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Occurrence of mycotoxins and other fungal metabolites in diets of lactating dairy cows in Austria: A 2020 survey

Diploma thesis

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1 Introduction

Mycotoxins posed a problem for the first time when the human being began to cultivate and store crops. This was about 10,000 years ago (Pitt & Miller, 2017). In 1985, the Food and Agriculture Organization (FAO) estimated that 25 % of worldwide food-crops were contaminated with mycotoxins, though the figure of contamination above the detectable levels appears to be considerably higher, i.e. up to 60-80 % (Eskola et al., 2019). In comparison, also feedstuffs are highly contaminated with mycotoxins (Selvaraj et al., 2015). This message was underlined by a review from 2004-2012 about mycotoxin contamination and co-occurrence in animal feed, whose outcome was that 75-100 % of samples from multi-mycotoxin studies contained more than one mycotoxin (Streit et al., 2012). In fact, nearly all of the agriculturally important fungal toxins were first described as animals diseases (Pitt & Miller, 2017). Monogastric animals (especially pigs and equine) are generally more sensitive to mycotoxins than ruminants (Frey & Althaus, 2007) because the rumen microflora are able to degrade, deactivate or bind some of the toxins (Gallo et al., 2015). However, mycotoxicoses are recurrent in cattle herds worldwide and are considered a diagnostic challenge (Mostrom & Jacobsen, 2020; Richard, 2007; Richard & Thurston, 2012). According to Baumgärtner and Gruber (2020), the most relevant mycotoxicoses for cows are:

(1) Aflatoxicosis, consisting of hepatotoxic, carcinogen, teratogenic, mitotic inhibiting, immunosuppressive. The acute form can cause petechiae, haemolysis, hepatic degeneration, cholestasis, icterus or death, the chronic form can cause hepatic cirrhosis.
(2) Ergotism which provokes vascular spasms, thrombose, acral necrosis and abortions.
(3) Phomopsin intoxication which causes hepatocyte degeneration.
(4) Stachybotryotoxicosis which induces skin and mucosal ulcers and necrosis.

Even often the sickness and/or suffering including related problems are not evident, mycotoxins can negatively affect dairy cattle in different ways. The key negative consequences of mycotoxicoses in ruminants are less feed intake, less nutrient absorption and disturbed metabolism, alterations in the endocrine and exocrine systems, suppression of the immune system (Rodrigues, 2014) and a modified microbial growth (Whitlow & Hagler Jr., 2020). Acute intoxications caused by secondary metabolites from fungi are rare, more often mycotoxins cause adverse economic losses due to fertility problems, reduced weight gain, drop in performance (e.g. less milk production) and increased susceptibility to infectious diseases (Löscher & Richter, 2016) which lead also to higher costs of veterinary care (Haque et al.,

2020). Testing feedstuffs regularly for mycotoxin contamination is crucial for understanding local and global prevalence and risks. Moreover, updated data about mycotoxin occurrence can be valuable for reducing negative impacts of mycotoxins on animal performance (Weaver et al., 2020). Surveys on mycotoxin contamination are necessary for the whole field of mycotoxin research because the results can be compared, they can help to answer questions and make decisions based on objective data (Gallo et al., 2015).

2 Literature Review

2.1 Mycotoxins – an overview

“All mycotoxins are low-molecular-weight natural products (i.e., small molecules) produced as secondary metabolites by filamentous fungi” (Bennett & Klich, 2003). Notably, not all secondary metabolites from fungi are called mycotoxins. The ones which are mainly toxic to bacteria are called antibiotics, those which are mainly toxic to plants are called phytotoxins and only those which are toxic to vertebrates and other animal groups in low concentrations are called mycotoxins (Bennett & Klich, 2003). Mycotoxins are toxic abiotic hazards with a biotic origin, but once the mycotoxins are produced and secreted from the fungi, they persist independently in feeds and foods due to their stability (Lauren & Smith, 2001). Additionally, they are odourless, tasteless and they do not change the organoleptic characteristics of foods and feeds, which makes them hard to detect. Moreover, mycotoxins are heat resistant and they tolerate a wide range of pH values (Winter & Pereg, 2019). Importantly, mycotoxins do not trigger an antibody mediated reaction like proteins, which makes them not recognisable for the immune system (Rodrigues, 2014). Currently, more than 500 mycotoxins from about 100 fungi have been reported as potentially toxigenic (Haque et al., 2020). It is not yet fully understood why fungi produce mycotoxins (Stroka & Gonçalves, 2019). The common knowledge is that fungal secondary metabolites are important players in ecological settings, as they provide protection in various ways. Their production is affected by a lot of things, for example light, nutrients, pH, endofungal bacteria and epigenetic reprogramming in microbial interactions (Venkatesh & Keller, 2019).

Mycotoxins can enter the feed chain at any point (**fig. 1**) and can be divided into two big groups. Initially, there are mycotoxins produced in the field by field fungi, which parasitize on forage crops in the field pre-harvest, mainly from *Fusarium* and *Claviceps* spp. Secondly, there are mycotoxins produced after harvest often by storage fungi, which infest the feed post-harvest, mainly from the genera *Aspergillus* and *Penicillium* (Löscher & Richter, 2016) but can be also produced by field fungi, which infested the feedstuff before. **Fig. 1** shows the roles of mycotoxin contamination (Haque et al., 2020). Important conditions for fungal growth are at least 15 % water content of the feed, a high humidity (Löscher & Richter, 2016) and for most fungi aerobic conditions (Reverberi et al., 2010). Also, extreme weather events (temperature, humidity, drought) or insect infestation, which lead to stress in plants promote pre-harvest contamination. Subsequently, poor harvesting and storage practices, as well as inadequate feeding management, can result in higher mould growth and post-harvest mycotoxin formation

(Bryden, 2012; Coulombe, 1993). Remarkably, fungal growth and mycotoxin production do not necessarily need the same conditions to thrive (Whitlow & Hagler Jr., 2020).

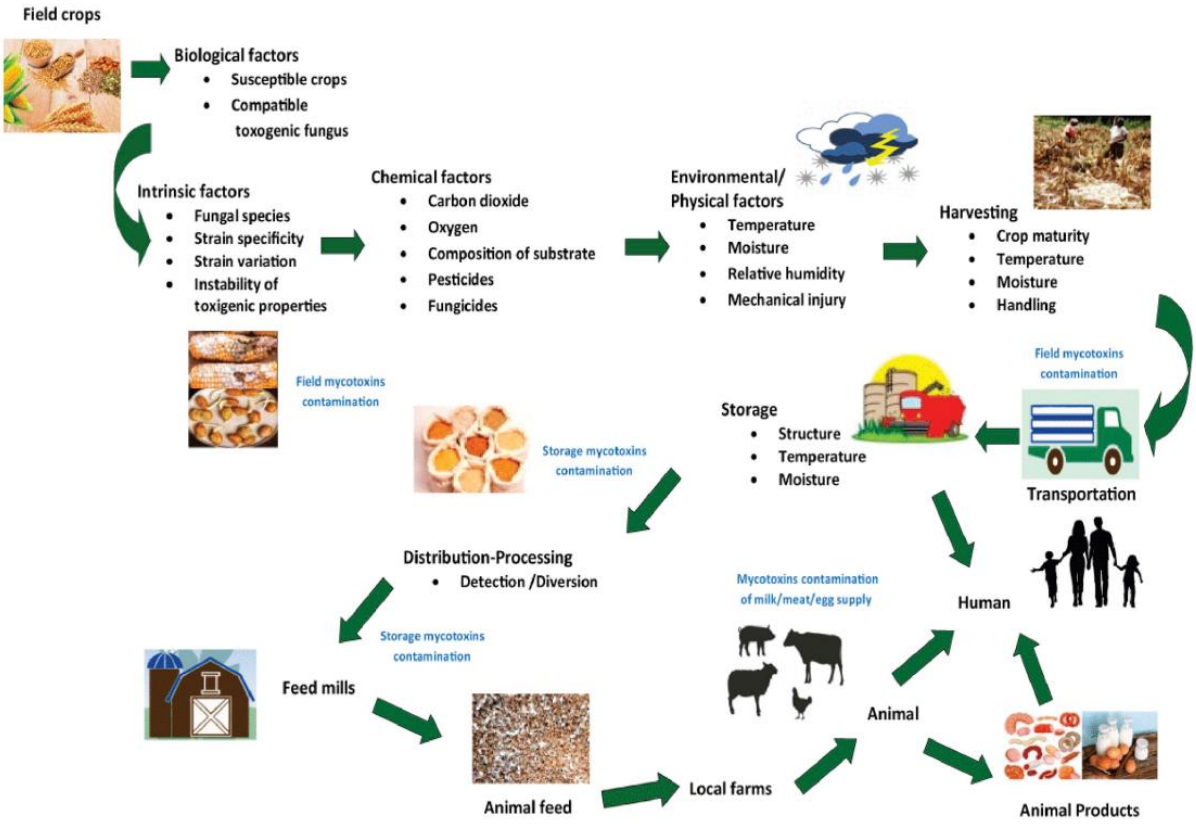

Fig. 1: Factors affecting mycotoxin occurrence in the food and feed chain. (Haque et al., 2020)

2.1.1 Regulated mycotoxins

In general, it is more complicated to define guidelines for mycotoxin contaminations in animal feeds because the negative effects of mycotoxins on animal health and productivity depends on many factors such as animal species, gender, age, duration of exposure, stress factors of the environment as well as production and dietary factors (Whitlow & Hagler Jr., 2020). Nevertheless, the laws of the European Union (EU) on feed contamination exist. Since this thesis deals with mycotoxin contamination in diets of lactating Austrian dairy cows, the laws of the EU must be considered and present the starting point for further discussions.

For products intended for animal feeds, some mycotoxins are regulated by the EU laws. **Tab. 1** lists the maximum content of regulated mycotoxin according to the directive 2002/32/EC of the European Parliament and of the Council, of 7 May 2002 on undesirable substances in animal feed in the version of 28.11.2019 (2002/32/EC, 2002/28.11.2019). As the table indicates, strict rules are only written for aflatoxin (AF) B_1 and the presence of rye ergot in

unground cereals. **Tab. 1** shows also that the maximum contents are significantly lower for dairy cows than for other animals, because of the risk that AF B_1 could pass over in the milk for human consumption.

***Tab. 1**: Parts of the annex 1 from the directive 2002/32/EC of the European Parliament and of the Council, of 7 May 2002 on undesirable substances in animal feed in the version of 28.11.2019, section 2: Mycotoxins (2002/32/EC, 2002/28.11.2019)*

Undesirable substances	Products intended for animal feed	Maximum content in mg/kg (ppm) relative to a feedingstuff with a moisture content of 12 %
Aflatoxin B_1	Feed materials	0.02
	Complementary and complete feed with the exception of:	0.01
	— compound feed for dairy cattle and calves, dairy sheep and lambs, dairy goats and kids, piglets and young poultry animals	0.005
	— compound feed for cattle (except dairy cattle and calves), sheep (except dairy sheep and lambs), goats (except dairy goats and kids), pigs (except piglets) and poultry (except young animals)	0.02
Rye ergot (Claviceps purpurea)	Feed materials and compound feed containing unground cereals.	1,000

Other mycotoxins, including Ochratoxins (OTs) A, Zearalenone (ZEA), Deoxynivalenol (DON), Fumonisins (FUMs), T-2 and HT-2 toxins in products intended for animal feeding are also regulated but with guidance values (2006/576/EC, 2006/02.08.2016). Regarding the European of legal acts, the difference between a directive and a recommendation is that the first outlines certain rules which must be met by the member states, whereas a recommendation is only an advice which can but not must be followed by the member states. As shown in **tab. 2** and **tab. 3** grains contaminated with this group of mycotoxins with higher values than the guidance values can be legally feed to animals, the European Commission only recommends staying below these values. This may explain the high prevalence of these mycotoxins in animal feeds. In contrast and as shown in **tab. 1**, it is illegal to feed animals grains contaminated with AF B_1 and rye ergot with higher values than the maximum content.

***Tab. 2**: Parts of the annex from the commission recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding in the version of 02.08.2016 (2006/576/EC, 2006/02.08.2016)*

Mycotoxin	Products intended for animal feed	Guidance value in mg/kg (ppm) relative to a feedingstuff with a moisture content of 12 %
Deoxynivalenol	Feed materials (*)	
	— Cereals and cereal products (**) with the exception of maize by-products	8
	— Maize by-products	12
	Compound feed with the exception of:	5
	— compound feed for pigs	0.9
	— compound feed for calves (< 4 months), lambs, kids and dogs	2
Zearalenone	Feed materials (*)	
	— Cereals and cereal products (**) with the exception of maize by-products	2
	— Maize by-products	3
	Compound feed for:	
	— piglets, gilts (young sows), puppies, kittens, dogs and cats for reproduction	0.1
	— adult dogs and cats other than for reproduction	0.2
	— sows and fattening pigs	0.25
	— calves, dairy cattle, sheep (including lamb) and goats (including kids)	0.5
Ochratoxin A	Feed materials (*)	
	— Cereals and cereal products (**)	0.25
	Compound feed for:	
	— pigs	0.05
	— poultry	0.1
	— cats and dogs	0.01
Fumonisin B_1 + B_2	Feed materials (*)	
	— maize and maize products (***)	60
	Compound feed for:	
	— pigs, horses (Equidae), rabbits and pet animals	5
	— fish	10
	— poultry, calves (< 4 months), lambs and kids	20

	— adult ruminants (> 4 months) and mink	50
T-2 + HT-2 toxin	Compound feed for cats	0.05
(*) Particular attention has to be paid to cereals and cereals products fed directly to the animals that their use in a daily ration should not lead to the animal being exposed to a higher level of these mycotoxins than the corresponding levels of exposure where only the complete feedingstuffs are used in a daily ration. (**) The term 'Cereals and cereal products' includes not only the feed materials listed under heading 1 'Cereal grains, their products and by-products' of the non-exclusive list of main feed materials referred to in part B of the Annex to Council Directive 96/25/EC of 29 April 1996 on the circulation and use of feed materials (OJ L 125, 23.5.1996, p. 35) but also other feed materials derived from cereals in particular cereal forages and roughages. (***) The term 'Maize and maize products' includes not only the feed materials derived from maize listed under heading 1 'Cereal grains, their products and by-products' of the non-exclusive list of main feed materials referred to in the Annex, part B of Directive 96/25/EC but also other feed materials derived from maize in particular maize forages and roughages.		

Adult ruminants also seem to tolerate higher levels of FUM B_1 + B_2 and ZEA. Their guidance contents are significantly higher than for other animals. We can see from **tab. 2**, that there are no guidance levels for OT A and T-2 + HT-2 toxin for dairy cows or other livestock species. These facts indicate that the species sensitivity is different.

Furthermore, the regulation also distinguishes the contamination of some mycotoxins among cereal and cereal products. In accordance, **tab. 3** gives an overview of the indicative levels (which are not feed and food safety levels) of T-2 and HT-2 toxin for cereal and cereal products from the commission recommendation of 27 March 2013. The list excludes food for human consumption (2013/165/EU, 2013). Indicative levels of these toxins are higher in oat and its products compared with other cereals.

***Tab. 3**: Parts of the annex from the commission recommendation of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products (2013/165/EU, 2013)*

Indicative levels for cereals and cereals products (*) (**)	
	Indicative levels for the sum of T-2 and HT-2 (µg/kg) from which onwards/above which investigations should be performed, certainly in case of repetitive findings (*)
1. Unprocessed cereals (***)	
1.1. barley (including malting barley) and maize	200
1.2. oats (with husk)	1,000
1.3. wheat, rye and other cereals	100
4. Cereal products for feed and compound feed (*****)	
4.1. oat milling products (husks)	2,000
4.2. other cereal products	500

4.3. compound feed, with the exception of feed for cats	250

(*) The levels referred to in this Annex are indicative levels above which, certainly in the case of repetitive findings, investigations should be performed on the factors leading to the presence of T-2 and HT-2 toxin or on the effects of feed and food processing. The indicative levels are based on the occurrence data available in the EFSA database as presented in the EFSA opinion. The indicative levels are not feed and food safety levels.
(**) For the purpose of this Recommendation rice is not included in cereals and rice products are not included in cereal products.
(***) Unprocessed cereals are cereals which have not undergone any physical or thermal treatment other than drying, cleaning and sorting.
(*****) The indicative levels for cereals and cereal products intended for feed and compound feed are relative to a feed with a moisture content of 12 %.

2.2 Mycotoxins and their influence on the health of dairy cows

This chapter provides an overview of the best described and investigated mycotoxins as well as mycotoxin mixtures, modified and emerging mycotoxins. Most of the time, undesirable effects from mycotoxins occur after the animals eat contaminated feed but they can also happen after contact or inhalation (Whitlow & Hagler Jr., 2020). The main fungal genera who produce mycotoxins are *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, and *Claviceps* (Marin et al., 2013). As stated before, ruminants are less susceptible than monogastric animals because of their ruminal microbial metabolism. Microbes in the rumen can detoxicate some of the mycotoxins. However, under certain conditions, microbial metabolism of mycotoxins could lead to more toxic derivatives, for example when ZEA is transformed into α-ZEL, which is four times more active than ZEA (Dell'Orto et al., 2015; Goncalves et al., 2015). Although ruminal degradation can prevent acute toxicity however it may lead to undetected chronic toxicity which is accompanied with more diseases, reproductive and milk performance problems (Whitlow & Hagler Jr., 2020).

2.2.1 Aflatoxins

AFs involve several chemical forms such as B_1, B_2, G_1, G_2, M_1 and M_2 (Juneja, 2010) where B_1 is the most toxic (Kupper et al., 2020a). Whereas the B_1, B_2, G_1 and G_2 AFs are produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*, the M_1 and M_2 AFs are developed when the B_1 and B_2 toxins are metabolised in animals (Juneja, 2010). There are further forms of AFs which are not mentioned because of their neglecting agronomical and acute toxicological concerns (Juneja, 2010). There are other *Aspergillus* species (*Aspergillus nomius*, *Aspergillus bombycis*, *Aspergillus pseudotamari* and *Aspergillus ochraceoroseus*) which produce AFs, but they have low occurrence (Da Rocha et al., 2014). Aflatoxigenic fungi infest feeds usually post-harvest, during transportation and storage, leading to an increased risk of more AF

contamination in imported grains (Kupper et al., 2020a). Still, the fungi can be found already pre-harvest, on the plants and in the soil (Marin et al., 2013). To produce AFs, the fungi need temperatures above 30 °C. Compared to *Fusarium* species, which are field fungi, *Aspergillus* prefers lower water activities and higher temperatures. Therefore, heat and drought stress as well as insect damage before harvest promote AF production (Whitlow & Hagler Jr., 2020). AFs are stable compounds and thermoresistant, thus high contamination levels maintain during long storage and they are not destroyed by heat during common food and feed processing methods (Kupper et al., 2020a).

Once the AFs are ingested by the animal, they are metabolised mainly in the liver by the P450 enzyme by hydroxylation (Kupper et al., 2020a). For example, AF $B_{1,}$ which is the most carcinogenic and potent mycotoxin known (Simion, 2017), is metabolised into AF M_1, AF Q_1, AF P_1, aflatoxicol, aflatoxicol H_1 and aflatoxin B_{2a}. AF M_1 and AF M_2 can be found in the milk from lactating animals exposed to AF B_1. The majority of AF B_1 is excreted via faeces within a week after the administration (Kupper et al., 2020a). Accumulations of AF B1 in different organs were also reported (Simion, 2017). The toxicity of AF metabolites is related to their ability to bind to the DNA, therefore they can affect the protein synthesis and they have oncogenic and immunosuppressive properties (Da Rocha et al., 2014). The focus lays on the DNA-damage, which leads, in extreme conditions, to liver degeneration, icterus, liver cirrhosis and hepatic tumours. Additionally, the microbial fermentation in the rumen could be impaired by AFs (Kupper et al., 2020a), reducing the ruminal motility, the capacity of digesting cellulose and the production of volatile fatty acids and proteolysis (Cook et al., 1986). There are cases of acute intoxications but the chronic course is more common (Kupper et al., 2020a). Acute symptoms would be inappetence, lethargy, ataxia, rough hair coat, and pale, enlarged fatty livers (Whitlow & Hagler Jr., 2020). Therefore, clinical adverse effects for cows are decreased feed intake, depletion of milk production and impaired hepatic function (Simion, 2017). According to Kupper et al. (2020a), the following AF B_1 concentrations in feed (regarded to wet basis) over several weeks led to chronic toxicity: calf 200 µg/kg, heifer 700 µg/kg, young bull 1,000 µg/kg, and cow 100 µg/kg. Adverse effected of chronic exposures include inefficient feeding (reduction of ruminal motility), immunosuppression, and reproductive problems. Dairy cows with high production could be more sensitive to the toxins (Simion, 2017).

2.2.2 Ergot alkaloids

Ergot alkaloids are produced mainly by the ergot fungus *Claviceps purpurea*, but can also be produced by the other fungi including *Acremonium*, *Balansia*, *Aspergillus* and *Penicillium* (Juneja, 2010). Ergot alkaloids are derivatives of the lysergic acid (Kupper et al., 2020d). Various ergot alkaloids, for example, ergovaline, ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornine are reported in grains (Marin et al., 2013). *Claviceps purpurea* (an ascomycete) infests many cereal grains (especially rye) and weeds (especially sweet-rases) in the field and forms a 1-4 cm, curved and dark blue-violet sclerotium in the ears. This sclerotium is called ergot or *Secale cornutum* (in German: Mutterkorn), and its formation thrives during dry weather conditions (Kupper et al., 2020d). Ergot alkaloids are commonly known because it was a cause of several deaths in the Middle Ages. The intoxication is called ergotism and two forms are described: the gangrenous form, which affects the supply of blood to the extremities of the body, and the convulsive form, which directly affects the central nervous system. Affected animals show symptoms of the gangrenous form and can additionally have abortions, convulsions, suppression of lactation, hypersensitivity and ataxia (Da Rocha et al., 2014; Marczuk et al., 2019). The explanation for the affected blood supply is that ergot alkaloids act on receptors in blood vessels, which lead to extreme and long-lasting vasoconstrictions. Abortions happen because some alkaloids (for example ergometrine) are causing contractions in the uterus, just like oxytocin does. The resorption after oral intake depends on the absorbed alkaloids, they are metabolized in the liver and the resulting metabolites are eliminated with the bile fluid. The symptoms usually occur after two to three weeks but can be soonest seen on day three after the intake. Once again, acute intoxications are described but chronic courses are more frequent (Kupper et al., 2020d).

2.2.3 Ochratoxins

OTs can be divided into three types – OT A, B and C. These toxins are produced by several moulds including *Aspergillus ochraceus*, *Aspergillus carbonarius*, *Penicillium verrucosum* among others (Juneja, 2010). OT A is of toxicological interest because it is the most toxic form (Marin et al., 2013). These fungi infest the feed after harvest when the feed is moist enough and they are able to multiply also in colder conditions. The minimum temperature for toxin production is 12 °C (Kupper et al., 2020e). OT A is very stable, it needs temperatures above 250 °C for several minutes to break it down (Marin et al., 2013). The excretion of the metabolites in the animals happens through the bile fluid and urine but also in small amounts through the milk. Residues of OT A can be found in the milk as well as in the muscles (Kupper

et al., 2020e). OT A is first of all known mycotoxins to be nephrotoxic, but also hepatotoxic, immunosuppressive, teratogenic and carcinogenic (Da Rocha et al., 2014). The minimum toxic concentration in feed for cattle is 20 mg/kg, it is 1 mg/kg for non-ruminating calves and the minimum lethal dose for cattle is 13 mg/kg body weight, for non-ruminating calves it is 4 mg/kg body weight (Kupper et al., 2020e). Cows can tolerate higher concentrations of OT A than other animals because the ruminal microbes, especially protozoa, are able to degrade OT A (Simion, 2017). The capacity however depends on the health of the microbial community, which is easily affected by the pH of the rumen and therefore diets play a major role in the avoidance of toxic effects from OT A. When the capacity is reached and OT A is accumulating in the rumen, symptoms of ochratoxicosis such as pulmonary edema and reduced animal health appear (Simion, 2017).

2.2.4 Fumonisins

FUMs involve the toxins FUM B_1, B_2 and B_3, which are produced mainly by *Fusarium* spp., like *Fusarium proliferatum* and *Fusarium verticillioides* (previous *Fusarium moniliforme*) (Juneja, 2010) as well as from *Fusarium nygamai* and *Alternaria alternata f.sp. lycopersici* (Da Rocha et al., 2014). Altogether, twelve FUMs analogues have been established, but the FUMs B are the most important ones whereby FUM B_1 is the most toxic (Marin et al., 2013). FUMs occur predominantly in cereal grains, especially in maize (Knutsen et al., 2018a), in warmer regions and are produced pre-harvest or in early stages of storage (Marin et al., 2013). FUMs are quite heat stable and only reduce slightly during fermentation (Marin et al., 2013). FUMs are known to have hepatotoxic, carcinogenic and apoptosis effects (Da Rocha et al., 2014). According to the European Food Safety Authority (EFSA), the risk of adverse health effects of feeds containing FUMs $B_{1\text{-}3}$ is considered very low for ruminants, which are less sensitive than horses and pigs. The EFSA determinated a no-observed-adverse-effect level of 31 mg FUMs $B_{1–3}$/kg feed for cattle, based on the following clinical findings: gross and histopathological lesions, increase in serum enzymes, cholesterol and bilirubin as well as the decrease in lymphocyte blastogenesis, which indicate an impairment of liver and possibly kidney function. The EFSA calculated that the highest concentrations of FUMs B are in the dietary of maize silage-based diets with the inclusion of cereal grains in the complementary compound feed for lactating dairy cows (between 368-1,894 µg/kg feed). The lowest concentrations of FUMs B are in the dietary of beef cattle on straw-based rations (between 14-270 µg/kg feed) (Knutsen et al., 2018a).

2.2.5 Trichothecenes

Trichotheces can be structurally divided into two groups – type A trichotheces and type B trichotheces. Type A trichotheces contains the toxins T-2 toxin, HT-2 toxin, diacetoxyscirpenol, monoacetoxyscirpenol and neosolaniol. Type B trichotheces involves the toxins nivalenol, DON (vomitoxin), fusarenon X and diacetylnivalenol (Juneja, 2010). They are produced by the fungi of the genera *Fusarium*, *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichoderma*, *Trichotecium*, *Verticimonosporium* and possibly others (Da Rocha et al., 2014). A lot of these species are soil fungi and infest the crops in the field (Marin et al., 2013). The fungi need min. 12 % moisture in the feed, a pH value between four to eight and enough oxygen to grow, which can be also in colder regions. The production of toxins can be increased through pest infestation or the use of pesticides (Kupper et al., 2020b). Trichotheces in general cause inhibited eukaryotic protein synthesis and peptidyl transferase activity (Da Rocha et al., 2014), leading to clinical manifestations such as gastrointestinal disorders, abortion, haemorrhages and immunosuppression (Simion, 2017). DON is the most recurrent of the thichothecenes toxins, but it is not the most toxic. In high doses DON can cause nausea, vomit and diarrhea, hence the name vomitoxin. In small doses it can cause weight loss and reduced feed intake (Da Rocha et al., 2014). In dairy cows, DON can cause a significant reduction of milk performance (Simion, 2017). Ruminants are less sensitive to DON than other animals because the ruminal microbial of healthy cows can detoxify DON into deepoxy-deoxynivalenol (Schelstraete et al., 2020). DON is an extremely stable toxin, which cannot be fully deactivated through heat. It is often found in maize, wheat, barley and oat and is primary attached on the outside of the outer hard cereal shell. This explains high DON levels discovered in bran (Kupper et al., 2020b). Like other toxins, DON in its pure form is less toxic than DON from naturally contaminated feed. This observation can possibly be explained by the interaction with other co-contaminated mycotoxins (Whitlow & Hagler Jr., 2020). Fusarial toxin T-2 can be found especially in grains. This toxin is hydrolysed in the liver and afterwards harmless, but it can partially be excreted unaltered via the milk. Resorption injures the cell proliferation in the bone marrow, causing severe immunosuppression (Kupper et al., 2020c). The effects of T-2 toxin in dairy cattle has not yet been fully researched, but gastrointestinal problems have been observed (Whitlow & Hagler Jr., 2020). The toxins produced by *Stachybotrys atra* are called satratoxine, verrucarine and roridine and belong to the group macrocyclic trichothecene. They occur especially in hay and straw, even more when it is stored outside during winter, but also when other feedstuffs are infested. The fungi produce gatherings of soot-black spores in

infested feed. The toxicological principles are the same as for T-2 toxin. For a sheep, 170 g of contaminated hay could be lethal, there are no data available for cattle (Kupper et al., 2020f).

2.2.6 Zearalenone

The toxin ZEA is produced by the fungi species *Fusarium spp.*, in grains often by *Fusarium graminearum* and *Fusarium culmorum* (Juneja, 2010) and also from *Fusarium equisetii* and *Fusarium crookwellense* (Da Rocha et al., 2014). Although it is classified as a toxin, ZEA is not highly toxic, but its structure resembles 7b-estradiol. Therefore it would fit into the classification of a non-steroidal oestrogen or a mycoestrogen (Da Rocha et al., 2014). α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) are the most important metabolites, whereby α-ZEL has a higher oestrogenic activity (Marin et al., 2013). Due to its structure ZEA has hormonal effects and causes hyperestrogenism and reproductive disorders (Da Rocha et al., 2014), including stillbirths, abortions and birth defects (Kupper et al., 2020g). Especially in cows, uterus hypertropia, swelling of the vulva and mammary glands and lower rates of ovulation and conception are reported (Simion, 2017). In some countries, synthetic ZEA is used as growth promoter (Kupper et al., 2020g). Its carcinogenic capacity is not yet verified (Da Rocha et al., 2014). After an oral intake of ZEA, the metabolite α-ZEL is excreted through the milk. α-ZEL is still biological (oestrogenic) active and that is the reason for strict thresholds of ZEA in the milk for consumer. ZEA is also called F-2 toxin. Although ZEA is a reduction product from F-2 toxin, it is three to four times more biologically active. Silage, maize or concentrated feed are typical feed sources with high fusarial contamination. The conditions for optimal fungal growth are similar as for trichothecenes producing fungi (Kupper et al., 2020g). Indeed, the fungi infest crops during blooming, but toxin production is likely to continue in storage under poor storage conditions (Marin et al., 2013). For cattle, the minimal toxicological concentrations of ZEA in feed (with respect to wet feed) are 12 mg/kg. The most frequent complications due to ZEA are related to reproductive problems. Symptoms appear four to seven days after the initiation of intake (Kupper et al., 2020g). ZEA is quite heat stable but more attackable under alkaline conditions (Marin et al., 2013).

The toxic effects in cattle from some of the key mycotoxins described above are summarized in **tab. 4**.

***Tab. 4**: Main toxic effects of the most important mycotoxins in cattle (Goncalves et al., 2015)*

Mycotoxins	**Toxic effects**
Aflatoxins	Affects immunological functions and rumen metabolism Decreases feeding efficiency in dairy cattle Reduces milk yield Causes damage to the liver Decreases feeding efficiency in dairy cattle Hepatotoxic, Immunosupressive
Ochratoxins	Causes kidney problems Reduces milk yield
Fumonisins	Causes reproductive problems Negatively affects the function of the immunological system Causes lesions in kidneys and liver
Zearalenone	Causes infertility Reduces milk yield
Deoxynivalenol	Causes hyperestrogenism Negatively affects the function of the immunological system Gastrointestinal effects
T-2 toxin and HT-2 toxin	Acute hemorrhagic enteritis Reduces feed intake

2.2.7 Mycotoxin mixtures

Single mycotoxin contamination in nature is unusual. It is scientifically proven that several mycotoxins occur simultaneously in feed. Several terms are used to refer to mycotoxin mixtures such as mycotoxin co-occurrence, mycotoxin combinations, mycotoxin cocktails and mycotoxin co-contamination (Battilani et al., 2020).

However, the majority of research studies has focused solely on single mycotoxin and its toxicology. As a result, their combined effects are less known especially in livestock and the legal regulations of feed contamination only focus on specific mycotoxins considered separately (Smith et al., 2016). In terms of risk assessment for humans, Speijers and Speijers (2004) suggested to define a group daily tolerable intake or a provisional tolerable weekly intake. Such suggestions would be similarly applicable for animals. Recent studies have investigated the co-occurrence of mycotoxins in animal feedstuffs (Palumbo et al., 2020; Smith et al., 2016; Streit et al., 2012). The most studied combinations were AFs + FUMs, DON +

ZEA, AFs + OT A, and FUMs + ZEA. Studies showed antagonist, additive, synergic or potential effects of combined mycotoxins (Smith et al., 2016), however additive or synergistic interactions are often reported, which should be alarming (Šegvić Klarić, 2012). **Fig. 2** gives an overview of factors which influence the presences of mycotoxin mixtures (Šegvić Klarić, 2012).

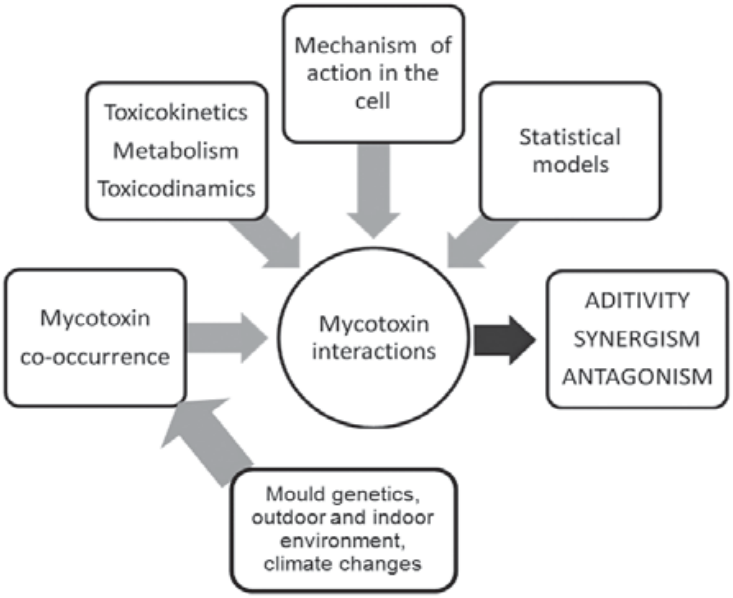

***Fig. 2**: Influence of various factors on mycotoxin (Šegvić Klarić, 2012)*

Another notion is that mycotoxins formed *in vivo* could be more toxic than mycotoxins formed *in vitro*, at least this was observed in two studies for AFs and DON (Applebaum et al., 1982; Foster et al., 1986). A drawn conclusion from these studies could be that there are a lot more mycotoxins (mixed mycotoxins) in the *in vivo* samples. Other authors reported findings which reinforces this message (Hagler et al., 1984; Smith & MacDonald, 1991).

2.2.8 Emerging mycotoxins

The term emerging mycotoxin is not entirely described in the literature but is used since 2008 for the *Fusarium* mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin (Gruber-Dorninger et al., 2017). Vaclavikova et al. (2013) defined emerging mycotoxins as “Mycotoxins, which are neither routinely determined, nor legislatively regulated; however, the evidence of their incidence is rapidly increasing”. In order to show their importance, an example will be given in the next paragraph.

Evidence has shown that there is high prevalence of emerging mycotoxins in feedstuffs. For instance, screenings of 83 feed samples detected beauvericin in 98 %, enniatins in 96 % and

moniliformin in 76 % of the screened samples (Fraeyman et al., 2017). So far, 29 different types of enniatins, produced by *Fusarium* spp., are currently known and have been identified. Enniatins are toxic *in vitro* but the toxicity *in vivo* is non-existing or low (Gruber-Dorninger et al., 2017). Beauvericin is also produced by several *Fusarium* species and toxic *in vitro* but not toxic to rodents and poultry *in vivo* (Gruber-Dorninger et al., 2017). The EFSA did not indicate enniatins and beauvericin of concern for human health, and no statement was given for ruminants (Maranghi et al., 2018). Moniliformin is produced by several *Fusarium* species and *Penicillium melanoconidium*. The toxin is primarily cardiotoxic, especially in poultry (Gruber-Dorninger et al., 2017) as well as haematotoxic (Knutsen et al., 2018b). The EFSA showed insecurity about the toxicity of moniliformin, but indicated a low or even negligible risk for animals from exposure to moniliformin in feed (Knutsen et al., 2018b). These are only a few examples of hundreds of emerging mycotoxins. Although as individual compounds, most of these emerging mycotoxins do not appear to have high toxicity. Again, associative effects with other mycotoxins are unknown and only a handful of studies have studied them thus far. Further investigations should not only focus on well-documented mycotoxins but also should stir towards understanding metabolism and toxicity of emerging mycotoxins.

2.2.8.1 Modified mycotoxins

Another classification of mycotoxins is termed modified mycotoxins that include the so-called masked mycotoxins. The original term “masked mycotoxin” first appeared in the year 1990 to describe a ZEA glucoside, which was not detected in routine analysis, but had effects in animals. Nowadays, the term is used for different definitions (Rychlik et al., 2014). The International Life Science Institute defined masked mycotoxins as follows: “Mycotoxin derivatives that are undetectable by conventional analytical techniques because their structure has been changed in the plant are designated masked mycotoxins” (Rychlik et al., 2014). Fusarial toxins (DON, nivalenol, fusarenon-X, T-2 toxin, HT-2 toxin, ZEA, OT A, destruxins and fusaric acid) are metabolised or bound by plants leading to masked mycotoxins (Berthiller et al., 2013). Rychlik et al. (2014) suggested the term modified mycotoxins should be used in the future instead of masked mycotoxins because it describes any modification of the basic chemical structure of mycotoxins (chemical or biological modifications). There are only a few toxicological studies about the risks of modified mycotoxins, but some of them indicate a potential threat against human and animals. For example, the conjugation of the modified mycotoxin ZEA-14-glucoside back into the parental molecule ZEA was observed in the

gastrointestinal tract of pigs, which means ZEA-14-glucoside could induce the same toxic effects as ZEA. Similarly, the modified mycotoxin DON-3-β-D-glucoside could be partly reversed into DON in the gastrointestinal tract of animals (Berthiller et al., 2013).

2.3 Occurrence of mycotoxins in dairy feed in European countries: from field to feed bunk

The weather and other environmental factors vary every year, influencing the mycotoxin occurrence and concentration (Coulombe, 1993). Dairy cattle in Europe, especially in Austria, often receive conserved forages (such as grass silage, maize silage and hay) as major forage sources and concentrates, which contain mostly cereals, protein sources and minerals. In general, concentrate feed, especially cereals, are viewed as feed sources with high risks of mycotoxin contamination and thus are a lot more examined than the forages. In fact, mycotoxins are already present in forage crops. Mycotoxin formation in Austria already starts in the field (AGES, 2020), which is supported by a recent study investigating contamination of mycotoxins and fungal metabolites in Austrian pastures (Penagos-Tabares et al., 2021). In the Czech Republic Štýbnarová et al. (2016) investigated a 3.6 ha cattle pasture for mycotoxin contamination, with the outcome of a mean concentration of DON being 667.82 µg/kg, of T-2 + HT-2 toxin being 49.96 µg/kg and of ZEA 66.69 µg/kg. Also Kononenko et al. (2015) found that multiple fungal and mycotoxin contamination were already formed in plant tissues by the moment of first mowing. Okabe et al. (2015) observed that the FUM levels in forage maize plants in agricultural fields increased four to eight weeks after silking, indicating that the risk of contamination increases with later harvest times. Since mycotoxins are highly stable compounds, once mycotoxins are already present in the forage crops, silage-making conditions might not significantly eliminate them. Moreover, even though silage is produced under anaerobic conditions, but if oxygen is available, storage moulds can easily grow, resulting in greater contamination of the silages. Furthermore, mould growth is also promoted in insufficiently dried hay and straw (Scudamore & Livesey, 1998). Common mycotoxins in silages are trichothecenes, FUMs, AFs, ZEA, mycophenolic acid, and roquefortine C (Ogunade et al., 2018).

There were already research attentions in contamination of mycotoxins in feedstuffs in Austria decades ago. During 1979-1981 there was a screening of 221 Austrian feed samples (74 oats, 67 mixed feed, 28 barley, 27 maize, 18 corn-silage and 7 wheat) for mycotoxins (Neuhold, 1982). It was observed that the highest DON levels were found in maize (5,000 µg/kg), followed by oats (2,220 µg/kg), mixed feed (1,500 µg/kg), corn-silage (1,200 µg/kg) and barley

(700 µg/kg). The highest ZEA levels were detected in mixed feed (1,200 µg/kg), followed by barley (450 µg/kg), oats (320 µg/kg), corn-silage (308 µg/kg) and maize (300 µg/kg) (Neuhold, 1982). A later study published in 1983 did research on mycotoxin contamination in Austrian animal feed, with the results that 46 % of 389 mycotoxin-suspected feed samples contained DON (25-21,500 µg/kg) and 30 % of 516 samples contained ZEA (5-17,500 µg/kg) and OT A as well as citrinin were also found in feed samples (Schuh, 1983). In the year 1990, 48 samples of oats, wheat and maize from different parts of Austria were screened for *Fusarium spp.*, with the outcome that *Fusarium poae*, *Fusarium avenaceum* and *Fusarium graminearum* were predominant in oat and wheat grains; *Fusarium sacchari var. subglutinans*, *Fusarium graminearum* and *Fusarium avenaceum* were most common in maize grains (Adler et al., 1990). Kovalsky et al. (2016) investigated mycotoxin occurrence in finished feed and maize from 2012-2015 with the results that DON, ZEA and FUMs showed large increases of annual medians in Europe. The same study showed that in Austria, median DON concentrations increased to 1,400 µg/kg whereas Germany stayed at 350 µg/kg in 2015. Similar to DON and ZEA, enniatins observed in 2014 was at the median concentration of 250 µg/kg in Europe. In Serbia during the period of 2004-2016, AF concentrations frequently exceeded the EU limits (Udovicki et al., 2018). A ten-year study from 2008-2017 for mycotoxin occurrence in feed examined 21,036 samples from the Central Europe. A brief summary of the outcome was as follows: Trichothecenes, which includes DON and T-2 toxin, were the most prevalent mycotoxins. Also, ZEA and FUMs were detected. Only 0.9 % of DON and 0.4 % of ZEA positive samples exceeded the EU guidance levels. In the year 2014, DON and ZEA concentrations in maize were significantly higher compared to the other years. They also reported the percentage of the positive samples and the median concentration of certain mycotoxins including AF B_1 (12.7 %, 1.6 µg/kg), FUMs (43.2 % 187 µg/kg), ZEA (45 %, 40 µg/kg), DON (69.8 %, 428 µg/kg), OT A (11.9 %, 2.8 µg/kg) and T-2 toxin (30.7 %, 11 µg/kg) (Gruber-Dorninger et al., 2019). **Tab. 5** gathers numbers of mycotoxin contaminations in Europe from different studies as cited below.

Streit et al. (2012) reported different surveys published since 2004-2012 about mycotoxin contamination in Europe, while Ogunade et al. (2018) reported those for silages and Gallo et al. (2015) for forages and other fibrous feeds throughout Europe. From the data presented from Streit et al. (2012), it is difficult to infer trends on recent developments regarding mycotoxin contamination in European feed, which underlines the importance of survey studies like this thesis. The data from Ogunade et al. (2018) reveals that the contribution of silage

mycotoxins to the total amount of mycotoxins ingested by cows can be greater than the maximum concentrations allowed or recommended in ruminant diets by the US Food and Drug Administration and EU. Consequently, in addition to cereal grains and compound feeds, more surveillance studies of mycotoxins in forage sources and importantly complete rations are needed.

***Tab. 5**: Occurrence of mycotoxins in Europe*

	Data Source	**AFs**	**ZEA**	**DON**	**FUMs**	**OT A**
Number of tests	1	79	453	512	51	80
	2	74	759	1,103	70	90
	3	253	320	359	239	233
	4	17	176	239	34	28
Percent positive (%)	1	4	33	53	29	46
	2	28	23	59	50	28
	3	2	36	48	19	14
	4	12	16	54	29	29
Average (µg/kg)	1	0	37	968	478	1
	2	2	26	907	1,131	5
	3	0	80	637	166	1
	4	1	120	1,049	2,449	45
Median of positive (µg/kg)	1	1.8	42.5	503.5	530	2.5
	2	1.7	78.4	560.5	1,807	2.7
	3	6	139	462	533	3
	4					
Maximum (µg/kg)	1	3	1,045	49,000	5,489	12
	2	103	1,045	49,000	7,260	331
	3	9	2,146	14,326	3,134	35
	4	1	665	26,121	6,770	331

1 = Central Europe; HPLC; 2009-2010; animal feed; own representation based on Rodrigues and Nährer (2012)
2 = All Europe; HPLC or ELISA; 2010; animal feed; own representation based on Borutova and Nährer (2012)
3 = All Europe; HPLC or ELISA; 2010; silage; own representation based on Borutova and Nährer (2012)
4 = Central Europe; HPLC or ELISA; 2010; animal feed; own representation based on Rodrigues et al. (2010)

It can be seen that there are data available for the most known mycotoxins like AFs, DON, FUMs, OTs and ZEA, but less data for co-contaminations of several other mycotoxins and fungal metabolites. Such comprehensive data began to emerge due to advancement in

analytical methods based on high performance liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS).

2.4 Climate change impacts

The climate and the environment have a great impact on the presence of fungal growth and their mycotoxin production. Factors such as temperature, relative humidity, insect attack, drought, stress condition of the plants, unseasonal rain during harvest times, and extreme weather events influence the mycotoxin production by moulds (FAO, 2020; Miraglia et al., 2009; Paterson & Lima, 2011). Common knowledge states that the man-made climate change is already happening and has been more rapidly in the last years (Avery et al., 2019; European Commission SWD, 2013; Conte et al., 2018) and climate variabilities are responsible for about a third observed yield variabilities globally (Ray et al., 2015). Conditions related to climate changes relevant for agriculture are the rising temperature, variation in precipitation, droughts, and the rising atmospheric carbon dioxide (CO_2). In Central Europe, land degradation (for example soil erosion or loss of soil organic matter), the thrive of pests and diseases (Olsson et al., 2019), heat waves due to rising temperature, increased atmospheric CO_2 levels, frequent spells of drought and higher precipitations are already observed (Galieni et al., 2021). These factors affect the frequency of infection as well as the plant's health to fight off pathogens. Therefore, fungal colonization and mycotoxin contamination of field crops and subsequently final feed could differ or even thrive as a result of climate changes. Such changes have already been documented. For instance, in northwest Europe *Fusarium culmorum* and *Fusarium nivale* were usually the major pathogens found in the disease head blight, but now *Fusarium graminearum* took over. The problem is that *Fusarium graminearum* produces more mycotoxins than the others (European Commission SWD, 2013). Furthermore, due to climate change, there will be a change in the utilization of arable land, rendering some regions to become suitable or unsuitable for crop production, which might have effects on the distribution of fungal pathogens and therefore mycotoxin production. Such distributions already happened, AFs have become a problem in some parts of Europe (FAO, 2020) in Italy, Hungary (Paterson & Lima, 2011) and Serbia (Udovicki et al., 2018). In the common predicted scenario of a global warming of 2 °C, higher levels of AFs in Europe are anticipated (Battilani et al., 2016) as well as higher levels of DON (Van der Fels-Klerx et al., 2012a). It is worth mentioning that what is favourable for one mycotoxin may be unfavourable for the production of another. Van der Fels-Klerx et al. (2012b) showed that DON and ZEA concentrations increased in wheat with higher temperatures, relative humidity and rainfall during cultivation, whereas nivalenol decreased.

Tab. 6 is adapted from Ksenija (2018) and gives an overview of the favoured temperatures and water activity for some filamentous fungi capable of producing mycotoxins.

***Tab. 6**: Mycotoxins, associated fungi and optimal production conditions (Ksenija, 2018)*

Mycotoxin	**Fungi**	**Temp [°C]**	**Water activity [a_w]**
Aflatoxins	*Aspergillus flavus, A. parasiticus*	33	0.99
Ochratoxins	*Aspergillus ochraceus, A. carbonarius,* *Penicillium verrucosum*	15-30	0.85-0.98
Fumonisins	*Fusarium verticillioides, F. proliferatum*	10-30	0.93
Patulin	*Penicillium expansum*	24	0.99
Zearalenone	*Fusarium graminearum, F. culmorum*	25-30	0.98
Deoxynivalenol	*Fusarium graminearum, F. culmorum,* *F. sporotrichioides, F. roseum,* *F. tricinctum, F. acuminatum*	15-25	0.97-0.99

Taking a closer look into Austria, temperature is rising in recent years. The year 2019 was the third-warmest year in the last 252 years of Austrian weather recording, only the years 2018 and 2014 were warmer (Chimani et al., 2020). June 2019 was the warmest June ever since record-keeping began. The rainwater total was on average 5 % above the expectation, whereas the north and southeast were exceptionally dry and the west and southwest were too damp. The 14 warmest years ever since record-keeping were all, apart from the year 1994, from the year 2000 onwards (Chimani et al., 2020). The year 2020 was 2 °C warmer compared to the reference period 1961-1990 and the fifth hottest year for 253 years of measuring, 2018, 2014, 2019 and 2015 were warmer. Although there were no strong heat waves in the summer, the weather fluctuated but it was relatively too warm, August was rich in precipitation (Hiebl et al., 2021). Not only climate change influences mould growth and mycotoxin contamination of crops but it could also adversely affect livestock health and well-being making them more vulnerable to diseases and toxins. For instance, problems related to heat stress are expected in the future affecting the health, growth and reproduction of cattle as well as there will be a change in the feed production for cattle, for example less pastures and more crop fields (Jaykus et al., 2008). Climate change will have direct and indirect effects on the dairy sector,

which will lead to higher mortality rates, impaired immune functions, greater distribution of infectious diseases, reproductive problems, alterations in feed intake and growth and reduced milk yields (Gauly & Ammer, 2020). Therefore, the animals could be more sensitive to mycotoxins and the adverse effects therefore exaggerate.

It is apparent that mycotoxin contamination of feed is an alarming issue that affects not only feed safety but also food safety. There are modelling studies for predicting mycotoxin occurrence, but such models need detailed climate data, which is often hard to collect (Ksenija, 2018; Sloth Madsen et al., 2012). Importantly, while climate is the key driver of fungal colonization and mycotoxin production in plants, there are many factors such as agricultural land use and crop management, global trading of grains as well as differences among farms in feed choices, post-harvest methods and farm managements that could affect the level and distribution of feed contamination. Therefore, mycotoxin contamination of feed is constantly changing and require surveillance programs. There is an urgent need for actualized data on a reasonable scale because the available data in Austria in the past is not representative for today. All in all, active surveillance and advanced research data are needed to characterize profiles of mycotoxin contamination of feeds intented to dairy cattle nutrition, promoting safety in the feed and food chain.

3 Study aim

Mycotoxins represent risks for feed and food safety, deserving urgent research. The aim of this thesis was to determine the current status of mycotoxin contamination in complete diets of lactating dairy cows in Austria. Therefore, the current thesis performed a survey of feed contamination of a total of 98 pilot dairy farms in three different federal states dominating the Austrian dairy sector including Lower Austria, Upper Austria and Styria.

Advanced analytical tools and technologies provide possibilities to identify broad spectrums of fungal compounds including regulated, emerging and modified mycotoxins in addition to other (potentially toxic) secondary metabolites, which pave the way for research to study the complexity of mycotoxin mixtures in feeds. Using LC-MS/MS this study characterized the profiles of mycotoxins and other fungal metabolites contaminating representative samples of diets of lactating dairy cows in Austria in the year 2020. This study presents valuable data at the national scale for further investigations regarding the impacts of feed contamination on health and productivity of dairy cattle.

This thesis is purley an observational study, therefore no hypothesis are formulated.

4 Material and methods

For this survey study, the candidate farms were screened according to the following criteria:

- Location: Styria, Lower and Upper Austria (Austrian federal states dominating the dairy sector)
- Moderate to large farm size with the number of lactating cows >50
- Farms with active recording of animal health, productive and reproductive data

In total 98 pilot farms were enrolled and the sampling took place from June to September 2020. The sample preparation was performed from October to November 2020 and the LC-MS/MS analysis from January to February 2021.

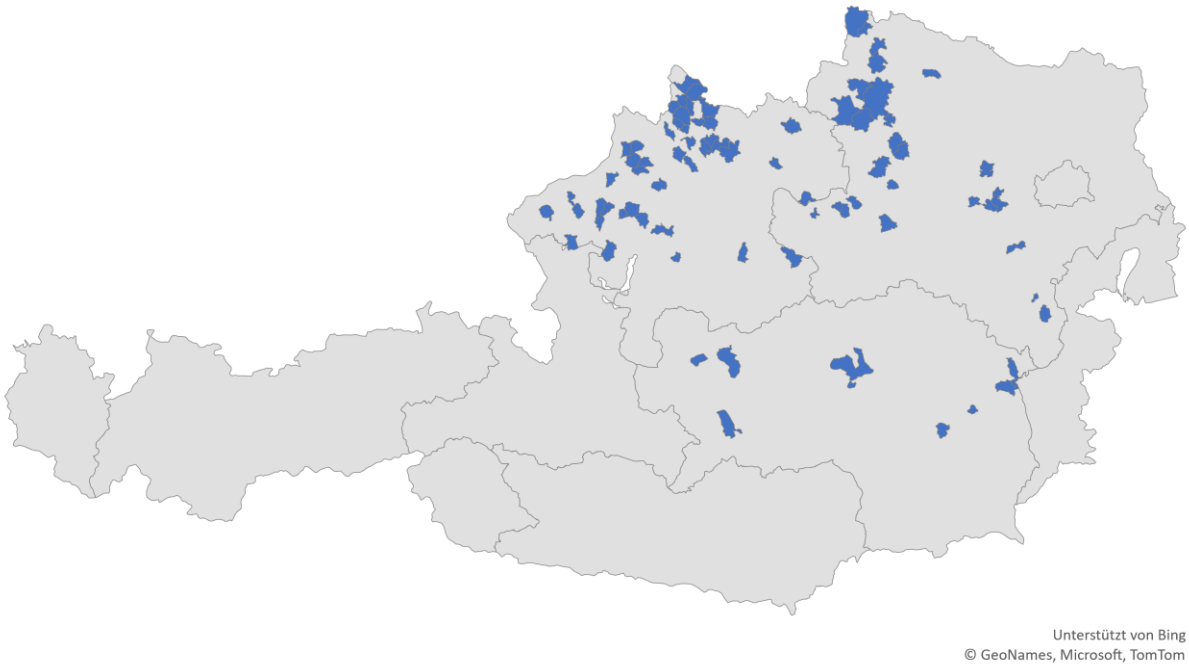

Fig. 3: Locations of sampled Austrian dairy farms

4.1 Feedstuff sample collection

Sample collection is very prone to error and therefore highly crucial for detection of mycotoxin contamination. As mycotoxins are not homogeneously distributed in feed, it is extremely difficult to collect a sample that accurately represents the mean concentration (Whitaker, 2003). Cows in Austria are often fed mixed rations either total mixed ration (TMR), where all feed components of the cow's diet are mixed and fed as a ration or partial mixed ration (PMR) that contains basal components and a proportion of concentrate feed which is given to each

cow separately (for example with transponder-controlled feed stations) depending on her level of performance. For PMR and TMR samples, it was necessary to collect at least handfulls of materials from numerous locations in order to collect a representative sample. Additionally, the sample gathering from the feed bunk must happen before the cows start to eat. This is due to fact that animal sorting of feed ingredients begins immediately after the feed delivery, which can falsify the outcome. Another factor contributing to a non-representative outcome is saliva contamination because it increases the wetness and spoilage of feed. As the collected samples of PMR or TMR are usually too large to submit for laboratory analysis, the samples must be mixed, quartered, and then bagged in smaller sizes (Robinson & Meyer, 2010). Fungi may grow and produce mycotoxins during shipment and storage, especially in wet feed. In order to prevent substantial fungi growth, the feed should be oven-dried to less than 13 % moisture. Known mycotoxins are not significantly degraded if the feed is dried at moderate temperatures (e.g. 60 to 70 °C). If wet feed cannot be dried immediately, it should be frozen (Carlson & Ensley, 2003).

Following these concerns and recommendations, the present study collected representative feed samples of the pilot farms. At each farm, two samples were collected. One sample of basal feed (PMR) and one sample of concentrate feed (i.e., grain mix). Only one farm had TMR and therefore, only one sample was gathered from this farm. Altogether, this resulted in a total of n=195 sub-samples. For collection of the basal feed (PMR or TMR) sample, 20-30 sub-samples from different spots of the feed bunk were collected manually in one bin before the cows started to eat. Each collection had to be done carefully to assure nothing gets lost and all sizes of particles were gathered. Afterwards, the collected sample was placed on a 1 m x 1 m plastic film and then mixed thoroughly by hand. After mixing, the sample was divided into four parts. One to two parts resulting in a total weight of 1 kg were sampled and vacuum-sealed in a plastic bag. To stop fungal growth and mycotoxin formation the vacuum-sealed samples were immediately stored at -20 °C until they were processed.

The samples of concentrate feed that was separately fed to cows were collected directly from milking robots, automatic feeders or silos. In case the cows were fed with more than one concentrate feed, a representative sample was composited considering the average intake of the different concentrates. The final sample size (at least 1 kg) of the representative concentrate feeds were vacuum-packed in a plastic bag and immediately stored at -20 °C until they were processed at the laboratory.

4.2 Feedstuff sample preparation

The samples were prepared prior to the mycotoxin analysis. Accordingly, the drying process was first performed for basal feed (PMR and TMR) samples because of their high water contents (>20 % H°). Each basal feed sample was divided into two parts and each one was first weighed, and then dried in an aluminium tray by an air-oven at 65 °C for 48 hours.

Thereafter, the dried basal feed samples as well as the concentrate feed samples were ground to a final particle size of 0.5 mm. For this procedure, the cutting mill SM 300® (Retsch GmbH, Germany) was used. First, every sample was ground with a 2 mm sieve. Subsequently, a 0.5 mm sieve was used. Everything that was not ground to 0.5 mm in the cutting mill SM 300® (Retsch GmbH, Germany), was later ground with the ultra centrifugal mill ZM 200® (Retsch GmbH, Germany) with a 0.5 mm sieve. Between the samples, everything which was used during the grinding process was properly cleaned with a vacuum cleaner to prevent cross contamination. The whole sample from the two machines was collected in a plastic bag and mixed.

After grinding, basal and concentrate feeds were pooled per farm according to their estimated intake data to create a representative sample of the complete diet of the farm, totalling to 98 representative samples for analysis. Finally, 5.00 g of each pooled sample were used for multi-mycotoxin analysis.

4.2.1 Liquid chromatography/tandem mass spectrometry analysis

Mycotoxin analysis was performed at the Institute of Bioanalytics and Agro-Metabolomics, the University of Natural Resources and Life Sciences (Tulln an der Donau, Austria) using a LC-MS/MS analysis (Spectrum 380®, Biomin GmbH, Tulln, Austria), which can detect and quantify over 400 compounds. This method allows accurate quantifications of several mycotoxins/metabolites because of its high reproducibility, sensitivity and selectivity. LC-MS/MS is useful especially for detection of modified and emerging mycotoxins (Lu et al., 2020).

For the LC-MS/MS analysis, an extraction solvent (acetonitrile/water/acetic acid 79:20:1) was added to each sample. Then the sample was extracted, and the resulting supernatant was transferred into auto-sampler vials and diluted with an equal volume of dilutions solvent (acetonitrile/water/acetic acid 20:79:1) and then a small fraction of the diluted raw extract was injected into LC-MS/MS instrument. Afterwards the compounds were quantified with a high degree of sensitivity and selectivity based on the unique mass/charge transitions of each compound of interest. Details regarding the instrument and analytical condition are described

by Sulyok et al. (2020). **Tab. 7** (see annex) contains a list about the apparent recovery, the limit of detection (LOD) as well as the limit of quantification (LOQ) of the LC-MS/MS analysis. Here the LOD is the smallest amount of a mycotoxin where there can be reliably distinguished if a mycotoxin occurs or not. The LOQ is the smallest amount of a mycotoxin that can be quantitatively detected with a stated accuracy and precision, so above this limit the mycotoxin will be stated quantified.

4.3 Statistical analysis

A descriptive statistical analysis was performed on the analysed mycotoxin data to characterize the occurrence and contamination levels in this thesis. Only compounds with values higher than the LOD were used, and those with values lower than the LOD were regarded as not detectable. Compounds with the concentrations below the respective LOQ were then computed as LOQ/2. The results are reported based on a dry matter (DM) basis in µg/kg and on a logarithmic scale (Log10). The graphs were generated using Microsoft Excel and for the co-occurrence analysis a matrix was constructed with the detection frequencies of the mycotoxins occurring ≥20 %.

5 Results

In total, there were 109 metabolites detected across all samples. The full list of all 109 metabolites including occurrence, average ± standard deviation, median, minimum and maximum concentration is presented in **tab. 8** (see annex)**.** Neither AFs nor OTs were found in the analysed samples. Only some highlighted findings are described below.

For the presentation of the results regarding concentration of metabolites, only the data of positive samples are used for the boxplots showing distribution of the concentration across all positive samples, bottom and top red dots indicate minimum and maximum concentration, grey crosses indicate average concentration. The bottom of the boxplot is the 25th percentile and the top is the 75th percentile, and the cross line represents the median concentration.

Fig. 4 provides an overview of levels of fungal metabolites summed up in different groups classified by their main producers: *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, ergot alkaloids, other fungal species, and *Lichen*. The groups have been set up to the current knowledge, but it is known that some mycotoxins and metabolites can be produced from multiple fungal groups. The fungi group *Alternaria* contained eleven different metabolites, *Aspergillus* 14, *Fusarium* 35, *Penicillium* 17, *Lichen* one and there were 18 compounds from other fungal species. The group ergot alkaloids contained 13 different metabolites.

All groups were detected in 100 % of the samples, except ergot alkaloids only in 33 % and *Lichen* in 2 %. The group of metabolites with highest levels of metabolites was *Fusarium* (mean: 1,663 µg/kg; Max: 5,271 µg/kg). Next to *Fusarium* was *Alternaria* showing the mean and max concentrations of 365 µg/kg and 1,350 µg/kg and mean concentrations of *Aspergillus* metabolites and ergot alkaloids were 3.4 and 19.6 times lower than *Alternaria*, respectively. Contamination levels of the groups *Alternaria, Aspergillus* and *Fusarium* showed a quite similar distribution, whereas ergot alkaloids, *Penicillium* and other fungal species had a more heterogeneously distribution across all samples. In total, the concentration of total fungal metabolites detected in the analysed samples ranged between 576 µg/kg and 6,230 µg/kg with an average of 2,599 µg/kg.

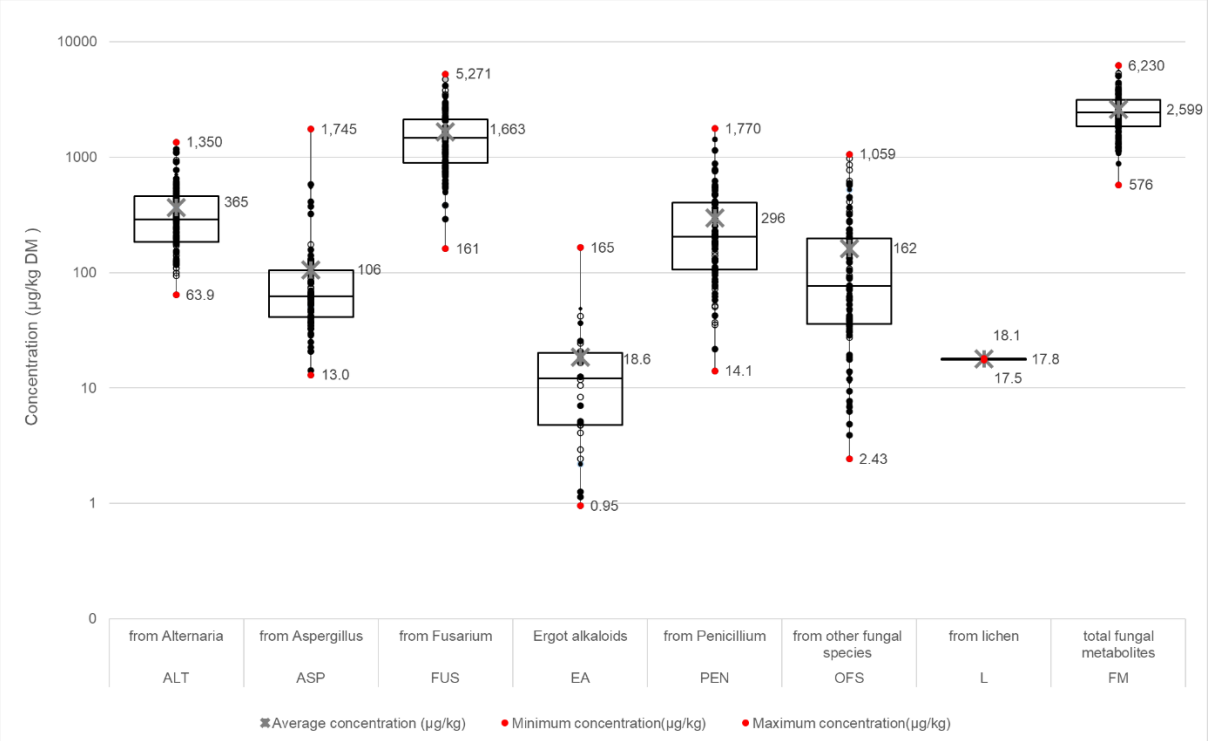

***Fig. 4**: Distribution of the concentration of fungal metabolites (by groups) detected in complete diets of Austrian dairy cows.*
Abbreviation: Alternaria (ALT), Aspergillus (ASP), Fusarium (FUS), Ergot alkaloids (EA), Penicillium (PEN), other fungal species (OFS), Lichen (L), total fungal metabolites (FM)

Fig. 5 and 6 show the occurrence and concentration of regulated mycotoxins by the EU recommendation (2006/576/EC, 2006/02.08.2016) detected in the collected samples. Only some of the mycotoxins regulated with the guidance value were detected, all of them were fusarial metabolites and none of the samples exceeded the guidance values. Among these mycotoxins, DON and FUM showed the higher mean concentrations than T-2 and HT-2 toxins and ZEA (**fig. 5**). Although T-2 and HT-2 toxins were presented together, almost all of the positive samples contained only HT-2 toxin and only one sample contained a detectable amount of T-2 toxin at 11.1 µg/kg. In terms of frequency, DON was most frequently detected (94 % occurrence) compared to the other three groups of regulated mycotoxins (**fig. 6**). All in all, DON was the most relevant mycotoxin in terms of occurrence and concentration among the regulated ones.

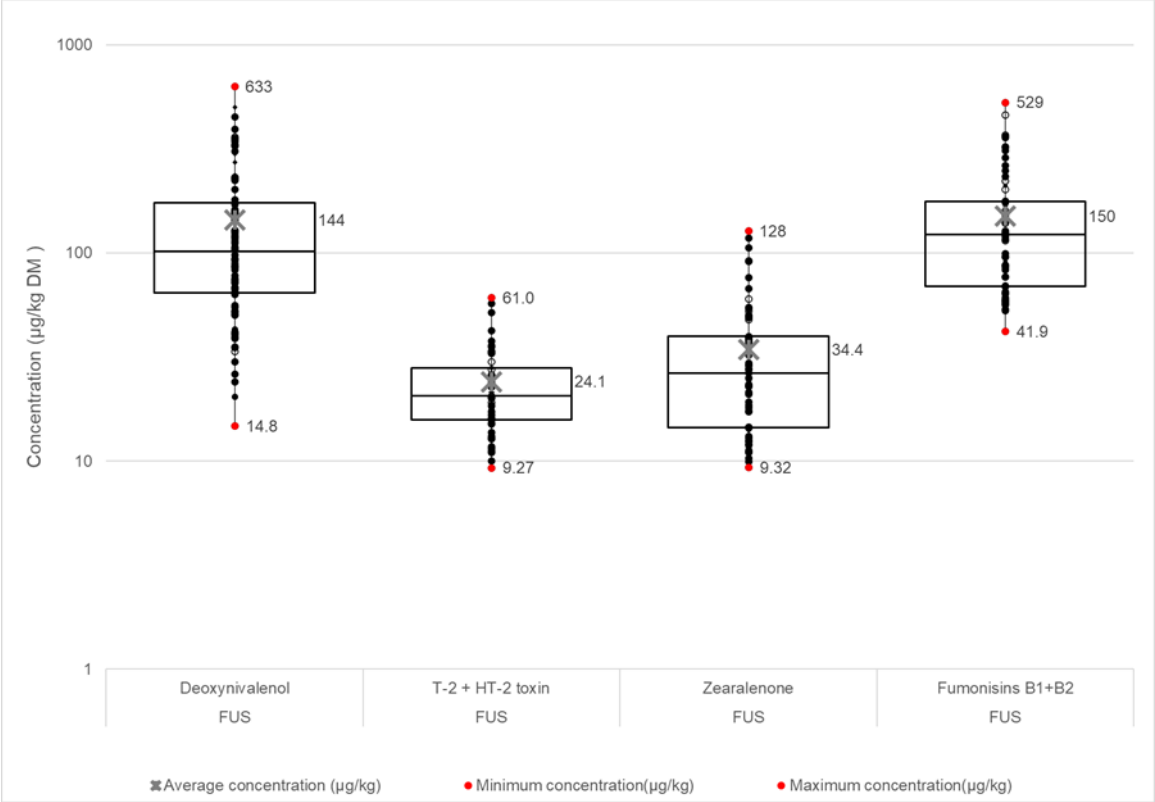

***Fig. 5**: Concentration of regulated mycotoxins by EU recommendations in diets of Austrian dairy cows*
Abbreviation: Fusarium (FUS)

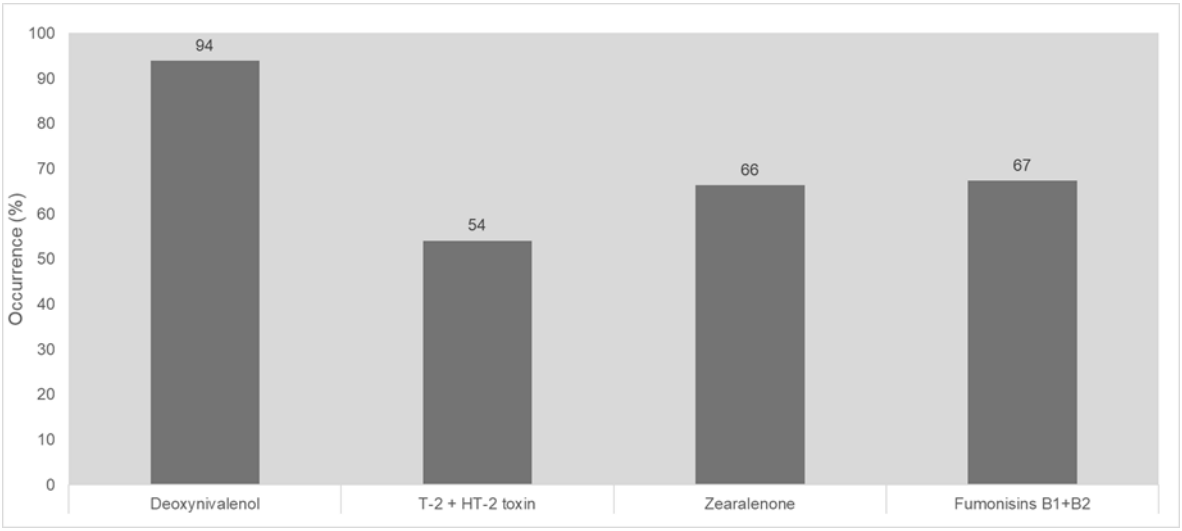

***Fig. 6**: Occurrence of regulated mycotoxins by EU recommendations in diets of Austrian dairy cows*

With respect to *Fusarium*, there were 35 fusarial metabolites detected and 13 metabolites are shown in **fig. 7**. DON-3-glucoside, nivalenol, T-2 toxin, HT-2 glucoside, monoacetoxyscirpenol, FUM B_3 and FUM B_4 were individually found only in one to seven samples. The other six fusarial metabolites had occurrences between 36 % and 94 %. All samples containing FUM B_3 or B_4 had levels >200 µg/kg of FUM B_1. The average concentration of most fusarial metabolites was between 10 µg/kg and 100 µg/kg, only DON, nivalenol and FUM B_1 were above 100 µg/kg. The concentration of the sum of all fusarial metabolites ranged from 41.9 µg/kg to 629 µg/kg with the average of 154 µg/kg.

***Fig. 7**: Concentration of Fusarium mycotoxins in diets of Austrian dairy cows*
Abbreviation: Fusarium (FUS)

As shown in **fig. 8**, there were 13 different ergot alkaloids detected across the samples. The distribution of ergot alkaloids concentrations was very heterogeneously. The most frequent was chanoclavin found in 15 out of 98 samples and it had also the highest maximum concentration with 46 µg/kg. The other ergot alkaloids were found less frequent and in lower concentrations. Only chanoclavin and festuclavine had an average concentration higher than

10 µg/kg. In the sample with the highest total amount of ergot alkaloids, eleven out of the 13 ergot alkaloids were detected. The concentration of the sum of all ergot alkaloids ranged from 0.95 µg/kg to 165 µg/kg with the average of 18.6 µg/kg.

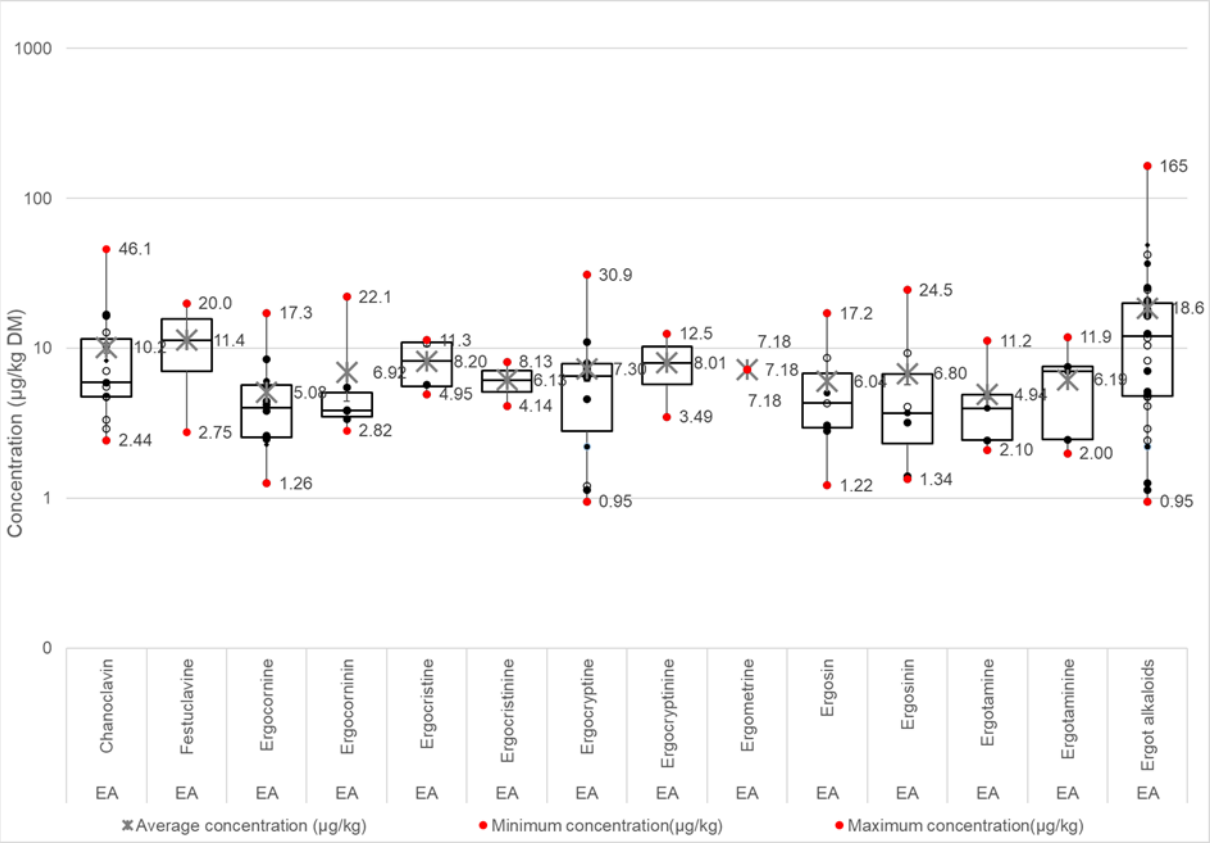

***Fig. 8**: Distribution of the concentration of ergot alkaloids in complete diets of Austrian dairy cows Abbreviation: Ergot alkaloids (EA)*

The concentrations of emerging fusarial mycotoxins are shown in **fig. 9**. These emerging mycotoxins were frequently presented in the samples. Beauvericin, enniatins, culmorin and 15-hydroxyculmorin were detected in more than 97% of samples (see **tab. 8** in annex). For most emerging fusarial mycotoxins, even their occurrence was very high, the respective average concentration stayed below 200 µg/kg, except culmorin with 425 µg/kg – the concentration 45.7 times higher than beauvericin. Moniliformin was found only in 14 samples and fusaproliferin in nine.

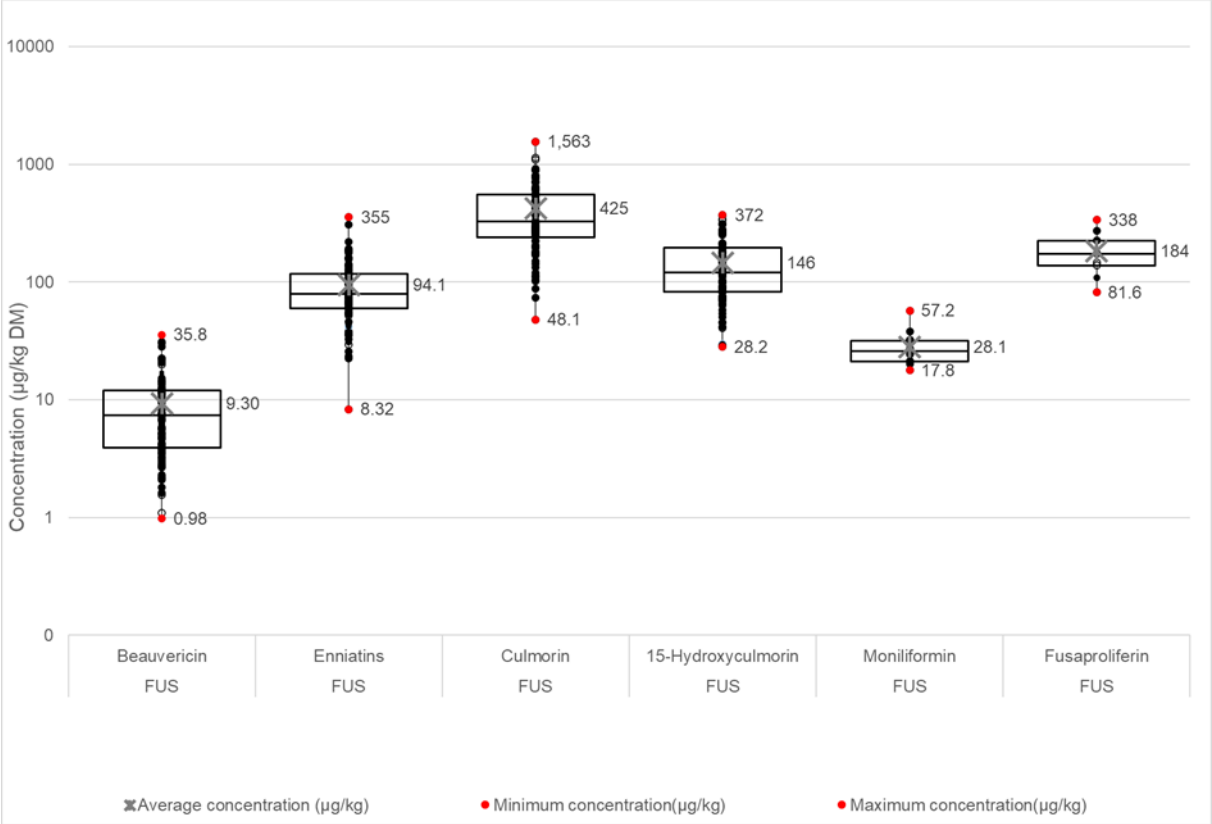

***Fig. 9**: The concentration of emerging Fusarium mycotoxins in complete diets of Austrian dairy cows Abbreviation: Fusarium (FUS)*

In **fig. 10** some other prevalent mycotoxins or fungal metabolites with high concentrations and high occurrence (≥95 %) are presented. These prevalent metabolites are produced by several fungal groups, in particular *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium*. All metabolites, except for phenopyrrozin, showed the average concentration between 100 µg/kg and 600 µg/kg in most of the tested samples. The concentration levels per farm of the *Penicillium* derived penicinoline and *Alternaria* derived infectopyron differed greatly, with 88 and 44 times different respectively between its minimum and maximum concentration.

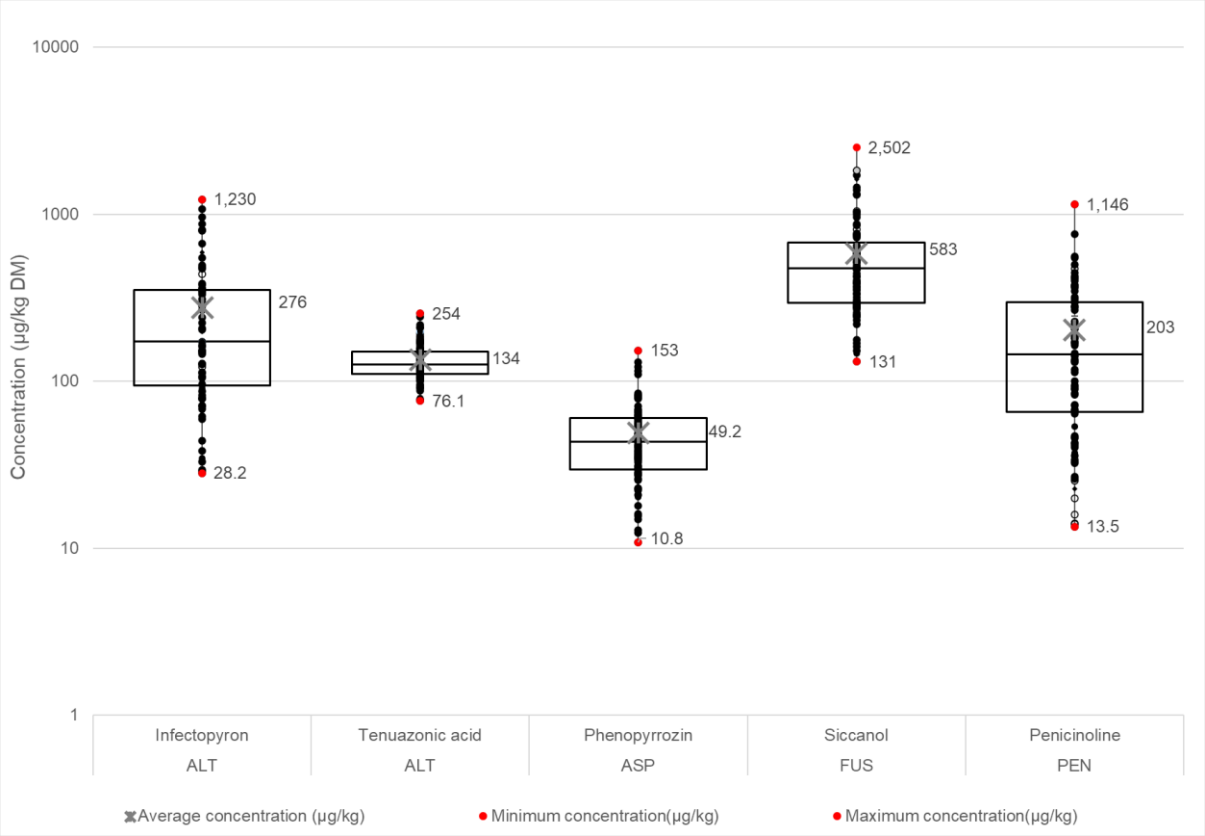

***Fig. 10**: Distribution of the concentration of other prevalent metabolites in complete diets of Austrian dairy cows*
Abbreviation: Alternaria (ALT), Aspergillus (ASP), Fusarium (FUS), Penicillium (PEN)

Concerning co-occurrence, on average, a sample co-contaminated with 33 metabolites. The majority (>50 %) of the farms showed a total of 28 metabolites in the feed sample. The farm with the highest number of co-contaminants had 53 fungal metabolites. **Fig. 11** presents the co-occurrence analyses of important fungal metabolites (metabolites with occurrence >20 %), excluding the groups *Penicillium* and other fungal metabolites. Co-occurrence is defined as the number of samples in total samples (in %) that contain a respective pair of mycotoxins and fungal metabolites. For example, within the regulated mycotoxins (yellow highlighted), 64 % of the samples co-contaminated with DON and FUM B_1 and only 37 % for DON and FUM B_2. The majority of considered mycotoxins and metabolites were of fusarial origin, especially emerging ones (aurofusarin, beauvericin, enniatin B and enniatin B1) and thus their very high co-occurrence levels. To take a look at the fusarial metabolites, it must be underlined that FUM B_1 did not always co-occur with FUM B_2. FUM B_1 was found in 64 samples and FUM B_2 in 36

samples. Furthermore, FUM B_2 was detected only in two samples without the occurrence of FUM B_1. As a result, FUM B_1 + B_2 co-occurred in 35 % of the samples.

Regarding co-occurrence with regulated mycotoxins, DON showed the highest co-occurrence with tenuazonic acid (93 %) from the group of *Alternaria*. DON also had higher levels of co-occurring with the two *Aspergillus* metabolites (phenopyrrozin and flavoglaucin) as well as with other emerging fusarial mycotoxins like beauvericin (94 %), enniatins (92 %), culmorin (92 %) and 15-hydroxyculmorin (93 %). The metabolites from the groups *Aspergillus* and *Alternaria* except for tentoxin were found often co-contaminating with emerging fusarial toxins (72-100 %).

Metabolites/Toxins with occurrence over 20% (without PEN and OFM)		from Alternaria			from Aspergillus		from Fusarium																			
		Infectopyron	Tentoxin	Tenuazonic acid	Phenopyrrozin	Flavoglaucin	15-Hydroxyculmorin	Antibiotic Y	Apicidin	Aurofusarin	Beauvericin	Bikaverin	Culmorin	Enniatin A	Enniatin A1	Enniatin B	Enniatin B1	Enniatin B2	Equisetin	Siccanol	W493	Deoxynivalenol	Fumonisin B1	Fumonisin B2	HT-2 toxin	Zearalenone	Zearalenone-sulfate
from Alternaria	Infectopyron		38	79	80	67	79	26	76	80	80	78	78	72	79	80	80	80	65	77	28	76	52	31	44	49	26
	Tentoxin	38		44	45	39	45	13	41	45	45	45	45	43	45	45	45	45	37	44	23	43	37	21	32	36	21
	Tenuazonic acid	79	44		99	80	98	32	91	99	99	97	97	90	97	99	99	98	79	94	38	93	64	37	53	66	36
from Aspergillus	Phenopyrrozin	80	45	99		81	99	32	92	100	100	98	98	91	98	100	100	99	80	95	38	94	65	37	54	66	36
	Flavoglaucin	67	39	80	81		80	24	73	81	81	80	80	73	80	81	81	81	62	77	31	77	57	34	45	53	30
from Fusarium	15-Hydroxyculmorin	79	45	98	99	80		32	91	99	99	97	97	90	97	99	99	98	79	94	38	93	64	36	54	66	36
	Antibiotic Y	26	13	32	32	24	32		31	32	32	32	31	28	32	32	32	32	24	31	17	30	19	6	15	24	14
	Apicidin	76	41	91	92	73	91	31		92	92	90	90	85	91	92	92	92	77	89	36	88	60	34	54	62	28
	Aurofusarin	80	45	99	100	81	99	32	92		100	98	98	91	98	100	100	99	80	95	38	94	65	37	54	66	36
	Beauvericin	80	45	99	100	81	99	32	92	100		98	98	91	98	100	100	99	80	95	38	94	65	37	54	66	36
	Bikaverin	78	45	97	98	80	97	32	90	98	98		97	89	96	98	98	97	79	94	38	93	65	37	54	66	35
	Culmorin	78	45	97	98	80	97	31	90	98	98	97		89	96	98	98	97	78	93	38	92	65	37	53	66	36
	Enniatin A	72	43	90	91	73	90	28	85	91	91	89	89		91	91	91	91	76	87	37	86	62	36	53	63	33
	Enniatin A1	79	45	97	98	80	97	32	91	98	98	96	96	91		98	98	98	80	94	38	93	64	37	54	66	35
	Enniatin B	80	45	99	100	81	99	32	92	100	100	98	98	91	98		100	99	80	95	38	94	65	37	54	66	36
	Enniatin B1	80	45	99	100	81	99	32	92	100	100	98	98	91	98	100		99	80	95	38	94	65	37	54	66	36
	Enniatin B2	80	45	98	99	81	98	32	92	99	99	97	97	91	98	99	99		80	95	38	94	65	37	54	66	35
	Equisetin	65	37	79	80	62	79	24	77	80	80	79	78	76	80	80	80	80		78	29	78	54	36	48	56	23
	Siccanol	77	44	94	95	77	94	31	89	95	95	94	93	87	94	95	95	95	78		37	92	63	37	52	64	32
	W493	28	23	38	38	31	38	17	36	38	38	38	38	37	38	38	38	38	29	37		35	22	12	24	32	22
	Deoxynivalenol	76	43	93	94	77	93	30	88	94	94	93	92	86	93	94	94	94	78	92	35		64	37	53	64	32
	Fumonisin B1	52	37	64	65	57	64	19	60	65	65	65	65	62	64	65	65	65	54	63	22	64		35	38	49	24
	Fumonisin B2	31	21	37	37	34	36	6	34	37	37	37	37	36	37	37	37	37	36	37	12	37	35		24	29	11
	HT-2 toxin	44	32	53	54	45	54	15	54	54	54	54	53	53	54	54	54	54	48	52	24	53	38	24		41	16
	Zearalenone	49	36	66	66	53	66	24	62	66	66	66	66	63	66	66	66	66	56	64	32	64	49	29	41		30
	Zearalenone-sulfate	26	21	36	36	30	36	14	28	36	36	35	36	33	35	36	36	35	23	32	22	32	24	11	16	30	

***Fig. 11**: Co-occurrence (%) of most recurrent mycotoxins and fungal metabolites detected in complete diets of Austrian lactating dairy cows*
Abbreviation: Penicillium (PEN), other fungal metabolites (OFM)

6 Discussion

6.1 Prevalence of mycotoxins

Using advanced technologies for analysis with high performance based on LC/MS-MS, in total 109 fungal metabolites were detected in diets used in 98 Austrian dairy farms. Individual samples contained on average 33 different metabolites. Once again, this study underlines the diversity of fungal metabolites; many of which are emerging mycotoxins. The validated method used in the present study is able to detect >500 different fungal secondary metabolites (Sulyok et al., 2020), this suggests the necessity to incorporate such analysis for routine surveillance of feeds.

The present survey of Austrian dairy diets showed that the fungal mycotoxins and metabolites based on their average concentration, the top fifteen with highest concentrations in descending order were integracin B (1076 µg/kg), siccanol (583 µg/kg), culmorin (425 µg/kg), kojic acid (359 µg/kg), infectopyron (276 µg/kg), integracin A (275 µg/kg), andrastin C (270 µg/kg), penicinoline (203 µg/kg), fusaproliferin (184 µg/kg), 15-hydroxyculmorin (146 µg/kg), nivalenol (145 µg/kg), DON (144 µg/kg), tenuazonic acid (134 µg/kg), FUM B_1 (124 µg/kg) and mycophenolic acid (97,7 µg/kg). Additionally, some of these had high maximum levels (>1000 µg/kg): siccanol (2,502 µg/kg), culmorin (1,563 µg/kg), infectopyron (1,230 µg/kg), penicinoline (1,146 µg/kg) and integracin B (1,076 µg/kg). Seven of these are mycotoxins and metabolites of *Fusarium*, and three of these toxins, including mycophenolic acid and roquefortine c, are from the fungi group *Penicillium,* indicating that contamination from this fungal group should not be overlooked. *Penicillium* toxins are considered the most relevant post-harvest toxins in conserved forages (Pahlow et al., 2003). Since they increase during ensilaging, they are even called “silage mycotoxins” (Oh et al., 2015), and silage is often a big part of the Austrian dairy cows diet. *Penicillium* toxins are known to have immunomodulatory and immunotoxin properties, cause reduction of the appetite as well as other negative effects on health (Gallo et al., 2015; Oh et al., 2015).

6.1.1 Regulated mycotoxins

Out of regulated mycotoxins, AF B_1 is the only mycotoxin for which maximum permitted levels have been set for feedstuffs under Directive 2002/32/EC. In the present study, *Aspergillus* produced AF B_1 was not detected in all samples. This is not surprising because AFs usually occurs in regions with tropical or subtropical climates. AFs in feedstuffs circulating in Austria can be found more often in imported feed (Streit et al., 2012). The forages from the farms in the study were produced nearly entirely in Austrian fields and forages are usually not the major

source of AF B_1 (Gallo et al., 2015). Many farms in the present study used commercial concentrate feeds which likely came from various origins. Nevertheless, because AF B_1 is strictly regulated by the EU, it also explains that feeds circulating in the EU markets are opted to have low or no AFs. Feedstuffs prone to AF contamination like peanuts and copra meal are not common ingredients used in dairy diets in Austria.

The present data pointed out regulated mycotoxins of *Fusarium* origin as the major contaminants in Austrian dairy feeds. As field fungi, *Fusarium* species thrive in the field and can continue to be active after harvest, especially when stored feeds are not properly dried (Kupper et al., 2020d; Whitlow & Hagler Jr., 2020). Hence, fusarial metabolites were found often across different types of dairy feedstuffs including pasture, conserved forages as well as cereal grains (Gallo et al., 2015; Knutsen et al., 2018a; Štýbnarová et al., 2016). *Fusarium* produced toxins have special relevance worldwide and some of them are regulated in several countries, including in EU guidelines. Streit et al. (2013) and Kemboi et al. (2020) as well as other authors determined that mixtures of *Fusarium* toxins are most commonly detected. Likewise, the findings of the present study also confirm the prevalence of *Fusarium* mycotoxins in dairy diets. In total, there were 35 different *Fusarium* toxins and metabolites, many of which with high occurrence levels identified, including emerging as well as new prevalent mycotoxins like siccanol (further discussed in **6.1.2**). Among regulated *Fusarium* mycotoxins, DON was the most prevalent in the present study, although the concentrations observed were never exceeding the EU guidance value. In comparison to older data, some differences could be outlined. Neuhold (1982) found DON levels between 700 µg/kg to 5,000 µg/kg in maize, oats, mixed feed, corn-silage and barley. Schuh (1983) detected DON in 46 % of mycotoxin-suspected feed samples. In the present study, the occurrence of DON in the complete dairy diets was more than twice as high, although the concentration levels were a lot lower than these old reports. A newer study from Kovalsky et al. (2016) reported median DON levels at 1,400 µg/kg in 2015 in Austria, which was many times higher than the current median concentration (102 µg/kg) observed in 2020 in the present study. It should be noted that it is not easy to compare studies because there were different surveyed ingredients and different methods of analysis used in the studies. DON is regarded as the most recurrent mycotoxin in silages and other forages (Gallo et al., 2015). In line with that, grass and maize silages were the main forages used in many of the pilot farms of the present study (visual observations, unreported data). The studies from Rodríguez-Blanco et al. (2021) and Venslovas et al. (2021) demonstrate that maize silage is most of the time heavier contaminated with mycotoxins than

grass silage, suggesting that increasing inclusion levels of maize silage could play a role in DON contamination. FUM B_1 + B_2 are also regulated *Fusarium* toxins. Though FUM B_1 was one of the fifteen metabolites with highest concentrations in this study, the concentrations never exceeded the guidance values.

Another regulated *Fusarium* mycotoxin is ZEA. The occurrence rates vary among studies. Schuh (1983) detected ZEA in 30 % of mycotoxin-suspected feed samples. In the present study, the occurrences of ZEA in the complete dairy diets were more than twice as high (66 %), although the ZEA concentration levels in the current study were relatively low compared to previous reports who studied mixed feed and other individual grains (Neuhold, 1982). When comparing with a newer study from Kovalsky et al. (2016) as well as with a long survey from 2008-2017 for mycotoxin occurrence in dairy feedstuffs in Central Europa (Gruber-Dorninger et al., 2019), the current ZEA medial levels were also lower but were found more often. The study from Panasiuk et al. (2019) showed that ZEA occurred more often and in higher levels in maize silage compared to grass silage, also Reisinger et al. (2019) found ZEA in 68 % of their tested maize silage samples. As maize made up a major component of the Austrian dairy cows' diet, it is not surprising that ZEA was frequently found in the samples. Interestingly, Vandicke et al. (2021) studied mycotoxin occurrence in maize silage in Belgium with the outcome that the mean concentration of every single detected mycotoxin decreased after ensiling, which points out that ensiling plays a role in reducing mycotoxin concentration. ZEA is known for its oestrogenic activity. Smith et al. (1990) conducted a study on ewes with the result that a ZEA intake of more than 3 mg/day impairs reproduction by lowering the ovulation rate and the percentage of lambings. In the current findings, the average concentration of ZEA was 34.4 µg/kg and the maximum concentration was 128 µg/kg. Assuming an approximate 20 kg feed DM intake per day and taking the average concentration observed, a cow would have an intake of 688 µg (or 0.69 mg) ZEA per day and an intake of 2.56 mg ZEA per day when taking the maximum concentration. By using the average ZEA concentration, one could claim a low risk for ZEA-associated fertility problems from the investigated Austrian dairy cows diets. However, the estimation based on the maximum value were only slightly below the toxicological limit, so it cannot be ruled out that in individual cases the ZEA contents from contaminated feed could reach toxicological values. Furthermore, one should not disregard possible interaction effects with metabolites with similar oestrogenic activity. These could influence ZEA contamination and thus also fertility. A synergistic effect seems plausible when

ZEA occurs together with other mycotoxins and xenoestrogens such as phytoestrogens as discussed by a current study (Penagos-Tabares et al., 2021).

Other regulated *Fusarium* toxins not yet discussed are T-2 + HT-2 toxin. Although the guidelines are written for the sum of these toxins, almost all of the positive samples in this study contained only HT-2 toxin and all of the combined samples were quite under the EU guidelines in cereals and cereal products. Only one farm was positive for T-2 toxin with the same mean concentration as Gruber-Dorninger et al. (2019) found in Central Europe. The other regulated mycotoxins, OT A and AF B_1 were not detected in the current study.

The EU regulate contamination of ergot sclerotia in unground cereals (maximum permitted level at 1,000 mg/kg) and recommend the monitoring of contamination levels of ergot alkaloids (2002/32/EC, 2002/28.11.2019). The fungi *Claviceps* and *Epichloë* produce ergot alkaloids in different plants including forage grasses and cereals (Penagos-Tabares et al., 2021). In particular, the fungi *Claviceps purpurea* are thriving and producing more mycotoxins during dry weather conditions (Kupper et al., 2020d). The diversity of ergot alkaloids in dairy feed is underlined by the present data showing 13 different ergot alkaloids detected, although they individually had often low contamination levels lesser than 10 µg/kg. Ergotism is primarily related to the *Claviceps* toxins such as ergotamine. This ergot alkaloid was detected in our samples with a greater mean concentration than most of the EAs detected. Coufal-Majewski et al. (2016) studied ergot alkaloids and published that feed contaminated with 250 µg/kg of ergot alkaloids should not be fed to pregnant animals as there is a higher risk of abortion and agalactia syndrome. In the present study, almost all samples had total ergot alkaloids concentrations <50 µg/kg, except for one with 165 µg/kg. Therefore, overall the Austrian dairy diet represents a low risk of toxic effects from ergot alkaloids. Still, sporadic cases of high ergot alkaloids concentration seem possible. The strict law in Europe controlling the contamination level of ergot sclerotia in unground grains will likely keep contaminantion of ergot alkaloids low in concentrate feeds. However, unregulated feed sources like forages and pastures also contribute to the level of contamination (Penagos-Tabares et al., 2021; Schiff, 2006) and therefore, dairy diets, especially unregulated feed sources, should be closely monitored for ergot alkaloids.

6.1.2 Emerging mycotoxins

Although the analysed dairy cow diets had regulated mycotoxin contamination levels below the EU guidance levels, a broad range of other mycotoxin and fungal metabolites was evident.

Still, reports for other mycotoxins than AFs, DON, FUMs, OTs and ZEA are generally scarce in the literature, possibly due to analytical limitations in the past research. With advanced technologies, new data began to emerge.

There are various methods for mycotoxin detection. Chromatographic methods like thin-layer chromatography and high-performance LC are commonly used for AF detection. Thin-layer chromatography was most used in the 1980s but it has low sensitivity and poor accuracy, and that is why high-performance LC replaced it. High-performance LC with immunoaffinity column clean-up is one ordinary method for the analysis of major mycotoxins. Enzyme-linked immunosorbent assay methods need confirmatory analysis using other procedures. Other "easier" methods like microplate reader or lateral flow strip still require expertise and well-trained operators (Singh & Mehta, 2020). More recently, LC-MS is being integrated with high sensitivity and accuracy as useful as high-performance LC. Moreover, it has a greater potential for multi-mycotoxin analysis in large number of samples.

In the present study testing complete dairy cow diets based on LC/MS-MS, many emerging and modified mycotoxins were detected, many of which occurred in high frequency. Most emerging *Fusarium* toxins had occurrences >97 %. Similar to the study from Fraeyman et al. (2017), we observed that none of the samples were free of enniatins and beauvericin. A much lower frequency was detected for moniliformin found only in 14 % of the farms, while Fraeyman et al. (2017) detected moniliformin in 76 % of their samples of grain and grain-based products. Relatively high occurrences (>70 %) of the mycotoxins emodin, culmorin, enniatin B1, enniatin B, and beauvericin were reported for maize silage samples (Reisinger et al., 2019). In addition to the high frequency, culmorin and 15-hydroxyculmorin were among the fifteen highest maximum values of all mycotoxins and metabolites detected in the present report.

Again, it must be underlined that the contamination levels presented here were for complete diets, not single feed sources. This fact must be kept in mind when comparing with other studies with different or single feed sources, as studies with complete diets were scarce in the literature. Various type of feeds represents different risks of contamination, which is influenced by, for example, type of fungus, type of feed, manufacturing process, and environmental influences. Geological, climate and weather situations are important environmental influences that affect the occurrence of mycotoxins and therefore may explain variations among studies. The year 2020 differed from 2014 when extreme weather conditions (high precipitation in July and August) were observed in Central Europe. The relatively high DON and ZEA contamination

levels in maize in 2014 reported by Gruber-Dorninger et al. (2019), as discussed by the authors, might be related to these extreme weather conditions. In terms of toxicology, there are not enough toxicity data available to evaluate the risks of emerging mycotoxins in ruminants, although there are studies about the metabolism of some fusarial emerging mycotoxin like enniatins and beauvericin in monogastric animals (Křížová et al., 2021). Nevertheless, due to their very high prevalence repeatedly observed in previous and present studies, there is an urgency to confirm their toxicological effects.

6.1.3 Co-occurrence of mycotoxins

Mycotoxin contamination is ubiquitous, which was confirmed in this study. Interestingly, the co-occurrence analysis (see **fig. 11**) showed that *Fusarium* metabolites, which were the most present, co-occurred frequently with some metabolites from *Alternaria* and *Aspergillus*, indicating the co-existence of multiple fungi in dairy diets. Specifically, *Alternaria*-produced infectopyron and tenuazoic acid and *Aspergillus*-produced phenopyrozin and flavoglaucin co-occurred with emerging *Fusarium* mycotoxins and, among the regulated mycotoxins, only DON in 75 % or more of the samples. This hints that the metabolism of fungi *Fusarium, Alternaria* and *Aspergillus* might be interlinked. In line with this presumption, according to Gavrilova et al. (2021), *Fusarium* and *Alternaria* adapt to each other in grain mycobiota and even some positive correlations between these two fungi and their mycotoxin production were observed. On the contrary, Hoffmann et al. (2021) showed in their experiment with wheat-ears that *Fusarium* and *Alternaria* show a competitive behaviour when they are present together, but the mycotoxin production of *Fusarium* in the presence of *Alternaria* stayed stable and interestingly, was not able to reduce the tenuazonic acid production. Both *Fusarium* spp. and *Alternaria* spp. are common colonizers of wheat (Schiro et al., 2018). Both fungi seem to favour similar climate conditions supported by the data from a recent study in pastures collected in 2020. Briefly, concentrations of metabolites from both fungi remained low at a temperature below 15 °C, and at warmer temperatures, the concentrations rose rapidly (Penagos-Tabares et al., 2021). Regarding the toxicity of *Alternaria* toxins, which were recurrent in diets of lactating dairy cows, the EFSA stated that there is limited research data and information on the toxic effects on farm and companion animals and their presence in feed, so the health risk to different animal species associated with *Alternaria* toxins in feed is unknown (Alexander et al., 2011).

To summarise, the contamination levels in Austrian lactating dairy diets were not alarmingly high and no EU regulations were exceeded. The most prevalent group of mycotoxins and metabolites is derived from *Fusarium*. Additionally, high prevalence of some emerging

mycotoxins, which risks are yet unknown, were found. While average concentrations did not raise a concern, attention must also be paid to maximum levels, which could lead to toxicological levels with high intake level as discussed before. This shows sporadic possibilities of high-risk diets in dairy farms in Austria. As already mentioned in the literature part, the assessment of the risk and safety of feed is based on regulations, recommendations and experimental data of individual mycotoxins. In nature, feeds contain a wide spectrum of fungal toxins and metabolites. Their levels, as an individual, might be small but they together acting accumulatively may influence the toxicology, but solid proofs of this require more research data. Besides, one should keep in mind that animals are continuously exposed to these metabolites and therefore, apart from acute effects, attention should also be paid to chronic effects. Another factor to keep in mind is the possible transfer of these compounds and their metabolites into edible products. For prevention measures, it is also important to recognise the causal factors that trigger increased levels of contamination in the first place. Many factors influence the frequency of mould infections as well as the health of the plants to the negative, for example, climate change implications like variation in precipitation, droughts, land degradation or the thrive of pests and diseases (for more, see chapter **2.4**). Due to the co-occurrence of metabolites combined with climate change implications and heat stress already possess a challenge on animal health, the adverse effects of mycotoxins could be higher than previously thought, may further jeopardize animal health and cause unexpected high economical losses for farmers. For future studies, data on the impact of chronic exposure to different mixtures of mycotoxins on health, reproductive and productive performance of dairy cattle would be beneficial.

7 Summary

Mycotoxins can be harmful to livestock and humans. The contamination of feed can lead to a reduction in animal welfare and productivity, which leads to economic losses. Mycotoxin contamination of feed commodities is on the rise worldwide. Notably, not single but complex fungal mycotoxins and metabolites are presented in feed commodities, many of which are not yet fully investigated. There are many influential factors and thus the issue of mycotoxin contamination is continuously evolving. The present study investigated the current situation regarding mycotoxin contamination in Austrian dairy farms. In total, 98 farms from three federal states dominating the dairy sector were investigated. The representative dairy cow diets were collected in summer 2020, prepared through drying and grinding for the applied analytical method (liquid chromatography combined with tandem mass spectrometry). In total, 109 fungal metabolites (from *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium* and others including ergot alkaloids) were detected. The co-contamination ranged from 20 to 53 metabolites per sample and, on average, a sample contained 33 different metabolites. Among regulated mycotoxins in the European law, deoxynivalenol, fumonisins B_1 + B_2 and zearalenone were the most occurring ones contaminating 94 %, 67 % and 66 % of the samples, respectively though none exceeded the European Union guidance level, and aflatoxins and ochratoxins were not found. A high prevalence of emerging mycotoxins predominantly from *Fusarium* was evident, some of which showed high concentrations. The three mycotoxins with the highest average concentrations in descending order were integracin B (1076 µg/kg, *Alternaria*), siccanol (583 µg/kg, *Fusarium*) and culmorin (425 µg/kg, *Fusarium*), where the maximum levels were a multiple of the average. Other emerging mycotoxins like beauvericin and enniatins were identified but with lower concentrations. Compared to literature, the contamination levels were not alarmingly high or could lead to acute toxicological effects. However, chronic effects due to the prolonged exposure as well as the transfer of toxic metabolites into food should not be ignored. Moreover, attention should also be paid to the interaction and accumulative effects of multiple mycotoxins and metabolites present in dairy cow diets.

8 Zusammenfassung

Mykotoxine können für Mensch und Tiere schädlich sein. Eine Kontamination von Futtermitteln kann zu vermindertem Tierwohl sowie reduzierter Leistung führen, was wirtschaftliche Verluste zur Folge hat. Jedoch steigt die Mykotoxinbelastung in Futtermitteln weltweit an. Insbesondere sind nicht einzelne, sondern mehrere komplexe Mykotoxine und Metaboliten in Futtermitteln vorhanden, von denen viele noch nicht vollständig erforscht wurden. Es gibt viele Einflussfaktoren, sodass sich die Problematik der Mykotoxinkontamination ständig verändert. Um Informationen über die aktuelle Situation bezüglich der Mykotoxinbelastung in österreichischen Milchviehbetrieben zu erlangen, wurde die vorliegende Beobachtungsstudie durchgeführt. Insgesamt wurden 98 Betriebe aus den drei Bundesländern untersucht, die den Milchsektor dominieren. Die Proben repräsentieren die gesamte Ration einer laktierenden Milchkuh und wurden im Sommer 2020 genommen, danach getrocknet und gemahlen, um sie für die angewandte Analysemethode (Flüssigchromatographie kombiniert mit Tandem-Massenspektrometrie) aufzubereiten. Insgesamt wurden 109 Pilzmetabolite (produziert von *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium* und anderen einschließlich Mutterkorn) nachgewiesen. Im Durchschnitt enthielt eine Probe 33 verschiedene Metabolite, die Co-Kontamination lag zwischen 20 und 53 Metaboliten. Alle Mykotoxine die in Rechtsquellen der Europäischen Union gelistet sind, außer Aflatoxine und Ochratoxine, wurden nachgewiesen. Davon waren Deoxynivalenol, die Fumonisine B_1 + B_2 und Zearalenon die am häufigsten vorkommenden, welche 94 %, 67 % und 66 % der Proben kontaminierten. Richtwerte wurden keine überschritten. Es zeigte sich eine hohe Prävalenz von „emerging mycotoxins“ (neuartige Mykotoxine), welche überwiegend von *Fusarium* spp. produziert werden und teilweise hohe Konzentrationen aufwiesen. Die drei Mykotoxine mit den höchsten Durchschnittskonzentrationen in absteigender Reihenfolge waren Integracin B (1076 µg/kg, *Alternaria*), Siccanol (583 µg/kg, *Fusarium*) und Culmorin (425 µg/kg, *Fusarium*), wobei die Maximalwerte ein Vielfaches der Durchschnittswerte betrugen. Ebenfalls konnten andere „emerging mycotoxins“ wie Beauvericin und verschiedene Enniatine identifiziert werden, jedoch in niedrigeren Konzentrationen. Im Vergleich zur Literatur waren die Kontaminationen nicht alarmierend hoch noch könnten sie zu akuten toxikologischen Wirkungen führen. Allerdings sollten chronische Auswirkungen aufgrund einer längeren Exposition sowie die Übertragung toxischer Metaboliten in Lebensmittel nicht außer Acht gelassen werden. Darüber hinaus sollten auch die Wechselwirkungen sowie akkumulativen Wirkungen mehrerer Mykotoxine und Metaboliten im Futter von Milchkühen berücksichtigt werden.

9 List of abbreviations

AF(s)	Aflatoxin(s)
DON	Deoxynivalenol
DM	Dry matter
EFSA	European Food Safety Authority
EU	European Union
FUM(s)	Fumonisin(s)
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MS/MS	Tandem mass spectrometry
OT(s)	Ochratoxin(s)
PMR	Partial mixed ration
TMR	Total mixed ration
ZEA	Zearalenone
ZEL	Zearalenol

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Tab. 7: Performance values of LC-MS/MS analysis

Metabolite	Apparent recovery (%)	LOD (µg/kg)	LOQ (µg/kg)	Metabolite	Apparent recovery (%)	LOD (µg/kg)	LOQ (µg/kg)	Metabolite	Apparent recovery (%)	LOD (µg/kg)	LOQ (µg/kg)
15-Hydroxyculmorin	93	7.0	23.4	Epiequisetin	138	1.0	3.2	Methylsulochrin	52	2.7	9.0
7-Hydroxypestalotin	78	1.6	5.2	Epoxycytochalsin C	77	1.2	4.0	Mevinolin	83	7.0	24.0
Acuminatum B	86	0.7	2.5	Equisetin	138	1.0	3.2	Moniliformin	97	5.3	17.7
Altenuisol	100	1.5	5.0	Ergocornine	96	0.3	0.9	Monoacetoxyscirpenol	66	3.3	11.1
Altemariol	45	3.4	11.3	Ergocorninin	66	0.7	2.4	Monocerin	67	1.3	4.2
Altemariolmethylether	52	3.3	11.0	Ergocristine	63	1.1	3.8	Mycophenolic acid	88	4.3	14.0
Altersetin	210	0.7	2.5	Ergocristinine	76	0.8	2.7	Myriocin	54	0.3	0.9
Andrastin A	50	0.5	1.5	Ergocryptine	55	0.1	0.4	Nivalenol	74	1.1	3.6
Andrastin B	52	2.5	8.5	Ergocryptinine	101	0.7	2.3	Penicinoline	74	2.8	9.0
Andrastin C	85	2.1	7.1	Ergometrine	77	0.3	1.0	Pestalotin	79	2.0	6.6
Antibiotic Y	95	5.7	19.0	Ergosin	58	0.2	0.6	Phenopyrrozin	112	0.8	2.6
Apicidin	65	0.5	1.5	Ergosinin	65	0.2	0.6	Pyrenophorol	74	2.4	8.1
Apicidin D2	37	4.2	13.9	Ergotamine	65	0.1	0.4	Questiomycin Derivat	100	1.0	3.0
Ascochlorin	42	2.3	7.5	Ergotaminine	65	0.1	0.4	Questiomycine	100	1.0	3.0
Aurofusarin	75	2.0	6.0	Festuclavine	57	0.6	1.9	Radicinin	100	0.5	1.5
Averufin	50	1.8	5.9	Flavoglaucin	47	0.4	1.3	Roquefortine C	41	3.5	12.0
Barceloneic acid	147	0.7	2.2	Fumigaclavine C	69	3.0	10.0	Roquefortine D	44	2.6	8.5
Bassianolide	58	1.6	5.4	Fumiquinazolin D	53	1.0	3.2	Rubellin D	49	1.7	5.6
Beauvericin	100	0.3	0.9	Fumonisin B1	119	16.0	53.0	Siccanol	111	7.0	23.0
Bikaverin	52	0.5	1.7	Fumonisin B2	106	11.0	36.0	Sporidesmolide II	100	0.5	1.5
Bis(methylthio)gliotoxin	58	1.0	3.3	Fumonisin B3	111	16.0	53.0	Sterigmatocystin	57	1.6	5.3
Calphostin C	88	0.7	2.3	Fumonisin B4	106	11.0	36.0	T-2 toxin	74	2.6	8.5
Chanoclavin	52	0.6	1.9	Fusaproliferin	100	10.0	30.0	Tentoxin	75	0.7	2.3
Chrysogin	92	0.7	2.4	Fusapyron	88	0.9	3.0	Tenuazonic acid	150	10.0	30.0
Citreohybridinol	38	0.4	1.2	HT-2 Glucoside	75	3.6	12.0	Ternatin	75	0.5	1.7
Culmorin	72	10.0	30.0	HT-2 toxin	72	2.3	7.6	Versicolorin C	33	2.3	7.5
Curvularin	77	0.7	2.3	Hydroxyandrastin C	56	1.9	6.2	W493	195	2.1	7.0
Cytochalasin B	59	1.7	5.5	Ilicicolin A	46	1.0	3.2	Zearalenone	70	2.8	9.2
Cytochalasin C	76	1.6	5.5	Ilicicolin B	36	2.7	8.9	Zearalenone-sulfate	100	1.0	3.0
Deoxygerfelin	100	0.5	1.5	Ilicicolin E	51	1.0	3.4	Zinndiol	77	0.6	2.1
Deoxynivalenol	83	3.6	12.0	Ilicicolin H	56	1.2	4.0	Zinniol	50	1.0	3.4
Destruxin B	51	0.4	1.4	Infectopyron	79	2.0	6.7				
DON-3-glucoside	100	11.0	38.0	Integracin A	87	1.2	3.9				
Emestrin	100	7.0	20.0	Integracin B	70	1.6	5.2				
Enniatin A	80	0.1	0.4	Kojic acid	58	87.0	290.0				
Enniatin A1	93	0.2	0.8	Lecanoric acid	57	2.9	9.5				
Enniatin B	71	0.9	2.8	LL-Z 1272e	48	2.5	8.2				
Enniatin B1	51	0.6	1.9	Marcfortine A	75	0.3	0.9				
Enniatin B2	103	0.1	0.2	Marcfortine C	75	0.3	0.9				

***Tab. 8**: List of all detected 109 metabolites in samples*

Group	Metabolite	Positive samples (%)[1]	Concentration (µg/kg)[2]		
			Average ± SD	Median	Range
Alternaria	Alternariol	14.29	27.5 ± 28.2	16.2	12.0 - 118
	Altenuisol	2.04	15.3 ± 5.31	15.3	9.96 - 20.6
	Alternariolmethylether	9.18	14.9 ± 2.94	14.2	11.7 - 20.0
	Altersetin	12.24	11.9 ± 5.92	10.8	4.16 - 24.1
	Infectopyron	79.59	276 ± 262	172	28.2 - 1,230
	Pyrenophorol	1.02	27.5 ± 0	27.5	27.5
	Tentoxin	44.90	4.52 ± 1.97	3.92	2.30 - 12.1
	Tenuazonic acid	98.98	134 ± 34.3	126	76.1 - 254
	Radicinin	2.04	4.44 ± 2.72	4.44	1.72 - 7.17
	Zinndiol	2.04	19.8 ± 1.91	19.8	17.9 - 21.7
	Zinniol	5.10	42.0 ± 23.7	36.4	22.4 - 87.7
Aspergillus	Averufin	1.02	8.03 ± 0	8.03	8.03
	Bis(methylthio)gliotoxin	5.10	12.8 ± 6.90	11.1	6.71 - 25.7
	Deoxygerfelin	3.06	12.2 ± 12.0	5.93	1.76 - 29.0
	Flavoglaucin	80.61	26.1 ± 53.8	8.59	1.57 - 348
	Fumigaclavine C	2.04	35.0 ± 23.6	35.0	11.4 - 58.6
	Fumiquinazolin D	3.06	24.0 ± 12.1	16.6	14.3 - 40.9
	Integracin A	1.02	275 ± 0	275	275
	Integracin B	1.02	1,076 ± 0	1,076	1,076
	Kojic acid	4.08	359 ± 92.1	314	293 - 516
	Methylsulochrin	2.04	18.2 ± 1.90	18.1	16.3 - 20.1
	Mevinolin	7.14	50.5 ± 31.5	42.8	28.6 - 126
	Versicolorin C	2.04	8.83 ± 0.88	8.83	7.95 - 9.72
	Phenopyrrozin	100.00	49.2 ± 29.0	43.5	10.8 - 153
	Sterigmatocystin	6.12	8.07 ± 1.55	8.21	5.95 - 10.3
Fusarium	Deoxynivalenol	93.88	144 ± 119	102	14.8 - 633
	DON-3-glucoside	2.04	53.5 ± 14.1	53.5	39.4 - 67.5
	Nivalenol	7.14	145 ± 102	100	34.6 - 329
	T-2 toxin	1.02	11.3 ± 0	11.3	11.3
	HT-2 toxin	54.08	23.9 ± 11.5	20.5	9.27 - 61.0
	HT-2 Glucoside	1.02	22.7 ± 0	22.7	22.7
	Monoacetoxyscirpenol	3.06	18.2 ± 3.25	16.8	15.2 - 22.7
	Zearalenone	66.33	34.4 ± 26.3	26.5	9.32 - 128
	Zearalenone-sulfate	35.71	17.0 ± 9.86	14.3	3.42 - 43.5
	Fumonisin B1	65.31	124 ± 78.3	93.7	53.0 - 420
	Fumonisin B2	36.73	55.8 ± 18.4	48.4	36.1 - 109
	Fumonisin B3	4.08	59.1 ± 3.40	60.9	53.2 - 61.2
	Fumonisin B4	1.02	38.2 ± 0	38.2	38.2
	Beauvericin	100.00	9.30 ± 7.29	7.38	0.98 - 35.8
	Enniatin A	90.82	1.40 ± 1.42	0.98	0.40 - 10.7
	Enniatin A1	97.96	6.29 ± 4.40	5.18	0.99 - 25.7
	Enniatin B	100.00	44.8 ± 29.9	35.9	4.34 - 175
	Enniatin B1	100.00	31.6 ± 20.2	25.5	2.42 - 126
	Enniatin B2	98.98	1.09 ± 0.74	0.87	0.22 - 4.70
	Culmorin	97.96	425 ± 291	325	48.1 - 1,563
	Apicidin D2	6.12	27.7 ± 13.9	22.5	17.5 - 57.2
	15-Hydroxyculmorin	98.98	146 ± 84.8	121	28.2 - 372
	Antibiotic Y	31.63	51.2 ± 37.2	38.0	19.7 - 175
	Apicidin	91.84	15.8 ± 16.8	10.6	2.29 - 105
	Aurofusarin	100.00	56.0 ± 38.8	43.6	6.79 - 214
	Acuminatum B	15.31	47.5 ± 18.7	38.8	23.2 - 80.7
	Chrysogin	5.10	25.6 ± 4.25	25.6	18.7 - 31.0
	Epiequisetin	19.39	6.07 ± 3.60	4.24	3.04 - 15.5
	Equisetin	79.59	8.53 ± 7.10	5.70	3.22 - 36.8
	Moniliformin	14.29	28.1 ± 9.81	25.8	17.8 - 57.2
	Siccanol	94.90	583 ± 415	473	131 - 2,502
	Bikaverin	97.96	28.2 ± 27.4	19.0	3.83 - 161
	Fusapyron	4.08	10.9 ± 9.61	6.42	3.49 - 27.5
	Fusaproliferin	9.18	184 ± 76.8	174	81.6 - 338
	W493	37.76	23.1 ± 13.3	18.6	8.07 - 55.8

Ergot alkaloids	Chanoclavin	15.31	10.2 ± 10.6	5.93	2.44 - 46.1
	Festuclavine	2.04	11.4 ± 8.62	11.4	2.75 - 20.0
	Ergocornine	12.24	5.08 ± 4.13	4.02	1.26 - 17.3
	Ergocorninin	6.12	6.92 ± 6.85	3.85	2.82 - 22.1
	Ergocristine	4.08	8.20 ± 2.88	8.26	4.95 - 11.3
	Ergocristinine	2.04	6.13 ± 2.00	6.13	4.14 - 8.13
	Ergocryptine	14.29	7.30 ± 7.18	6.55	0.95 - 30.9
	Ergocryptinine	2.04	8.01 ± 4.52	8.01	3.49 - 12.5
	Ergometrine	1.02	7.18 ± 0	7.18	7.18
	Ergosin	7.14	6.04 ± 5.02	4.32	1.22 - 17.2
	Ergosinin	7.14	6.80 ± 7.64	3.70	1.34 - 24.5
	Ergotamine	5.10	4.94 ± 3.31	3.97	2.10 - 11.2
	Ergotaminine	5.10	6.19 ± 3.65	7.01	2.00 - 11.9
Penicillium	Mycophenolic acid	11.22	97.7 ± 181	33.6	14.1 - 661
	Roquefortine C	16.33	56.1 ± 91.9	25.0	12.3 - 387
	Roquefortine D	1.02	20.6 ± 0	20.6	20.6
	Questiomycine	58.16	9.88 ± 9.74	6.44	3.03 - 49.2
	Questiomycin Derivat	37.76	58.4 ± 153	32.6	9.82 - 973
	Andrastin A	19.39	32.6 ± 39.9	14.0	1.80 - 140
	Andrastin B	8.16	68.8 ± 66.6	48.4	16.5 - 238
	Andrastin C	7.14	270 ± 170	247	43.4 - 603
	Hydroxyandrastin C	4.08	14.7 ± 5.32	14.6	8.68 - 20.8
	7-Hydroxypestalotin	2.04	7.96 ± 1.11	7.96	6.86 - 9.07
	Barceloneic acid	35.71	35.2 ± 29.4	23.7	7.84 - 133
	Citreohybridinol	1.02	5.38 ± 0	5.38	5.38
	Curvularin	5.10	6.09 ± 3.22	5.30	2.54 - 12.2
	Marcfortine A	27.55	12.0 ± 18.7	4.00	1.10 - 81.0
	Marcfortine C	11.22	3.32 ± 3.26	1.71	1.03 - 12.1
	Penicinoline	97.96	203 ± 186	145	13.5 - 1,146
	Pestalotin	12.24	8.55 ± 1.47	8.50	6.62 - 11.3
Other fungal species	Ascochlorin	1.02	8.25 ± 0	8.25	8.25
	Bassianolide	1.02	24.4 ± 0	24.4	24.4
	Calphostin C	1.02	8.34 ± 0	8.34	8.34
	Cytochalasin B	4.08	35.2 ± 13.4	35.0	16.5 - 54.3
	Cytochalasin C	2.04	8.77 ± 0.87	8.77	7.90 - 9.64
	Destruxin B	36.73	6.97 ± 8.68	3.39	1.44 - 44.1
	Emestrin	4.08	25.8 ± 3.28	25.0	22.3 - 31.0
	Epoxycytochalsin C	7.14	7.00 ± 2.71	5.46	4.20 - 12.2
	Ilicicolin A	4.08	5.97 ± 2.42	4.99	3.86 - 10.1
	Ilicicolin B	9.18	16.9 ± 4.83	17.1	9.27 - 23.0
	Ilicicolin E	6.12	6.79 ± 2.14	6.02	3.93 - 10.2
	Ilicicolin H	40.82	16.6 ± 21.0	10.7	4.18 - 123
	LL-Z 1272e	2.04	15.1 ± 6.21	15.1	8.89 - 21.3
	Monocerin	57.14	77.6 ± 173	15.1	4.75 - 893
	Myriocin	2.04	41.4 ± 24.0	41.4	17.4 - 65.3
	Rubellin D	65.31	51.3 ± 63.3	22.3	6.19 - 301
	Sporidesmolide II	98.98	68.7 ± 116	24.0	1.62 - 617
	Ternatin	1.02	16.14 ± 0	16.1	16.1
lichen-associated fungi	Lecanoric acid	2.04	17.8 ± 0.30	17.8	17.5 - 18.1

[1] n= 98 samples, samples with values > LOD; [2]Excluding data < LOD. In case values >LOD and < LOQ, LOQ/2 was used for calculation