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Changes in the nutritional profile, mycotoxins, and phytoestrogens from spring till late summer in selected horse pastures in Lower Austria

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Content

1. Introduction, aims and hypotheses

Pastures traditionally have played an important role in equine's lives. Not only do they serve as a feed resource, but they also act as exercise areas while grazing. If managed properly, they supply the horse with energy, protein, vitamins, minerals and other nutrients (Cunha 1991). However, due to climate conditions (i.e., cold and snow in northern Europe, droughts, and low precipitation in southern Europe) it is not everywhere possible to ensure grazing all year round. Therefore, meadows are used to produce hay in order to overcome times with limited or no access to pasture (Geor 2013).

Evolutionary, horses are herbivores and are used to a constant intake of forage feed. With unlimited access to pastures, horses spend at least twelve hours a day grazing to meet their needs of nutrients. When there is no access to pastures, their needs have to be met by hay, which means a daily dry matter (DM) intake of 1.5-2.0 % of bodyweight (Jeroch et al. 2020).

The nutritional composition of pastures is important, considering that they provide a horses' basal dietary component, the roughage. Roughages (i.e., grass, hay, haylage) play an important role for the functioning of the equine GI-tract, too. Therefore, it should be ensured that a horse gets at least 1.5 kg/100 kg body mass of DM per day (Coenen and Vervuert 2020). Moreover, depending on the nutritional composition, the supply and the equine's health can be affected. To give some examples: energy-rich grass may lead to obesity and high concentrations in fructans can trigger the onset of laminitis (Geor 2009). Mineral and/or trace element deficiency can influence the reproductive, immune and nervous system (Coenen and Vervuert 2020). High fibre or protein concentrations lead to lower digestibility and dysbiosis, respectively. Furthermore, among others, deficiencies in protein and zinc, are known to have a bad influence on hoof development and quality (Coenen and Vervuert 2020). In addition, the contaminants in the pasture also may pose a risk for the horse health. Therefore, analyses on the occurrence of mycotoxins and secondary plant compounds (i.e., phytoestrogens (PE)) on pastures are of great interest, since little is known about the contamination with these substances when compared to data in grains and conserved feed. However, most investigations concentrate on the regulated mycotoxins by the European legislation such as aflatoxin B1, deoxynivalenol (DON), zearalenone (ZEN). Hence, the modified and emerging mycotoxins (metabolites from the parent forms) are left out of consideration although their presence may have detrimental effects on animal health and reproduction (Penagos-Tabares et al. 2021).

Depending on different developmental stages of the plants and weather conditions, both the nutritional composition and the contaminant load varies. Since every individual horse has specific dietary needs, in particular horses with certain health conditions or at different ages, research on the nutritional profile of pastures is of great interest. This applies to equine veterinarians and horse owners. In this regard, the main aim of the current study was to determine the nutritional profile of horse pastures in Lower Austria and to formulate recommendations on which forage is most suitable for horses. For this purpose, the chemical proximate composition, minerals and trace elements, as well as mycotoxins, metabolites and plant derived compounds were analysed. This thesis has four main hypotheses such as:

1. Pastures have different nutritional profiles corresponding with the presence of different plant species: A high ratio of legumes leads to high concentrations of crude protein (CP) and calcium (Ca).
2. The developmental stage of a plant influences the nutritional profile of the pasture.
    a. The younger the plant, the higher the concentrations of fructans, minerals and trace elements.
    b. During the process of maturing, plants become richer in fibre.
3. Environmental conditions influence nutritional profiles of pastures:
    a. Low average temperatures stimulate the production and storage of fructans.
    b. High average temperatures cause a lower yield.
4. Mycotoxins and other contaminants are always present on pastures, though in varying concentrations, depending on the growth stage of the plants and weather conditions.

2. Literature review

2.1. Pastures and meadows in horse feeding

With pastures and meadows as the main contributors to an equines' diet, it is important to know which plant species they contain, which nutrients the plants provide, how this profile is affected by environmental conditions, and how certain nutrients can affect an equines' health.

2.2. Botanical composition of pastures and meadows

The typical botanical composition, or the nature and amount of plant species that make up a grassland (Gibson 2009), involves three main plant families: grasses, legumes and herbs, with each having a different suitability for dietary use (Coenen and Vervuert 2020, Kamphues 2014). This botanical composition is highly dependent on the climate and soil conditions, as well as on the type of use (i.e., grazing or haying) and the use of fertilizers (Jeroch et al. 2020). The higher the diversity of the botanical composition of a grassland, the higher the palatability, the yield and nutrient supply. Ideally, grasses make up about 70-80 % of the composition, whereas legumes and herbs contribute between 10 % and 15 % each. Table 1 shows the three main plant families and some of their subspecies. As mentioned before, soil and climate have a major influence on growing conditions. For example, grasses are overrepresented in moist areas with a lot of precipitation, while legumes and herbs prefer dry and calcareous soils (Coenen and Vervuert 2020). Moreover, roughage consists of vegetative fibres (stems, leaves) or a mix of plant and generative tissue (flower, seeds). During the growth process, the percentages of these tissues in the plants change. Therefore, the nutritional value of roughage depends on plant species, botanical composition, growth conditions, plant maturity and time of use. Additionally, factors like soil, climate, fertilisation, stage of growth, and time of utilisation can influence the botanical composition of grassland (Jeroch et al. 2020).

Tab. 1: Plant families and some of their subspecies commonly found on horse pastures (modified after Coenen und Vervuert 2020).

Plant family	Subspecies
Grasses	Upper grasses: *Frestuca pratensis, Phleum pratense pratense, Alopecurus pratensis, Arrhenatherum elatius, Trisetum flavescens,*

	Dactylis glomerata, Bromus inermis, Phalaris arundinacea Lower grasses: *Lolium perenne, Poa trivialis pratensis, Poa trivialis trivialis, Agrostis alba, Festuca rubra, Poa trivialis annua*
Legumes	*Trifolium repens, Trifolium pratense, Trifolium hybridum, Vicia sepium sepium, Vicia sepium cracca, Lythyrus pratenis*
Herbs	*Taraxacum officinale officinale, Leontodon autumnalis, Achillea millefolium, Pimpinella major, Plantago lanceolata, Sanguisorba officinalis, Rumex acetosa acetosa* etc.

2.2.1. Grasses

Grasses, a large family of monocotyledonous plants (Frame 2005), are the dominant species in pastures and therefore determine the amount of forage for many species of herbivorous animals like horses and equines (Coenen and Vervuert 2020). Typically, grasses consist of jointed stems and leaves. The leaves are divided in leaf sheath and leaf blade. The latter can be lanceolate, linear, ovate or oblong (Gandhi et al.). Upper grasses are characterized by high flowers with minor leaves, whereas lower grasses have more leaves and shorter stems. Due to their low crude fibre content, lower grasses are easier to digest and richer in energy. Depending on the species and on environmental aspects, different grasses have a different taste and nutritional value (Coenen and Vervuert 2020). In terms of their most favourable growing environment and metabolic characteristics/metabolic processes, two types of grasses can be distinguished: 1) cool season grasses, also known as temperate grasses or C3 grasses, and 2) warm season grasses, also called subtropical grasses or C4 grasses (Richards et al. 2021). Accordingly, C3 grasses are adapted to wet and dry areas, whereas C4 grasses are established in moist and dry areas (Saastamoinen 2012).

2.2.2. Legumes

Legumes are dicotyledons, which means that two leaves emerge from the soil (Frame 2005). In particular, alfalfa and various clovers are the most widely used species (Richards et al.

2021). In general, legumes are rich in CP, Ca and magnesium (Mg) and are easily digestible (Coenen and Vervuert 2020, Richards et al. 2021). Based on their lower fibre content than grasses, legumes are a more energy-rich forage. Unlike grasses, the leaves of legumes fulfil only a minor part of the structural function, which makes them easier to digest. The higher content of proteins and Ca lead to a higher pH, thus a more alkaline gastric fluid. This condition provides a higher buffering capacity against the development and/or the progression of gastric ulcers in horses and equines (Saastamoinen 2012).

2.2.3. Herbs

Herbs are a heterogeneous group that, next to edible food plants, also include weeds and poisonous species. In contrast to grasses, herbs partly contain more trace elements and Ca. However, if present in excess on a pasture, herbs will reduce the yield (Coenen and Vervuert 2020).

2.3. Chemical composition of pastures and meadows

In general, green plants produce their own energy through the process of photosynthesis. This is the process whereby sunlight and water are used to convert atmospheric carbon dioxide into glucose, which is subsequently metabolized to build various other compounds. This process mainly takes place in the chlorophyll granules of the leaves and is the most efficient in the middle developmental stage of a plant because more photosynthate is being produced than used. Whereby unused photosynthates are distributed to other plant parts. The products of photosynthesis are distributed throughout the plant and used for the metabolization of reserve carbohydrates, for storage in seeds and the integration into cell walls for structural purposes (Geor 2013).

In general, plant cells can be divided into 1) cell content (sugars, starches, soluble carbohydrates, pectin, non-protein nitrogen, proteins, and lipids) and 2) cell wall-components (hemicellulose, cellulose, and lignin). Carbohydrates are the most important nutrient group in forages and make up to 80 % of DM (Ellis 2006). They contribute the most to the energy supply of equines (Cunha 1991).

2.3.1. Water

In general, a significant part of forage consists of water (approximately 65-85 %), making it bulky but at the same time easily tainted if conserved inappropriately (Jeroch et al. 2020). This

should be considered when performing a nutritional analysis. Therefore, the grass samples should be pre-dried for homogenisation because initially, the proportion of water would be too high for that procedure (Jeroch et al. 2020).

2.3.2. Carbohydrates

Depending on their localisation in plants, carbohydrates can be divided into structural and non-structural (NSC) carbohydrates (Fig. 1). The latter composes of cell contents like simple sugars, starches, and soluble fibres. These are digestible to mammals through endogenous enzymes, whereas cell wall components can only be fermented by microbes in the hindgut (Geor 2010).

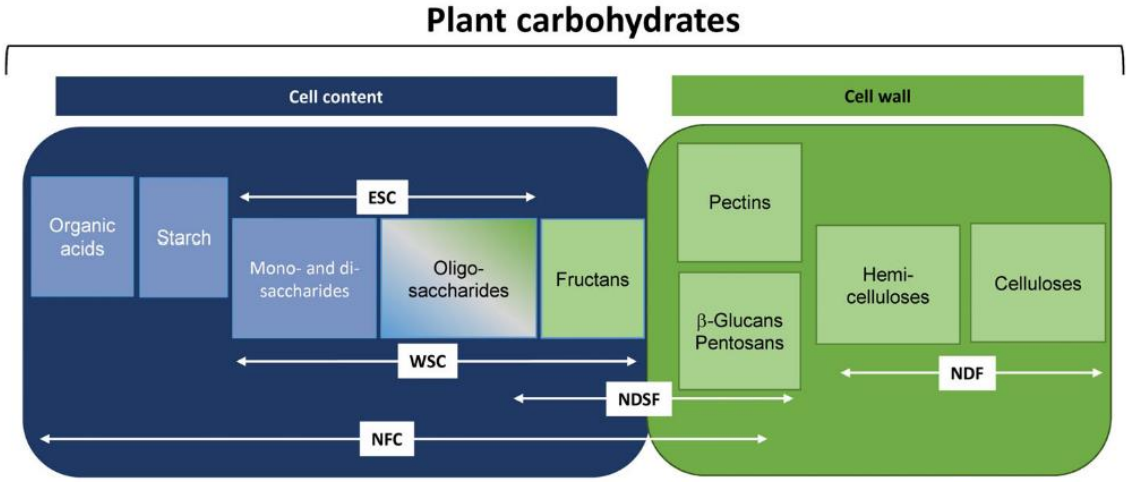

Fig. 1: Distribution of carbohydrates in the plant after Klevenhusen, Zebeli et al (2021). The blue coloured carbohydrates represent the structural carbohydrates in the cell walls (structural function), the green coloured carbohydrates represent the storage carbohydrates within the plant cells (energy source). Abbreviations: ESC, ethanol-soluble carbohydrates; WSC, water-soluble carbohydrates; NFC, non-fibre carbohydrates; NDSF, neutral-detergent-soluble fibre; NDF, neutral-detergent fibre.

Above all, plants use storage polysaccharides for metabolic processes, thus these sugars must be rapidly degradable and ready to use. Within the cell walls, the major carbohydrates are cellulose and hemicellulose, which both fulfil a structural function. In contrast, intracellular, the mono-, di- and oligosaccharides as well as the storage polysaccharides starch and fructan have an important function as energy reserve. Green plants hardly accumulate starch (Klevenhusen and Zebeli 2021), but in C4 grasses and legumes it is the main storage carbohydrate

(Longland and Byrd 2006). Furthermore, starch can be found in endosperm of grains, seeds, and tubers. In contrast, cool season grasses (C3) store water-soluble carbohydrates (WSC) uniquely during daytime and use these energy sources for different growing processes in the absence of daylight (Klevenhusen and Zebeli 2021). WSC include simple sugars and fructans. Together with starch they form the group of NSC (Longland and Byrd 2006). Regardless of their occurrence, both starch and WSC are easily available in the equine GI-tract and they are resorbed in the small intestine after degradation by digestive enzymes (Ellis 2006).

Concerning starch, two different chemical constructed polymers can be found: the α 1-4 linked linear amylose and the α 1-4 linked amylopectin (Ellis 2006). The latter one has a branched structure due to α 1-6 chemical bonds. Both, amylose and amylopectin are located in water-insoluble granules (Klevenhusen and Zebeli 2021) and are in particular degraded by the enzyme α-amylase (Ellis 2006). Sites of starch production and storage are the chloroplasts of leaves. The chloroplasts of the leaves are the cell structures where starch is produced and stored. Both processes, production and storage of starch, are self-limited because once the chloroplasts are saturated, the production stops (Longland and Byrd 2006).

The WSC are chemically very heterogenous and contain ethanol-soluble carbohydrates (ESC) and water-soluble carbohydrates. The ESC include mono-, di- and oligosaccharides together with some fructans. WSC, however, include the all the ESC plus all fructans (Klevenhusen and Zebeli 2021). Fructans are a group of naturally occurring oligo- and polysaccharides in the plant. They are built up of sucrose and serve as storage carbohydrates (Zimmermann et al. 2021). Fructans are the predominant WSC in C3 grasses. Plants draw on them when the need of carbon exceeds the supply. This need occurs especially at night (Klevenhusen and Zebeli 2021) because fructan synthesis is modulated by sunlight, affecting the sucrose availability in the plant (Vijn and Smeekens 1999). Moreover, next to carbon storage, fructans have multiple other functions like protection against abiotic stress (e.g., water deficit, low temperatures). Chemically they are built up from sucrose - a disaccharide made up from fructose and glucose- which is synthetized in the cytoplasm. However, fructans are produced and stored in the vacuoles of the plant and accumulation is not self-limited. That is because vacuolar fructan synthesis reduces the amount of sucrose in the cell, preventing sucrose-induced feedback photosynthesis-inhibition. In higher plants, five structurally different types of fructan can be distinguished depending on their fructosyl residues: inulin, levan, mixed levan, inulin neoseries and levan neoseries (Vijn and Smeekens 1999). Concerning the nutrition of horses and equines, fructans play an important role. They can be quickly fermented by microbes localized in the

hindgut. On one side, low concentration of fructans may have an advantageous effect by balancing the intestinal lumen matrix. On the other side, excessive levels of fructans being available to the hindgut flora, have been shown to favour the gram-positive microbes' proliferation and to impact the epithelial mucosa negatively by enhancing the risk for carbohydrate-induced laminitis (Ellis 2006). The type of fructan content depends on the grass species. Fructans with lower molecular weight and shorter chain length (e.g., in perineal ryegrass) are easier to ferment than others with longer chains (e.g., in timothy). Accordingly, it is suggested that the fructans with shorter chains involve a higher risk for pasture associated laminitis (Geor 2009).

Fibre in food products is defined as the proportion of cell wall components, including cellulose and related carbohydrates, as well as lignin (Cunha 1991). Chemically, cellulose is a water-insoluble glucose polymer (Jeroch et al. 2020), containing about ten thousand glucose monomers per molecule and is linked with β1,4-bonds (Ellis 2006). Cellulose is the main structural polysaccharide in plant cell walls, and can only be broken down by microbial cellulases in the large intestine of the horse (Jeroch et al. 2020). Hemicellulose is a group of polysaccharides with β1,4-linked chains, each building side chains with arabinose, xylose or uronic acid with 1-2, 1-3 or 1-4 bond. Furthermore, hemicellulose can bond with lignin, lowering the extent of cell wall degradation (Ellis 2006). Lignin itself is not a true carbohydrate but is the first limiting factor when it comes to digestive cell wall-breakdown. It is a condensation product of phenylpropanoid compounds (Ellis 2006) and of no nutritional value except that it contributes to the bulk supply (Cunha 1991). Lignin is mainly found in woody parts of plants, with an increasing amount in later stages of the development. Being indigestible to humans and animals, the fact that lignin can bond with carbohydrates like hemicellulose, makes it nutritionally interesting. That is because the breakdown of such carbohydrate-lignin complexes improves the accessibility of carbohydrate-degrading enzymes to their substrates (Jeroch et al. 2020).

2.3.3. Protein

Proteins are naturally occurring, complex substances consisting of amino acids joined by peptide bonds. Proteins are present in all living organisms and play a role in many essential biological compounds such as enzymes, hormones, antibodies, contractile proteins actin and myosin in the muscle, and they buffer the regulation of blood pH. An amino acid is built by an α-carbon attached to a carboxylic acid group (COO-), an amino group (+H3N) and a side chain group (R) (Fig. 2). The side chain group is unique for every amino acid. A common classification is based on whether amino acids can be produced by the mammalian body itself, or not.

Hence, two groups are distinguished: 1) indispensable (essential) amino acids, which must be provided by the diet, and 2) dispensable (non-essential) amino acids, which can be produced by the body itself. However, a grey area remains regarding amino acids for which the metabolic pathways are available but who cannot be produced in sufficient quantities under various conditions (e.g., growth, illness, injury, or physiological stress). These are called conditionally indispensable amino acids. Of the 21 mammalian amino acids, nine are strictly essential and depend on food intake: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Geor 2013).

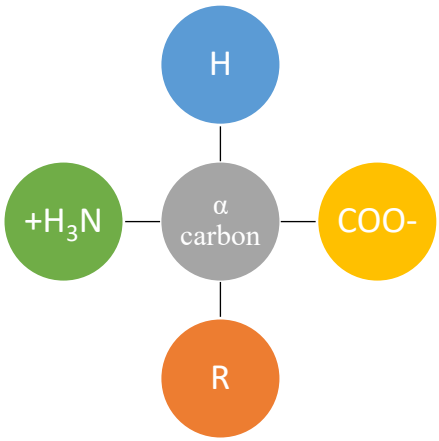

Fig. 2: Schematic description of the composition of amino acids: an α-carbon attached to a carboxylic acid group (COO-), an amino group (+H3N) and a side chain group (R) (modified after Geor 2013)

2.3.4. Fat

Forages and grains, which make up the bulk of a horses diet, are generally low in fat (Geor 2013, Jeroch et al. 2020). Whereby two groups of fats in forages and grains can be distinguished: 1) simple lipids (di- and triacylglycerol, non-esterified fatty acids, waxes and sterols) and 2) complex lipids (glycolipids, and phospholipids) (Geor 2013). In forage grasses, most of the fat is situated in the chloroplasts of the leaves (Jeroch et al. 2020). Besides ether extract (EE), fatty acids (Geor 2013) and accompanying fatty substances like provitamins, vitamins and colouring agents make up the fat content (Jeroch et al. 2020).

Depending on the degree of saturation or unsaturation, as well as the length of their hydrocarbon chain, fatty acids can be divided into two subgroups (Geor 2013): 1) saturation: saturated (no double bonds) and unsaturated (≥ 1 double bonds) fatty acids, 2) length: esterified (2 to 28

carbon molecules) and non-esterified (1 carbon molecule, i.e. unbound from the glycerol backbone) fatty acids. Plant-based fats contain more unsaturated (Geor 2013, Khiaosa-ard et al. 2020) and long chain fatty acids (Khiaosa-ard et al. 2020).

2.3.5. Minerals, trace elements, provitamins and vitamins

Minerals are essential substances (intake with food is obligatory) and can be subdivided into macro and trace elements, distinguished by their daily required amount in the diet: ≥ 100 ppm = macro element, < 100 ppm = trace element. Both oversupply and undersupply of minerals can have a negative impact on health and performance of the horse. However, the daily required amount depends on permanent endogenous losses (e.g., faeces, urine, and skin), utilization, and the individual condition. Concerning the latter, growth, pregnancy, lactation, and exercise are associated with higher requirements due to higher mineral losses through weight gain, milk production and sweat (Geor 2013). Especially on grass-only meadows, at the beginning of the season, shortages of the macro element Ca, rarely also Mg, can occur. Phosphorus (P) is normally sufficient unless the soil is poor in P or unfertilised. Potassium (K) concentrations are generally high (particularly after rich fertilisation), whereas sodium (Na) is mostly marginal or insufficient. Trace element supply depends highly on soil condition and fertilisation. Usually the minerals Ca, copper (Cu), zinc (Zn), and selenium (Se) are insufficient in grasses for the maintenance of foals and pregnant mares (Coenen and Vervuert 2020). Overall, feeding of plants or plant products often need to be supplemented with minerals because sufficient supply is not guaranteed (Geor 2013).

Vitamins are organic substances with essential vital functions (e.g., cofactor for metabolic or immune reactions), but they are not used for energy production. Depending on their solubility, two classes of vitamins can be differentiated: 1) fat-soluble vitamins (A, D, E, K) and 2) water-soluble vitamins (B-complex, C) (Geor 2013, Richards et al. 2021). Most vitamins are essential, meaning intake with food is indispensable for sufficient supply. However, some vitamins are an exception: vitamin D3 can be produced in the skin when exposed to UV-light, vitamin C can be produced in mammal livers (excluding guinea pigs, bats, and humans), some B vitamins can be produced from microbes in the hindgut, whereas it is not known how efficient the absorption from the hindgut is (Geor 2013).

Pro-vitamins are precursors of vitamins which can be enzymatically metabolized to vitamins by the body (Geor 2013). In plants, vitamin A is only present in the form of its precursors – the carotenoids – with β-carotene as the major representative. Furthermore, grasses contain

tocopherols as well as vitamin K and many vitamins of the B-complex. In general, most forages are rich in pro-vitamins and vitamins (Jeroch et al. 2020).

2.3.6. Mycotoxins as possible contaminants

Mycotoxins are metabolic products from various mould fungus, which have a negative effect on health and performance of animals (Jeroch et al. 2020). The intake of mycotoxins poses a risk for equine health (Geor 2013). Since mycotoxins are not essential for mould survival, their production is not consistent. However, certain environmental factors such as drought, and unusual temperature or humidity, induce fungi to produce mycotoxins as protective mechanism. Furthermore, carbohydrates (i.e., starch or cellulose), moisture, oxygen and temperatures between 12 °C and 25 °C are necessary for mould growth (Osweiler 2001). In their study, Pengos-Tabares et al. investigated mycotoxins, PE, and other secondary metabolites in Austrian pastures. Concerning mycotoxins, *Fusarium* was the major present fungi in the samples. Furthermore, *Alternaria* and ergot alkaloids (EA) were also found in high amounts. However, the concentrations of these mycotoxins were influenced by geoclimatic (e.g., temperature) and botanical (e.g., plant species) factors as well as year variations (Penagos-Tabares et al. 2021).

2.4. Nutritional variation in pastures and meadows

During the maturation process, the parts of plants change in terms of quantity, with a decrease in the amount of cell content and an increase in the amount of cell wall (stem proportion). As a result, the chemical composition, and thus the nutritional value of the plant, differs at various stages of the development (Geor 2013).

The varying concentration of NSC is of great interest for equine nutrition. This variation depends on the plant species, environmental factors, and stage of development. When plants endure stress, NSC-accumulation takes place, making the plant more viable. That is because the NSC serve as an energy reserve. Accordingly, cool season grasses with NSC as their main storage carbohydrates, cumulate them most. In contrast, warm season grasses are in no need of NSC to survive because they go dormant under conditions of stress (Watts 2010). Stressful conditions are for instance plant growth restricting factors such as low temperatures, frosts, non-lethal herbicides or low soil fertility (Geor 2009). Another important factor for nutritional variation in pastures is the type of soil, especially concerning the amount of nitrogen (Jeroch et al. 2020) and minerals (Geor 2013) in plants.

2.4.1. Species

As mentioned before, three main grass species are common on pastures: grasses, legumes, and herbs. Accordingly, each species has its unique/specific nutritional characteristics.

2.4.1.1. Non-structural carbohydrates

First and foremost, it should be emphasised, that the production of NSC in grass species is not inherited, but part of their genetic constituency. This means, certain environmental conditions (e.g., water deficit, low temperatures) are needed to trigger the NSC-production. Most grass species are able to build up NSC (Watts 2010). Nonetheless, it is known that C3 grasses have a higher concentration of NSC than C4 grasses, because they gather fructans in a non-self-limiting process as their main reserve carbohydrates. In C4 grasses and in legumes, starch functions as reserve carbohydrate (see chapter 2.3.2) (Geor 2009, Longland and Byrd 2006). In conclusion, when seeking low sugar concentrations in grasses, prevention of triggering conditions have to be favoured (Watts 2010).

2.4.1.2. Proteins

Firstly, the leaf-stem-ratio has an impact on CP concentrations with a decrease during the development of the plant. The fewer leaves, the lower the proportion of CP. In general, herbs have a higher leaf mass than grasses or legumes. Consequently, they contain more protein (Jeroch et al. 2020) but lower fibre than grasses, making them rich in energy (Richards et al. 2021). Secondly, young grass has a high nitrogen intake for building its biomass during maturation, which makes it rich in CP. Nitrogen fertilisation supports this process even more (Jeroch et al. 2020). Thirdly, also legumes have naturally higher CP concentrations than grasses because they bind atmospheric nitrogen through a symbiotic relationship with root microbes. (Richards et al. 2021). In conclusion, both legumes and herbs can influence the overall CP concentration in pastures (Jeroch et al. 2020).

2.4.1.3. Minerals

Green plants are naturally rich in K. This can be strengthened with additional K-fertilisation (e.g., slurry). In contrast, Na is only present in low concentrations. Other than grasses, legumes and herbs have a higher mineral content due to their deeper root system (Jeroch et al. 2020).

Furthermore, legumes usually have a higher concentration of Ca and Mg than grasses (Richards et al. 2021).

2.4.2. Temperature, daytime, and season

Storage of fructans is highly influenced by day/night temperatures due to temperature-dependant activity of respiratory facilitating enzymes. The activity of these enzymes slows down below 5 °C and completely stops below freezing temperature. Under warm conditions the enzymes are more efficient (Watts 2010). Taking this into account, NSC concentrations are prone to rise in the morning, peak in the afternoon and plunge during night. Furthermore, the values are the highest in spring, the lowest in summer, and intermediate in fall (Geor 2009). Concerning mycotoxins, Penagos-Tabares et al. have shown that temperature plays a significant role for their existence in Austrian pastures. With increasing temperature, the quantity of metabolites increases, whereas below 15 °C *Fusarium*, *Alternaria* and total fungal metabolites were low (Penagos-Tabares et al. 2021).

2.4.3. Soil and soil conditions

Depending on local soil conditions, not only the nutritional but also the botanical composition can be influenced with the application of different fertilisers (Coenen and Vervuert 2020). For example, C4 grasses have a more efficient water-utilisation than C3 grasses. Therefore, C4 grasses can grow in areas with higher temperatures and dryer soil conditions (Richards et al. 2021). Furthermore, different fertilisers can be used on pastures. For example, nitrogen fertilisers favour the growth of grasses. In contrast, legumes prevail with the application of K- or P-fertilisers. Concerning soils low in mineral concentration (e.g., sandy soil and marshland), they can be enriched with fertilisers including the missing elements (Coenen and Vervuert 2020).

2.4.4. Stage of growth

During the different stages of development, the nutritional composition of plants varies depending on the (im-)maturity of the plant (Geor 2013) and complexity of the cell wall (Cunha 1991).

2.4.4.1. Fibre

Fibre is a limiting factor when it comes to digestibility. Hence, the more a plant matures, the more complex the cell walls are built and therefore become tougher to digest. Therefore, the nutrients which are localised in the cell are less available to the horse (Cunha 1991). Grass in

spring, late summer, and autumn is low in structural carbohydrates (many leaves and less stem) and consequently, is low in fibre but rich in energy and protein. In summer it is the other way around (Coenen and Vervuert 2020). Furthermore, herbs due to their high leave mass are lower in fibre than grasses, making them rich in energy (Richards et al. 2021).

2.4.4.2. Protein

In early stages of the development, plants contain the highest CP concentrations (Geor 2013), as younger plants have more leaves and less stem. However, the more mature a plant, the less CP it contains (Jeroch et al. 2020).

2.4.4.3. Fat

In the course of maturation and with the transition into the reproductive state of growth, not only the fat content but also the amount of unsaturated fatty acids decrease (Jeroch et al. 2020).

2.4.4.4. Minerals, pro-vitamins, and vitamins

In early stages of development, plants are rich in minerals, pro-vitamins, and vitamins. However, Mg in these stages is very low. Though the amount of minerals decreases with ongoing development during the first cut, minerals are generally higher at the second cut. Also the concentration of carotenoids decline with progressing maturation (Jeroch et al. 2020). The reason behind is that the leaves contain more of β-carotene than the stem of a plant (Richards et al. 2021).

2.5. Pastures and meadows related to equine health

As pastures and meadows are the fundament of an equine diet, they contribute to the health and welfare of horses. However, contaminants like mycotoxins as well as an under- or over-supply with nutrients can cause diseases and maldevelopment.

2.5.1. Health effects of mycotoxins

Mycotoxins are secondary fungal metabolites which can be divided into two groups depending on their time of occurrence: Contamination and toxin production from 1) field-fungi (i.e., *Alternaria ssp., Fusarium ssp.*) happen before cropping, whereas with 2) storage-fungi (i.e.,

Penicillium ssp., Aspergillus ssp.) this happens after the crop. Mycotoxins can effect different organ-systems such as the GI-tract, the urogenital-tract and the central nervous system (Coenen and Vervuert 2020). A selection of the effects on equine health are listed in Table 2.

Tab. 2: A selection of mycotoxins produced by specific fungi, their effects in horses and their main metabolites (modified after Coenen and Vervuert 2020).

Mycotoxin	Fungal species	Effect in horses	Main metabolites
Trichothecene	*Fusarium spp.*	reduced food intake, immunosuppression, cytotoxic	Deoxynivalenol (DON), T 2/HT 2-Toxin
Zearalenon	*Fusarium spp.*	Fertility disorders	Zearalenon, α-ZON, (β-ZON)
Fumonisine	*Fusarium verticillioides*	Equine Leukoencephalomalacia, liver- and cardiovascular damage	Fumonisin B1 (B2, B3)
Ergot alkaloids	*Claviceps purpurea*	contractions of visceral- and uterine-musculature	Ergometrine, Ergosin, Ergotamine, Ergocornine, Ergocrytpine, Ergocristine

2.5.2. Ergotism

EA are a group of toxic fungal substances which parasitise on various grass species (Jeroch et al. 2020) and are localised in their seed heads (Geor 2013). The EA are mainly produced by the ergot fungi *Claviceps* et *Epichloë*. Among other monocotyledonous plants, they attack forage grasses and cereals. Intake of these alkaloids, with ergotamine as the major disease-causing toxin, can cause ergotism (Penagos-Tabares et al. 2021). Ergotism is a disease with either hallucinations, paranoia, and peripheral spasms (convulsive ergotism), or symptoms like peripheral sensation loss, oedema, and ultimately necrosis and loss of the affected tissue (gangrenous ergotism) (Grzybowski et al. 2021). However, in horses not many data on intoxication with different EA is available. The only better researched health effects on horses are the adverse effects of Ergovaline, an EA originating from contamination of pasture grasses with *Neotyphodium* spp.. Clinical signs include delayed parturition and agalactia in mares

(caused by altered prolactin levels), and incidentally neurotoxic symptoms (EFSA, European Food Safety Authority 2005a).

2.5.3. Dental problems

Teeth are the first instance of making nutrients available for the equine metabolism. Chewing breaks down feeding stuff into smaller pieces and provides a larger surface for digestive enzymes to work. Especially the abrasion and/or the damage of the molars in older aged horses limits their grinding capability. Therefore, tooth problems need to be taken seriously and have to be met with feeding adjustment to high digestibility (Coenen and Vervuert 2020).

2.5.4. Hoof function and development

The hoof plays a major role in equine health. Not only is it vital for supportive functions and leverage, but also acts as shock absorption and facilitates the reflow of venous blood from the distal limbs to the heart (Silva et al. 2022). Up to now, not much is known about the interaction between food supply and ungulate vitality. However, some nutrients are known to influence the growth and the quality of the hoof. Accordingly, protein deficiency (especially sulfuric amino acids) prolongs the growth of the hoof horn, Zn deficiency changes the hoof horn quality, and a deficiency of β-carotene or vitamin A leads to a loose and fragile hoof horn. Furthermore, Se in abundance disturbs keratin synthesis (Coenen and Vervuert 2020). Recently, Silva et al. found in their study that levels of Ca, Cu and Zn in the hoof capsule from pre- and postweaning Criollo foals, are influenced by the season and different physiographic regions. This suggests that practitioners need to consider different soil and pasture conditions when evaluating whether mineral supplementation in these developmental stages is needed (Silva et al. 2022).

2.5.5. Orthopaedic diseases in foals and adult horses

In foals, malnutrition primarily concerns developmental disorders. Especially the supply with energy, Ca, P and Cu must be considered at this young age. The intake of feed rich in carbohydrates – and therefore high in energy – supports chondrodystrophic conditions in the growth disc. This is due to a higher concentration of insulin and growth factors, facilitating proliferation and differentiation of chondrocytes. Furthermore, the deep and superficial digital flexor tendons contract due to the higher growth rate of the bones. Moreover, a lack of Ca, which has a key role in bone growth, leads to disturbances in the maturation of the skeleton. Especially if foals are only receiving food in form of young pasture grasses, a Ca deficiency can emerge. Also,

osteochondrosis, a disturbance in the transformation from cartilage to bone due to a disruption of the supplying blood vessels (Handbuch Pferdepraxis, 2017), can be caused by 1) insufficient Cu supply of the mother (intrauterine and colostrum), 2) sparse Cu-storage in the liver of the foal, 3) intake of food poor in Cu, or 4) antagonistic influence of Ca, Zn, and Fe (Coenen and Vervuert 2020).

Concerning adult horses, prevention of adiposity and Ca deficiency are of orthopaedic interest. Accordingly, the energy-supply for instance needs to be restricted in arthritic horses because more body weight puts more pressure on the affected joints. Furthermore, lameness can be caused by a shortage of Ca which leads to skeletal demineralisation and tension-stress of the periost (Coenen and Vervuert 2020).

2.5.6. Pasture associated laminitis

In previous times, grass breeders have been keen on cultivating plants being able to maximize their NSC accumulation. All of this because it leads to a high-caloric content, a stimulation of microbial fermentation and an increasing use of nitrogen in cattle's rumens. However, for horses this approach seems to be mainly a drawback (Watts 2010).

Laminitis is a syndrome, which can be caused by various conditions and leads to a destruction of the connective tissue between the inner hoof wall and the distal phalanx. Three primary aetiologies are differentiated up until now: 1) sepsis/inflammatory condition (e.g., colic, carbohydrate overload), 2) endocrine/metabolic dysfunctions (e.g., insulin resistance, obesity, pituitary pars intermedia dysfunction (PPID), equine metabolic syndrome (EMS)), and 3) mechanical stress (e.g., relieving posture of the contralateral foot) (Geor 2013). Considering equine nutrition, a high intake of feeding stuff rich in starch (e.g., grains) and sugar (e.g., pasture grass) is most relevant. In grasses, an overload with WSC (first and foremost fructans) can either induce strong glucose- and insulin-reactions, especially in horses and ponies with metabolic dysregulations, or lead to dysbiosis and epithelial damage in the hindgut (Coenen and Vervuert 2020).

According to current knowledge, the pathogenesis of pasture associated laminitis is as follows: If the amount of NSC exceeds the small intestines' digestive capacity, they get to the hindgut undigested and undergo rapid microbial fermentation, increasing the risk for laminitis (Ellis 2006, Geor 2010, Longland and Byrd 2006). Large amounts of both starch and fructan lead to a higher risk for laminitis (Longland and Byrd 2006). The influence of fructan as the dominating

WSC in grasses (Klevenhusen and Zebeli 2021) was proven by a study where the application of a bolus of high concentrations of fructans induced laminitis (Garner et al. 1977). However, large amounts of NSC entering the large intestine, induces a shift of the microflora becoming dominated by lactic acid producing microorganisms. This acid environment can induce lactate acidosis (Ellis 2006, Longland and Byrd 2006), laminitis, colic, and diarrhoea (Ellis 2006). Furthermore, endo- and exotoxin release from dying microbes and production of vasoactive amines, are factors involved in the development of laminitis: the epithelial barrier gets leaky due to the acidic conditions, releasing the toxins and amines into the circulation. All of this results in a systemic inflammation, leading to lamellar injury and failure due to the destruction of lamellar epithelium and extracellular matrix (Geor 2010). Until now, the direct correlation of NSC-intake and the development of laminitis has not been proven, but it is very likely (Longland and Byrd 2006).

Another predisposing factor influencing the occurrence of laminitis, is insulin resistance (IR) (Coenen and Vervuert 2020, Geor 2013, Longland and Byrd 2006). IR is a condition of the body, where insulin has little or no effect on the muscle-, liver- and fat-cells. Therefore, these cells hardly take up any glucose from the blood. As compensation, the pancreatic beta-cells produce even more insulin to help the cells getting at least a bit of glucose into them (Ighbariya and Weiss 2017). As for that, it is known that especially horses with EMS, an endocrine symptom complex including adiposity, are at higher risk to develop laminitis (Geor 2010). On the one hand, proinflammatory cytokines from the fat tissue antagonise insulin-activity on its receptors. (Coenen and Vervuert 2020, Geor 2013, Longland and Byrd 2006). On the other hand, insulin activates endothelin I (vasoconstrictive) and inhibits nitrogen monoxide (NO) (vasodilative) (Coenen and Vervuert 2020, Geor 2013). However, both conditions lead to insufficient glucose supply of the cells, including the ones of the hoof (Longland and Byrd 2006), increasing the risk for lamellar injury (Geor 2013).

Considering the impact of nutritional factors on the aetiology of pasture-associated laminitis, not only pasture management (reducing the intake of large NCS-amounts), but also horse management (avoiding IR hence, obesity and EMS) need to be taken into account for the prevention of pasture-associated laminitis (Longland and Byrd 2006). Actions that need to be taken are denying access to grass pastures for predisposed equines and limiting the amount of excessive NSC-intake (see chapter 2.4.) (Longland and Byrd 2006, Watts 2010).

3. Materials and Methods

3.1. Pasture data

For this study, seven randomly chosen horse pastures in Lower Austria were sampled for analyses on their nutritional profile and contamination levels with mycotoxins and PE. Horse stable owners were contacted by the author and asked for the permission to sample grass on their properties and process the data. Five out of seven consented to provide their meadows. Three owners provided one pasture each and two owners provided two pastures each, leading to a total of seven pastures. Five of the properties (1, 2, 3, 4, 7) were used as meadows (haymaking) and two (5, 6) as pastures (grazing). Due to data privacy reasons, the pastures were assigned with the numbers 1 to 7. The locations are disposed in Figure 3 and Table 3.

Tab. 3: Locations of the sampled pastures in the province of Lower Austria.

owner 1	**owner 2**	**owner 3**		**owner 4**		**owner 5**
pasture 1	pasture 2	pasture 3	pasture 4	pasture 5	pasture 6	pasture 7
Bad Vöslau, Baden	Weissenbach an der Triesting, Baden	Göllersdorf, Hollabrunn	Göllersdorf, Hollabrunn	Sonnberg, Hollabrunn	Sonnberg, Hollabrunn	Unterrohrbach

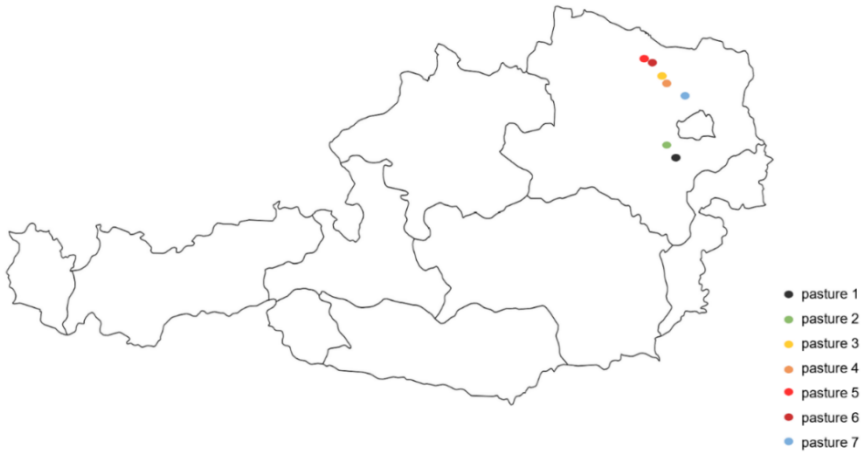

Fig. 3: Locations of the selected pastures (n = 7) in Lower Austria.

Under informed consent of the owners, three representative grass samples of each pasture were collected by the author and an employee of the institute during the grazing and haying season 2022 (May to August 2022). Gathered information included type and time of fertilisation (Tab. 4), native species and at the day of sample collection, the stage of growth, height of plants and botanical composition (see chapter 3.2.).

Tab. 4: Types and time of fertilisation performed on each pasture.

Pasture	Type of fertilisation	Time of fertilisation
1	cattle manure	March 2021
2	chicken manure	March 2022
3	none	-
4	horse manure	February 2022
5	horse manure	during grazing
6	horse manure	during grazing
7	horse manure	August 2021

3.2. Sample Collection

To evaluate the effect of the developmental stages of the plant on the nutritional composition of the pastures, each pasture was sampled three times during the haying/grazing season of the year 2022. However, from pasture 6 only two samples were taken because of massive drought damage at the third sampling. According to the botanical stage of development, the sampling season was classified into ear emergence (1st sample taking, 7 samples), early- till full-bloom (2nd sample taking, 7 samples) and drought-induced damage (3rd sample taking, 6 samples). The first two sample takings took place at the beginning and end of May before the first cut for haymaking or grazing, respectively. The third sampling was performed in mid-July (pasture 1 and 2) and in the beginning of August (pastures 3, 4, 5, 7). Hence, before the second cut. Pastures 1 and 2 were sampled on one day and pastures 3 to 7 on another. The cutting of each sample was performed in the same order and at approximately the same time of the day, ranging from morning until midday (Tab. 5).

Tab. 5: Sampling data of the seven pastures including information about time of sampling, developmental stage of the plants, date, and daytime.

Cut	Sampling	Pasture	Date	Daytime	
				From	To
Before 1st cut	1 (Ear emergence)	1	2nd May 2022	08:25	09:15
		2		09:50	10:30
		3	4th May 2022	08:30	09:15
		4		09:25	09:45
		5		10:10	10:35
		6		10:45	11:05
		7		12:10	12:30
Before 1st cut	2 (Early- till full-bloom)	1	16th May 2022	08:20	09:10
		2		09:35	10:15
		3	23rd May 2022	07:55	08:25
		4		08:45	09:10
		5		09:30	09:55
		6		10:00	10:30
		7		11:10	11:35
Before 2nd cut	3 (Drought-induced damage)	1	20th July 2022	08:00	08:55
		2		09:15	09:45
		3	10th August 2022	07:50	08:55
		4		09:05	09:40
		5		09:55	10:15
		7		11:15	11:30

Before cutting, the grass height was evaluated at the day of sampling with a tape measure. The sampling locations were randomly selected over the entire pasture area and were cut 5-7 cm above the soil level using an electric (ISIO, Bosch) and a manual grass shear (Gardena, Husqvarna Austria GmbH, Linz, Austria), both depictured in Figure 4. From each pasture an overall quantity of 5 kg was collected and dry stored in cardboard boxes until further preparation back at the institute (Institute of Animal Nutrition and Functional Plant Compounds. Department for.

Fig. 4: Manual (above) and electric (below) grass shear used for grass cutting.

3.3. Botanical characterisation

The botanical characterisation was conducted at the pastures when the samples were taken. The primarily occurring grass specimen were determined by their morphological characteristics with the help of an expert. He showed the author and her assistant how to determine the main species on pastures 1 and 2. Afterwards the author and the assistant investigated the plants on the other pastures themselves. The different developmental stages of the plants and the weather conditions influenced the extent of species determination (i.e., Fig. 5 and Fig. 6). At the stage of ear emergence, no determination was made because of the immaturity of the plants. At the stage of early- till full-bloom the different species could be best evaluated.

Fig. 5: Comparison of growing conditions between the first (left) and third (right) sampling of pasture 1.

Fig. 6: Comparison of growing conditions between the first (left) and third (right) sampling of pasture 4.

3.4. Sample preparation

First, the collected samples were weighed into aluminium grilling trays with a precisions scale (ME 4002, Mettler Toledo, Vienna, Austria). The trays were labelled so that they would not be mixed up. To fit the size of the grilling trays, the grass was cut into adequate portions with the same manual grass shear which was used for sample taking. Then, the sample-containing trays were put into a dry cabinet (Memmert GmbH & Co, Schwabach, Germany) for 48 hours at a temperature of 45 °C. After a cooling time of approximately two hours, the samples were weighed out (ME 4002, Mettler Toledo, Vienna, Austria) and stored in sealable plastic bags. Next, the hay was roughly cut with a conventional grass shear and pulverized to a particle size of 1 mm by a cutting mill (SM 300, Retch GmbH, Haan, Germany). The milling was conducted in 3 steps by using different sizes of sieves: firstly 6 mm, secondly 2 mm and thirdly 1 mm. The resulting homogenate was subsequently used for further analyses.

3.5. Nutritional characterisation

The following parameters concerning the nutritional composition of the pastures were analysed: Chemical proximate composition (DM, crude ash (CA), CP, EE, neutral detergent fibre (NDF), acid detergent fibre (ADF), NFC), sugars (WSC, ESC), minerals (Ca, P, Mg, K, Na, Fe, Mn, Zn, Cu), as well as mycotoxins and contaminants.

3.5.1. Proximate and fibre analysis

With certain measurement methods (proximate analysis and Anthrone method), the grass samples were analysed.

3.5.1.1. Dry matter (DM) and raw water (RW)

The tare weight of a porcelain pan was determined with an analytic scale (MS205DU, Mettler Toledo, Vienna Austria). Then, 5 g of the homogenate were weighed into the porcelain pan and put into a dry cabinet (Trockenschrank Serie FD 240, Binder-GmbH, Germany) at 100 °C for at least four hours. Afterwards, the porcelain pan was removed from the dry cabinet and cooled in an exicator (Vakuum-Exsikkatoren, VWR International LLC, USA). Again, the weight was determined with the above-mentioned analytic scale.

DM (dry matter) = oS (original substance) – RW (raw water)

3.5.1.2. Crude ash (CA) and organic matter (OM)

The weighed sample from DM-determination was put into a muffle kiln (Standard Kammeröfen CWF 11/23, Carbolite GmbH, Germany) at 580 °C for six hours. Afterwards, the sample was cooled down and the remnant was weighed with an analytic scale (MS205DU, Mettler Toledo, Vienna Austria).

CA = DM – oS

3.5.1.3. Crude protein (CP)

For sample digestion, 1 g of the fine-grained homogenate was weighed into a glass sample tube with an analytic scale (MS205DU, Mettler Toledo, Vienna Austria). Next, two Kjehldahltabs containing NaSO4, CuCO4 and Se (Kjeldahl Katalysator nach Wieninger, AppliChem GmbH, Germany) and 20 ml of concentrated sulphuric acid (Sulfuric acid, puriss. p.a., for determination of Hg, ACS reagent, reag. ISO, reag. Ph. Eur., 95.0–97.0 %, Sigma-Aldrich Co. LLC, USA) were added to the tube. Thereafter the mixture was heated and cooked for three and a half hours with a Büchi Digestion Unit (Digestion Unit K-424/ K-435, Büchi Labortechnik AG, Switzerland) until the solution was almost colourless. After cooling down, distillation with the Büchi Distillation Unit (Distillation Unit B-323, Büchi Labortechnik AG, Schweiz) followed. The device was loaded with 33 % sodium hydroxide solution. Furthermore, an Erlenmeyer flask was filled with 20 ml of 0.25 mol sulphuric acid and 80 ml water. The glass sample tube with the solution as well as the filled Erlenmeyer flask were put into the Distillation Unit and the installed programme started. At last, the sample solution was titrated using a pH-electrode (702 SM Titrino, Deutsche METROHM GmbH & Co. KG, Germany). The percentage of CP was calculated by the device and displayed on its screen.

3.5.1.4. Ether extract (EE)

With an analytic scale (MS205DU, Mettler Toledo, Vienna Austria) 3.5 g of the homogenate were weighed into a glass sample tube. After adding 5 g of Celite (Celite® 545 filter aid, treated with sodium carbonate, flux calcined, Sigma-Aldrich Co. LLC, USA) and 50 ml of 4 mol hydrochloric acid, the components were mixed. Furthermore, glass frits were filled with 50 g of quartz sand and 5 g of Celite. Both, the sample tubes, and the glass frits were put into the Büchi Hydrolysis Unit (Hydrolysis Unit E-416/ B-411, Büchi Labortechnik AG, Switzerland) and the installed programme was started. Afterwards, the glass frits were dried at 100 °C in the dry cabinet (Trockenschrank Serie FD 240, Binder-GmbH, Germany) over night. Finally, extraction of the fat took place. At first, the fatty pots, which later contained the extracted EE, were weighed empty and then filled with 130 ml petroleum ether. Another 20 g of quartz sand were added to the sample in the glass frit. Then, the Büchi Universal Extraction System B-811 (Extraction System B-811/ B-811 LSV, Büchi Labortechnik AG, Schweiz) was loaded with both, the glass frits, and the fat pots. When the installed programme was finished, the fat pots with the extracted EE were put into the dry cabinet (Venticell 222 - ECO line, MMM Medcenter, Munich Germany) over night. The final cooled down fat pots were weighed.

3.5.1.5. Neutral detergent fibre (NDF) & Acid detergent fibre (ADF)

First, the tare weight of a fibre bag was evaluted with an analytic scale (MS205DU, Mettler Toledo, Vienna Austria). Second, 1 g of the homogenized sample was put into the fibre bag. Next, a spread finger out of glass was inserted in the fibre bag and together they were placed in the Fibretherm (Fibretherm; C. Gerhardt GmbH & Co. KG, Germany). In there, fat was washed out of the sample by flushing with petroleum ether. During the following installed programme, the sample was boiled, washed, and dried. Afterwards, the spread finger was removed from the fibre bag. The fibre bag itself, containing the filtrate, was put into heated porcelain pots, and was dried in the dry cabinet (Trockenschrank Serie FD 240, Binder-GmbH, Germany) at 100 °C for 24 hours. Finally, the fibre bag with the filtrate was incinerated in the muffle kiln (Standard Kammeröfen CWF 11/23, Carbolite GmbH, Germany) at 580 °C for two hours, cooled and the remnant weighed.

3.5.1.6. Non-fibre carbohydrates (NFC)

By subtracting all the previously determined groups of substances from 100 %, NFC result.

$$NFC = 100 – (CA + CP + EE + NDF)$$

3.5.2. Water-soluble carbohydrates (WSC)

Exactly 100 mg of the fine-grained homogenate was weighed with an analytic scale (MS205DU, Mettler Toledo, Vienna Austria) into a 50 ml Tube and mixed with 200 ml double-distilled water (dd.H2O) (Adrona-Biopak Reinstwasseranlage, Primelab GmbHV, Vienna Austria). The mixture was incubated in a water bath (Wasserbad GFL 1086, Eppendorf, Vienna Austria) by 80 °C for two hours. Then, the WSC-extract was cooled down, transferred into a 25 ml volumetric flask (Primelab GmbH) and mixed with 25 ml of dd.H2O. Subsequently, the mixture was centrifugated (Zentrifuge Eppendorf R5810, Eppendorf, Vienna Austria) for ten minutes at 4000 rpm (rounds per minute). Afterwards, a small amount of the translucent WSC-extract was additionally filtered in a 5 ml disposable syringe (institution pharmacy Vetmed Uni Vienna, Vienna Austria) with a 0.45 µm syringe filter (Spritzenvorfilter RC 0,45µm, Primelab GmbH, Vienna Austria) and stored in the fridge until the measurement. Finally, the measurements were taken by use of an ELISA-method (ELISA-Reader xMark, BioRad GmbH, Vienna Austria).

3.5.3. Ethanol-soluble carbohydrates (ESC)

Exactly 100 mg of the fine-grained homogenate was weighed into a 15 ml Tube with an analytic scale (MS205DU, Mettler Toledo, Vienna Austria) and mixed with 10 ml ethanol (80 % v / v). The mixture was incubated in a waterbath (Wasserbad GFL 1086, Eppendorf, Vienna Austria) by 80 °C for ten minutes. Then the supernatant was collected in a 25 ml volumetric flask (Primelab GmbH). The volumetric flask was filled up with ethanol (80 % v / v) until the 25 ml-mark was reached. The resulting mixture was then centrifugated (Zentrifuge Eppendorf R5810, Eppendorf, Vienna Austria) for ten minutes at 4000 rpm. Afterwards, a small amount of the translucent WSC-extract was additionally filtered in a 5 ml disposable syringe (institution pharmacy Vetmed Uni Vienna, Vienna Austria) with a 0.45 µm syringe filter (Spritzenvorfilter RC 0,45µm, Primelab GmbH, Vienna Austria) and stored in the fridge until the measurement. Finally, the measurements were taken by use of an ELISA-method (ELISA-Reader xMark, Bio-Rad GmbH, Vienna Austria).

3.5.4. Mycotoxins and contaminants

Various toxins and pesticides in the pasture samples were analysed using the liquid chromatography tandem mass spectrometry (LC–MS/MS), a system enabling the accurate quantification of > 1200 biotoxins, pesticides and veterinary drugs in complex feed. The article from Steiner et al. 2020 gives a detailed description of the method (Steiner et al. 2020). The apparent recovery ranged from 30 % (Tryptophol) to 210 % (Altersetin) and the recovery of extraction reached from 49 % (Enniatin B1) up to a value of 106 % (Formonetin). For the analysis, the same fine-grained homogenate (dried with Memmert GmbH & Co, Schwabach, Germany; milled with SM 300, Retch GmbH, Haan, Germany) as for the previous mentioned analyses was used. The milled samples were homogeneously mixed into one representative sample per pasture. Aliquots of 5 g of each sample were then used for the analysis which was carried out at the Department of Agrobiotechnology (IFA-Tulln) at the University of Natural Resources and Life Sciences Vienna (BOKU) in Tulln, Austria.

Each group of metabolites is characterized by their main producing fungi including *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, other (non-identified) and unspecific metabolites (i.e., metabolites produced by fungi, bacterial and/or plants), or other kinds of metabolites (i.e., EA, PE, and cyanogenic glycosides).

3.6. Weather data

The climatic data were collected from the website of the Austrian Agency of Meteorology and Geodynamics (Zentralanstalt für Meteorologie und Geodynamik-ZAMG, https://data.hub.zamg.ac.at/dataset/). These include monthly data as well as daily data (data from the day before sample taking). The considered parameters are listed in Table 6. Accordingly, the first and second sample taking have the same monthly data as they both took place in May. For the third sampling, recorded ZAMG-data of July (pastures 1 and 2) and August (pastures 3 to 7) were considered.

Tab. 6: Considered weather parameter for this thesis.

	Parameter (unit)
previous day parameter	air temperature 2 m daily average (°C) air temperature 2 m daily maximum (°C) air temperature 2 m daily minimum (°C) relative humidity daily average (%) duration of sunshine 24 h-sum (h)
month parameter	air temperature 2 m monthly average (°C) air temperature 2 m monthly maximum (°C) air temperature 2 m monthly minimum (°C) relative humidity monthly average (%) duration of sunshine monthly sum (h) days without sunshine

3.7. Statistical analysis

The effect of botanical stage of development on the nutritional composition of the pastures was analysed using a mixed model of the MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA). The statistical model included the fixed factor of botanical stage and the random effect of pasture fields. Accordingly, the values reported are least-squares means and the standard error of the mean (SEM). The significant differences among botanical stages are deemed significant when $p < 0,05$ and tend to be significant when $0,5 \leq p < 0,10$. The sampling time was grouped as already mentioned above: ear emergence, early- till full-bloom and drought-induced damage.

For the descriptive statistics of the mycotoxins only the positive values ($x \geq$ limit of detection (LOD)) were considered. Data below LOD were deemed not detectable.

The location of the pastures was defined according to the nearest weather station of ZAMG.

4. Results

4.1. Botanical characterisation

There was a variation in botanical composition between the pastures 1-7 which is listed in Table 7. At the stage of early- till full-bloom, the species could be determined best. The sampled pastures contained mixtures of *Gramineae* (Family: *Poaceae*, including *Lolium perenne, Dactylis glomerata, Poa trivialis, Festuca pratensis, Alopecurus pratensis* and *Phleum pretense*) and *Leguminosae* (Family: *Fabaceae*, including *Trifolium pratense, T. repens and Medicago sativa*). As identified, on 1 and 2 was a homogenous mixture of common horse pasture plants from the *Fabaceae*-, *Poaceae*- and *Taraxacum*-family found. Pasture 3 inhabited mainly *Poaceae* with isolated spots of *Fabaceae*. The pastures 4-7 were dominated by plants of the *Fabaceae*-family (Fig. 7). Visually, some pastures were dominated by certain plant species, but no exact proportions were determined.

Tab. 7: Botanical composition of the pastures in alphabetical order (printed in bold: dominating species).

	1st sampling	**2nd sampling**	**3rd sampling**
	Ear emergence	**Early- till full-bloom**	**Drought-induced damage**
Pasture 1		*Alopecurus pratensis, Arrhenatherum elatius, Cirsium vulgare, Cruciata laevipes, Dactylis glomerata, Festuca pratensis, Longifolia, Medicago sativa, Mentha longifolia, Phleum pratense, Plantago lanceolata, Poa trivialis, Rumex acetosa, Taraxacum officinale, Trifolium incarnatum, Trifolium pratense, Trifolium repens, Vicia sepium*	*Alopecurus pratensis, Cirsium vulgare, Dactylis glomerata, Festuca pratensis, Medicago sativa, Mentha longifolia, Plantago lanceolata, Poa trivialis, Rumex acetosa, Taraxacum officinale, Trifolium pratense, Trifolium repens*

Pasture 2		*Alopecurus pratensis, Arrhenatherum elatius, Bromus hordeaceus, Crepis biennis, Dactylis glomerata, Galium mollugo, Lolium perenne, Poa trivialis, Ranunculus acris, Rumex acetosa, Taraxacum officinale, Tragopogon, pratensis, Trisetum flavescens, Vicia sepium*	*Alopecurus pratensis, Arrhenatherum elatius, Bromus hordeaceus, Crepis biennis, Dactylis glomerata, Galium mollugo, Lolium perenne, Poa trivialis, Ranunculus acris, Rumex acetosa, Taraxacum officinale, Tragopogon pratensis, Trisetum flavescens, Vicia sepium*
Pasture 3		*Dactylis glomerata, Medicago sativa, Plantago lanceolata, Salvia pratensis, Trifolium pratense, Trifolium repens, Trisetum flavescens*	*Plantago lanceolata, Trifolium repens*
Pasture 4		*Arrhenatherum elatius, Bromus hordeaceus,* ***Dactylis glomerata,*** *Lolium perenne,* ***Medicago sativa,*** *Trisetum flavescens*	***Medicago sativa***
Pasture 5		***Dactylis glomerata,*** *Lolium perenne,* ***Medicago sativa, Onobrychis viciifolia,*** *Plantago lanceolata, Poa trivialis, Trifolium incarnatum, Trifolium pratense, Trifolium repens*	***Medicago sativa***
Pasture 6		Arrhenatherum elatius, Bromus hordeaceus, Dactylis glomerata, Festuca pratensis, Lathyrus pratensis, **Medicago lupulina,**	-

		Medicago sativa, **Melilotus officinalis,** Onobrychis viciifolia, Poa trivialis, Sisymbrium officinale, **Trifolium pratense, Trifolium repens,** Vicia sepium	
Pasture 7		Capsella bursa-pastoris, Dactylis glomerata, **Lolium perenne,** Poa trivialis, Trifolium pratense, Trifolium repens, **Triticum spp.**	Cirsium vulgare, Matricaria chamomilla, **Trifolium repens**

Fig. 7: Cut-outs of pasture 4 (left), pasture 5 (middle) and pasture 7 (right) at the third sampling.

4.2. Nutritional (chemical) composition of the pastures

The results of the performed analyses are as follows.

4.2.1. Chemical proximate composition

Data of chemical proximate analysis are shown in Table 8. It shows the average content of WSC, ESC, fructans, DM, CA, CP, EE, NDF, ADF, NFC and ADF/NDF ratio (ADF:NDF) as well as the differences between the stages of botanical development.

The average DM content in the pasture samples collected at ear emergence growth stage was 20.5 % DM (27.3 % to 15.3 %). When harvested at early- till full-bloom, the DM content averaged 24.3 % DM (30.2 % to 18.4 %). In both cases, the DM content was lower ($p = 0.002$) than

DM content of the samples taken in summer at the stage of drought-induced damage, being 35.8 % DM (23.4 % to 50.0 %).

The differences in CA concentrations between the three different stages of growth, were not statistically significant (p = 0.056) but could point out a tendency. The CP concentrations between the first and the second sample and between the first and the third sample, decreased significantly (p = 0.002). Between the second and third sample, no significant difference was found.

It appeared, that the NDF concentrations at the early- till full bloom stage were significantly higher than in ear emergence and drought-induced damage stages (p < 0.001).

There was a statistically significant difference in ADF concentrations between the stage of ear emergence and the two other stages of development. With the lowest average values at the stage of ear emergence (p = 0.005).

The amount of WSC was significantly higher in the first samplings than in the following two (p = 0.008). The fructans, as a subgroup of the WSC, followed the same tendency (p = 0.014). However, the ESC concentrations differed significantly between all three stages of development (p = 0.009).

Tab. 8: Results of the chemical proximate analysis.

Variable	**Ear emergence**	**Early- till full-bloom**	**Drought-induced damage**	**SEM**	**p-level**
	n=7	**n=7**	**n=6**		
Month	**2nd/4th May**	**16th/23rd May**	**20th July/10th August**		
Cut	Before 1st cut	Before 1st cut	Before 2nd cut		
Sampling	1	2	3		
DM (%)	20.5^{b}	24.3^{b}	35.8^{a}	2.29	0.002
CA (% DM)	9.30^{ab}	8.29^{b}	11^{a}	0.686	0.056
CP (% DM)	21.7^{a}	15.5^{b}	16.6^{b}	1.55	0.002
CF (% DM)	2.4	2.4	2.4	0.138	1.000

NDF (% DM)	45.12^{b}	53.8^{a}	48.8^{b}	2.30	<0.001
ADF (% DM)	24.2^{b}	30.7^{a}	32.8^{a}	1.65	0.005
NFC (% DM)	21.2	19.8	21.0	2.04	0.786
ADF:NDF	0.53^{b}	0.57^{b}	0.66^{a}	0.023	0.004
WSC (g/kg DM)	160^{a}	94.8^{b}	77.7^{b}	19.34	0.008
ESC (g/kg DM)	110^{a}	81.1^{ab}	65.3^{b}	10.75	0.009
Fructans (g/kg DM)	46.8^{a}	13.7^{b}	12.2^{b}	10.98	0.014

SEM = standard error of the mean, n = number of pasture samples.

Superscripts ^{abc} means significant differences ($p < 0.05$) based on Tukey's method.

4.2.2. Minerals and trace elements

As shown in Table 9, the concentration of minerals and trace elements in the pastures varied between the three developmental stages. However, Ca, P, Mg, K, Na, and Fe showed significant differences.

Tab. 9: Results of mineral analysis.

Variable	**Ear emergence**	**Early- till full-bloom**	**Drought-induced damage**	**SEM**	**p-level**
	n=7	**n=7**	**n=6**		
Month	**2nd/4th May**	**16th/23rd May**	**20th July/10th August**		
Cut	Before 1st cut	Before 1st cut	Before 2nd cut		
Sampling	1	2	3		
Calcium (g/kg DM)	7.27^{b}	7.36^{b}	12.6^{a}	1.36	<0.001

Phosphorus (g/kg DM)	3.79^{x}	3.22^{y}	3.50^{xy}	0.363	0.090
Magnesium (g/kg DM)	1.98^{b}	1.96^{b}	3.18^{a}	0.169	<0.001
Potassium (g/kg DM)	32.36^{a}	27.89^{ab}	23.79^{b}	1.61	0.003
Sodium (g/kg DM)	0.4	0.3	0.3	0.088	0.393
Iron (mg/kg DM)	93.3^{a}	49.6^{b}	109^{a}	8.93	0.001
Manganese (mg/kg DM)	47.1	36.6	43.4	5.63	0.268
Zinc (mg/kg DM)	24.4	20.9	22.4	2.29	0.150
Copper (mg/kg DM)	8.0	6.1	7.4	0.767	0.127

SEM = standard error of the mean, n = number of pasture samples.
Superscripts ^{abc} means significant differences ($p < 0.05$) based on Tukey's method.

4.2.3. Detected Mycotoxins and metabolites

The occurrence and concentrations (average, standard deviation, median, minimum, and maximum, expressed in µg/kg on a DM basis) of individual and grouped metabolites, are shown in Table 10. In total, 94 out of 148 targeted fungal, plant, unspecific and other metabolites, were detected in the analysed pasture samples. These include 72 fungal compounds, one bacterial metabolite, nine plant metabolites, ten unspecific metabolites, and two cyanogenic glycosides. Furthermore, six pesticides were found.

Tab. 10: Occurrence and concentrations of mycotoxins and metabolites (SD = standard deviation).

			Concentration (µg/kg sample)		
Group	**Metabolite**	**Positive samples (%)**	**Average ± SD**	**Median**	**Range**
Major mycotoxins and derivatives	T-2 toxin	5	1.4 ± 6.4	0.00	0-28.5
	3-Acetyl-T-2 Toxin	5	0.3 ± 1.4	0.00	0-6
	Zearalenone	20	12.9 ± 32.3	0.00	0-111.2
	Fumonisin B1	5	5.7 ± 25.3	0.00	113
	total		0.35 ± 0.9	0.00	0-4
Further *Fusarium* metabolites	Moniliformin	10	2.1 ± 6.5	0.00	0-23
	Beauvericin	45	8.7 ± 31	0.00	0-139
	Enniatin A	20	0.6 ± 1.7	0.00	60-.7
	Enniatin A1	30	3.2 ± 8.9	0.00	0-37.2
	Enniatin B	35	13 ± 35.6	0.00	0-158
	Enniatin B1	40	14.5 ± 40	0.00	0-172
	Enniatin B2	10	0.3 ± 1.2	0.00	0-5.3
	Culmorin	10	17.3 ± 57.1	0.00	0-237
	15-Hydroxyculmorin	5	9.9 ± 44.2	0.00	0-198
	Antibiotic Y	40	163 ± 455	0.00	0-2033
	Apicidin	25	6.3 ± 12.2	0.00	0-39.5
	Aurofusarin	30	31.9 ± 80	0.00	0-300
	Epiequisetin	5	0.2 ± 1	0.00	0-4.4
	Equisetin	45	61.6 ± 206	0.00	0-922
	Fungerin	5	0.9 ± 4	0.00	0-18
	Siccanol	25	94.9 ± 204	0.00	0-765
	W493	15	3.3 ± 8.9	0.00	0-33.9
	total	60	4 ± 4.7	1.50	0-13

Ergot alkaloids	Ergocornine	5	0.7 ± 3.3	0.00	0-14.7
	Ergocorninine	5	0.18 ± 0.8	0.00	0-3.6
	Ergocristine	5	1.7 ± 7.4	0.00	0-33.2
	Ergocristinine	5	0.2 ± 0.9	0.00	0-4.1
	Ergocryptine	5	0.7 ± 2.9	0.00	0-13.1
	Ergocryptinine	5	0.2 ± 0.9	0.00	0-4
	Ergometrine	5	0.4 ± 2	0.00	0-8.9
	Ergometrinine	5	0.2 ± 0.7	0.00	0-3.2
	Ergosin	5	1.7 ± 7.4	0.00	0-33.3
	Ergosinin	5	1.5 ± 6.7	0.00	0-29.8
	Ergotamine	5	1.4 ± 6.2	0.00	0-27.8
	Ergotaminine	5	0.9 ± 4.2	0.00	10-8.7
	Ergovalin	10	51248 ± 160946.6	0.00	0-611040
	total	10	0.7 ± 2.9	0.00	0-13
Aspergillus metabolites	3-Nitropropionic acid	10	358 ± 1309	0.00	0-5760
	Kojic acid	5	50.4 ± 225	0.00	0-1008
	Phenopyrrozin	35	7.3 ± 12.1	0.00	0-37.4
	total	50	0.5 ± 0.5	0.50	0-1
Penicillium metabolites	Barceloneic acid	15	46 ± 136.2	0.00	0-580.3
	Bilaid A	45	5.2 ± 7.1	0.00	0-22.7
	Chanoclavin	25	4.1 ± 10.8	0.00	0-45.1
	Curvularin	15	1.3 ± 3.1	0.00	0-9.8
	Deoxygerfelin	10	0.2 ± 0.6	0.00	0-2.1
	Pyrenocin A	5	7.6 ± 33.8	0.00	0-151.3
	Questiomycin	50	50.2 ± 141.7	2.48	0-635.8
	Secalonic acid D	5	1.8 ± 7.9	0.00	0-35.2
	total	95	1.7 ± 1.2	1.00	0-5
Alternaria metabolites	Tenuazonic acid	15	13 ± 34.2	0.00	0-128.2
	Alternariol	20	6.3 ± 14.8	0.00	0-57

	Alternariol-methylether	20	5.7 ± 13.8	0.00	0-54.1
	Tentoxin	10	2.3 ± 7.1	0.00	0-24.3
	Altersetin	75	43 ± 70.3	14.74	0-244.3
	Infectopyron	10	214 ± 730.7	0.00	0-3112
	Pyrenophorol	20	4 ± 9.2	0.00	0-34.6
	Radicinin	30	20.9 ± 81.6	0.00	0-367
	total	80	2 ± 2.2	1.00	0-8
Metabolites from other fungal genera	Abscisic acid	100	3007 ± 1573	3128	675-5816
	Ascochin	5	0.4 ± 1.8	0.00	0-8.1
	Cercosporamide	5	0.8 ± 3.5	0.00	0-15.8
	Cytochalasin B	5	6.2 ± 27.5	0.00	0-123.1
	Cytochalasin D	5	0.9 ± 4.2	0.00	0-18.9
	Epoxycytochalasin C	20	10.6 ± 29.1	0.00	0-121
	Ilicicolin B	20	10.5 ± 25.4	0.00	0-95.6
	Ilicicolin H	5	0.6 ± 2.5	0.00	0-11.1
	Monocerin	25	7.8 ± 19.2	0.00	0-75.8
	Rubellin D	15	3 ± 9.1	0.00	0-39
	Sporidesmolide II	50	58.5 ± 169	1.38	0-751
	Sporidesmolide III	20	5.2 ± 17.2	0.00	0-75.7
	Sydowinin A	40	133 ± 216	0.00	0-725
	total	100	3.2 ± 2.7	2.00	1-9
Bacterial metabolites	Cereulide	5	0.7 ± 3.1	0.00	0-14
	total	5	0.1 ± 0.2	0.00	0-1
Plant metabolites / toxins (phytoestro-gens)	Biochanin	75	9168 ± 10941	3156	0-31224
	Coumestrol	80	347 ± 596	101.64	0-1960
	Daidzein	40	327 ± 562	0.00	0-2063
	Daidzin	55	2023 ± 4621	208	0-20752

	Formonetin	55	9565 ± 10959	8956	0-31212
	Genistein	55	524 ± 747	198	0-2684
	Genistin	70	9914 ± 14634	3972	0-51529
	Ononin	85	8902 ± 11068	4685	0-34174
	Prunasin	5	44.4 ± 198	0.00	0-887
	total	100	5.2 ± 2.6	6.00	1-8
Cyanogenic glycosides	Linamarin	70	70201 ± 112380	9494	0-366632
	Lotaustralin	90	96636 ± 141250	13533	0-402162
	total	90	166837 ± 249645	22880	0-764578
Pesticides	Dichlorprop	5	0.2 ± 0.9	0.00	0-4
	Dimethomorph	5	0.7 ± 3.4	0.00	0-15
	Fluopyram	5	0.4 ± 1.9	0.00	0-8.3
	Iprovalicarb	5	0.5 ± 2.2	0.00	0-9.9
	Metrafenon	5	0.6 ± 2.5	0.00	0-11.1
	Spiroxamin	10	0.4 ± 1.3	0.00	0-45
	total	15	0.4 ± 1.1	0.00	0-5
Unspecific metabolites	Brevianamid F	55	11.5 ± 14	8.96	48.2
	Chlorocitreorosein	5	0.3 ± 1.3	0.00	0-5.7
	Chrysophanol	20	51.5 ± 123	0.00	0-483
	Citreorosein	30	36.2 ± 86.4	0.00	0-313
	cyclo(L-Pro-L-Tyr)	45	50.6 ± 68.9	0.00	0-235
	cyclo(L-Pro-L-Val)	100	163 ± 150	113	16.2-585
	Emodin	65	170 ± 240	38	0-771
	Iso-Rhodoptillometrin	5	0.5 ± 2.5	0.00	0-11

	Norlichexanthone	40	27.1 ± 58.2	0.00	0-249
	Tryptophol	80	653 ± 447	584	0-1473
	total	100	4.5 ± 1.2	4.00	2-7

Between the three times of sampling there was a significant difference in the number of metabolites per sample and the concentrations of several groups of mycotoxins and metabolites (Tab. 11). Samples collected at the stage of drought-induced damage had higher levels of co-contamination of fungal metabolites without Ergovaline ($p = 0.004$) compared to those of either ear emergence or early- till full-bloom. A similar trend occurred with the number of total fungal metabolites ($p = 0.00$), *Fusarium* ($p < 0.001$) and *Alternaria* ($p = 0.013$), which resulted in higher concentrations in the pastures during the last sampling than in the two early samplings.

Tab. 11: Results of occurrences and concentrations of detected mycotoxin metabolites.

Category	**Variable**	**Ear emergence**	**Early- till full-bloom**	**Drought-induced damage**	**SEM**	**p-level**
		n=7	**n=7**	**n=6**		
	Month	**2nd/4th May**	**16th/23rd May**	**20th July/10th August**		
	Cut	Before 1st cut	Before 1st cut	Before 2nd cut		
	Sampling	1	2	3		
Contaminant concentration (µg/kg sample)	*Fusarium* metabolites	64.6[b]	34.9[b]	1390[a]	300.04	0.013
	Emerging *Fusarium* metabolites[2]	12.7[b]	8.03[b]	839[a]	156.63	0.004
	Aspergillus metabolites	214	5.4	1130	499.64	0.293
	Penicillium metabolites	104	156	104	76.85	0.827
	Alternaria metabolites	40.3	11.7	971	313.53	0.095

	Other fungal metabolites	3369	2804	4165	622.50	0.127
	Total fungal metabolites	3657	90168	76523	64097.33	0.592
	Total fungal metabolites (without Ergovaline)	3656[b]	2827[b]	7537[a]	815.17	0.004
	Phytoestrogens[3]	33247	53437	52195	18981.67	0.401
	Plant metabolites	219113	191245	299025	118445.00	0.474
	Total metabolites	464023	514972	715490	267077.33	0.612
	Total metabolites (without Ergovaline)	476811	440469	658432	250208.00	0.485
number of contaminants	*Fusarium* metabolites	1.1[b]	2.0[b]	10.7[a]	1.20	<0.001
	Penicillium metabolites	1.3	1.6	2.3	0.446	0.282

	Alternaria metabolites	1.5[ab]	0.6[b]	4.0[a]	0.673	0.013
	Total fungal metabolites	6.9[b]	6.4[b]	25.7[a]	3.37	0.003
	Total metabolites	17.7[b]	19.0[b]	37.2[a]	4.15	0.012

SEM = standard error of the mean, n = number of pasture samples.

Superscripts [abc] means significant differences ($p < 0.05$) based on Tukey's method.

[1]Values are least-squares mean (LS means) and SEM is the standard error of the LS means

[2]Concentration of Emerging Fusarium metabolites 15-ydroxculmorin, Apcidin, Aurofusarin, Beauvericin, Bikaverin, Culmorin, all Enniatins, Epieq-uisetin, Equisetin, Fusapyron, Moniliformin and Siccanol according to Penagos-Tabares 2022 Toxin 14, 493

[3]Concentration of phytoestrogens (Biochanin, Coumestrol, Daidzein, Daizin, Formonetin, Genistein, Genistin and Ononin) according to Penagos-Tabares 2022 Toxin 14, 493

None of the pastures was free of either *Fusarium* or *Penicillium* metabolites. As evident from Figure 8, the contamination with *Fusarium* metabolites was highest during the stage of drought-induced damage. In two pastures EA were detected. In both, Ergovalin was the dominating EA. In total, three *Aspergillus* metabolites were detected, of which Phenopyrrozin occurred most frequently. *Alternaria* metabolites were remarkably frequent present in two pastures in the third sampling with the highest portion being Infectopyron. Abscisic acid was found in all pastures and reached the highest concentrations of the group of other fungal metabolites in each pasture. Cereulide, an emetic toxin produced by virulent *Bacillus cereus*, was detected in one pasture during the stage of ear emergence. Moreover, various plant metabolites/toxins (PE), two cyanogenic glycosides and some unspecific metabolites (i.e., analytes produced by different and unrelated species of fungi, bacteria and/or plants) were detected in every pasture. Regarding pesticides, a cocktail of five different pesticides was detected in the third sampling.

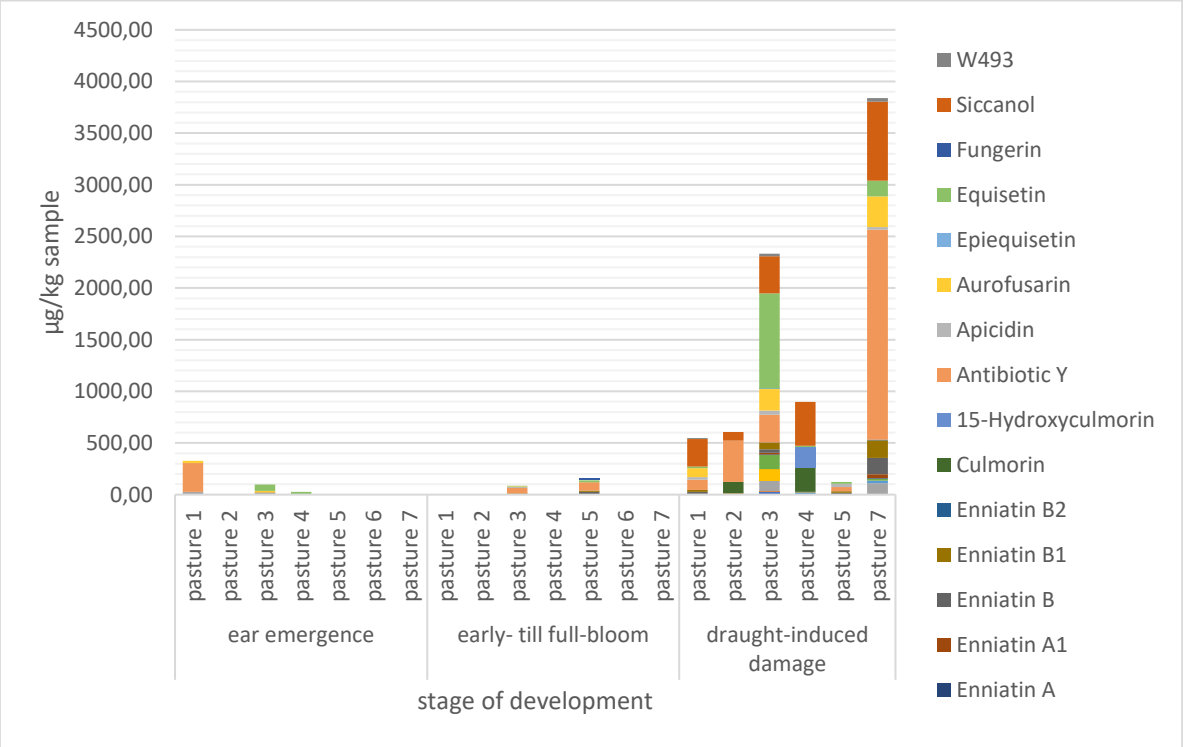

Fig. 8: Detected Fusarium metabolites.

4.3. Weather conditions

As with the season, the weather conditions changed over the time when the pasture samples were taken. The collected ZAMG-data are listed in the table below (Tab. 12).

4.3.1. Daily weather data from the day before sampling

There were statistically significant differences of all considered parameters between the three times of sampling. The daily average ($p < 0.001$) and minimum ($p < 0.001$) temperature increased from the first to the second and further to the third sampling. The daily maximum temperature was lower during the first sampling than during the second and third ($p < 0.001$). With no significant change during the latter ones. The average relative humidity ($p = 0.002$) showed a decline, whereas the 24-hour sum of sunlight were rising ($p = 0.03$) throughout the sampling period.

4.3.2. Monthly weather data

As evident from Table 12, the monthly average ($p < 0.001$), maximum ($p < 0.001$) and minimum ($p < 0.001$) temperature differed significantly between the three times of sampling. All three showed a similar trend, with higher values during the third sampling than during the two earlier ones. However, there were no significant changes regarding humidity, precipitation, and sunlight.

Tab. 12: Weather data from the website of ZAMG.

Category	**Variable**	**Ear emergence**	**Early- till full-bloom**	**Drought-induced damage**	**SEM**	**p-level**
		n=7	**n=7**	**n=6**		
	Month	**2nd/4th May**	**16th/23rd May**	**20th July/10th August**		
	Cut	Before 1st cut	Before 1st cut	Before 2nd cut		
	Sampling	1	2	3		

Climate day data	Mean temperature (°C)	13.2^{c}	16.6^{b}	20.7^{a}	0.476	<0.001
	Maximum temperature (°C)	21.6^{b}	23.7^{a}	29.5^{a}	0.954	<0.001
	Minimum temperature (°C)	4.66^{c}	9.43^{b}	11.9^{a}	0.739	<0.001
	Humidity (%)	59.4^{a}	52.0^{ab}	41.8^{b}	2.71	0.002
	Sunlight (h)	7.34^{b}	10.9^{ab}	12.2^{a}	1.14	0.030
Climate month data		May data	May data	July and August data		
	Mean temperature (°C)	16.2^{b}	16.2^{b}	21.3^{a}	0.160	<0.001
	Maximum temp (°C)	30.9^{b}	30.9^{b}	35.7^{a}	0.164	<0.001
	Minimum temp (°C)	3.33^{b}	3.33^{b}	10.1^{a}	0.756	<0.001
	Humidity (%)	65.6	65.6	63.7	0.945	0.312
	Precipitation (mm)	55.1	55.1	65.7	4.55	0.196
	Sunlight (h)	259	259	242	5.96	0.120

SEM = standard error of the mean, n = number of pasture samples.

Superscripts ^{abc} means significant differences ($p < 0.05$) based on Tukey's method.

5. Discussion

This study researched on the nutritional profile and possible contamination with mycotoxins of horse pastures in Lower Austria. The aim was to find out, how developmental stages of the plants, botanical composition of the pastures and weather conditions influence the chemical composition of forages. Finally, the results of this study can serve as a guideline for nutritional recommendations for horses at different ages, with different health status or with different performance levels.

5.1. Materials and methods

The seven randomly chosen pastures in Lower Austria were divided into two groups due to their geographical proximity. Therefore, pastures 1 and 2 (Industry Quarter) were sampled on one day and pastures 3-7 (Wine Quarter) were sampled on another. To ensure comparable data of each pasture between the three times of sample collection, the pastures were sampled in the same order and about the same time of day, respectively. According to their geographical location, the pastures were assigned to the nearest weather station of ZAMG. Hence, the station in Berndorf provided the weather data for pastures 1 and 2, the station in Schöngrabern provided the weather data for pastures 3-6, and the station in Stockerau provided the weather data for pasture 7. Weather data from the previous day of sample taking was raised to evaluate the short-term influence on the amount of fructans in the plants. However, monthly weather data was raised to evaluate the influence of seasonal weather changes on growth and development of the plants. As the first two samplings took place in the same month (May), monthly weather data is identical.

Weather data and information about the pastures' management (type and time of fertilisation, native species) as well as the pastures' constitution (botanical composition, stage of growth, height of plants) were gathered to draw a conclusion about their influence on the development of the plants as well as their nutritional composition.

The times of sampling were chosen depending on the time of mowing by the farmers. To analyse the changes in the nutritional composition between earlier and later stages of development, the first two samples of each pasture were taken before the first cut which happened at end of May. To evaluate the changes in the nutritional composition between two growths, the third sample of each pasture was taken before the second cut which happened in the middle

of August. Due to drought-induced damage and grazing, the required amount of grass for the analyses could not be taken from pasture 6 at the third sampling.

5.2. Botanical characterisation

At the first sample taking, the immaturity of the plants made it impossible to identify distinct species because none or little blossoms and/or seedheads were developed at that stage. However, at the second sampling, the extent of the botanical diversity could clearly be perceived. For the current study, the main species on each pasture were registered to compare their known characteristics with the analysed nutritional profile. All three main groups of plants (grasses, legumes, and herbs) were present on all pastures, albeit in a different ratio. Visually, the highest diversity and most balanced distribution of upper and lower grasses could be found on pasture 1 and, although less distinct, on pasture 2. A higher variety of different species is not only more palatable for the horse, but also ensures both digestibility, and fibre supply (Coenen and Vervuert 2020, Cunha 1991, Richards et al. 2021). Pastures 4 and 5 were very rich in *Fabaceae* since the farmers' pointedly used seed containing a high amount of this plant species.

Remarkably, rising temperatures during the succession of the seasons caused a lower yield, confirming the study's hypothesis. However, during the hot summer weeks, the drought-resistant *Fabaceae* grew lush compared to other species. That is because they are more accommodated to dry environmental conditions (Coenen and Vervuert 2020). With climate change in mind, it therefore can be economically beneficial to include *Fabaceae* in the applied seed to ensure a higher yield even after a dry period.

5.3. Chemical proximate composition

Research on nutritional values of pastures in general, and in Austria in particular, is scarce. Therefore, the few available studies were used for comparison even though the environmental conditions may be different due to different climate zones.

5.3.1. Protein

The results in this study showed, that the CP concentrations in general were high, and particularly were higher in the first samples than in the second. Jeroch et al. (2020) attributed this phenomenon to a faster absorption than utilisation of nitrogen for the build-up of biomass, by younger plants. The measured CP values at different developmental stages are in line with the

values found by Ball et al. (2002) for alfalfa and for cool season grasses (Tab. 13) (Geor 2013). A review from Hoskin and Gee (2004) on the nutritional value of horse pastures in New Zealand (Tab. 13), also found higher CP concentrations in younger plants. However, these researchers analysed three types of pastures with either ryegrass and white clover, red clover or lucerne. Therefore, data must be extrapolated to be comparable with the current results. Ryegrass belongs to the family of *Poaceae*, white clover to the family of *Fabaceae*. Considering that the sampled pastures in this study contained a mixture of *Fabaceae* and *Poaceae*, comparison with Hoskin and Gee's values of ryegrass and white clover pastures is most appropriate.

Tab. 13: CP concentrations of different pastures/plants at different stages of development.

Species	Stage of development	% CP (DM basis)
Alfalfa[a]	Pre-bud/bud	22-26
	Early- till full-bloom	9-22
Cool season grasses[a]	Vegetative	16-20
	Boot	12-16
	Head	8-12
Ryegrass and white clover[b]	Spring	15-24
	Summer	10-22

[a]Geor (2013); [b]Hoskin, Gee (2004)

The hypothesis, that a high ratio of legumes leads to higher CP concentrations, could be confirmed with this study, since average CP concentrations were higher in samples from pastures with a high ratio of *Fabaceae*. This is most likely due to the unique ability of *Fabaceae* to bind atmospheric nitrogen and therefore making them more independent from soil nitrogen (Jeroch et al. 2020). Another interesting finding was, that CP values steadily decreased until the third sampling in all pastures except in pastures 5 and 7. The explanation for this divergent result of pasture 5 could be the high ratio of *Fabaceae* in the third sampling. Furthermore, after the second sampling, horses were grazing on the pasture which may led to nitrogen supply through urination and droppings. On pasture 7, a higher ratio of *Trifolium repens* (also from the family of *Fabaceae*) was measured compared to the first two samples, which can explain the higher CP concentration. CP rich forages are appropriate to cover increased protein requirements of high-performance horses, lactating mares, and foals. However, an excessive amount may lead to gastrointestinal dysbiosis and is too rich in energy for horses with maintenance requirements (Coenen and Vervuert 2020).

To summarise, grass harvested at an early stage of development and/or pastures rich in *Fabaceae*, is most suitable for horses with a high requirement of amino acids (protein) and Ca for building up body mass (high performance horses) and for growing processes (lactating mares, foals). However, for horses who just need to fulfil the demands of basal metabolism, early harvested plants are too rich in these two nutrients.

5.3.2. Carbohydrates

Another finding was the steadily decreasing WSC concentration in the plants from the first to the third sampling. Kramer, Kagan et al. (2020) also concluded that the WCS concentration went down from the beginning to the end of the season (Kramer et al. 2020). A possible explanation is the temperature-dependant activity of fructan-storing enzymes which slow down below 5 °C and completely stop working below freezing temperature. Under warm conditions, the enzymes function more efficient (Watts 2010). Furthermore, in early stages of development, plants are rich in leaves and characterised by a high amount of cell content-components and therefore contain more WSC. However, until blooming, the WSC concentration decreases on account of the elongation of the plant. For the elongation, the plant needs a higher amount of cell wall-contents for stabilisation during its growing process (Jeroch et al. 2020). These findings confirm the study's' hypotheses, that high WSC concentrations are related to low average temperatures and early developmental stages of the plants. However, varying WSC concentrations could also be measured when the botanical composition changed: pastures with a high percentage of *Fabaceae* showed a lower WSC concentration. This approves the common knowledge that *Fabaceae* have generally lower WSC values than other grasses (Jeroch et al. 2020). This is ascribed to the fact that legumes mainly use starch as a storage carbohydrate (Longland and Byrd 2006) and not WSC such as C3 grasses (Klevenhusen and Zebeli 2021).

In conclusion, for horses with a history or a predisposition for equine laminitis, it is recommended to use late-cut hay due to its lower WSC concentrations. In addition, grazing should be avoided or restricted early in the grazing season and postponed to a later period for horses with a risk for laminitis. In case of the sampled pastures in Lower Austria from 2022, this would mean to use hay from a late first (end of May) or second cut (July-August) and allow grazing from the end of May until August, respectively. A low concentration of WSC is beneficial because, as pointed out earlier, high concentrations of fructans in the equine diet can trigger the onset of equine laminitis. This specifically applies to horses with EMS who suffer from insulin

resistance. This condition leads to an insufficient supply of the cells, including the ones of the hoof (Longland and Byrd 2006), leading to lamellar injury (Geor 2013). However, it is important to keep in mind that depending on the weather conditions, WSC values can change annually. Therefore, further analyses would be helpful to evaluate those changes. Concerning hay harvest, it would be beneficial for the first cut to wait until the plants mature and likewise are lower in fructans. However, a later first cut could mean that due to dry conditions in late summer, the second cut may fall out which consequently leads to economic losses for the farmer.

5.3.3. Fibre

When plants mature, the percentage of cell wall-components increase and consequently NDF concentrations rise (Jeroch et al. 2020). In this study, the average NDF and ADF concentrations of the second samples were the highest. Therefore, the hypothesis of mature plants containing more fibre, was confirmed. After the second sampling, the hay was harvested. However, because of a drought-induced stress during summer, a notable rise in the ADF/NDF ratio occurred in the samples measured in the third sampling, although the NDF content was not different from the samples collected at the first sampling. An explanation for this could be, that the third sample concerned plants which did not develop to their full extent and the NDF values were therefore more like the ones of the first cut. Moreover, the plants could not fully develop due to the drought-induced damage. Overall, the higher ADF/NDF ratio in late cut hay is an important dietary concern because high ADF concentrations cause a lower digestibility in the equine GI-tract. Consequently, hay harvested late in the season is not suitable for high-performance horses, horses with dental problems or foals, which all require a highly digestible forage. However, it is appropriate for horses with basal nutritional requirements and healthy older horses, because both groups do not need an extra amount of energy (Coenen and Vervuert 2020).

5.4. Minerals and trace elements

As plants develop, concentrations of minerals and trace elements decrease until the first cut (Jeroch et al. 2020). During the maturation, the amount of cell wall-components in the plant increase at the cost of the cell content (Geor 2013), including the minerals and trace elements. This decrease was also perceived in the present study for all minerals and trace elements from the first to the second sample taken, except for Ca and Mg, which stayed at the same level.

Hence, the hypothesis that younger plants contain generally higher concentrations of minerals and trace elements was not confirmed with this study.

However, in the second cut, according to Jeroch, Drochner et al. (2020), minerals and trace elements are generally higher. This fact was only partially confirmed in the results of this study. The concentrations of Ca, Mg and Fe increased, but the concentrations of P, K, Mn, Zn and Cu were lower in the samples taken at the stage of drought-induced damage.

In Table 14, an overview of the minimum requirements for minerals and trace elements for a 500 kg horse as prescribed by Eichberg (2014), is given (Eichberg 2014). However, these data are only based on a small number of horses and therefore have a low reliability and may not be generalizable to the population level. Furthermore, little or nothing is known about the bio-availability of minerals and trace elements in fresh forage and reliable data about optimal intake are lacking (Hoskin and Gee 2004). Moreover, many factors influence the amount of minerals and trace elements in pastures during their development. These include plant species, soil (type and pH) as well as fertilisation, weather conditions (temperature, rainfall) and industry emissions (Jeroch et al. 2020).

Tab. 14: Minimum requirements of minerals and trace elements for a 500 kg horse according to Eichberg (2014).

g/d					**mg/d**			
Cal-cium	**Phos-phorus**	**Magne-sium**	**Potas-sium**	**So-dium**	**Iron**	**Manga-nese**	**Zinc**	**Copper**
17.4	12.0	5.6	14.7	2.9	422.9	422.9	422.9	105.7

Results from our analyses were compared to studies conducted in New Zealand (Hoskin and Gee 2004) and Poland (Jachimowicz-Rogowska et al. 2022) which are listed in Table 15.

Tab. 15: Comparison of mineral and trace element concentrations from pastures in Lower Austria, New Zealand, and Poland.

	This study in Lower Austria (average over all samplings)	**New Zealand (tall fescue and ryegrass/white clover)[a]**	**Poland (group A)[a]**	**Poland 2 (group)[a]**
Ca (g/kg DM)	7-13	3-4.2		
P (g/kg DM)	3-4	3-3.8		
Mg (g/kg DM)	2-3	1.6-2.1		
Na (g/kg DM)	0.3-0.4	1-2.7		
K (g/kg DM)	24-32	24-39		
Cu (mg/kg DM)	6-8	6.3-7.9	4±1	7±1
Fe (mg/kg DM)	50-109	132-225	47±6	48±2
Zn (mg/kg DM)	21-24	22-37	11±3	20±2
Mn (mg/kg DM)	37-47	55-87	99±14	205±2

[a]Hoskin, Gee (2004); [b]Jachimowicz-Rogowska, Topczewska et al. (2022)

Data from our study indicate, that pastures in Lower Austria meet the required concentrations of Ca, P, Mg, and K based on the need of a 500 kg horse. Yet, there is a deficiency of Na, Fe, Mn, Zn and Cu (Tab. 16).

Tab. 16: Detected concentrations and the intake of selected minerals and trace elements in the pastures compared to the requirement of a 500 kg horse at a maintenance level, assuming a DM-intake of 10 kg/d (calculated based on the recommendations of GfE, 2014).

		g/kg DM	g/d	mg/kg DM	mg/d	mg/kg DM	mg/d	mg/kg DM	mg/d	mg/kg DM	mg/d
S	**P**	**Na**		**Fe**		**Mn**		**Zn**		**Cu**	
1	1	0.2	2.0	84.1	841	37.3	373	23.4	234	7.5	75
	2	0.3	2.8	100.2	1002	34.5	345	33.4	334	8.6	86
	3	0.2	1.6	111.7	1117	66.0	660	23.4	234	7.4	74
	4	0.3	2.5	109.9	1099	55.5	555	24.5	245	8.5	85
	5	0.5	4.7	95.9	959	51.2	512	24.5	245	9.6	96
	6	0.3	2.8	76.6	766	42.6	426	20.2	202	6.4	64
	7	0.8	8	74.4	744	47.4	474	19.4	194	5.4	54
2	1	0.2	1.9	48.7	487	32.8	328	21.2	212	6.3	63
	2	0.3	3.2	52.0	520	31.8	318	27.6	276	6.4	64
	3	0.1	1.2	45.5	455	45.5	455	20.1	201	6.3	63
	4	0.2	2	41.3	413	37	370	22.2	222	6.3	63
	5	0.4	4	63.2	632	40.7	407	20.3	203	7.5	75
	6	0.2	1.8	52.3	523	30.9	309	18.1	181	5.3	53
	7	0.4	3.5	44.5	445	42.4	424	14.8	148	4.2	42
3	1	0.2	1.6	78.8	788	26.6	266	19.2	192	6.4	64
	2	0.2	1.6	108.6	1086	29.5	295	35.9	359	7.4	74
	3	0.1	1.2	84.9	849	53.1	531	15.9	159	4.2	42
	4	0.3	2.8	83.5	835	36.4	364	16.1	161	6.4	64
	5	0.3	2.7	119.8	1198	37.1	371	25.5	255	12.7	127
	7	0.9	8.9	180	1800	85.2	852	22.4	224	7.5	75
requirements 500 kg horse			**2.9**		**422.9**		**422.9**		**422.9**		**105.7**

S = sampling, P= pasture

High concentrations of Ca were detected on pastures with a high ratio of *Fabaceae*, confirming the study's hypothesis. However, against the declaration that Ca concentrations decrease with high precipitation rates (Jeroch et al. 2020), in our study the highest average Ca concentrations coincide with the lowest monthly sum of rainfall over the sampling period. As Ca plays an important role in vital processes such as blood coagulation, bone mineralisation and intercellular communication, its supply is essential. As far as our analyses show, the pastures provide enough Ca for the requirements of a 500 kg horse (Tab. 16). However, this does not consider its final bioavailability to the horse.

The minerals P and Mg are usually sufficient in forages (Coenen and Vervuert 2020, Jeroch et al. 2020). This was also valid for the samples taken in this study. Considering the minimal requirements of a 500 kg horse, both P and Mg were abundant in all samples (Tab. 16). The pasture with the highest P concentrations throughout all sample takings was pasture 2, which is probably due to the fertilisation with chicken manure two months before the start of sample taking (March 2022). Chicken manure is known to be very rich in P (Topcu et al. 2022). Another reason could be a chalky soil, which would have to be examined through soil analysis (Jeroch et al. 2020).

It is generally known, that forage is rich in K but very low in Na (Jeroch et al. 2020). This also applies for the pastures in Lower Austria that were part of the study. Thus, considering the minimal requirements, a spare amount of K but a lack of Na was detected (Tab. 16). Therefore, K does not need to be supplemented, whereas free access to salt lick stones containing sodium chloride (NaCl) for all horses is recommended. For horses with physical activity especially during summer and high-performance horses with an increased sweat loss through exercise, the specific supplementary feeding of NaCl is recommended, based on the level of exercise and seating. This is because Na and Cl are eliminated from the body with sweat which leads to deficiencies in the bodily stores of these electrolytes. Concerning K, whereas a deficiency is unlikely, an oversupply via grass/hay is generally well tolerated by horses as it is mainly excreted through faeces and urine. The solely indication for a restricted K supply is for Quarter Horses with the autosomal-recessive genetic defect called Hyperkaliaemic Paralysis (Coenen and Vervuert 2020).

Average concentrations of Cu were low throughout the sampling period. The measured values were comparable to the results of studies in New Zealand (Hoskin and Gee 2004) and Poland (Jachimowicz-Rogowska et al. 2022) (Tab. 15). Since the minimal required concentrations are

not met (Tab. 16), supplementation should be considered. Deficiencies have a negative effect equine health. Foals may suffer from anaemia and skeletal deformation due to the influence of Cu on haemoglobin-, myelin- and collagen-formation. In adult horses, loss of pigmentation and a disposition for vessel rupture could be a consequence of an insufficient Cu supply (Coenen and Vervuert 2020).

Surprisingly, Mn concentrations in our study were very low compared with data from New Zealand (Hoskin and Gee 2004) and Poland (Jachimowicz-Rogowska et al. 2022). Low levels of Mn can be caused by circumstances such as chalky soils, Ca rich fertilisation or longer periods of drought (Jeroch et al. 2020). However, the last two can be excluded as the values showed no correlation with the raised temperature data and none of the used fertilizers suggested being rich in Ca. Which leaves the chalky soil as the most likely cause of low Mn-levels. To evaluate this factor, analyses of soil samples would be necessary. Mn has many important functions in the body: co-factor for many enzymes in the mineral- and fat-metabolism, maintenance of ovary and chondroitinsulfat function, immune system activity, and protection against oxidative stress (Coenen and Vervuert 2020). Since the minimal required concentrations for Mn are not met (Tab. 16), horses fed with forage from the sampled pastures in Lower Austria, may require supplementation of Mn through mineral feed.

As none of the pastures meet the recommended minimum Zn concentrations either (Tab. 16), and as Zn is influenced by the same environmental factors as Mn, the assumption that a chalky soil is the cause for low values of Mn and Zn, seems to be confirmed. With Zn having various functions in the body (i.e., cell regeneration, maintenance of genetic information, creatine synthesis), mineral supplements should always contain Zn to compensate the deficiency in forage (Coenen and Vervuert 2020).

5.5. Mycotoxins and contaminants

Very little is known about the prevalence and potential adverse effects of mycotoxins on pastures used for horse feed. To the best of our knowledge, no systematic review on mycotoxins in forage for horses has been published. The present study is the first in Europe that documents the occurrences, not only of mycotoxins, but also of some relevant plant derived compounds (PE and cyanogenic glycosides) as well as unspecific metabolites in pastures used for horse grazing and hay production. However, there are three surveys on mycotoxins on Austrian pastures used for dairy production (Penagos-Tabares et al. 2021, Penagos-Tabares et al. 2022), and one study from Germany on mycotoxins in commercial horse feed preparations

(complementary feeds) (Liesener et al. 2010). Since mycotoxins, their metabolites and plant derived compounds can have negative effects on equine health, this lack of data is quite surprising. The only mycotoxin that has been studied more in equine science, is Fumonisin B1 (FB1), which, in high concentrations, can cause Equine Leukoencephalomalacia (EFSA, European Food Safety Authority 2005b, Liesener et al. 2010, Osweiler 2001) and, in low concentrations, can induce liver toxicity. Furthermore, FB1 is the only regulated mycotoxin with guidance values for horses by the European Food Safety Authority (EFSA) (EFSA, European Food Safety Authority 2005b).

One important finding of the current study was, that the concentrations of *Fusarium* metabolites and total fungal metabolites (without Ergovaline) were the highest at the stage of drought-induced damage, indicating a correlation to dry weather conditions. The values of total fungal metabolites, were corrected by excluding Ergovaline because very high values of Ergovaline in two single samples, falsified the correlation analysis. However, according with temperature changes, the concentrations of *Fusarium* metabolites and total fungal metabolites remained comparably low when the monthly average temperature was about 16 °C (first and second sampling) and rose when the monthly average temperature was around 21 °C (third sampling). A similar trend occurred with the fungal metabolite contamination in the study from Penagos-Tabares et al. (2021), where, over the critical temperature of 15 °C concentrations of *Fusarium*, *Alternaria* and total fungal metabolites rapidly rose (Penagos-Tabares et al. 2021).

Another interesting result of this study was that not only the quantity but also the variety of different metabolites increased when temperatures were highest and rainfall was lowest in the foregoing month. This means, that samples collected late in the season had higher levels of co-contamination with fungal metabolites. Furthermore, the number of total fungal metabolites (without Ergovaline) increased compared to those of early sampling.

Both regulated and emerging *Fusarium* metabolites were detected in the samples. Among the EU-regulated mycotoxins, FB1, the mycoestrogen ZEN, and the cytotoxic type A trichothecene T-2 were present in some samples. For horses, there are only specific guidance values for FB1, with maximum allowed levels of 5000 µg/kg in corn and corn byproducts. In experimental studies, adverse effects of FB1 (i.e., neurological abnormalities) were observed at a minimum intravenous dose of 0.01-0.05 mg/kg body weight pure FB1. With the assumption of a bioavailability of 5 % in horses, this would mean an oral dose of 0.2-1.0 mg/kg body weight per day. In the current study, only pasture 3 had once a FB1-positive sample with a measured

value of 0.1 mg/kg sample. Assuming a 500 kg horse with a daily intake of 1.5 kg hay/kg body weight and a consistent incidence of FB1 over the whole pasture, the oral dose would reach 0.002 mg/kg bodyweight. Therefore, health consequences are very unlikely (EFSA, European Food Safety Authority 2005b). Although the adverse effects after the intake of regulated and emerging mycotoxins are not properly characterized, these compounds can interact with other well-recognised fungal toxins, increasing their toxicological activity (Penagos-Tabares et al. 2022).

It is interesting to note, that in the samples of pasture 7 at the third sampling, a total of 13 EA were detected. Most of them were present in low concentrations (3-33 mg/kg sample), whereas Ergovaline reached a value as high as 414 mg/kg sample. This high contamination with Ergovaline, seems to bear major health effects, as EFSA concluded in its evaluation concerning ergots in animal feed, that mares are sensitive to even lower values, starting from 0.05-0.1 mg/kg. Ergovaline is known to cause delayed parturition, dystocia, and agalactia in mares (Blodgett 2001), as well as neurological symptoms in both sexes. For other EA, data related to equine health is very scarce and quantitative data are lacking (EFSA, European Food Safety Authority 2005a). The only other pasture with a contamination of EA, was pasture 3 at the second sampling. There, the analyses detected only Ergovaline, but in even higher concentrations (611 mg/kg sample) than on pasture 7. EA are mainly produced by the fungi *Claviceps* and *Epichloë* spp. which parasitise on a wide range of monocotyledonous plants, including the family of *Poaceae*. Since *Poaceae* were present on all pastures and the weather conditions were roughly the same at all locations, it remains unclear, why only pasture 7 showed such a high number of EA in the last sample. Ergovaline is associated with the presence of fescue grass (i.e., *Festuca spp.*), as its producing fungi parasitises on these plants (Penagos-Tabares et al. 2021). Penagos-Tabares et al. (2021) explained in their study, that the absence of Ergovaline in their samples, was due to the low occurrence of *Festuca pratensis*. However, in the current study pasture 3 and 7 with high Ergovaline concentrations, contained visibly no *Festuca spp.* when compared to others. This suggests that not only *Festuca spp.* produces Ergovaline, but also other plant species do so.

A notable number of PE were present in all samples. PE are plant compounds, which show a resemblance to the hormone 17β-estradiol (E2). Therefore, they are able to bind on estrogen receptors, which can lead to endocrine disorders (i.e., influence on the estrous cycle) (Ferreira-Dias et al. 2013). In sheep, cattle and horses, the PE coumestrol is known to cause a declining reproductive efficiency (Penagos-Tabares et al. 2021). The legumes alfalfa and clover are two

types of estrogenic plants, producing PE (Ferreira-Dias et al. 2013, Penagos-Tabares et al. 2021). This would suggest that a high ratio of alfalfa and clover on pastures, lead to high PE concentrations. Surprisingly, the current study showed the opposite: PE concentrations were low on pastures with a high legume ratio (pasture 4 and 5) compared to pastures containing a more diverse botanical variety (pastures 1 and 2). This suggests that not only plant species, but also other, yet unknown, factors determine the presence of PE in forage. Research on health effects of PEs is scarce and guidance values for horse feed are missing. Therefore, more research needs to be done on this issue.

Pesticides were only present in very low amounts. However, it was remarkable, that in the third sample of pasture 3, five pesticides were detected even though this pasture is declared as a conservation area, where the use of pesticides is not allowed. Since the farmer declared, that no pesticides were used, a rational explanation could be the airborne contamination from nearby potato fields. Five out of six detected pesticides were fungicides, the fungal contamination was not lower.

To sum up, the analyses confirmed the hypothesis that mycotoxins and other contaminants are always present on pastures, though in varying concentrations. However, the co-contamination with different mycotoxins, PE, and other metabolites occurring in the sampled pastures, suggest undiscovered and unpredictable synergistic and antagonistic toxic effects. In this study, the analyses showed that the contamination was higher, when the hay was cut later in the season. Since this finding was related to dry weather conditions, it is not unreasonable that with ongoing climate change, contamination levels will become higher even earlier in the year. However, because not much is known about minimum values causing adverse effects in horses, further research is necessary. Overall, the use of drought-damaged grass for grazing or hay production should be made with caution.

6. Summary

The aim of this thesis was to evaluate the nutritional profile (i.e., fibre, protein, carbohydrates, minerals, and trace elements) and common contaminants (i.e., mycotoxins, their metabolites, and plant-derived compounds such as PE) in selected horse pastures in Austria from spring (2nd May 2022) to late summer (10th August 2022). Particular attention was on the influence of weather conditions and developmental stages of the plants. Therefore, the pastures were assigned to the nearest weather station of the ZAMG, and grass samples of each pasture were taken on three different times during the seasons.

The results showed that the digestibility of hay and grass is related to the amount and type of fibre. High ADF concentrations in late cut hay and grass in late summer, respectively, lead to a lower digestibility. Therefore, horses in need of a highly digestible diet (i.e., foals, high performance horses, horses with dental problems) should be fed hay of the earlier season with a more balanced ratio of ADF and NDF. Furthermore, high CP concentrations co-occurred with early developmental stages of the plants and with a high ratio of *Fabaceae* on the pastures.

Regarding the influence of different weather conditions, lower monthly average temperatures and early stages of plant development were associated with high WSC concentrations. Therefore, the risk for laminitis is expected to be lowest for horses by consuming summer-hay/grass.

Since minerals and trace elements play an important role in many body functions, a deficiency of them likely leads to health problems. The sampled pastures in Lower Austria showed high concentrations of Ca, P, Mg, K and Fe, but where low in Na, Mn, Zn and Cu. Therefore, a supplementation of the latter four in the form of food additives is suggested when feeding a mere hay/grass-diet.

The analyses of this thesis underlined the presence of a broad number of mycotoxins (most of them unregulated), and other metabolites occurring in diets of horses in Austria. Since little is known about the effects of different mycotoxins and other fungal or plant derived compounds, the occurrence of these substances in horse feed should be further investigated since the co-contamination with different mycotoxins, PE, and other metabolites suggest undiscovered and unpredictable synergistic and antagonistic toxic effects.

In summary, the drought and high weather temperatures in summer affected the nutritional and hygienic value of the pastures, as evidenced by higher contents of ADF and CP, as well as the higher contamination with mycotoxins and other metabolites, respectively. Further

research is required to evaluate the effects of such strong changes in the nutritional, safety, and hygienic status of the pasture on equine nutrient supply and health.

7. Zusammenfassung

In dieser Arbeit wurden das Nährstoffprofil (u.a. Fasergehalt, Proteine, Kohlenhydrate, Mineralstoffe) sowie das Vorkommen verbreiteter Kontaminanten (z.B. Mykotoxine, deren Metaboliten, pflanzliche Substanzen wie Phytöstrogene) von ausgewählten Pferdeweiden in Österreich untersucht. Der Untersuchungszeitraum erstreckte sich von Frühling (2. März 2022) bis Spätsommer (10. August 2022). Besonderes Augenmerk lag dabei auf dem Einfluss von Witterungsbedingungen und dem Entwicklungsstadium der Pflanzen. Um dies zu untersuchen, wurden die einzelnen Weiden jeweils der nächstgelegenen Wetterstation der ZAMG zugeteilt und von jeder Weide wurden Proben an drei verschiedenen Zeitpunkten während des Untersuchungszeitraums genommen.

Die Ergebnisse zeigten, dass die Verdaulichkeit von Heu/Gras mit der Menge und dem Typ an Faserstoffen verbunden ist. Hohe Mengen an ADF in spätgeschnittenem Heu, beziehungsweise Gras im Spätsommer, führen zu niedriger Verdaulichkeit. Aus diesem Grund sollten Pferde, die hochverdauliches Heu benötigen (z.B. Fohlen, Sportpferde, Pferde mit Zahnproblemen), frühgeschnittenes Heu gefüttert bekommen, das ein ausgeglicheneres Verhältnis von ADF und NDF aufweist. Des Weiteren wurde gezeigt, dass Weiden mit einem hohen Anteil an *Fabaceae* sowie Pflanzen in frühen Entwicklungsstadien, höhere Proteinkonzentrationen aufwiesen als andere.

Hinsichtlich der Einflüsse von Witterungsverhältnissen und dem Entwicklungsstadium der Pflanzen ging aus den gesammelten Daten hervor, dass niedrige durchschnittliche Monatstemperaturen und junges Alter der Pflanzen in Zusammenhang mit höheren Konzentrationen an wasserlöslichen Kohlenhydraten standen. Aus diesem Grund ist das Risiko für die Entstehung von Hufrehe am niedrigsten bei der Aufnahme von Heu/Gras aus den Sommermonaten.

Mineralstoffe (Mengen- und Spurenelemente) haben wichtige Funktionen im Körper und eine mangelnde Versorgung hat oft gesundheitliche Konsequenzen. Die beprobten Weiden in Niederösterreich wiesen hohe Mengen an Ca, P, Mg, K und Fe, aber niedrige Mengen an Na, Mn, Zn und Cu auf. Folglich ist es ratsam, die vier letztgenannten Mineralstoffe bei einer alleinigen Heu-/ Grasfütterung in Form von Ergänzungsfuttermitteln zu supplementieren.

Weiters zeigten die Analysen dieser Arbeit das Vorkommen einer großen Bandbreite an Mykotoxinen und anderen Metaboliten in den beprobten Pferdeweiden. Da wenig zu den Wirkungsweisen der verschiedenen Mykotoxine und Metaboliten bekannt ist, sollte das Auftreten

dieser Substanzen weiter untersucht werden. Vor allem, weil die Co-Kontamination mit verschiedenen Mykotoxinen, Phytöstrogenen und anderen Metaboliten bis dato unbekannte und unvorhersehbare synergistische und antagonistische toxische Effekte haben kann.

Zusammenfassend kann gesagt werden, dass Trockenheit und hohe Lufttemperaturen im Sommer den nutritiven und hygienischen Status von den Weiden beeinflusst haben. Dies ging sowohl aus den höheren Konzentration von Rohprotein und ADF als auch aus der höheren Kontamination mit Mykotoxinen und anderen Metaboliten hervor. Zukünftig sind weitere Untersuchungen indiziert, um eine Aussage über die gesundheitlichen Effekte dieser starken nutritiven und hygienischen Veränderungen von Weiden auf die Nährstoffversorgung und Gesundheit von Pferden treffen zu können.

8. Abbreviations

ADF	Acid detergent fibre
Ca	Calcium
CA	Crude ash
CP	Crude protein
Cu	Copper
DM	Dry matter
DON	Deoxynivalenol
EA	Ergot alkaloid(s)
EE	Ether extract
EMS	Equine metabolic syndrome
ESC	Ethanol-soluble carbohydrate
FB1	Fumonisin B1
Fe	Iron
GI-tract	Gastrointestinal tract
IR	Insulin resistance
K	Potassium
LOD	Limit of detection
Mn	Manganese
Na	Sodium
NaCl	Sodium chloride
NDF	Neutral detergent fibre
NFC	Non-fibre carbohydrate
NfE	Nitrogen-free extract
NSC	Non-structural carbohydrate
oM	Organic matter
oS	Original substance
P	Phosphorus
PE	Phytoestrogen(s)
PPID	Pituitary pars intermedia dysfunction
RW	Raw water
Se	Selenium
SEM	Standard error of the mean

WSC	Water-soluble carbohydrate
ZAMG	Zentralanstalt für Meteorologie und Geodynamik
ZEN	Zearalenone
Zn	Zinc

9. Index of tables

10. Index of figures

11. Literature

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