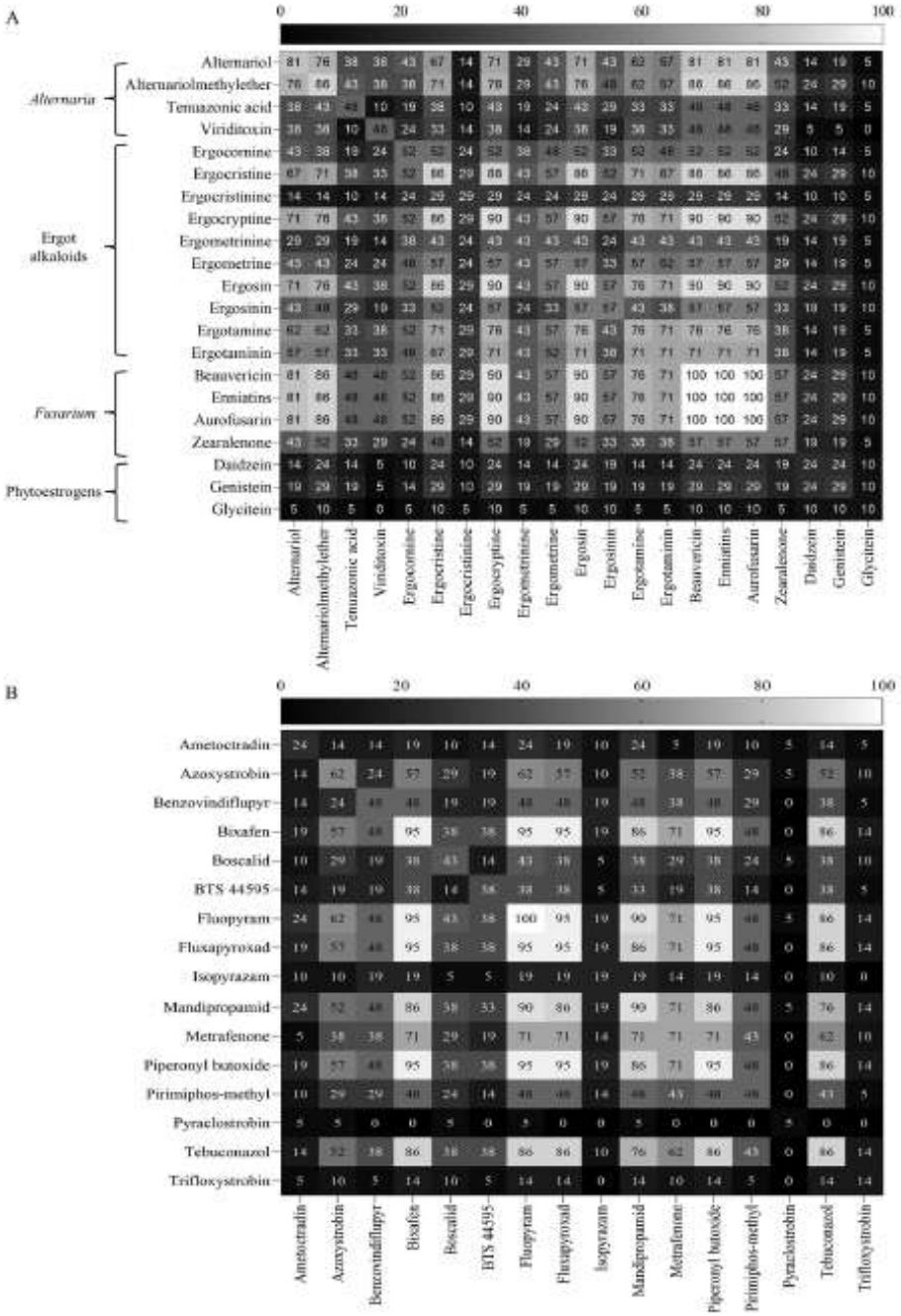

Figure 3. Heatmaps of the co-occurrence (%) of (A) fungal contaminants (with occurrence rate > 25%) and phytoestrogens as well as (B) of pesticide residues detected in wet brewery's spent grains intended for the nutrition of dairy cattle in Austria.

detected and co-occurred with ZEN in 57% of the samples. More than 50% of the BSG presented co-contamination with several ergot alkaloids. Combinations between mycoestrogens derived from *Alternaria* AOH, AME, and TeA with ZEN were 43%, 52% and 33%, respectively. Co-occurrences between the mentioned mycoestrogens and the detected phytoestrogens (daidzein, genistein and glycitein) were lower than 30% (Figure 3(A)). Concerning pesticide residues, over 60% of the samples presented mixtures of fluopyram, fluxapyroxad, mandipropamid, metrafenone, piperonyl butoxide and tebuconazole. All the samples containing the insecticide for storage pirimiphos-methyl (48%) contained the semisynthetic synergist piperonyl butoxide: both compounds has been recently found in cereal samples from Croatia (Kovač et al. 2021).

These outcomes evidenced the ubiquitous presence of mixtures of multiple natural and synthetic chemicals in this by-product, linked to the feed and food supply chain. Although the occurrence of several contaminants was high, the concentrations found were low and under the legal limits (GVs and MRLs). The individual concentrations indeed do not represent an acute or critical risk for farm animals and human consumers. However, it is known that the combined effect of several co-occurring toxins and endocrine disruptors may be additive, synergistic or antagonistic, varying by type of compound or/and concentration (Guo et al. 2020). Such biological effects of toxin mixtures on animal and human health have been growing notably in recent years, but related knowledge is still overall scarce (Battilani et al. 2020; Gil-Serna et al. 2014; Smith et al. 2016; Weaver et al. 2020). Toxicological interactions have been described among mycotoxins, phytoestrogens and pesticides (Hessenberger et al. 2017; Vejdovszky, Hahn, et al. 2017a, Vejdovszky, Schmidt, et al. 2017a,b; Eze et al. 2019). For example, it is known that the interaction of diverse kinds of natural and synthetic xenobiotics, such mycotoxins, plant metabolites and chemical biocides, can also shape microbiota composition, which influences the health and metabolic status of the host (Lindell et al. 2022). The relevance of the co-occurrence (in real-world situations) of natural and synthetic chemicals has to be addressed by toxicologists (Warne and Hawker 1995; Groten et al. 2001; Mattsson 2007). Nowadays, advances in analytic methods allow for evaluating hundreds of natural and synthetic pollutants, achieving high performances (LOD, LOQ and recovery) (Steiner et al. 2020; Sulyok et al. 2020; Steiner et al. 2021). Multi-toxin and multi-metabolites analysis has been used during the last decade to bring more insights into the complex field of mixture toxicology (Groten et al. 2001; Battilani et al. 2020; Martin et al. 2021). The evidenced ubiquitous presence of mixtures of pesticides suggests an extended application of this kind of substances in barley intended for beer production. This could also indicate that multiple biocides are being incorporated constantly at low levels in the feed and food chain, which can result in negative ecological and toxicological consequences (Mishra et al. 2014; Rivera-Becerril et al. 2017; Vanbergen 2021; Panico et al. 2022). Pesticide interactions lead mainly to synergic effects. Mixture effects differ depending on the dose and/or physiological target. Thus, more research and data for this important and exciting field are still required (Rizzati et al. 2016).

Conclusion

This study provides insights into the widespread occurrence of cocktails of mycotoxins, phytoestrogens and pesticides in wet BSG. Mycotoxins/metabolites produced by the genera *Fusarium*, *Aspergillus*, and *Alternaria* were detected in all the samples. Ergot alkaloids were also frequently found (90%). *Penicillium* secondary metabolites, associated primarily with storage contamination, were present in 48% of the samples and showed the highest average concentration among the groups of fungal compounds. The storage-associated contamination leads to the necessity to improve strategies for preserving wet BSG in the farms. Additionally, we demonstrate the ubiquitous co-occurrence of several pesticide residues (at least six per sample, primarily fungicides). Two of them (ametoctradin and mandipropamid) exceeded the EU MRLs. Some pesticides (azoxystrobin, bixafen, fluopyram, fluxapyroxad, mandipropamid, metrafenone, piperonyl butoxide and tebuconazole) showed high occurrences (>60%),

which could suggest a common and extended use on food/feed crops of the mentioned pesticides and incorporation of these biocides into the feed/food chain and into the agroecosystems. Although the vast majority (88%) of the detected pesticides presented low concentrations, the potential combined effects of such biocide mixtures and natural toxins are unpredictable and should be subject to future studies. Further investigations with a larger number of samples and evaluation of BSG together with other feeds/foods is highly advocated.

Acknowledgments

The authors appreciate enormously the excellent technical support and cooperation provided by Dr. Manfred Hollmann, Anita Dockner, Sabine Leiner (Institute of Animal Nutrition and Functional Compounds, Vetmeduni, Vienna) for the excellent technical assistance during the sample preparation.

Disclosure statement

N.V. and J.F. are employed by BIOMIN Holding GmbH, part of DSM Animal Nutrition and Health, which operates the BIOMIN Research Center and produces animal feed additives. This, however, did not influence sampling, analyses or interpretation of data.

Funding

This study is part of the Project "D4Dairy-Digitalization, Data Integration, Detection and Decision support in Dairying" supported by the Austrian Federal Ministry for Climate Action, Environment, Energy, Mobility, Innovation and Technology (BMK), Austrian Federal Ministry for Digital and Economic Affairs (BMDW), and the provinces of Lower Austria and Vienna within the framework of COMET-Competence Centers for Excellent Technologies, which is handled by the Austrian Research Promotion Agency (FFG).

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Phytoestrogens
Aspergillus metabolites
Ergot alkaloids
Fusarium metabolites
Penicillium metabolites
Metabolites from other fungi

3.5. Publication 5:

Residues of pesticides and veterinary drugs in diets of dairy cattle from conventional and organic farms in Austria

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*Environmental Pollution (*2022) 120626. https://doi.org/10.1016/j.envpol.2022.120626

Environmental Pollution 316 (2023) 120626

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Residues of pesticides and veterinary drugs in diets of dairy cattle from conventional and organic farms in Austria

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ARTICLE INFO

Keywords:
Pesticides
Veterinary drugs
Residues
Animal feed
Milk production
Mixtures toxicology

ABSTRACT

Modern agriculture depends highly on pesticides and pharmaceutical preparations, so controlling exposure to these substances in the feed and food chain is essential. This article presents the first study on residues of a broad spectrum of pesticides and veterinary drugs in the diets of dairy cattle. One hundred and two representative samples of the complete diets, including basal feed rations and additional fed concentrate, were collected in three Austrian provinces (Styria, Lower and Upper Austria) in 2019 and 2020. The samples were tested for >700 pesticides, veterinary drugs and related metabolites using a validated method based on liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS). In total, 16 residues (13 pesticides and three veterinary drug residues) were detected. > 90% of the diets contained pesticide residues and <10% veterinary drug residues, whereas banned pesticides were not found. The most frequent pesticide residues were fluopyram (62%), piperonyl butoxide (39%) and diethyltoluamide (35%). The following pesticides exceed the default EU maximum residue level (MRL) (10 μg kg^{-1}) for products exclusively used for animal feed production: Benzovindiflupyr (proportion of samples > MRLs: 1%), bixafen (2%), fluopyram (6%), ipconazole (1%) and tebuconazole (3%). Three residues (dinitrocarbanilide, monensin and nicarbazin) of veterinary drugs were identified, all below the MRLs. Over 60% of the evaluated samples contained mixtures of two to six residues/sample. Only one pesticide (diethyltoluamide) presented a significant difference among regions, with higher concentrations in Upper Austria. Brewery's spent grains were the dietary ingredient that showed the strongest correlation to pesticide residues. These findings evidence the realistic scenario of highly occurrent low doses of pesticides cocktails in the feed/food chain, which may affect the animal, human and environmental health. Since the risk assessments are based on single pesticides, the potential synergistic effect of co-occurring chemicals ("cocktail effect") requires further investigations.

1. Introduction

Milk and dairy products represent one of the most important food commodities for all the age groups of the human population in several countries around the globe (Kubicova et al., 2019). The dairy industry is the second-largest agricultural sector in the European Union, corresponding to more than 12% of its total agricultural output (Augère-Granier, 2018). Specifically in Austria, the dairy industry is the most relevant agricultural sector, representing 13% of the national agricultural production (BMLRT, 2021). In modern agriculture (including dairy farming), the production of crops and animal-derived foods is highly dependent on pesticides and veterinary pharmaceutical preparations, which are the foundation of the called conventional agriculture systems. These substances have been essential for protecting

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https://doi.org/10.1016/j.envpol.2022.120626
Received 16 August 2022; Received in revised form 26 October 2022; Accepted 7 November 2022
Available online 9 November 2022

crops and livestock from pest infestation and diseases (Beyene, 2016; Özkara et al., 2016).

Pre-and post-harvest use of pesticides safeguards crops and controls pests (like insects, weeds and plant pathogens), improving production quantity (Özkara et al., 2016). However, residues of pesticides can be accumulated in crops and the environment, affecting human, animal and environmental health (Igbedioh, 1991; Damalas and Eleftherohorinos, 2011; Cozma et al., 2017; Silva et al., 2019; Jepson et al., 2020; Kruse-Plaß et al., 2021; Zaller et al., 2022). For example, it is known that pesticides are stress factors affecting health and raising the mortality of bees and other insects worldwide (Hallmann et al., 2017; El Agrebi et al., 2020; Barmentlo et al., 2021; Bruinenberg et al., 2022). The global decline of insect populations is a big concern affecting complete ecosystems because of their critical role in several ecological functions like pollination, nutrient cycling, pest control and food sources for multiple species (Wilson et al., 1999; Yang and Gratton, 2014). Pesticides have also been related to the decline of bird populations (Goulson, 2014). Regarding the impacts on human health, chronic pesticide exposure has been linked to carcinogenicity, neurodegenerative diseases, infertility, malformation, hormonal disruption and alteration in the immune system (Parrón et al., 2011; Mai et al., 2014; Karalexi et al., 2021; de Barros Rodrigues et al., 2022; Palaniyappan et al., 2022; Singh et al., 2022).

The extensive use of veterinary drugs, which are added to the feed of food-delivering animals for prophylaxis and metaphylaxis purposes, and growth promoters is also a big concern (Anadón and Martínez-Larrañaga, 1999; Beata, 2016; Anadón et al., 2018). Antibiotics, anti-parasitic drugs and non-steroidal anti-inflammatory drugs have been broadly utilized in livestock feeds, associated with the appearance of residues in animal products such as milk, meat and eggs (Beyene, 2016; Rana et al., 2019). Incorporating pharmaceutical preparations can affect feed/food safety, contributing mainly to public health problems like multidrug resistance, carcinogenicity, teratogenicity and disruption of normal gut microbiota (Ortelli et al., 2018; Rana et al., 2019). In particular, antimicrobial resistance represents an increasing threat to global public health that requires appropriate action across governments and society (Hao et al., 2014; Baynes et al., 2016; Lekshmi et al., 2017; Ortelli et al., 2018).

Organic agriculture has been developed to respond to problems generated by conventional industrial agriculture on the environment, animal and human health (Röös et al., 2018). In 2019, 8.5% of total EU agricultural land (approx. 13.8 million hectares) was under organic farming, which represented an increment of 66% compared with 2009 (8.3 million hectares). Austria presented the highest proportion of organic agriculture at the EU level, with 25.3% of the agricultural land under this productive system (Commission, 2022). The "organic" label guarantees a production that avoids synthetic fertilizers, hormones and pesticides as well as minimizing the use of veterinary drugs (Prache et al., 2022); however, pesticide and veterinary drugs residues have been detected in milk (Ghidini et al., 2005; Gutiérrez et al., 2012; Wanniatie et al., 2019), other commodities (Bursić et al., 2021; Schusterova et al., 2021) and soils of organic farming systems (Geissen et al., 2021). Monitoring the exposure to pesticides and veterinary drug residues in the feed and food chain is essential and required to enforce legislation and guarantee food safety (Masiá et al., 2016; Kumar et al., 2019). The European Union has one of the strictest legislation concerning pesticides and veterinary drug residues in the feed and food chain (EC, 2004; Anastassiadou et al., 2019; Kuchheuser and Birringer, 2022). The European Commission (EC) has been promoting low pesticide-input farming in the Member States and individual governments, and it has been expected to create the necessary conditions for farmers to implement Integrated Pest Management (IPM) (Hillocks, 2012).

More recently, the European Green deal, lined with the Farm to Fork and the Zero Pollution strategies, aims to reduce pesticide utilization by 50%, eliminate soil pollution and establish at least 25% organic farmland in Europe by 2030 (EC, 2020a, 2020b; Silva et al., 2022). To achieve the goals of these strategies, a diagnosis of the current situation and regular monitoring of the use of pesticides and veterinary drugs in different segments of the feed and food chain is crucial. Thus, this study aimed to characterize a broad spectrum (>700) of pesticide and veterinary drug residues in the complete dietary rations of lactating cows in Austrian organic and conventional dairy farms. It was achieved by employing a validated multi-metabolite liquid chromatography/electrospray ionization-tandem mass spectrometric (LC/ESI–MS/MS) method. Additionally, correlation analysis was performed between the most occurrent analytes and the main dietary ingredients. Moreover, the geographical distribution patterns of the residues were explored.

2. Material and methods

2.1. Sampling and data collection

This research was performed in the framework of a project that aimed to survey feed safety aspects in the Austrian dairy sector, which also included investigations on natural contaminants and metabolites (such as mycotoxins, phytoestrogens, plant toxins and other secondary metabolites) recently published (Penagos-Tabares et al., 2021, 2022a; 2022b, 2022c). After signing a confidentiality and data protection agreement with the involved Austrian dairy farmers, one representative sample of lactating cows' diet per farm was collected (n = 102, 93 rations of conventional and nine organic farms). The included organic farms followed the BIO AUSTRIA regulations for organic farming in Austria (available at. https://www.bio-austria.at/app/uploads/RiliEnglish20121.pdf). The relation of organic/conventional farms was not balanced due to the low availability and acceptance of organic farms to participate in this study during the recruiting. Moreover, because it was not included in the project's overall goal. The sampling was performed between May 2019 and September 2020 in the three provinces with the country's major dairy production: Upper Austria (n = 53), Lower Austria (n = 32) and Styria (n = 17) (Fig. 1). On average, the herd sizes of the visited farms were 59 ± 15 standard deviation (SD) lactating cows per farm, fluctuating from 32 to 140. Each representative sample of the complete diet involved the separate collection of fresh mixed rations from the feeding table and concentrate feed from the automatic feeders. A minimum of 30 sub-samples of the above mentioned feeds were manually collected using nitrile gloves to avoid cross-contamination. The final sample of each kind of feed was at least 1 kg, which was vacuum-packed and stored at −20 °C until sample preparation. Additionally, information concerning the farming system (organic or conventional), basal feed composition (main components and their respective proportions), estimated total intakes (of mixed rations and concentrate feed), use of pesticides (in the feed crops) and veterinary drugs (in the rations) were obtained via a questionnaire-guided interview.

Fig. 1. Map showing the locations of the dairy farms of complete dietary rations (n = 102) of Austrian dairy cattle.

2.2. Sample preparation

Once the sampling period finished, the frozen mixed ration samples were dried at 65 °C in an electric fan oven for 48 h. Once dried, the mixed rations and concentrate feeds were milled to a final particle size of ≤0.5 mm. They were firstly milled using the cutting mill (SM 300, Retsch GmbH, Haan, Germany) at 1,500 rpm for approximately 1 min. Subsequently, using an ultra-centrifugal mill (ZM 200, Retsch GmbH, Haan, Germany) at 10,000 rpm for about 30 s, the remnants (non-milled residues, mainly corresponding to hard fragments of seeds) were milled. Both milled fractions were combined, mixed and packed in plastic bags. The processed, mixed rations and concentrated feeds were composited according to the average intake proportions (data provided by the farmers) to obtain 20 g (±0.01 g) of the whole diet representative sample. Finally, 5 g (±0.01 g) of the homogenized complete diet samples were stored in 50-mL polypropylene conical tubes (Sarstedt, Nümbrecht, Germany) and kept at −20 °C until analysis.

2.3. Analysis of multiple pesticides and veterinary drug residues

Following the protocol described by Steiner et al. (2020), the previously prepared sample (5 ± 0.01 g) was put into a 250 ml Erlenmeyer flask with 20 ml of extraction solvent. Next, homogenization was performed using a GFL 3017 rotary shaker (GFL, Burgwedel, Germany) for 90 min. Quantification was established on external calibration utilising a serial dilution of a multi-analyte stock solution. The solvent solution-sample mixture was centrifuged for 2 min at 2,012 × g on a GS-6 centrifuge (Beckman Coulter Inc., Brea, CA, USA). The extract, along with dilution solvent, was diluted at one to one proportion. The injection volume of both diluted sections of the samples and the standard analyte solutions was 5 μl. Identification and quantification of each analyte were performed in two separate chromatographic runs using a QTrap 5500 LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with a TurboV electrospray ionization (ESI) source coupled to a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Quantitative analysis of all the analytes was performed using a validated method based on LC-ESI-MS/MS described by Steiner et al. (2020). Results were corrected for apparent recoveries determined during method validation, according to Steiner et al. (2020). Values related to the method performance (apparent recoveries, the limit of detection (LOD) and the limit of quantification (LOQ) of each analyte as well as the specific chemical class are described in Table S1. The targeted pesticides (660), veterinary drugs (129) and their respective related metabolites along with the compound identification numbers (PubChem CID) are enlisted in Table S2 and Table S3. Analyses, analytical quality control and method validation were performed in accordance with DG SANTE guidelines for pesticide and veterinary drug residues analysis in food and feed. (EC, 2019).

2.4. Data analysis

Concentrations of all detected residues and related metabolites (i.e., markers such as dinitrocabanilide) were presented on a dry matter (DM) basis in μg kg^{-1}. Descriptive statistics, i.e., frequencies, mean, median and ranges of the concentration of analytes, were calculated considering only the positive results (x ≥ LOD). Results below the LOQ were computed as LOQ/2. Normality test of the data was performed via D'Agostino & Pearson test, Anderson-Darling test, Shapiro-Wilk test and Kolmogorov-Smirnov test. All the tests indicated the non-normal distribution of the handled data. The Kruskal-Wallis test (the non-parametric alternative of the ANOVA) was runned to analyse significant differences in the concentration and number of compound residues among the three Austrian provinces. In case of significant differences among the three provinces, these differences were re-evaluated via non-parametric Mann-Whitney *U* test between pairs of provinces (Upper Austria Vs Lower Austria, Upper Austria Vs. Styria, and Lower Austria Vs. Styria, respectively). This applied only for diethyltoluamide. Additionally, to confirm our findings, was performed a two-stage step-up method of Benjamini, Krieger and Yekutieli test as multiple comparisons test for controlling the False Discovery Rate (FDR). Subsequently, Spearman correlation analysis between the compound residues as well as between compound residues and the proportions of the dietary ingredients were performed. The correlation analysis was interpreted considering only substantial correlations with coefficients (rho[ρ]) ≥ 0.3, based on Hinkle et al. (2003). The tables were made using Microsoft Excel®. The mentioned statistical analyses and figures were performed and elaborated using GraphPad Prism® version 9.1 (GraphPad Software, San Diego, California, USA).

3. Results and discussion

3.1. Diet composition (main ingredients)

The dairy farms in this investigation fed mixed rations (consisting mostly of forages but also mineral supplementation and concentrate feed) with an additional amount of concentrate feed (given to the animals via automatic feeders). The most common dietary components incorporated in the diets were: concentrate feed (with a frequency of inclusion of 100%), grass silage (97%), maize silage (84%), straw (58%), brewery's spent grains (26%), hay (19%) and other silages (including wheat, oats, barley, sunflower and beep pulp) (12%) (Fig. 2). Regarding the proportions in the diet, the most relevant dietary ingredient incorporated in the analysed dietary rations was grass silage, which represented on average 40.6% (SD ± 15%) of the complete ration, fluctuating from 10.4% to 86.8%. The inclusion rate of concentrate feeds was, on average, 35.3% (SD: ± 9.6), varying from 11% to 67.6%. On average, maize silage accounted for 26.7% (SD ± 10.6%) of the total diet, ranging from 1.7% to 59%. On average, the other mixed rations' ingredients corresponded to ≤5% of the diet. Such as other silages (average: 5%; SD: ± 4.8%; range: 0.5%–15.8%), hay (4.3%; ±5.9%; 0.6%–28.5%), brewery's spent grain (3.6%; ±1.8%; 0.3%–8.1%) and straw (2.7%; ±1.9%; 0.2%–10.1%). The mean proportion of forage in the ration (understood as the sum of silages, straw and hay) was 64.7% (SD: ± 9.6; range: 32.4%–89%) (Fig. 2). The respective rates and proportions of the conventional and organic farms are in Table 1. Since the unbalanced sample size of conventional and organic farms (due to the complexity and difficulty of recruiting organic farms for this study), no statistical comparison was performed. However, as a general trend, it can be observed that the diets of organic farms did not include brewery's spent grain and other silages in their formulations. Additionally, the diets of organic farms presented a higher inclusion rate of hay (33%) compared to conventional farms (17%). Regarding silage inclusion, the organic farms

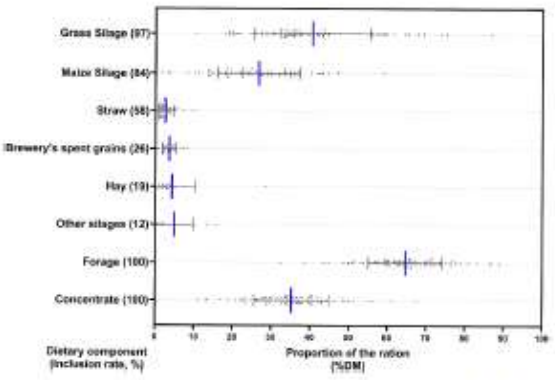

Fig. 2. Frequency and proportion of inclusion the main components of dietary rations of Austrian dairy cattle.

Table 1
Frequencies and proportion of inclusion of the main components incorporated in complete dietary rations of Austrian dairy cattle under conventional and organic farming systems.

Dietary ingredient	Conventional farms (n = 93)						Organic farms (n = 9)					
	Inclusion	Proportion in the diet (% DM)					Inclusion	Proportion in the diet (% DM)				
	(%)	Average ± SD			Range		(%)	Average ± SD			Range	
Maize Silage	91	26.8	±	10.6	1.7	– 59.0	11.1	18.5	±	0.0	18.5	– 18.5
Grass Silage	97	38.4	±	13.4	10.4	– 73.7	100	62.3	±	12.4	43.7	– 86.8
Straw	58	2.6	±	2.0	0.2	– 10.1	56	3.5	±	1.4	1.6	– 5.0
Hay	17	4.5	±	6.3	0.9	– 28.5	33	3.3	±	3.0	0.6	– 7.5
Brewery's grains silage	29	3.6	±	1.8	0.3	– 8.1	0		0			0
Other silages	13	5.0	±	4.8	0.5	– 15.8	0		0			0
Forage	100	64.5	±	9.3	32.4	– 89.0	100	67.4	±	11.5	51.3	– 86.8
Concentrate	100	35.5	±	9.3	11.0	– 67.6	100	32.6	±	11.5	13.2	– 48.7

incorporated, on average, a higher proportion of grass silage (62.3%) and lower maize silage (18.5%) compared with the conventional diets (38.4% and 26.8%, respectively). As a general trend, the forage-to-concentrate ratio in both groups' was very similar; however, the organic farm contained slightly more forage (Table 1). For future studies, it would be required to have a balanced (higher sample size) of organic farms to get a more representative and accurate view of this farming system, which, as mentioned previously, should be promoted and established in at least 25% of the agricultural land of the European Union by 2030 (EC, 2020a, 2020b.; Silva et al., 2022). In 2020, 25.5% (6,631 of 25,872) of the Austrian dairy farms corresponded to organic farms, and 19.2% (649,368 of 3,384,412 t) of the milk produced was organic (BMLRT, 2021).

Based on our results and according to the FAO's report "world mapping of animal feeding systems in the dairy sector" (2014), the kind of diets analysed during this study are classified in the feeding system of "year-round silage", which is the most relevant in the country. This feeding system has been implemented in around 40% of the Austrian dairy farms, the equivalent to 50% of the national milk production, at the time of the report (FAO, IDF, IFCN, 2014). The other 50% of the production for 2014 was "green fodder + silage" (35%) and "haymilk" (15%)(FAO, IDF, IFCN, 2014). The forage proportion of this kind of feeding system (year-round silage) was in 2014 of 78%, and 22% of concentrate feeds (specifically, cereal grains (15%), by-products (6%) and compound feed (1%)). To the best of our knowledge, no current or more recent data on the proportion of the feeding system of the Austrian dairy sector are available. According to the cited report, grass silage (average: 53%), concentrated feeds (22%) (cereal grains (15%), by-products (6%) and compound feeds (1%)), maize silage (19%) and hay (6%) were the main dietary components (FAO, IDF, IFCN, 2014). Although the proportion of the main ingredients differs from the mentioned report, the order and relevance of the main dietary components are similar. The FAO's report also evidenced that the feeding system of the here targeted farms was (during the last decade) and surely is the most relevant in Austria in terms of the amount of produced milk and quantity of producing units (farms) (FAO, IDF, IFCN, 2014).

3.2. Information regarding the use of pesticides and veterinary drugs in Austrian dairy farms

Among the conventional farms, 62% of the interviewed farmers reported the application of pesticides. Around 33% of the farmers that confirmed the use of pesticides (equivalent to 19% of all the conventional farms) did not provide additional specific information (such as applied products or active substances). In total, 32 commercial pesticide products were indicated across the farms, consisting of 16 fungicides, 15 herbicides and one insecticide. According to the provided data, on average, two commercial pesticide products for feed crops per farm were applied, varying from one to ten (specific data not shown. As expected, the organic farmers stated that no pesticides were used in their crops. None of the farmers reported the incorporation of veterinary drugs in the rations.

Regarding the reported applied active substances, 12 were non-persistent, six were persistent, four were persistent and three were very persistent (PPDB, 2022). Of the reported active substances, 11 were fungicides, 13 were herbicides and one was an insecticide (Table S4). According to the interviews, the pesticides were applied on cereals (maize for silage, wheat, rye and triticale for concentrate feed). Three of the compounds described as applied (specifically, chlortoluron, esfenvalerate and S-metolachlor) were not targeted by implemented multi-pesticide analytic method (Table S2 and Table S4).

3.3. Occurrence and concentration of pesticides and veterinary drug residues in diets of Austrian dairy cattle

In total, residues of 15 active substances (13 pesticides and two veterinary drugs) were detected. Most of the samples (90%) presented some kind of residue. 89% of dietary rations contained pesticide residues and 8% of veterinary drugs. Among the pesticide residues were identified nine fungicides (benzovindiflupyr, bixafen, fluopyram, fluxapyroxad, ipconazole, metrafenone, pyraclostrobin, tebuconazole and trifloxystrobin), three insecticides (piperonyl-butoxide, pirimiphos-methyl, diethyltoluamide) and one herbicide (metolachlor). Two veterinary drug residues were detected: monensin and nicarbazin. The marker of nicarbazin, dinitrocarbanilide, was also detected. Dinitrocarbanilide [N,N'-bis(4-nitrophenyl)urea)] and 4,6-dimethyl-2(1H)-pyrimidinone in a ratio 1:1 conform to the molecular complex nicarbazin (an antiprotozoal compound used as a feed additive) (Tarbin et al., 2005). The pesticide residues detected in the highest occurrences were fungicides fluopyram (62%), the insecticide synergist piperonyl-butoxide (39%) and the repellent diethyltoluamide (35%). Residues of the other detected pesticides showed occurrences below 20%. Residues of veterinary substances (monensin, nicarbazin and dinitrocarbanilide) showed occurrences lower than 5%. The directive 2009/8/EC states that monensin and nicarbazin are authorized for use as feed additives by the regulation (EC) No 1831/2003 (EC, 2003, 2009; Anadón et al., 2018).

The pesticide residues with the highest average concentration were piperonyl-butoxide (27.1 $\mu g\ kg^{-1}$), diethyltoluamide (24.2 $\mu g\ kg^{-1}$) and fluopyram (7.07 $\mu g\ kg^{-1}$). Diethyltoluamide showed the maximum concentration detected among pesticides (1475 $\mu g\ kg^{-1}$). The average concentrations of the veterinary drug residues were less than 2.5 $\mu g\ kg^{-1}$. The highest concentration of the veterinary drug residues was 142 $\mu g\ kg^{-1}$ of monensin. Concerning the maximum residue levels (MRLs), no veterinary drugs but five pesticides exceeded the EU-MRLs (EC, 2009; EU Pesticide Database, 2022). Specifically, the pesticides that exceeded the EU-MRLs definitely (taking into consideration the expanded measurement uncertainty of 50%) were: benzovindiflupyr (1% of the investigated samples), bixafen (2%), fluopyram (6%), ipconazole (1%) and tebuconazole (8%)(Table 2). In the European Union, pesticide

Table 2
Occurrences and concentrations of the pesticides and veterinary drug residues detected in complete dietary rations of lactating dairy cattle in Austria.

Analyte		Occurrence[a] (%)	> MRL[b] (%)	Concentrations (μg kg^{-1} DM)					Type[c]	Persistence[c,d]	WHO classification by hazard[e]/Enlisted as highly hazardous pesticides by PAN[f]
				Average ± SD		Range					
Pesticides	Benzovindiflupyr	10	1	1.05	±4.28	1.40	–	32.4	Fungicide	VP	II
	Bixafen	10	2	0.99	±4.21	4.65	–	29.3	Fungicide	VP	N/A
	Diethyltoluamide	35	N/A	24.2	±151	2.57	–	1475	Insecticide (repellent)	No data*	N/A
	Fluopyram	62	6	7.07	±11.2	2.30	–	78.3	Fungicide, nematicide	P	III
	Fluxapyroxad	10	0	0.46	±1.63	2.65	–	8.66	Fungicide	P	III
	Ipconazole	10	1	1.29	±4.43	2.40	–	25.5	Fungicide	MP	N/A
	Metolachlor	2	0	0.05	±0.37	2.65	–	2.65	Herbicide	MP	III
	Metrafenone	10	0	0.33	±1.43	0.90	–	12.8	Fungicide	P	U
	Piperonyl butoxide	39	N/A	27.1	±72.0	7.50	–	572	Insecticide (synergist)	NP	U
	Pirimiphos-methyl	13	0	0.73	±2.13	1.65	–	11.5	Insecticide	MP	II/+
	Pyraclostrobin	1	0	0.04	±0.38	3.85	–	3.85	Fungicide	MP	II
	Tebuconazole	12	3	3.87	±17.2	4.68	–	118	Fungicide, plant growth regulator	MP	U/+
	Trifloxystrobin	1	0	0.05	±0.46	4.62	–	4.62	Fungicide	NP	U
Veterinary drugs	Dinitrocarbanilide	4	N/A	2.3	±12.3	23.0	–	89	Marker of nicarbazin	N/A	N/A
	Monensin	4	0	1.75	±14.1	4.70	–	142	Antibiotic/ Anticoccidial	NP	N/A
	Nicarbazin	3	0	1.32	±8.35	19.8	–	69.4	Anticoccidial	P	N/A
Total pesticides		91	N/A	67.2	±164	2.30	–	1482	N/A	N/A	N/A
Total drug residues		8	N/A	5.36	±24.1	4.70	–	158	N/A	N/A	N/A
Total residues		90	N/A	72.6	±165	2.30	–	1482	N/A	N/A	N/A

[a] n = 102 representative samples of complete diets of lactating dairy cows from Austria, values considered as positive were > limit of detection (LOD); In case values > LOD and < limit of quantification (LOQ), LOQ/2 was used for the calculation.

[b] Maximal residue level of pesticides (MRL) for products or part of products exclusively used for animal feed production according to the European Union guidelines is 10 μg kg^{-1} expressed at 88% DM (11.36 μg kg^{-1} DM basis)(EU Pesticide Database, 2022). In Europe, MRL of the detected veterinary drugs are dictated by the Commission Directive 2009/8/EC of February 10, 2009 (EC, 2009). For instance the MRL of monensin and nicarbazin for compound feed for dairy are 1250 μg kg^{-1}, and 1500 μg kg^{-1}, expressed at 88% DM basis (and 1420 μg kg^{-1}, and 1705 μg kg^{-1} at DM basis).

[c] Data retrieved from Pesticide Properties DataBase (PPDB, 2022) and Veterinary Substance DataBase (VSDB, 2022) of the University of Hertfordshire.

[d] Based on the typical disappearance time 50 (DT50); VP = very persistent, P = persistent, MP = moderately persistent, NP = non-persistent; * No data found in the PPDB. According to an assessment report of the EU: "Diethyltoluamide does not meet any of the criteria for Persistent, Bioaccumulative and Toxic (PBT)" (Kern, 2010).

[e] WHO classification of pesticides by hazard. Ia (Extremely hazardous, Ib (highly hazardous), II (moderately hazardous), III (slightly hazardous) and U (unlikely to present an acute hazard) (WHO, 2019) (Organization, 2020).

[f] + = highly hazardous, according to pesticide action network international (PAN, 2021) (PAN, 2021), N/A: Not available/not apply.

residue levels, particularly in plant and animal-derived foods and feeds, have been set by Commission (EC) No 396/2005 (EC, 2022). Information concerning MRLs and toxicity is available in the EU Pesticide database (EU Pesticide Database, 2022). However, feedstuffs, compound feeds and dietary rations exclusively used for animal feed purposes have not yet established harmonized EU MRLs for pesticides. For that reason, the general default MRL value of 0.01 mg kg^{-1} (10 μg kg^{-1}) expressed at 88% DM applies (EU Pesticide Database, 2022). The distribution of the residue levels is illustrated in Fig. S1a.

Concerning the samples collected from conventional farms, 97% (90/93) contained pesticide residues and 8% (7/93) contained veterinary drug residues. On the other hand, only one of the nine dietary samples derived from organic farms (corresponding to 11%) was positive for pesticide residue, particularly for benzovindiflupyr (13.8 μg kg^{-1} DM). Likewise, other sample from an organic farm (also 11%) presented residues of dinitrocarbanilide (64.6 μg kg^{-1}) (Fig. S1a).

Regarding the environmental persistence, among the detected compound residues (15, not including the nicarbazin marker dinitrocarbanilide), two were classified as very persistent, three as persistent, four as moderately persistent and three as non-persistent (Table 2). Two of the detected pesticides are enlisted as highly hazardous by pesticide action network international, for instance pirimiphos-methyl (added since January 2009) and tebuconazole (added since March 2019) (PAN, 2021). According to the WHO classification by hazard, none of the detected compound residues were cataloged as extremely or highly hazardous. However, three of the detected pesticides (benzovindiflupyr, pyraclostrobin and pirimiphos-methyl) were considered moderately hazardous, also three (fluopyram, fluxapyroxad and metolachlor) as slightly hazardous and four as unlikely to present an acute hazard (WHO, 2019).

Four of the detected compound residues (metolachlor, piperonyl butoxide, pirimiphos-methyl and diethyltoluamide) are not approved as plant protection products on the European Union's market by the Regulation (EC) 1107/2009 (PPDB, 2022). The herbicide metolachlor is widely used in the USA and is linked to human carcinogenicity (EPA, 1995; Rusiecki et al., 2006). Metolachlor was also related to poor semen quality in men (Swan et al., 2003). Piperonyl butoxide, as an insecticide synergist, increases the potency of certain insecticides such as carbamates and pyrethrins (Basak et al., 2021). Piperonyl butoxide is not a cholinesterase inhibitor and has low toxicity; consequently, it is not only used for crop protection. Piperonyl butoxide-containing products are applied to crops both pre- and post-harvest. Facilities and storage areas where produce and livestock are processed may also be treated and can be a source of contamination (Daise and Edwards, 2006; Keane, 1999). Its broader purpose of use may explain its higher detection frequency compared to the majority of detected residues. Pirimiphos-methyl is an organophosphate fumigant insecticide that controls many insects and mites (PPDB, 2022). This moderately persistent insecticide is considered highly toxic for bees (Berjawi et al., 2020) and was added to the HHP list in 2009 (PAN, 2021).

Diethyltoluamide is an insect repellent applied to human and animal skin to protect from insects. It is moderately toxic to aquatic life (PPDB,

2022). Not enough data is available regarding its environmental fate. According to an assessment report of the EU, this compound "does not meet any of the criteria for Persistent, Bioaccumulative and Toxic (PBT)" (Kem, 2010). The other detected pesticides approved as plant protection products are fungicides (see Table 2) and are usually used for crop protection against foliar diseases of cereals, legumes and other crops (PPDB, 2022). Residues of diethyltoluamide have been reported in several food commodities (such as chanterelle, blueberry and raspberry) in several countries like Germany, the Russian Federation, Poland, Belarus, Bulgaria and the Czech Republic (Scherbaum and Mraka, 2019). It was concluded that the residual contamination with diethyltoluamide was usually the result of contact with the hands of the picker who had sprayed himself with the repellent (Scherbaum and Marka, 2019). Diethyltoluamide has also been found in Avena in Poland (Malinowska et al., 2015) and was the most abundant "pharmaceutical and personal care product (PPCP)" in leachates of the USA and Poland, showing a high risk for the environment (Yu et al., 2020). In the case of our study, we speculate that spraying this substance on the stable and the animal is probably the source of the residues in the dietary rations.

3.4. Comparison of concentrations of residues by the geographical localization (province)

Table S5 shows the occurrences and concentrations of pesticide and veterinary drugs residues in Lower Austria, Styria and Upper Austria. The major occurrence of residues was in Upper Austria (96%), followed by Styria (82%). Lower Austria (78%). Relating to the average concentration of total residues, Upper Austria presented the highest concentration (89.7 µg kg^{-1}), subsequently Lower Austria (59.8 µg kg^{-1}) and finally Styria (43.3 µg kg^{-1}); however, no significant differences were evidenced (Tables S5 and S6, Fig. S1b). At the concentration of the individual analytes, only the diethyltoluamide levels presented substantial differences between provinces (Kruskal-Wallis test, p-value = 0.017). Upper Austria showed significantly higher levels compared with the respective levels of Lower Austria and Styria (Mann-Whitney Test, p-values = 0.016 and 0.046) (Fig. S1c, Table S5). The multiple comparisons test (two-stage step-up method of Benjamini, Krieger and Yekutieli) confirmed the findings suggesting that only diethyltoluamide levels presented significant differences among provinces (Table S6).

3.5. Cocktails of residues in diets of Austrian dairy cattle

This study shows that Austria dairy cattle's complete diets usually contain mixtures of pesticides. For instance, 62% of the complete diets of lactating dairy cattle evaluated contained combinations (of two to six) of different residues. The diets contained, on average, two compounds, and no significant differences among Austrian provinces were detected (p-value = 0.4013) (Fig. 3a). Specifically, 23% of the samples contained two residues, 17% three residues, 13% four residues, 9% five residues and 1% six different residues (Fig. 3b). These findings confirm once again the idea that multiple biocides are being incorporated at low levels in the feed/food chain and subsequently in the environment, implicating negative toxicological and ecological consequences (Márquez et al., 2005; Relyea, 2009; Mishra et al., 2014; Panico et al., 2022). Interactions of pesticide mixtures lead mainly to synergic effects, which differ depending on the dose and physiological target (Rizzati et al., 2016). Thus, although the detected levels of individual residues do not seem to be a risk, the effects of the detected biocide mixtures are unpredictable because such may imply multiple potential interactions amongst different pesticides. More research and data available in this exciting field are still highly required (Rizzati et al., 2016; Hernández et al., 2017). This kind of exposure to multiple pesticides could implicate adverse effects on health. It might contribute to an increased risk of long-term diseases, including cancer and neurodegenerative diseases, reproductive and developmental disturbances, and emerging threats such as developmental neurotoxicity and immunotoxic effects (Parrón et al., 2011, 2014; Gonzalez-Alzaga et al., 2014; Mokarizadeh et al., 2015; Hernández et al., 2017).

3.6. Relationships between the detected residues and main dietary ingredients

Significant Spearman's correlation coefficients (ρ) between residues as well as among the levels and the number of residues with the main

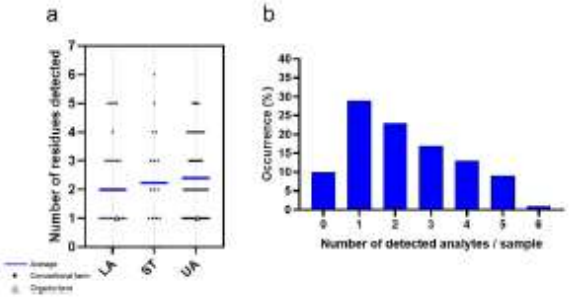

Fig. 3. Number of residues/sample of dairy cow's diet. (a) In the provinces (LA: Lower Austria; ST: Styria; UA: Upper Austria). (b) Occurrence by number of residues/sample of dairy cow's diet.

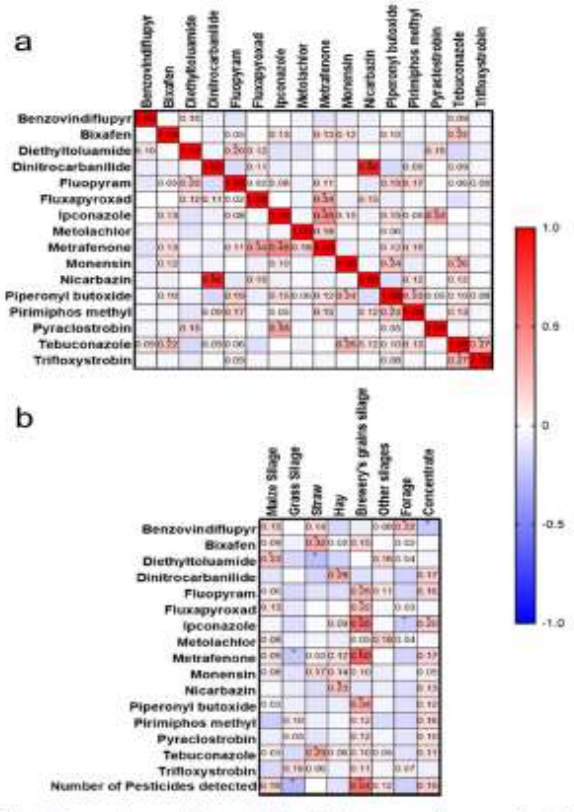

Fig. 4. Spearman's correlation coefficients (ρ) (a) among residues as well as (b) among residues with the main dietary components. The asterisk (*) indicates a significant coefficient (p-value < 0.05). All Spearman's correlation coefficients (ρ) and the exact p-values are available in Tables S7 and S8, respectively.

dietary components are shown in Fig. 4a and b, respectively. All Spearman's correlation coefficients (ρ) and all the exact p-values are available in Table S7 and Table S8, respectively. Firstly, the correlation analysis among the different compound residues showed a highly significant correlation ($\rho = 0.86$; p-value < 0.001) between nicarbazin and its marker residue dinitrocarbanilide (Danaher et al., 2008). Low positive correlations were detected between the fungicides metrafenone with ipconazole ($\rho = 0.45$; p-value <0.001), metrafenone and fluxapyroxad ($\rho = 0.34$; p-value $= 0.001$) as well as pyraclostrobin and ipconazole ($\rho = 0.33$; p-value $= 0.001$) (Fig. 4a). The correlation between the other compound residues were negligible ($\rho < 0.3$). Regarding the relationship between diet composition and residue levels, moderate positive correlations were found between brewery's spent grains with the fungicides metrafenone ($\rho = 0.60$; p-value < 0.001) and ipconazole ($\rho = 0.55$; p-value < 0.001). Brewery's spent grains also showed a moderate positive correlation with the number of detected pesticide residues ($\rho = 0.55$; p-value < 0.001). The other dietary components presented negligible correlations with the residues and the number of residues detected (Fig. 4b).

The most relevant dietary component related to pesticide residue levels was the brewery's spent grains, which was previously reported by its capacity of absorption (after mashing) of pesticides, which reduced the concentration of these substances in beer production (Inoue et al., 2011; Xi et al., 2014; Wei et al., 2020). Brewery's spent grains were not included in the rations of organic farms visited. The farms ($n = 27$) that incorporated this by-product presented average levels of ipconazole (4.87 μg kg^{-1}) and metranone (1.25 μg kg^{-1}), which are 3 times higher compared with general average (1.29 μg kg^{-1} and 0.33 μg kg^{-1}). The number of detected residues per sample was also higher in farms with brewery's spent grains inclusion (four residues/sample) than the overall of the farms (two residues/sample). Several of the pesticides detected in this study such as benzovindiflupyr, bixafen, fluopyram, fluxapyroxad, metrafenone, piperonyl butoxide, pirimiphos-methyl, pyraclostrobin, tebuconazole and trifloxystrobin were also detected recently in samples of brewery's spent grains intended for feeding of dairy cows in Austria (Penagos-Tabares et al., 2022c). It is known that barley (cereal mostly used for beer production) is a crop frequently contaminated with traces of fungicides (Palladino et al., 2021). Given the incorporation of commercial concentrate feeds and other feedstuffs non-produced at the farm, the pesticides detected in this study can also be different from the ones reported by the farmers on the crop feeds (cereals, like maize, wheat, rye and others).

4. Conclusions

- To the best of our knowledge, this work is the first quantitative LC/ESI–MS/MS-based method covering a vast amount of pesticides (660) and veterinary drug residues (129) in complete dairy cattle diets. Consequently, it enabled data on the occurrences and levels of multiple residues in the diets of food-delivering animals.
- Mixtures of pesticides presented high occurrences (>60%) in the complete diets of Austrian dairy cows.
- Organic dairy farms presented lower occurrences (22%) and fewer residues (up to one per sample) than conventional dairy farms (97%, up to six per sample).
- In some cases, the complete diets of Austrian dairy cows exceed the default EU MRL (10 μg kg^{-1}) for pesticides in products or part of products exclusively used for animal feed production.
- Four detected compound residues (metolachlor, piperonyl butoxide, pirimiphos-methyl and diethyltoluamide) are not approved as plant protection products on the European Union's market.
- Veterinary drug residues in the diets of Austrian dairy cows were detected in very low frequencies (<10%) and were not detected above the EU MRLs.
- Brewery's spent grains were the most correlated ingredient to pesticide residues.
- Similar studies are required to estimate the current situation regarding pesticides and veterinary drug residues in animal feed and animal-derived products.
- Cocktails of pesticides are a realistic scenario in the diets of Austrian dairy cattle. Their potential long-term synergistic effects on animal, human and environmental health should be subject to further investigations.

Credit author statement

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Funding

The current study is part of the Project "D4Dairy – Digitalization, Data Integration, Detection and Decision support in Dairying" supported by BMK, BMWFJ, the province of Lower Austria and the city of Vienna, within the framework of COMET – Competence Centers for Excellent Technologies., which is handled by the FFG.

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

The authors do not have permission to share data.

Acknowledgments

The authors appreciate enormously the excellent technical support and cooperation provided by Dr. Manfred Hollmann, Anita Dockner, Sabine Leiner (Institute of Animal Nutrition and Functional Compounds, Vetmeduni, Vienna) for the excellent technical assistance. The authors thank the anonymous reviewers for their suggestions to improve the quality and presentation of the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.120626.

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Residues of pesticides and veterinary drugs in diets of dairy cattle from conventional and organic farms in Austria

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Table S1. Performance values of liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI–MS/MS) analysis for multiple pesticides and veterinary drugs residues detected in complete dietary rations of dairy cattle in Austria.

Table S2. List of 660 targeted pesticides and metabolites via a validated liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI–MS/MS). Chemical structure, chemical formula, CAS/IUPAC numbers, molecular weights and additional physicochemical characteristics are available by searching the compound identification (CID) number at the PubChem database (available at: https://pubchem.ncbi.nlm.nih.gov/).

Table S3. List of 129 targeted veterinary drugs and metabolites via a validated liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI–MS/MS). Chemical structure, chemical formula, CAS/IUPAC numbers, molecular weights and additional physicochemical characteristics are available by searching the compound identification (CID) number at the PubChem database (available at: https://pubchem.ncbi.nlm.nih.gov/).

Table S4. The list reported pesticides (active substances) applied in feed crops of Austrian dairy farms.

Table S5. Occurrences and comparison of levels of the pesticide and veterinary drug residues detected in complete dietary rations of lactating dairy cattle in Lower Austria, Styria, and Upper Austria via Kruskal-Wallis test.

Table S6. Multiple comparison tests of levels of the pesticide and veterinary drug residues detected in complete dietary rations of lactating dairy cattle in Lower Austria, Styria, and Upper Austria via the two-stage step-up method of Benjamini, Krieger and Yekutieli for controlling the False Discovery Rate (FDR).

Table S7. Spearman's correlation coefficients (ρ) between detected residue levels and main components of complete dietary rations of lactating dairy cattle.

Table S8. P-values of Spearman's correlation coefficients between detected residue levels and main components of complete dietary rations of lactating dairy cattle.

Figure S1. Distribution of the (a) individual and (b) accumulated concentrations of pesticides and veterinary drugs residues detected in complete dietary rations of Austrian dairy cattle. (c) Levels of diethyltoluamide in the provinces (LA: Lower Austria; ST: Styria; UA: Upper Austria). ns: No significant. The asterisk (*) indicates significant differences ($p < 0.05$) detected via the Mann-Whitney test, corroborated via the two-stage step-up method of Benjamini, Krieger and Yekutieli for controlling the False Discovery Rate (FDR) (see Table S6).

Table S1. Performance values of liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI–MS/MS) analysis for multiple pesticides and veterinary drugs residues detected in complete dietary rations of dairy cattle in Austria.

Analyte	Apparent recovery (%)	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	Type of compound / Chemical classification of the compound	Reference
Benzovindiflupyr	73	0.8	2.8	Fungicide / Aromatic amide, organochlorine, organofluorine, pyrazol, olefinic phospholipid and bridged compound	PPDB, 2022; NCBI, 2022a
Bixafen	56	2.8	9.3	Fungicide / Aromatic amide, organofluorine, pyrazol, biphenyls, dichlorobenzene and anilide	PPDB, 2022; NCBI, 2022b
Diethyltoluamide	94	0.7	2.5	Unclassified pesticide, repellent / Aromatic amide and esther	PPDB, 2022; NCBI, 2022c
Dinitrocarbanilide	48	2.5	8.2	Marker and part in ratio 1:1 of the nicarbazin (anticoccidial) structure (4,4'-Dinitrocarbanilide and 2-Hydroxy-4,6-dimethylpyrimidine)	Tarbin etal., 2005
Fluopyram	6	2.3	7.5	Fungicide/ organochlorine, pyridine, (trifluoromethyl)benzene and benzamide. Unclassified nematicide	PPDB, 2022; NCBI, 2022d
Fluxapyroxad	57	2.2	5.3	Fungicide/ Aromatic amide, a member of biphenyls, a member of pyrazoles, a trifluorobenzene and an anilide fungicide	PPDB, 2022; NCBI, 2022e
Ipconazole	43	1.5	4.8	Fungicide/ cyclopentanol, monochlorobenzene, triazole, tertiary alcohol, conazole	PPDB, 2022; NCBI, 2022f
Metolachlor	74	1.6	5.3	Herbicide / Chloroacetanilide, aromatic amide, ether, benzene and organochlorine	PPDB, 2022; NCBI, 2022g
Metrafenone	64	0.5	1.8	Fungicide/ benzophenone, aromatic ether, organobromine and aryl phenyl ketone	PPDB, 2022; NCBI, 2022h
Monensin	123	0.9	3	Antibiotic and anticoccidial/ Polyether (ionophore), monocarboxylic acid, cyclic hemiketal, spiroketal	PPDB, 2022; NCBI, 2022i
Nicarbazin	49	3.4	12	Anticoccidial/ Unclassified equimolar complex of 4,4'-Dinitrocarbanilide and 2-Hydroxy-4,6-dimethylpyrimidine.	PPDB, 2022; NCBI, 2022j
Piperonyl butoxide	100	5	15	Inscticide synergist / Cyclic aromatic and benzodioxol	PPDB, 2022; NCBI, 2022k
Pirimiphos methyl	97	1	3.3	Insecticide and acaricide/ Organophosphate and aminopyrimidine	PPDB, 2022; NCBI, 2022l
Pyraclostrobin	62	2.3	7.7	Fungicide/ Strobilurin, carbanilate, phenylpyrazole, aromatic ether, monochlorobenzene, methoxycarbanilate	PPDB, 2022; NCBI, 2022m
Tebuconazole	68	1.1	3.7	Fungicide/ Triazole, conazole, monochlorobenzene and tertiary alcohol.	PPDB, 2022; NCBI, 2022n
Trifloxystrobin	67	1.2	4	Fungicide/ Strobilurin, methoxyiminoacetate, oxime O-ether, organofluorine, methyl ester and methoxyiminoacetate, benzeneacetic acid and methyl ester	PPDB, 2022; NCBI, 2022o

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Table S2. List of 660 targeted pesticides and metabolites via a validated liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI–MS/MS). Chemical structure, chemical formula, CAS/IUPAC numbers, molecular weights and additional physicochemical characteristics are available by searching the compound identification (CID) number at the PubChem database (available at: https://pubchem.ncbi.nlm.nih.gov/).

Compound	PubChem CID	Compound	PubChem CID	Compound	PubChem CID
1,2,3,6-Tetrahydrophthalimid	12403423	Anilofos	91687	Bifenthrin	6442842
1,4-Dimethylnaphthalene	11304	Anthrachinon	6780	Binapacryl	10234
2,3,5-Trimethacarb	25550	Aspon	18609	Biphenyl	7095
2,4,5-T	1480	Asulam	18752	Bitertanol	91656
2,4,6-Trichlorphenol	6914	Atrazine	2256	Bixafen	11434448
2,4-D	1486	Atrazin-Desisopropyl	13878	Boscalid	213013
2,4-DB	1489	Avermectin B1b	6858005	Bromacil	9411
2,4-Dimethylaniline	7250	Azaconazole	43233	Bromobutide	53079
2-Naphthyloxyacetic acid	76313	Azadirachtin	5281303	Bromocyclen	15583
2-Phenylphenol	7017	Azamethiphos	71482	Bromophos	16422
3,5-Dichloraniline	7257	Azinphos-ethyl	17531	Bromophos-ethyl	20965
3-Chloroaniline	7932	Azinphos-methyl	2268	Bromopropylate	28936
4,4'-Dichlorobenzophenone	7034	Azoxystrobin	3034285	Bromoxynil	15531
4-Chlorophenoxyacetic acid	26229	Beflubutamide	6451159	Bromoxynil Methyl Ether	2743079
8,9-Z-Abamectin B1a	6443270	Benalaxyl	51369	Bromuconazole	3444
Acephate	1982	Benalaxyl-M	176648	BTS 27271 (Amitraz Metabolite)	36326
Acequinocyl	93315	Bendiocarb	2314	BTS 40348 (Prochloraz Metabolite)	3842173
Acetamiprid	213021	Benfluralin	2319	BTS 44596 (Prochloraz Metabolite)	57472173
Acetochlor	1988	Benfuracarb	54886	BTS 9608 (Prochloraz Metabolite)	11331
Acibenzolar-S-methyl	86412	Benodanil	27195	Bupirimate	38884
Acifluorfen	44073	Benoxacor	62306	Buprofezin	50367
Aclonifen	92389	Bentazon	2328	Butachlor	31677
Acrinathrin	6436606	Benthiavalicarb-isopropyl	53297381	Butafenacil	11826859
Alachlor	2078	Benzovindiflupyr	53491464	Butamifos	37419
Aldicarb	9570071	Benzoximat	34475	Butocarboxim	36879
Aldicarb-Sulfone	9570093	Benzoylprop-ethyl	31068	Butocarboxim-sulfoxide	9576739
Aldicarb-Sulfoxide	9568700	Benzyldimethyldodecylammonium chloride (BAC-C12)	124204256	Butoxycarboxim	9571009
Aldrin	12310947	Benzyldimethylhexadecylammonium chloride (BAC-C16)	31202	Butraline	36565
Allethrin	11442	Benzyldimethyltetradecylammonium chloride (BAC-C14)	8755	Buturon	19587
alpha-Endosulfan	12309460	Benzyldimtehyloctadecylammonium chloride (BAC-C18)	31204	Butylate	16181
Ametoctradin	15604010	Benzyldimethyldecylammonium chloride (BAC C10)	13762	Cadusafos	91752
Ametryn	13263	Benzyldimethyloctylammonium chloride (BAC C8)	13740	Captafol	17038
Amidosulfuron	91777	beta-Endosulfan	12309460	Carbaryl	6129
Aminocarb	16247	Bicyclopyrone	11188745	Carbendazim	25429
Amitraz	36324	Bifenazat	176879	Carbetamide	152031
Ancymidol	25572	Bifenox	39230	Carbofuran	2566

Table S2. (Continued)

Compound	PubChem CID	Compound	PubChem CID	Compound	PubChem CID
Carbofuran-3-Hydroxy	27975	Climbazole	37907	Demeton-S-methyl	13526
Carbophenothion	13081	Clodinafop-propargyl	92431	Demeton-S-Methylsulfone	28213
Carbosulfan	41384	Clofentezine	73670	Demeton-S-Methylsulfoxide	4618
Carboxin	21307	Clomazone	54778	Desethylatrazine	22563
Carfentrazon-ethyl	86222	Clopyralide	15553	Desmedipham	24743
Chinomethionat	17109	Cloquintocet-Mexyl	93528	Dialifos	25146
Chlorantraniliprole	11271640	Clothianidin	86287519	Diallat	5284376
Chlorbensid	7639	Coumaphos	2871	Diazinon	3017
Chlorbenzilat	10522	Crufomate	9300	Dichlobenil	3031
Chlorbromuron	25912	Cyanazine	30773	Dichlofenthion	7328
Chlorbufam	16073	Cyanofenphos	25669	Dichlofluanid	14145
Chlordan, cis	91746601	Cyanophos	17522	Dichlormid	37829
Chlordan, gamma	21732	Cyazofamid	9862076	Dichlorprop	8427
Chlordimeform hydrochloride	12336279	Cycloate	14337	Dichlorvos	3039
Chlorfenapyr	91778	Cycloxydim	135438605	Diclobutrazole	53309
Chlorfenprop-methyl	26693	Cycluron	16554	Diclofop	38687
Chlorfenson	6635	Cyflufenamid	135515530	Diclofop-methyl	39985
Chlorfenvinphos	5377791	Cyfluthrin	104926	Dicloran	7430
Chlorfluazuron	91708	Cyhalofop-butyl	180089	Dicofol	8268
Chloridazon	15546	Cymiazole	43714	Dicrotophos	5371560
Chlormephos	32739	Cymoxanil	5364079	Didecyldimethylammonium chloride	23558
Chlormequat chloride	13836	Cypermethrin	2912	Didodecyldimethylammonium bromide	18669
Chloroneb	17581	Cyproconazole	86132	Dieldrin	969491
Chloropropylate	22094	Cyprodinil	86367	Diethofencarb	91742
Chlorothalonil	15910	Cyprosulfamide	11707647	Difenoconazole	86173
Chlorotoluron	27375	Cyromazine	47866	Diflubenzuron	37123
Chloroxuron	16115	DDD, o,p-	4211	Diflufenican	91735
Chlorpropham	2728	DDD, p,p-	6294	Dimefuron	91612
Chlorpyrifos	2730	DDE, o,p-	246598	Dimepiperate	91679
Chlorpyrifos-methyl	21803	DDE, p,p-	3035	Dimethachlor	39722
Chlorthal-dimethyl	2943	DDT, o,p-	13089	Dimethenamide	91744
Chlorthiamide	2734819	DDT, p,p-	20570	Dimethipin	41385
Chlorthiophos	30859	DEET (Diethyltoluamide)	4284	Dimethoate	3082
Chlozolinate	51574	Deltamethrin	40585	Dimethomorph	5889665
Cinidon-ethyl	5851439	Demeton-O	9273	Dimethyldioctylammonium bromide	76408
Cinosulfuron	92420	Demeton-S	24723	Dimoxystrobin	10936292

Table S2. (Continued)

Compound	PubChem CID	Compound	PubChem CID	Compound	PubChem CID
Diniconazole	6436605	Ethiprole	9930667	Fenthion	3346
Dinoseb	6950	Ethirimol	135424354	Fenthion-Oxon	23046
Dinotefuran	100958102	Ethofumesate	33360	Fenthion-Oxon-sulfone	26449
Dinoterb	14994	Ethofumesat-2 keto	590774	Fenthion-Oxonsulfoxid	23047
Dioxabenzofos	19657	Ethoprophos	3289	Fenthion-sulfone	19578
Dioxacarb	23421	Etofenprox	71245	Fenthion-sulfoxide	19577
Dioxathion	6531	Etoxazole	153974	Fentinacetate	16682804
Diphenamide	13728	Etridiazole	17432	Fenuron	7560
Diphenylamin	11487	Etrimfos	37995	Fenvalerate	3347
Disulfoton	3118	Famoxadon	213032	Fipronil	3352
Disulfoton-Sulfone	17241	Famphur	5859	Fipronil-sulfide	9953940
Disulfuton-Sulfoxide	17242	Fenamidon	10403199	Fipronil-Sulfone	3078139
Ditalimfos	21207	Fenamiphos	31070	Flamprop-methyl	40521
Dithianon	18771	Fenamiphos-sulfone	36028	Flazasulfuron	93539
Dithiopyr	91757	Fenamiphos-sulfoxide	36027	Flonicamid	9834513
Diuron	3120	Fenarimol	43226	Florasulam	11700495
DMF	6228	Fenazaquin	86356	Fluacrypyrim	9954185
DMSA	2724354	Fenbutatin oxide	16683004	Fluazifop	91701
DMST	738302	Fenchlorphos	9298	Fluazifop-P-butyl	3033674
Dodemorph	61899	Fenchlorphos-oxon	77602	Fluazinam	91731
Dodine	17110	Fenclorim	77338	Fluazuron	65651
Edifenphos	28292	Fenitrothion	31200	Flubendiamide	11193251
Emamectin-benzoate	133634285	Fenobucarb	19588	Fluchloralin	36392
Endosulfansulfate	13940	Fenoprop	7158	Flucythrinate	50980
Endrin	12358480	Fenothiocarb	44178	Fludioxonil	86398
EPN	16421	Fenoxaprop-ethyl	47938	Fluensulfone	11534927
Epoxiconazole	3317081	Fenoxaprop-P	11949285	Flufenacet	86429
EPTC	12968	Fenoxycarb	51605	Flufenoxuron	91766
Esprocarb	91740	Fenpiclonil	91724	Flufenzine	153985
Ethalfluralin	41381	Fenpropathrin	47326	Flumetralin	62210
Ethametsulfuron-methyl	91756	Fenpropidin	91694	Flumetsulam	91759
Ethidimuron	91596	Fenpropimorph	93365	Flumioxazin	92425
Ethiofencarb	34766	Fenpyrazamine	11493665	Fluometuron	16562
Ethiofencarb-sulfone	119490	Fenpyroximat	9576412	Fluopicolid	139594118
Ethiofencarb-Sulfoxide	3035207	Fenson	6636	Fluopyram	11158353
Ethion	3286	Fensulfothion	8292	Fluotrimazole	91600

Table S2. (Continued)

Compound	PubChem CID	Compound	PubChem CID	Compound	PubChem CID
Fluoxastrobin	11048796	Hexazinon	39965	Lactofen	62276
Flupyradifurone	16752772	Hexythiazox	13218777	lambda-Cyhalothrin	6440557
Fluquinconazole	86417	Hymexazole	24781	Lenacil	16559
Fluridone	43079	Imazalil	37175	Leptophos	30709
Flurochloridone	91677	Imazamox	86137	Lindane	727
Fluroxypyr	50465	Imazapyr	54738	Linuron	9502
Flurprimidol	73668	Imazaquin	54739	Lufenuron	71777
Flurtamone	91755	Imazethapyr	54740	Malaoxon	15415
Flusilazole	73675	Imazosulfuron	92433	Malathion	4004
Fluthiacet-methyl	93542	Imibenconazole	93483	Mandipropamid	11292824
Flutolanil	47898	Imidacloprid	86287518	MCPA	7204
Flutriafol	91727	Indoxacarb	107720	MCPB	7207
Fluxapyroxad	16095400	Iodosulfuron-methyl-sodium	16760189	Mecarbam	17434
Fomesafen	51556	Ioxynil	15530	Mecoprop	7153
Fonofos	13676	Ipconazole	86211	Mecoprop-P	185588
Foramsulfuron	11419598	Iprobenfos	33294	Mefenpyr-diethyl	10937610
Forchlorfenuron	93379	Iprodione	37517	Mepanipyrim	86296
Formetanate Hydrochloride	31899	Iprovalicarb	10958189	Mepiquat Chlorid	62781
Fosthiazate	91758	Isazofos	39223	Mepronil	41632
Fuberidazole	19756	Isocarbophos	90479	Meptyldinocap	5284389
Furalaxyl	42504	Isodrin	12310946	Mesotrione	175967
Furathiocarb	47759	Isofenphos	32872	Metaflumizone	11614934
Halosulfuron-methyl	91763	Isofenphos-methyl	127394	Metalaxyl	42586
Haloxyfop	50895	Isofenphos-oxon	35736	Metalaxyl-M	11150163
Haloxyfop-2-ethoxyethyl	91743	Isofetamid	71657865	Metamitron	38854
Haloxyfop-P	448979	Isoprocarb	17517	Metazachlor	49384
Haloxyfop-P-methyl	13363033	Isopropalin	36606	Metconazole	86210
HCH, alpha-	90476870	Isoprothiolane	39681	Methabenzthiazuron	29216
Heptachlor	3589	Isoproturon	36679	Methacrifos	3034435
Heptachlor endo-epoxid	71317194	Isopyrazam	25271089	Methamidophos	4096
Heptachlor exo-epoxide	13930	Isoxaben	73672	Methidathion	13709
Heptenophos	62773	Isoxadifen-ethyl	6451155	Methiocarb	16248
Hexachlor-1,3-butadien	6901	Isoxaflutole	84098	Methiocarb-Sulfone	16589
Hexachlorbenzol	8370	Jodfenphos	28935	Methiocarb-Sulfoxide	17521
Hexaconazole	66461	Karbutilate	312440	Methomyl	5353758
Hexaflumuron	91741	Kresoxim-methyl	6112114	Methoprotryne	13290

Table S2. (Continued)

Compound	PubChem CID	Compound	PubChem CID	Compound	PubChem CID
Methoxychlor	4115	Oxamyl	9595287	Pinoxaden	210326
Methoxyfenozide	105010	Oxycarboxin	21330	Piperonyl butoxide	5794
Metobromuron	18290	Oxyfluorfen	39327	Piperophos	32230
Metolachlor	4169	p,p′-Methoxychlor-olefin	75048	Pirimicarb	31645
Metolcarb	14322	Paclobutrazole	616765	Pirimicarb desmethyl	93139
Metosulam	86422	Paraoxon ethyl	9395	Pirimiphos ethyl	31957
Metoxuron	29863	Paraoxon methyl	13708	Pirimiphos methyl	34526
Metrafenone	6451057	Parathion	991	Pretilachlor	91644
Metribuzin	30479	Parathionmethyl	4130	Primisulfuron-methyl	101525
Metsulfuron-methyl	52999	Penconazole	91693	Prochloraz	73665
Mevinphos	5355863	Pencycuron	91692	Prochloraz desimidazole-amino (BTS44595)	1475957
Mirex	16945	Pendimethalin	38479	Procymidon	36242
Molinate	16653	Penflufen	11674113	Profenofos	38779
Momflurothrin	25193220	Pentachloranilin	10693	Profluralin	33500
Monocrotophos	5371562	Pentachloranisol	15767	Profoxydim Lithium salt	23592723
Monolinuron	15629	Pentachlorphenol	992	Promecarb	17516
Monuron	8800	Pentanochlor	16826	Prometon	4928
Myclobutanil	6336	Penthiopyrad	11388558	Prometryn	4929
Napropamide	27189	Permethrin	40326	Propachlor	4931
Neburon	11145	Perthan	6295	Propamocarb	32490
Nicosulfuron	73281	Pethoxamide	6450826	Propanil	4933
Nitenpyram	3034287	Phenmedipham	24744	Propaquizafop	16213016
Nitrapyrin	16004	Phenothrin	4767	Propargite	4936
Nitrofen	15787	Phenthoat	17435	Propazine	4937
Nitrothal-isopropyl	43704	Phorate	4790	Propetamphos	5372405
Novaluron	93541	Phorat-sulfone	17425	Propham	24685
Noviflumuron	9828359	Phorat-sulfoxide	17424	Propiconazole	43234
Nuarimol	91683	Phosalon	4793	Propoxur	4944
Octachlorodipropyl-ether	518659	Phosmet	12901	Propoxycarbazone-Natrium	12056759
Ofurace	42850	Phosmet-oxon	77323	Propyzamide	32154
Omethoat	14210	Phosphamidon	3032604	Proquinazid	11057771
Orbencarb	36867	Phoxim	9570290	Prosulfocarb	62020
Oryzalin	29393	Phthalimide	6809	Prothioconazole-desthio	119361
Oxadiargyl	94498	Picaridin	125098	Prothiofos	36870
Oxadiazon	29732	Picolinafen	3294375	Pymetrozin	9576037
Oxadixyl	53735	Picoxystrobin	11285653	Pyraclostrobin	6422843

Table S2. (Continued)

Compound	PubChem CID	Compound	PubChem CID	Compound	PubChem CID
Pyraflufen-ethyl	182951	Spirotetramat enol-glucoside	139600668	TFNG	46835486
Pyrazophos	26033	Spirotetramat-enol	54708610	Thenylchlor	443036
Pyrethrine	5281045	Spirotetramat-keto-hydroxy	71312325	Thiabendazole	5430
Pyributicarb	93486	Spirotetramat-mono-hydroxy	9879500	Thiacloprid	115224
Pyridaben	91754	Spiroxamine	86160	Thiamethoxam	5821911
Pyridafol	92316	Sulcotrion	91760	Thifensulfuron-methyl	73674
Pyridalyl	11488729	Sulfentrazone	91760	Thiocyclam Hydrogenoxalat	35969
Pyridaphenthion	8381	Sulfotep	19395	Thiodicarb	9601227
Pyridate	41463	Sulfoxaflor	16723172	Thiofanox	38235
Pyrifenox	55790	Sulprofos	37125	Thiofanox-Sulfone	38230
Pyrimethanil	91650	Tau-Fluvalinat	91768	Thiofanox-Sulfoxide	38229
Pyriofenone	23082663	Tebuconazole	86102	Thiometon	12541
Pyriproxyfen	91753	Tebufenozide	91773	Thionazin	9272
Pyroquilon	91665	Tebufenpyrad	86354	Thiophanate-methyl	3032791
Pyroxasulfone	11556910	Tebutam	92299	Tolclofos-methyl	91664
Pyroxsulam	11571555	Tecnazene	8330	Tolfenpyrad	10110536
Quinalphos	26124	Teflubenzuron	91734	Tolylfluanide	12898
Quinclorac	91739	Tefluthrin	11534837	Topramezone	11302979
Quinmerac	91749	Tembotrione	11556911	Tralkoxydime	135492483
Quinoxyfen	3391107	TEPP	7873	Transfluthrin	656612
Quintozene	6720	Tepraloxydim	135585373	Triadimefone	39385
Quizalofop	178795	Terbacil	22188	Triadimenol	41368
Quizalofop-ethyl	53518	Terbucarb	15967	Triallate	5543
Rimsulfuron	91779	Terbufos	25670	Triasulfuron	73282
Rotenone	6758	Terbufos-Sulfone	41718	Triazophos	32184
Saflufenacil	11571392	Terbufos-Sulfoxide	25355	Tribenuron-methyl	153909
Sebuthylazine	23712	Terbumeton	36584	Trichlorfon	5853
Secbumeton	33443	Terbuthylazine	22206	Trichloronat	9477
Sedaxane	11688533	Terbuthylazine-desethyl	108201	Triclopyr	41428
Silafluofen	92430	Terbutryn	13450	Tricyclazole	39040
Simazine	5216	Tetrachlorvinphos	5284462	Trifloxystrobin	11664966
Spinetoram	53297414	Tetraconazole	80277	Triflumizole	91699
Spinosad	56841558	Tetradifon	8305	Triflumizole Metabolite FM-1-1	15708953
Spirodiclofen	177863	Tetramethrin	83975	Triflumizole Metabolite FM-6-1	13783710
Spiromesifen	9907412	Tetrasul	16685	Triflumuron	47445
Spirotetramat	9969573	TFNA	2777549	Trifluralin	5569

Table S2. (Continued)

Compound	PubChem CID	Compound	PubChem CID	Compound	PubChem CID
Triflusulfuron-methyl	92434	Tritosulfuron	11657899	Vamidothion-Sulfone	21123472
Triforine	33565	Uniconazole	6436604	Vamidothion-Sulfoxide	16212160
Trinexapac-ethyl	92421	Valifenalate	11338509	Vinclozolin	39676
Triticonazole	6537961	Vamidothion	560193	Zoxamide	122087

Table S3. List of 129 targeted veterinary drugs and metabolites via a validated liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI–MS/MS). Chemical structure, chemical formula, CAS/IUPAC numbers, molecular weights and additional physicochemical characteristics are available by searching the compound identification (CID) number at the PubChem database (available at https://pubchem.ncbi.nlm.nih.gov/).

Compound	PubChem CID	Compound	PubChem CID	Compound	PubChem CID
Albendazole	2082	Eprinomectin	6450531	Nigericin	34230
Albendazole 2 aminosulfone	88125	Erythromycin A	12560	Nitroxynil	15532
Albendazole sulfone	53174	Ethopabate	6034	Norfloxacin	4539
Albendazole sulfoxide	83969	Fenbendazole	3334	Ornidazole	28061
Amoxicillin	33613	Fenbendazole sulfone	162136	Oxacillin	6196
Ampicillin	6249	Firocoxib	208910	Oxibendazole	4622
Bacitracin A	10909430	Florfenicol	114811	Oxolinic acid	4628
Cambendazole	33309	Flubendazole	114811	Oxolinsäure	5483939
Carnidazole	3032998	Flumequine	3374	Oxyclozanide	16779
Carprofen	2581	Flumethasone	16490	Oxytetracycline	54675779
Cefalexin	27447	Flunixin	38081	Penicillin G	5904
Cefalonium	21743	Halofuginone	62894	Penicillin V	6869
Cefapirin	30699	Ibuprofen	3672	Phenylbutazone	4781
Cefazolin	33255	Irgasan	5564	Phthalylsulfathiazole	4806
Cefoperazone	44187	Josamycin	5282165	Praziquantel	4891
Cefquinome	5464355	Ketoprofen	3825	Prednisolone	5755
Ceftiofur	6328657	Levamizol	26879	Rafoxanide	31475
Chlortetracyclin	54675777	Lincomycin	3000540	Robenidine hydrochloride	16212175
Ciprofloxacin	2764	Maduramicin	68595	Robenidine	9570438
Clazuril	58901	Marbofloxacin	60651	Rofecoxib	5090
Clopidol	18087	Mebendazole	4030	Ronidazole	5094
Clorsulon	43231	Mebendazole amine	40320	Salinomycin	3085092
Closantel	42574	Mefenamic Acid	4044	Sarafloxacin	56208
Cloxacillin	6098	Meloxicam	54677470	Spiramycin	6419898
Danofloxacin	71335	Methylprednisolone	6741	Sulfacetamide	5320
Decoquinate	29112	Metronidazole	4173	Sulfachloropyridazine	6634
Dexamethasone	5743	Monensin	441145	Sulfaclozine	66890
Diclazuril	456389	Morantel	5353792	Sulfadiazine	5215
Diclofenac	3033	Moxidectin	9832912	Sulfadimethoxin	5323
Dicloxacillin	18381	Nafcillin	8982	Sulfadimidine	5327
Difloxacin	56206	Nalidixin acid	4421	Sulfadoxine	17134
Dimetridazole	3090	Naproxen	156391	Sulfaguanidine	5324
Dinitrocarbanilide	9509	Narasin	65452	Sulfamerazine	5325
Doramectin	9832750	Nequinate	26383	Sulfameter	5326
Doxycyclin	54671203	Nicarbarzin	9507	Sulfamethazine	5327
Enrofloxacin	71188	Niclosamide	4477	Sulfamethizole	5328

Table S3. (Continued)

Compound	PubChem CID	Compound	PubChem CID	Compound	PubChem CID
Sulfamethoxazole	5329	Sulfisoxazole	5344	Triclabendazole	50248
Sulfamethoxypyridazine	5330	Tetracycline	54675776	Triclabendazole sulfone	10340439
Sulfamonomethoxine	5332	Thiamphenicol	27200	Triclabendazole sulfoxide	127657
Sulfamoxole	12894	Tiamulin	656958	Trimethoprim	5578
Sulfaphenazole	5335	Tilmicosin	5282521	Tulathromycin	9832301
Sulfapyridine	5336	Tolfenamic acid	610479	Tylosin	5280440
Sulfaquinoxaline	5338	Toltrazuril	68591	Valnemulin	9850878

Table S4. The list reported pesticides (active substances) applied in feed crops of Austrian dairy farms.

Active substances	Reported in (%)	Tested	Detected	Type	Persistence[a]
Amidosulfuron	1	Tested	No detected	Herbicide	NP
Azoxystrobin	1	Tested	No detected	Fungicide	MP
Benzovindiflupyr	5	Tested	Detected	Fungicide	VP
Bixafen	7	Tested	Detected	Fungicide	VP
Boscalid	1	Tested	No detected	Fungicide	VP
Chlorthalonil	5	Tested	No detected	Fungicide	NP
Chlortoluron	1	No tested	No detected	Herbicide	MP
Epoxiconazole	4	Tested	No detected	Fungicide	P
Esfenvalerate	2	No tested	No detected	Insecticide	MP
Florasulam	1	Tested	No detected	Herbicide	NP
Flufenacet	5	Tested	No detected	Herbicide	NP
Fluopyram	5	Tested	Detected	Fungicide	P
Foramsulfuron	5	Tested	No detected	Herbicide	NP
Iodosulfuron	2	Tested	No detected	Herbicide	NP
Isopyrazam	1	Tested	No detected	Fungicide	P
Metribuzin	1	Tested	No detected	Herbicide	NP
Nicosulfuron	2	Tested	No detected	Herbicide	NP
Pendimethalin	3	Tested	No detected	Herbicide	P
Pinoxaden	1	Tested	No detected	Herbicide	NP
Propiconazole	1	Tested	No detected	Fungicide	MP
Prothioconazole	15	Tested	No detected	Fungicide	NP
Pyridate	1	Tested	No detected	Herbicide	NP
S-Metolachlor	2	No tested	No detected	Herbicide	MP
Tebuconazole	15	Tested	Detected	Fungicide	MP
Tembotrione	5	Tested	No detected	Herbicide	NP

[a] data based on the typical disappearance time 50 (DT50) and retrieved from Pesticide Properties DataBase (PPDB, 2022) of the University of Hertfordshire; VP= very persistent, P= persistent, MP=moderately persistent, NP=non-persistent.

Table S5. Occurrences and comparison of levels of the pesticide and veterinary drug residues detected in complete dietary rations of lactating dairy cattle in Lower Austria, Styria, and Upper Austria via Kruskal-Wallis test.

		Lower Austria (n=32)					Styria (n=17)					Upper Austria (n=53)						
	Analyte	Occurrence (%)	> MRL (%)	Average ± SD (µg kg^{-1} DM)		Max (µg kg^{-1} DM)	Occurrence (%)	> MRL (%)	Average ± SD (µg kg^{-1}) DM		Max (µg kg^{-1} DM)	Occurrence (%)	> MRL (%)	Average ± SD (µg kg^{-1} DM)		Max (µg kg^{-1} DM)	Kruskal-Wallis test P-value	
Pesticides	Benzovindiflupyr	6	0	0.47	± 2.40	13.8	6	0	0.34	± 1.35	5.73	13	1	1.62	± 5.53	32.4	0.467	ns
	Bixafen	9	6	1.92	± 6.88	29.3	0	0	0.00	± 0	0.00	13	0	0.76	± 2.17	12.2	0.299	ns
	Diethyltoluamide	22	N/A	3.40	± 10.1	54.0	18	N/A	2.66	± 6.91	27.7	49	N/A	43.7	± 207	1475	0.017	*
	Fluopyram	53	0	3.37	± 4 57	14.0	59	0	4.94	± 5.52	15.7	68	6	9.99	± 13.9	78.3	0.057	ns
	Fluxapyroxad	13	0	0.62	± 1.85	8.47	12	0	0.65	± 2.04	8.44	8	0	0.31	± 1.31	8.66	0.717	ns
	Ipconazole	13	0	1.46	± 4.63	20.8	0	0	0.00	± 0	0.00	11	1	1.60	± 4.92	25.5	0.331	ns
	Metolachlor	3	0	0.08	± 0.46	2.65	6	0	0.16	± 0.62	2.65	0	0	-	-		0.270	ns
	Metrafenone	16	0	0.77	± 2.37	12.8	12	0	0.19	± 0.59	2.41	6	0	0.11	± 0.48	2.56	0.268	ns
	Piperonyl butoxide	28	N/A	34.8	± 112	572	53	N/A	22.9	± 28.4	93.8	42	N/A	23.8	± 45.3	187	0.215	ns
	Pirimiphos methyl	9	0	0.39	± 1.23	4.71	24	0	1.53	± 3.24	11.5	11	0	0.68	± 2.04	8.50	0.310	ns
	Pyraclostrobin	0	0	0.00	± 0	0.0	0	0	0.00	± 0	0.00	2	0	0.07	± 0.52	3.85	0.630	ns
	Tebuconazole	19	6	7.04	± 22.8	118	12	0	1.37	± 4.45	18.6	8	1	2.76	± 15.5	112.7	0.287	ns
	Trifloxystrobin	0	0	0.00	± 0	0.0	0	0	0.00	± 0	0.00	2	0	0.09	± 0.63	4.62	0.630	ns
Veterinary drugs	Dinitrocarbanilide	0	N/A	0.00	± 0	0.0	12	N/A	4.74	± 14.3	57.5	4	N/A	2.90	± 14.8	89.0	0.144	ns
	Monensin	9	0	5.42	± 24.7	141	0	0	0.00	± 0	0.00	2	0	0.09	± 0.64	4.70	0.144	ns
	Nicarbazin	0	0	0.00	± 0	0.0	12	0	3.84	± 11.4	45.5	2	0	1.31	± 9.44	69.4	0.060	ns
	Total residues	78	N/A	59.8	± 120	587	82	N/A	43.3	± 40.1	111	96	N/A	89.7	± 206	1482	0.0503	ns

[1] values considered as positive were > limit of detection (LOD); In case values > LOD and < limit of quantification (LOQ), LOQ/2 was used for the calculation; [2] maximal residue level for products or part of products exclusively used for animal feed production according to the European Union guidelines is 10 µg/kg expressed at 88% DM (11.36 µg/kg DM basis) (EU-MRL-Database, 2022; EURL; 2022); [3] WHO classification of pesticides by hazard. Ia (Extremely hazardous, Ib (highly hazardous), II (moderately hazardous), III (slightly hazardous), and U (unlikely to present acute hazard)[1]; [4] according to pesticide action network international (PAN) (2019) [2], N/A: Not available / not apply; ns: non-significant; * Significant ($p<0.05$).

Table S6. Multiple comparison tests of levels of the pesticide and veterinary drug residues detected in complete dietary rations of lactating dairy cattle in Lower Austria, Styria, and Upper Austria via the two-stage step-up method of Benjamini, Krieger and Yekutieli for controlling the False Discovery Rate (FDR).

Compound	Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli		
Benzovindiflupyr	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	0.2289	-3.652	-3.881
Discovery*	No	No	No
q-value	>0.9999	0.5698	0.5698
Individual p-value	0.9602	0.2853	0.3618
Bixafen	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	4.984	-1.629	-6.613
Discovery*	No	No	No
q-value	0.4353	0.6651	0.3779
Individual p-value	0.2764	0.6335	0.12
Diethyltoluamide	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	1.222	-13.83	-15.05
Discovery*	No	Yes	Yes
q-value	0.6104	0.0304	0.0342
Individual p-value	0.872	0.0145	0.0325
Dinitrocarbanilide	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	-5.882	-1.962	3.92
Discovery*	No	No	No
q-value	0.154	0.3973	0.2481
Individual p-value	0.0489	0.3784	0.1576
Fluopyram	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	-5.677	-15.05	-9.371
Discovery*	No	No	No
q-value	0.5346	0.0598	0.379
Individual p-value	0.5091	0.019	0.2406
Fluxapyroxad	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	0.3768	2.585	2.209
Discovery*	No	No	No
q-value	0.9812	0.9507	0.9507
Individual p-value	0.9344	0.4492	0.6036
Ipconazole	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	6.344	0.5513	-5.792
Discovery*	No	No	No
q-value	0.2732	0.9155	0.2732
Individual p-value	0.1662	0.8719	0.1735
Metolachlor	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	-1.406	1.594	3
Discovery*	No	No	No
q-value	0.5351	0.4984	0.4091
Individual p-value	0.5096	0.3164	0.1299
Metrafenon	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	2.62	5.504	2.884
Discovery*	No	No	No
q-value	0.5958	0.338	0.5958
Individual p-value	0.5674	0.1073	0.4979

Compound	Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli		
Monensin	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	4.828	3.894	-0.934
Discovery*	No	No	No
q-value	0.1669	0.1669	0.7731
Individual p-value	0.106	0.0805	0.7363
Nicarbazin	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	-5.941	-0.9811	4.96
Discovery*	No	No	No
q-value	0.0629	0.6435	0.0629
Individual p-value	0.0223	0.6128	0.0399
Piperonylbutoxid	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	-13.06	-7.299	5.758
Discovery*	No	No	No
q-value	0.2991	0.332	0.4493
Individual p-value	0.095	0.2108	0.4279
Pirimiphos-methyl	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	-8.129	-1.603	6.526
Discovery*	No	No	No
q-value	0.2709	0.7101	0.2709
Individual p-value	0.1141	0.6763	0.172
Pyraclostrobin	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	0	-0.9623	-0.9623
Discovery*	No	No	No
q-value	>0.9999	0.7784	0.7784
Individual p-value	>0.9999	0.3947	0.4942
Tebuconazol	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	3.864	5.857	1.993
Discovery*	No	No	No
q-value	0.6879	0.3592	0.6991
Individual p-value	0.4368	0.114	0.6658
Trifloxystrobin	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	0	-0.9623	-0.9623
Discovery*	No	No	No
q-value	>0.9999	0.7784	0.7784
Individual p-value	>0.9999	0.3947	0.4942
Accumulated	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	-10.71	-11.42	-0.7108
Discovery*	No	No	No
q-value	0.3514	0.2274	0.9762
Individual p-value	0.2231	0.0722	0.9297
Co-contamination	**LA x ST**	**LA x UA**	**STx UA**
Mean rank difference	-5.86	-3.035	2.825
Discovery*	No	No	No
q-value	0.754	0.754	0.754
Individual p-value	0.4926	0.6229	0.7181

LA: Lower Austria; ST: Styria; UA: Upper Austria; *"Discovery" is the equivalent to "statistically significant" used with the false discovery rate approach.

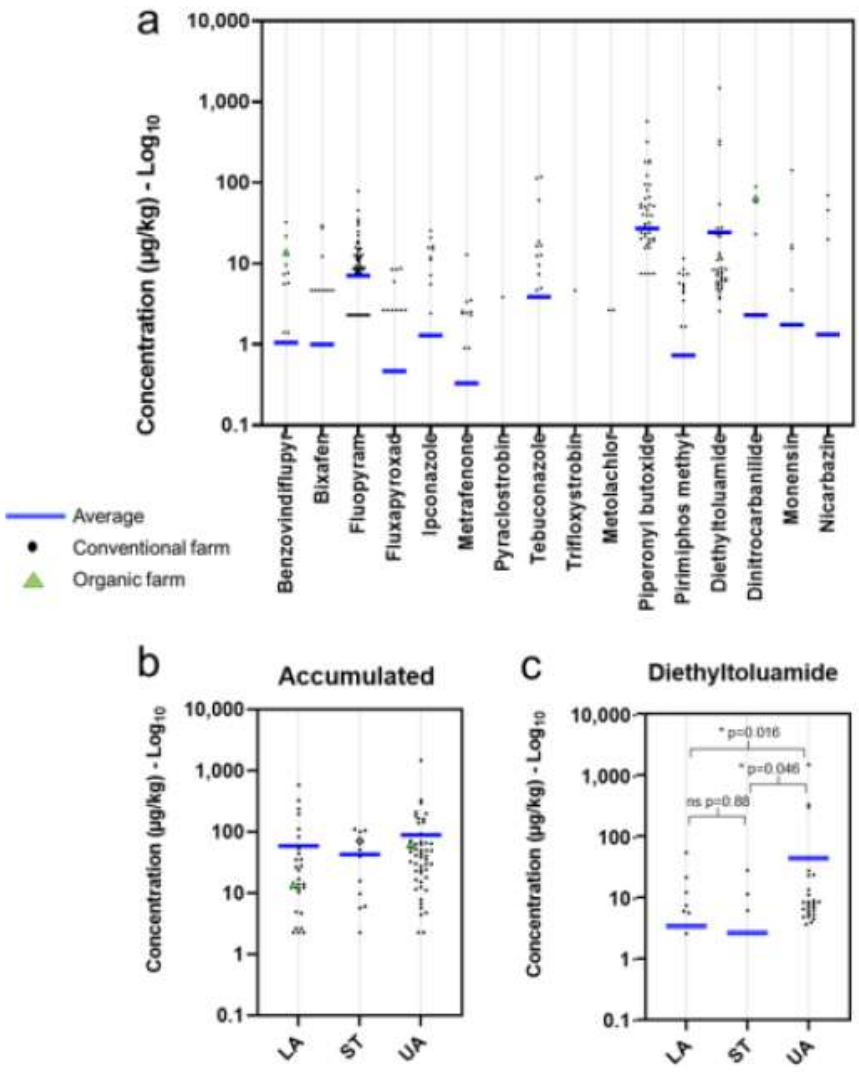
Table S7. Spearman's correlation coefficients (ρ) between detected residue levels and main components of complete dietary rations of lactating dairy cattle.

Spearman's correlation coefficient (ρ)	Benzovindiflupyr	Bixafen	Diethyltoluamid	Dinitrocarbanilide	Fluopyram	Fluxapyroxad	Ipconazole	Metolachlor	Metrafenon	Monensin	Nicarbazin	Piperonyl butoxide	Pirimiphos-methyl	Pyraclostrobin	Tebuconazole	Trifloxystrobin	N° of Pesticides detected	Maize Silage	Grass Silage	Straw	Hay	Brewer's grains silage	Other silages	Forage
Benzovindiflupyr	1.00	-0.01	0.10	-0.07	-0.09	0.00	0.00	-0.05	-0.11	-0.07	-0.06	-0.01	-0.02	-0.03	0.09	-0.03	0.18	0.15	0.00	0.14	-0.16	-0.07	0.08	0.22
Bixafen	-0.01	1.00	0.00	-0.07	0.05	-0.01	0.13	-0.05	0.13	0.12	-0.06	0.10	-0.04	-0.03	0.22	-0.03	0.33	0.09	-0.06	0.32	0.02	0.15	-0.01	0.02
Diethyltoluamide	0.10	0.00	1.00	-0.04	0.20	0.12	-0.11	-0.10	-0.02	-0.08	-0.01	-0.06	-0.10	0.15	-0.15	-0.07	0.34	0.23	-0.16	-0.25	-0.16	-0.02	0.16	0.04
Dinitrocarbanilide	-0.07	-0.07	-0.04	1.00	-0.14	0.11	-0.07	-0.03	-0.07	-0.04	0.86	-0.16	0.09	-0.02	0.09	-0.02	-0.16	-0.10	-0.01	-0.17	0.28	-0.12	-0.07	-0.17
Fluopyram	-0.09	0.05	0.20	-0.14	1.00	0.02	0.08	-0.06	0.11	0.00	-0.10	0.19	0.17	-0.01	0.06	0.09	0.55	0.06	-0.15	-0.11	-0.13	0.25	0.11	-0.16
Fluxapyroxad	0.00	-0.01	0.12	0.11	0.02	1.00	0.00	-0.05	0.34	-0.07	0.15	-0.07	-0.03	-0.03	-0.12	-0.03	0.23	0.13	-0.09	-0.01	0.00	0.22	-0.03	0.03
Ipconazole	0.00	0.13	-0.11	-0.07	0.08	0.00	1.00	-0.05	0.45	0.10	-0.06	0.15	0.05	0.33	0.00	-0.03	0.42	-0.04	-0.14	-0.02	0.09	0.55	-0.02	-0.20
Metolachlor	-0.05	-0.05	-0.10	-0.03	-0.06	-0.05	-0.05	1.00	0.18	-0.03	-0.02	0.06	-0.05	-0.01	-0.05	-0.01	0.03	0.08	-0.06	-0.04	-0.07	0.05	0.18	0.04
Metrafenon	-0.11	0.13	-0.02	-0.07	0.11	0.34	0.45	0.18	1.00	-0.07	-0.06	0.12	0.15	-0.03	0.00	-0.03	0.46	0.09	-0.22	0.02	0.12	0.60	-0.01	-0.17
Monensin	-0.07	0.12	-0.08	-0.04	0.00	-0.07	0.10	-0.03	-0.07	1.00	-0.04	0.24	-0.08	-0.02	0.26	-0.02	0.14	0.08	-0.11	0.17	0.14	0.10	-0.07	-0.05
Nicarbazin	-0.06	-0.06	-0.01	0.86	-0.10	0.15	-0.06	-0.02	-0.06	-0.04	1.00	-0.13	0.12	-0.02	0.12	-0.02	-0.10	-0.03	-0.07	-0.14	0.23	-0.10	-0.06	-0.13
Piperonyl butoxide	-0.01	0.10	-0.06	-0.16	0.19	-0.07	0.15	0.06	0.12	0.24	-0.13	1.00	0.23	0.05	0.10	0.08	0.53	0.03	-0.07	-0.11	-0.07	0.34	-0.04	-0.12
Pirimiphos-methyl	-0.02	-0.04	-0.10	0.09	0.17	-0.03	0.05	-0.05	0.15	-0.08	0.12	0.23	1.00	-0.04	0.13	-0.04	0.29	-0.19	0.10	-0.06	-0.04	0.12	-0.14	-0.16
Pyraclostrobin	-0.03	-0.03	0.15	-0.02	-0.01	-0.03	0.33	-0.01	-0.03	-0.02	-0.02	0.05	-0.04	1.00	-0.04	-0.01	0.16	-0.07	0.05	-0.10	-0.05	0.12	-0.04	-0.10
Tebuconazole	0.09	0.22	-0.15	0.09	0.06	-0.12	0.00	-0.05	0.00	0.26	0.12	0.10	0.13	-0.04	1.00	0.27	0.27	0.03	-0.15	0.29	0.06	0.10	0.05	-0.11
Trifloxystrobin	-0.03	-0.03	-0.07	-0.02	0.09	-0.03	-0.03	-0.01	-0.03	-0.02	-0.02	0.08	-0.04	-0.01	0.27	1.00	0.08	-0.15	0.15	0.06	-0.05	0.11	-0.04	0.07
N° of Pesticides detected	0.18	0.33	0.34	-0.16	0.55	0.23	0.42	0.03	0.46	0.14	-0.10	0.53	0.29	0.16	0.27	0.08	1.00	0.18	-0.28	-0.01	-0.12	0.54	0.12	-0.19
Maize Silage	0.15	0.09	0.23	-0.10	0.06	0.13	-0.04	0.08	0.09	0.08	-0.03	0.03	-0.19	-0.07	0.03	-0.15	0.18	1.00	-0.71	0.04	-0.09	0.10	0.19	0.19
Grass Silage	0.00	-0.06	-0.16	-0.01	-0.15	-0.09	-0.14	-0.06	-0.22	-0.11	-0.07	-0.07	0.10	0.05	-0.15	0.15	-0.28	-0.71	1.00	-0.08	-0.15	-0.26	-0.28	0.41
Straw	0.14	0.32	-0.25	-0.17	-0.11	-0.01	-0.02	-0.04	0.02	0.17	-0.14	-0.11	-0.06	-0.10	0.29	0.06	-0.01	0.04	-0.08	1.00	-0.17	0.07	-0.03	0.14
Hay	-0.16	0.02	-0.16	0.28	-0.13	0.00	0.09	-0.07	0.12	0.14	0.23	-0.07	-0.04	-0.05	0.06	-0.05	-0.12	-0.09	-0.15	-0.17	1.00	0.06	0.00	-0.20
Brewery's grains silage	-0.07	0.15	-0.02	-0.12	0.25	0.22	0.55	0.05	0.60	0.10	-0.10	0.34	0.12	0.12	0.10	0.11	0.54	0.10	-0.26	0.07	0.06	1.00	0.00	-0.19
Other silages	0.08	-0.01	0.16	-0.07	0.11	-0.03	-0.02	0.18	-0.01	-0.07	-0.06	-0.04	-0.14	-0.04	0.05	-0.04	0.12	0.19	-0.28	-0.03	0.00	0.00	1.00	-0.03
Forage	0.22	0.02	0.04	-0.17	-0.16	0.03	-0.20	0.04	-0.17	-0.05	-0.13	-0.12	-0.16	-0.10	-0.11	0.07	-0.19	0.19	0.41	0.14	-0.20	-0.19	-0.03	1.00
Concentrate	-0.22	-0.02	-0.04	0.17	0.16	-0.03	0.20	-0.04	0.17	0.05	0.13	0.12	0.16	0.10	0.11	-0.07	0.19	-0.19	-0.41	-0.14	0.20	0.19	0.03	-1.00

Table S8. P-values of Spearman's correlation coefficients (ρ) between detected residue levels and main components of complete dietary rations of lactating dairy cattle.

p-values	Benzovindiflupyr	Bixafen	Diethyltoluamide	Dinitrocarbanilide	Fluopyram	Fluxapyroxad	Ipconazole	Metolachlor	Metrafenon	Monensin	Nicarbazin	Piperonyl butoxide	Pirimiphos-methyl	Pyraclostrobin	Tebuconazole	Trifloxystrobin	N° of pesticides detected	Maize Silage	Grass Silage	Straw	Hay	Brewer's grains silage	Other silages	Forage	Concentrate
Benzovindiflupyr		0.925	0.303	0.507	0.346	0.974	0.981	0.642	0.279	0.507	0.567	0.904	0.810	0.744	0.349	0.744	0.071	0.121	0.961	0.164	0.117	0.505	0.425	**0.027**	**0.027**
Bixafen	0.925		0.994	0.507	0.594	0.958	0.184	0.642	0.190	0.229	0.567	0.310	0.681	0.744	**0.027**	0.744	**0.001**	0.364	0.579	**0.001**	0.859	0.130	0.889	0.856	0.856
Diethyltoluamide	0.303	0.994		0.658	**0.047**	0.212	0.269	0.311	0.805	0.440	0.917	0.577	0.327	0.126	0.131	0.477	**0.000**	**0.019**	0.113	**0.010**	0.098	0.881	0.106	0.697	0.697
Dinitrocarbanilide	0.507	0.507	0.658		0.159	0.273	0.507	0.776	0.507	0.684	**0.000**	0.118	0.394	0.841	0.392	0.841	0.100	0.295	0.912	0.086	**0.004**	0.232	0.462	0.084	0.084
Fluopyram	0.346	0.594	**0.047**	0.159		0.824	0.404	0.552	0.265	0.993	0.334	0.050	0.084	0.931	0.542	0.393	**0.000**	0.559	0.128	0.266	0.202	**0.011**	0.277	0.098	0.098
Fluxapyroxad	0.974	0.958	0.212	0.273	0.824		0.992	0.642	**0.001**	0.507	0.140	0.456	0.758	0.744	0.230	0.744	**0.020**	0.189	0.378	0.940	0.967	**0.024**	0.775	0.747	0.747
Ipconazole	0.981	0.184	0.269	0.507	0.404	0.992		0.642	**0.000**	0.335	0.567	0.127	0.642	**0.001**	0.994	0.744	**0.000**	0.712	0.174	0.827	0.343	**0.000**	0.856	**0.041**	**0.041**
Metolachlor	0.642	0.642	0.311	0.776	0.552	0.642	0.642		0.064	0.776	0.806	0.522	0.591	0.888	0.607	0.888	0.768	0.442	0.533	0.709	0.502	0.600	0.066	0.719	0.719
Metrafenon	0.279	0.190	0.805	0.507	0.265	**0.001**	**0.000**	0.064		0.507	0.567	0.223	0.137	0.744	0.976	0.744	**0.000**	0.352	**0.028**	0.839	0.243	**0.000**	0.889	0.091	0.091
Monensin	0.507	0.229	0.440	0.684	0.993	0.507	0.335	0.776	0.507		0.726	**0.016**	0.442	0.841	**0.008**	0.841	0.153	0.439	0.259	0.094	0.174	0.334	0.462	0.621	0.621
Nicarbazin	0.567	0.567	0.917	**0.000**	0.334	0.140	0.567	0.806	0.567	0.726		0.178	0.222	0.863	0.224	0.863	0.342	0.731	0.470	0.176	**0.019**	0.304	0.527	0.207	0.207
Piperonyl butoxide	0.904	0.310	0.577	0.118	0.050	0.456	0.127	0.522	0.223	**0.016**	0.178		**0.022**	0.618	0.328	0.432	**0.000**	0.797	0.496	0.288	0.477	**0.001**	0.689	0.227	0.227
Pirimiphos-methyl	0.810	0.681	0.327	0.394	0.084	0.758	0.642	0.591	0.137	0.442	0.222	**0.022**		0.705	0.179	0.705	**0.004**	0.056	0.305	0.554	0.719	0.211	0.164	0.119	0.119
Pyraclostrobin	0.744	0.744	0.126	0.841	0.931	0.744	**0.001**	0.888	0.744	0.841	0.863	0.618	0.705		0.718	0.921	0.102	0.488	0.625	0.300	0.637	0.214	0.718	0.303	0.303
Tebuconazole	0.349	**0.027**	0.131	0.392	0.542	0.230	0.994	0.607	0.976	**0.008**	0.224	0.328	0.179	0.718		**0.005**	**0.006**	0.750	0.142	**0.003**	0.532	0.305	0.625	0.250	0.250
Trifloxystrobin	0.744	0.744	0.477	0.841	0.393	0.744	0.744	0.888	0.744	0.841	0.863	0.432	0.705	0.921	**0.005**		0.416	0.144	0.131	0.539	0.637	0.267	0.718	0.510	0.510
N° of pesticides detected	0.071	**0.001**	**0.000**	0.100	**0.000**	**0.020**	**0.000**	0.768	**0.000**	0.153	0.342	**0.000**	**0.004**	0.102	**0.006**	0.416		0.073	**0.004**	0.920	0.249	**0.000**	0.231	0.060	0.060
Maize Silage	0.121	0.364	**0.019**	0.295	0.559	0.189	0.712	0.442	0.352	0.439	0.731	0.797	0.056	0.488	0.750	0.144	0.073		**0.000**	0.661	0.381	0.297	0.051	0.054	0.054
Grass Silage	0.961	0.579	0.113	0.912	0.128	0.378	0.174	0.533	**0.028**	0.259	0.470	0.496	0.305	0.625	0.142	0.131	**0.004**	**0.000**		0.418	0.142	**0.009**	**0.004**	**0.000**	**0.000**
Straw	0.164	**0.001**	**0.010**	0.086	0.266	0.940	0.827	0.709	0.839	0.094	0.176	0.288	0.554	0.300	**0.003**	0.539	0.920	0.661	0.418		0.087	0.502	0.791	0.168	0.168
Hay	0.117	0.859	0.098	**0.004**	0.202	0.967	0.343	0.502	0.243	0.174	**0.019**	0.477	0.719	0.637	0.532	0.637	0.249	0.381	0.142	0.087		0.526	0.986	**0.042**	**0.042**
Brewery's grains silage	0.505	0.130	0.881	0.232	**0.011**	**0.024**	**0.000**	0.600	**0.000**	0.334	0.304	**0.001**	0.211	0.214	0.305	0.267	**0.000**	0.297	**0.009**	0.502	0.526		0.970	0.056	0.056
Other silages	0.425	0.889	0.106	0.462	0.277	0.775	0.856	0.066	0.889	0.462	0.527	0.689	0.164	0.718	0.625	0.718	0.231	0.051	**0.004**	0.791	0.986	0.970		0.764	0.764
Forage	**0.027**	0.856	0.697	0.084	0.098	0.747	**0.041**	0.719	0.091	0.621	0.207	0.227	0.119	0.303	0.250	0.510	0.060	0.054	**0.000**	0.168	**0.042**	0.056	0.764		**0.000**
Concentrate	**0.027**	0.856	0.697	0.084	0.098	0.747	**0.041**	0.719	0.091	0.621	0.207	0.227	0.119	0.303	0.250	0.510	0.060	0.054	**0.000**	0.168	**0.042**	0.056	0.764	**0.000**	

Figure S1. Distribution of the (a) individual and (b) accumulated concentrations of pesticides and veterinary drugs residues detected in complete dietary rations of Austrian dairy cattle. (c) Levels of diethyltoluamide in the provinces (LA: Lower Austria; ST: Styria; UA: Upper Austria). ns: No significant. The asterisk (*) indicates significant differences ($p < 0.05$) detected via the Mann-Whitney test, corroborated via the two-stage step-up method of Benjamini, Krieger and Yekutieli for controlling the False Discovery Rate (FDR) (see Table S6).



4. GENERAL DISCUSSION

Mycotoxins are one of the most harmful types of contaminants in animal feeds. Their economic repercussion on livestock production involves the cost of eliminating contaminated feed and decreasing productivity (Magnoli et al., 2019). Most studies concerning mycotoxins and animal feeds have focused on monogastric animals and their primary dietary sources (cereal grains) (Figure 5). Additionally, due to technical limitations and limited knowledge, mycotoxin research was conventionally focused on the named "regulated mycotoxins" (e.g., AFB1, ZEN, DON, OTA and FUMs). With the recent technical developments in multi-metabolite analysis and rising data on other mycotoxins and fungal metabolites, the spectrum of fungal contaminants and the interest in their co-occurrences and toxicological impacts has been increasing (Battilani et al., 2020). So far, studies targeting feeds of dairy cows with wide-spectrum multi-mycotoxin analysis in Austria and Europe have been minimal. Since dairy production is a key sector of the Austrian economy, this thesis is focused on screening >700 fungal mycotoxins and metabolites as a first step for the obtention of data, contributing to future risk assessment and prevention strategies, searching for the optimization and safety of dairy production. A positive aspect evidenced by these investigations is that the most toxic and strongly regulated carcinogenic toxin AFB1 was not detected in feeds of Austrian dairy cows in the evaluated farms during 2019 and 2020. However, the cocktails of mycotoxins and other substances detected in feeds and diets of dairy cattle show the necessity to assess the effects of such mixtures on animal health, production and reproduction.

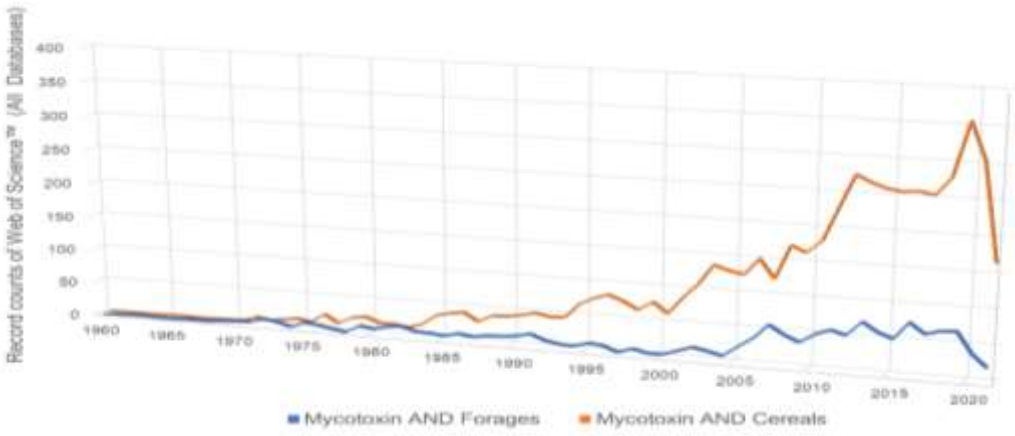


Figure 5 Number of publications found in all the databases of Web of Science™ by searching the topics "Mycotoxin AND Forage" as well as "Mycotoxin AND cereals" (Search performed in July 2021, based on Gallo et al., 2015).

4.1. Ubiquitous co-occurrences of mycotoxins in diets of dairy cows: the realistic scenario

The omnipresence of co-occurring mycotoxins was evidenced in the analysed feedstuffs (for instance: pastures, mouldy silages and BSG) (Penagos-Tabares, 2021, Penagos-Tabares et al., 2022a; Penagos-Tabares et al., 2022c;) and consequently in the complete dietary rations of Austrian dairy cows (Penagos-Tabares et al., 2022b). These findings corroborate again the statement of Schatzmayr and Streit (2013): "Mycotoxins are ubiquitously present in agricultural commodities". The data generated in the here presented publications allow an amplified diagnosis of exposome profiles of Austrian dairy cattle, thanks to the exceptional multi-mycotoxin analysis employed (Spectrum 380 ®). Some decades ago, the technical capacities were much more limited. For example, before 1985, the Food and Agriculture Organization (FAO) estimated global food crop contamination with mycotoxins to be 25%, which was a sub-estimation, as stated, given the technical capacities of that time (Eskola et al., 2020). The ubiquitous presence of toxic fungal metabolites in the diets of dairy cattle shows the necessity for more research to determine the impacts of such mycotoxin mixtures and the relevance of a constant risk assessment in dairy herds, and the consideration of these contaminants in the herd management as well as in the veterinary practice.

Pastures and conserved forages (like hay, straw, and silage) are essential for livestock systems, including dairy farming. The scientific publications generated during this doctoral study also revealed that forages (like pastures and silages) could be relevant contributors to the total dietary burden of complex mycotoxin mixtures (Penagos-Tabares et al., 2021a, 2022b; 2022c). The occurrence of forages contaminated with toxic fungal metabolites and their consequences have been vastly underestimated and remains poorly studied (Smith et al., 1994; Fink-Gremmels, 2005; Storm et al., 2008; Jouany et al., 2009; Nichea et al., 2015; Gallo et al., 2015b). Research of mycotoxins focused on fibrous feeds has usually been underestimated compared with cereal grains. However, in recent years, the investigation in this field has been increasing (Figure 5). The traditional focus on monogastric mycotoxicology and their primary dietary sources (cereal grains) is probably due to the concept that ruminants are less susceptible to the negative effects of fungal toxins (Fink-Gremmels, 2005).

4.2. *Fusarium*-derived mycotoxins and metabolites: Dominant fungal contaminants in feeds and diets of Austrian dairy cattle

Except for the mouldy silage spots (contaminated mainly by *Penicillium* spp. and related mycotoxins/metabolites), the analysed feedstuffs (pastures and BSG) and the complete diets of Austrian dairy cows showed that the accumulated *Fusarium*-derived mycotoxins/metabolites were the most relevant in terms of frequency as well as contamination levels. Such fact corroborates the status of *Fusarium* genera as one of the most widespread fungi in agriculture commodities and the principal contributor to mycotoxin contamination in animal feeds (D'mello et al., 1999; Nesic et al., 2014; Santos Pereira et al., 2019). The high co-occurrences (>70%) of fusarial regulated mycotoxins (like DON, ZEN and FUM B1) and emerging ones like ENN B; ENN B1, BEA, culmorin and aurofusarin indicate that the research emphasis, risk assessment and prevention should be focused on these recurrent toxic metabolites and their mixtures. These toxins were neglected in cattle for several years compared to other zootechnical species. Still, now it is well known that the idea that fusarial mycotoxins are only detrimental to monogastric animals is obsolete and antiquated (Gallo et al., 2022).

4.3. Geo-climatic factors influencing the pre-harvest contamination in Austrian dairy farms

Fungal colonization and growth, as well as the diversity and production of (toxic) secondary metabolites, are determined by the fungal strains as well as by environmental factors such as the temperature, water activity, relative humidity, pH, crop (substrate), agricultural practices and presence of other microorganisms (CAST, 2003; Jouany et al., 2009; Milani, 2013; Daou et al., 2021). These parameters also influence plant growth, strength, and health (CAST, 2003). If well several factors can influence the mycotoxin contamination of plants, the temperature is a primary factor influencing fungi growth and toxin production. The role of the environmental temperature during the growing seasons of pastures and maize silage was corroborated in two of the presented articles (#1 and #3) (Penagos-Tabares, 2021a, 2022b). Suppose well toxigenic fungi typically grow under optimal temperatures varying from 10 to 30 °C in substrates with pH from 4 to 8 and aw above 0.70. In that case, each fungal species has optimal growth conditions and toxin production (Jouany et al., 2009; Battilani et al., 2020). Thus, differences in fungal activity and toxigenic potential among geographical regions are apparent (Daou et al., 2021). For instance, some of the most minor water-demanding fungi as *Aspergillus* and derived

mycotoxins, such as AFs, occur mainly in countries with hot and relatively dry climates (tropics and subtropics). As previously mentioned, these carcinogenic compounds were not detected in the feeds and dietary rations of Austrian dairy cows collected during 2019 and 2020 (Penagos-Tabares, 2021a, 2022a; 2022b; 2022c). Although European reports of AFB1 are unusual, recently, it has been reported that 61% of TMR from Lithuanian dairy farms tested positive for this carcinogen, with an average of 2.42 μg/kg, ranging from 1.03 μg/kg -5.00 μg/kg (Vaičiulienė et al., 2021). The incidence of AFB1 has also been reported in TMRs from Spain (90% of the samples were positive) (Hernandez-Martinez and Navarro-Blasco, 2015), and 8.1% of Italy's TMR samples were contaminated (Decastelli et al. 2007). The findings related to the effect of the environmental temperature on mycotoxin levels reaffirm the idea that climate change plays a role in the increment of mycotoxin contamination (Dragan et al., 2019; Miraglia et al., 2009; Magan et al., 2011; Medina et al., 2015a; Medina et al., 2015b; Battilani et al., 2016; Medina et al., 2017; Van der Fels-Klerx et al., 2019; Perrone et al., 2020).

4.4. Silage-spoiling fungal organisms: Potential risk for animal and human health

Regarding the study entitled “Fungal species and mycotoxins in mouldy spots of grass and maize silages in Austria” (Penagos-Tabares et al., 2022a), this study did not employ the cutting-edge molecular techniques for fungal identification and microbiome characterization of the called “mouldy hot spots”. However, the dominant fungal species colonizing Austrian grass and maize silages were identified via the conventional fungal culture technique. *Penicillium roqueforti* was the predominant fungal species in mouldy fragments of Austrian grass and maize silage, occurring in > 70% of the hot spots. The mycotoxin cocktails in such hot spots were also described (Penagos-Tabares et al., 2022a). Such mycotoxin cocktails can implicate toxicological risks for the fed animals and farmworkers.

Among the wide range of toxins detected in mouldy spots of silage are included toxins with antibiotic activity like mycophenolic acid and roquefortine, which could affect the bacteria rumen populations and disturb the digestive process (Kopp-Holtwiesche and Rehm, 1990; Bentley, 2000; Gallo et al., 2015a). Such alterations on the rumen microbiota could affect the process, like the degradation of other (myco) toxins. Additionally, some *Penicillium* toxins' hepatotoxic activity can also affect this organ's detoxification activity (Noto et al., 1969; Bentley, 2000). Some *Penicillium* mycotoxins also have immunotoxic activity (Oh et al., 2012;

Oh et al., 2015a; Oh et al., 2015b). Neurotoxic effects have also been reported after ingesting mouldy silage (Niederberger et al., 2011). A “mouldy silage syndrome” was previously reported, described as a non-specific disorder, characterized by increasing oxidative stress parameters and cholesterol values, impairing the rumen and liver function and showing immunosuppressive effects (Santos and Fink-Gremmels, 2014). Animals affected by this syndrome presented increased oxidative stress and alterations in lipid metabolism. The “mouldy silage syndrome” was characterized by significantly decreased levels of glutathione peroxidase (GSH-Px) activity, glucose-6-phosphate-dehydrogenase (G6PD) concentrations and Trolox equivalent antioxidant capacity (TEAC) and increments in concentrations of free cholesterol in plasma, together with a decreased activity of phospholipid transfer protein (PLTP) and lecithin-cholesterol acyltransferase (LCAT) (Santos and Fink-Gremmels, 2014). Given that the specific toxic metabolites (and their concentrations) implicated in such syndrome have not been described, more research on this topic still required.

Regarding the risk for humans, the idea of acute intoxications (mycotoxicosis) in workers handling highly contaminated mouldy silage is rare but cannot be ignored and rejected. For example, there is a case report of a human patient who, shortly after exposure to mouldy silage, developed neurological symptomatology consisting of dementia and a remarkable tremor. This disorder resolved within one week. It was proposed, but not confirmed that this patient's illness resulted from exposure via inhalation to a tremorgenic mycotoxin (Gordon et al., 1993). The risks of mouldy spots are not only related to mycotoxicosis but also mycosis because among the detected species were found opportunist fungal pathogens (like *Aspergillus fumigat*us, *Mucor circinelloides*, *Rhizomucor* spp., *Lichtheimia* spp. and *Pseudallescheria boydii*) (Eucker et al., 2001; Alonso et al., 2013; de Hoog et al., 2020;). Another recognized clinical entity called Farmer’s lung disease, a form of hypersensitivity pneumonitis, is probably underdiagnosed and has high mortality rates. Its clinical presentation follows the inhalation of dust from mouldy feeds such as hay, silage or grain and is characterized by highly variable respiratory symptomatology (i.e., acute, sub-acute and chronic) (Cano-Jiménez et al., 2016; Malmberg et al., 1993; Rask-Andersen, 1989; Wuhrmann et al., 1965). Cases of pulmonary mycotoxicosis (also termed organic dust toxic syndrome or silo unloader's syndrome), an occupational disease of farmers who inhale enormous quantities of mycotoxins and other chemicals from contaminated silage, has been reported (Emanuel et al., 1975). The most essential preventive

measures consist of improving the storage conditions and utilising respiratory protective equipment in the presence of mouldy spots of feeds to avoid antigens inhalation (Cano-Jiménez et al., 2016).

The growth of the toxigenic moulds during ensiling, storage and feed-out is an inevitable process. Still, inadequate silage management (primarily oxygen availability) accelerates and exacerbates spoilage considerably (Driehuis et al., 2018). The findings of these studies show the necessity to avoid as much as possible the formation and the ingestion of mouldy spots and the associated high mycotoxin burdens of silage through several strategies for good silo management throughout the entire ensiling process from filling to unloading. The prevention strategies include good practices in the field and at harvest, compaction, sealing and unloading, and silage additives improving aerobic stability, antagonistic bacteria (*Lactobacillus*) and yeasts (Wambacq et al., 2016). Remediation strategies could also include manual remotion and cleaning (usually costly due to high bulk or volume), absorbents, microbial degradation, detoxification by exogenous microorganisms, and natural or recombinant enzymes (Alonso et al., 2013; Wambacq et al., 2016; Ogunade et al., 2018).

4.5. Maize silage and straw: Major contributors to the dietary mycotoxin contamination

Two of the studies included in this thesis, specifically regarding the mycotoxicological screening of mouldy grass and maize silages (Penagos-Tabares et al., 2022a), as well as complete diets (Penagos-Tabares et al., 2022a), showed that maize silage could be a potentially risky feedstuff in the ration of Austrian dairy cows. This has also been suggested by multiple studies, like "Mycotoxin Occurrence in Maize Silage—A Neglected Risk for Bovine Gut Health?" by Reisinger et al. (2019), which demonstrated a broad spectrum of mycotoxin in silages from several European countries (Finland, U.K., Germany, Denmark, The Netherlands, Poland, Hungary, Italy, Turkey and Austria). Regarding the mouldy spots of silage, the maize silage presented significantly higher levels of *Fusarium*-derived mycotoxins/metabolites and ergot alkaloids (associated with field contamination) and *Penicillium* toxins/metabolites (more related to storage contamination). Thus, the total concentrations of fungal (toxic) metabolites were superior in mouldy maize silages. In the same line, maize silage samples presented a higher number of co-contamination (number of metabolites/samples) of mycotoxins, total *Fusarium* metabolites, unspecific metabolites, and total fungal metabolites (Penagos-Tabares

et al., 2022b). In relation with the complete diets, maize silage and straw were the main forage components that led to the increased concentrations of *Fusarium* mycotoxins (like DON, ZEN and BEA) and total *Fusarium* metabolites (Penagos-Tabares et al., 2022b), which matched with previous results in other European countries, like the Netherlands (Driehuis et al., 2008a; Driehuis et al., 2008b) and Spain (Rodríguez-Blanco et al., 2019). These trends could be explained based on the chemical composition of the substrates, which in maize silage case has a higher content of water-soluble carbohydrates, including starch typically found in grasses, legumes, and their mixtures. This can be speculated based on experimental evidence that showed that starch content increases the biosynthesis of some *Fusarium* mycotoxins (like TCTs and FUMs) (Oh et al., 2016).

4.6. Co-occurrence of mycotoxins with other contaminants in diets of dairy cattle feeds and possible toxicological interactions

The attached manuscripts evidenced the presence of mixtures of multiple natural but also synthetic toxic and endocrine disrupting compounds (EDCs) in individual feed ingredients (like BSG) (Felipe Penagos-Tabares, 2022c) and the complete diet of dairy cows (Penagos-Tabares et al., 2022b, 2022d). Specifically, the presence of cocktails of mycotoxins, phytoestrogens, pyrrolizidine alkaloids, cyanogenic glucoside and pesticides was higher than 90%, demonstrating that such combinations are integrated into the feed chain and indeed in the food supply (if we consider that BSG is a by-product derived of human edible products: beer and barley). Even though the individual concentrations were low and usually under legal limits (GVs and MRLs), the combinations of such toxic/EDCs make unpredictable adverse long-term effects possible. This idea makes sense if we consider the “cocktail effect” derived from complex exposomes and not only from concentrations of individual substances (Mantovani and Proietti, 2011; Mantovani, 2012, 2016; Shaw, 2014; Le Magueresse-Battistoni et al., 2018; Kelly et al., 2020; Jamnik et al., 2022). If well, such cocktails do not represent an acute, critical or prominent risk for farm animals and human consumers. The long-term combined effects of several co-occurring toxins and EDCs can be highly complex, with additive, synergistic, potentiation or antagonistic interactions varying by compound or/and concentration (Guo et al., 2020). Toxicological interactions have been described among mycotoxins, phytoestrogens, and pesticides (Eze et al., 2019; Hessenberger et al., 2017; Vejdovszky et al., 2017a; 2017b). For

example, it is known that the interaction of diverse kinds of natural and synthetic xenobiotics, such as mycotoxins, plant metabolites, and chemical biocides, can also shape microbiota composition, which influences the health and metabolic status of the host (Lindell et al., 2022). The relevance of the co-occurrence (also denominated the real-world mixtures) of natural and synthetic chemicals has to be addressed by mixture toxicologists (Warne and Hawker, 1995; Groten et al., 2001; Mattsson, 2007). Multi-toxin and multi-metabolites analysis has been used during the last decade to bring more insights into this complex field. Nowadays, the advances in analytic methods allow for evaluating hundreds of natural and synthetic pollutants, achieving high performances (LOD, LOQ, and apparent recovery) (Steiner et al. 2020; Sulyok et al. 2020; Steiner et al. 2021). Research and interest in toxin/EDCs cocktails and their long-term biological effects on animal and human health have been growing notably in recent years (Mantovani and Proietti, 2011; Mantovani, 2012, 2016; Shaw, 2014; Marín et al., 2018; Le Magueresse-Battistoni et al., 2018; Kelly et al., 2020; Weaver et al., 2020; Jamnik et al., 2022;), but related knowledge is still overall scarce. Also, significant developments and advances in exposomic biomonitoring and combined risk analysis have been reported (Jamnik et al., 2022), being a research field with enormous perspectives in human and veterinary medicine.

5. CONCLUSIONS

This doctoral thesis highlighted the omnipresence of a broad number of mycotoxins (most of them unregulated), other toxic substances and EDCs like phytoestrogens and pesticides in the feeds and diets of dairy cows in Austria. The most common regulated mycotoxins in the diets of Austrian dairy cattle were DON, ZEN and FUM B1. Although the detected mycotoxins levels were below the guidance values of the EU commission, previous studies have proven that even dietary contamination under the guidance values can negatively affect the performance, digestion, and immunity of dairy cattle. Maize silage and straw showed to be the most effective dietary ingredients in the total burden of mycotoxins and fungal metabolites. The increased environmental temperature during the growing phases of pastures and maize was evidenced as a pivotal trigger of mycotoxin contamination, which should be considered in the current context of climate change.

Additionally, the potential “cocktail effect” of such mixtures of toxins and EDCs cannot be ignored and should be addressed. The fact that the effects of most of the mycotoxins and metabolites detected, associated impacts and risks are not well-known in animals reinforces the idea of approaching this subject with cutting-edge innovative methodologies. The data presented here evidenced the importance of surveillance and monitoring programs for a broad spectrum of metabolites in the dairy feed chain in Austria, Europe, and other global regions to understand their toxicological interactions, effects, and associated risks. Moreover, the outcomes of this doctoral dissertation increase the awareness of the significance of feed management reduction and prevention strategies for mycotoxin contamination in dairy production.

6. SUMMARY

6.1. English summary

Some decades ago, ruminants were widely assumed to be resistant to dietary mycotoxins; however, this conception is now obsolete and antiquated. Although over 400 metabolites have been reported as mycotoxins, most investigations have focused on regulated fungal toxins. Consequently, the negative implications of these metabolites have been neglected and underestimated by dairy farmers and veterinarians worldwide. In addition, the metabolic and dietary characteristics of high-yielding dairy cattle (e.g., high energy density diets and higher passage rate) seem to reduce the detoxifying capacity of the rumen, thereby increasing the risk of subclinical and clinical health disorders, impairing fertility, and affecting productivity. This doctoral thesis focused on the assessment of the contamination levels of a broad spectrum of (toxic) fungal secondary metabolites and other contaminants in dairy feeds (pastures, mouldy silages and BSG) and complete dietary rations in 100 farms in the three Austrian regions leading milk production: Styria, Lower- and Upper Austria.

The analytic method LC–MS/MS provided profiles of (toxic) metabolites derived from the genera *Fusarium*, *Alternaria*, *Aspergillus* and *Penicillium,* as well as ergot alkaloids. The mycotoxin profiles of the analysed pastures, mouldy spots of maize and grass silage, BSG and complete rations of dairy cattle evidenced the ubiquitous presence of complex mixtures of toxic fungal metabolites, dominated by *Fusarium*-derived ones. The regulated mycotoxins DON, ZEN and FUM B1, occurred >70% in the diets. The predominant factors influencing the content of mycotoxins in the diets of Austrian dairy cows were the co of maize silage and straw, as well as the environmental temperature. Additionally, it was demonstrated that maize silage and straw, as well as the environmental temperature, influenced the contamination with *Fusarium*-derived toxins and metabolites in total rations. Mouldy silage and BSG can also risk of exposition to *Penicillium* mycotoxins. Taken together, further studies are needed to evaluate the long-term implications of mixtures of mycotoxins and other contaminants on animal health, fertility and food safety.

6.2. Zusammenfassung

Vor einigen Jahrzehnten ging man allgemein davon aus, dass Wiederkäuer gegen Mykotoxine resistent seien; diese Auffassung ist jedoch inzwischen überholt und veraltet. Obwohl über 400 Mykotoxinmetaboliten bekannt sind, haben sich die meisten Untersuchungen auf regulierte Mykotoxine fokussiert. Infolgedessen wurden die negativen Auswirkungen dieser Metaboliten von Milchviehhaltern und Tierärzten weltweit vernachlässigt und unterschätzt. Darüber hinaus scheinen die Stoffwechsel- und Ernährungsmerkmale von Hochleistungsmilchkühen (z. B. Futter mit hoher Energiekonzentration und höherer Passagerate) die Entgiftungskapazität des Pansens zu verringern, wodurch das Risiko subklinischer und klinischer Gesundheitsstörungen erhöht und die Fruchtbarkeit und Produktivität beeinträchtigt wird. Im Rahmen dieser Dissertation wurde in 100 Betrieben in den drei österreichischen Leitregionen der Milchproduktion (Steiermark, Nieder- und Oberösterreich) die Belastung eines breiten Spektrums von (toxischen) Pilzsekundärmetaboliten und anderen Kontaminanten in Milchviehfutter (Weiden, verschimmelte Silagen und Biertreber) und in Gesamtrationen untersucht.

Die Analysemethode LC-MS/MS lieferte Profile von (toxischen) Metaboliten der Gattungen *Fusarium*, *Alternaria*, *Aspergillus* und *Penicillium* sowie Mutterkornalkaloide. Die Mykotoxinprofile der untersuchten Weiden, der Schimmelflecken in Mais- und Grassilage, des Biertrebers und der Gesamtrationen für Milchkühe zeigten das allgegenwärtige Vorhandensein komplexer Mischungen toxischer Pilzmetaboliten, wobei die von Fusarien stammenden dominierten. Die regulierten Mykotoxine DON, ZEN und FUM B1 kamen zu mehr als 70 % in den Futtermitteln vor. Die wichtigsten Faktoren, die den Mykotoxingehalt im Futter österreichischer Milchkühe beeinflussten, waren der Anteil von Maissilage und Stroh sowie die Umgebungstemperatur. Darüber hinaus wurde nachgewiesen, dass Maissilage und Stroh sowie die Umgebungstemperatur die Kontamination mit Fusarientoxinen und Metaboliten in den Gesamtrationen beeinflussen. Schimmelige Silage und BSG können ebenfalls ein Risiko für eine Exposition gegenüber *Penicillium*-Mykotoxinen darstellen. Insgesamt sind weitere Studien erforderlich, um die langfristigen Auswirkungen von Mischungen aus Mykotoxinen und anderen Kontaminanten auf die Tiergesundheit, Fruchtbarkeit und Lebensmittelsicherheit zu bewerten.

7. LIST OF ABBREVIATIONS

AF	Aflatoxin
BEA	Beauvericin
BSG	Brewery's spent grain
DAS	Diacetoxyscirpenol
DM	Dry matter
DON	Deoxynivalenol
E2	17β-estradiol
EDC	Endocrine disrupting compounds
ENN	Enniatin
FUM	Fumonisin
NIV	Nivalenol
OTA	Ochratoxin A
PA	Patulin
TCT	Trichothecene
ZEN	Zearalenone
α-ZEL	α-Zearalanol
β-ZEL	β-Zearalenol

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