Aus dem Institut für Tierernährung
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

und dem

Bundesinstitut für Risikobewertung

Transfer of plant toxins from feed into milk – a challenge for future sustainable milk production systems?

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Anna Maria Engel
Tierärztin aus Holzminden

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Meiner Familie

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List of Abbreviations

ADI	Acceptable daily intake
AM	Atypical pasture myopathy
AP	Adaptation period
BMEL	German Federal Ministry of Food and Agriculture
BIC	Bayesian information criterion
BfR	German Federal Institute for Risk Assessment
BSL	Blue sweet lupin
BW	Bodyweight
CK	Creatinkinase
CoA	Coenzyme A
DLQ	German Association for Performance and Quality Testing e.V.
DM	Dry matter
DP	Depuration period
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization of the United Nation
HBGV	Health based guidance value
HGA	Hypoglycin A
LC-HR-MS	Liquid-Chromatographie High-Resolution mass spectrometry
LC/MS-MS	High-Performance Liquid-Chromatographie-Tandem mass spectrometry
LFGB	German Food and Feed Code
LOQ	Limit of quantification
MCPrG	Methylencyclopropylglycine
MCPA	Methylencyclopropylacetyl
MCPA-C	Methylenecyclopropylacetylcarnitine
MCPA-CoA	Methylenecyclopropylacetyl-Coenzyme A
MCPA-G	Methylenecyclopropylglycine
MCPF	Methylencyclopropylformyl
MCPF-C	Methylenecyclopropylformylcarnitine
MCPF-CoA	Methylenecyclopropylformyl-Coenzyme A
MCPF-G	Methylenecyclopropylformylglycine
ME	Metabolizable energy
MoE	Margin of exposure
MRM	Multiple Reaction monitoring
NADH	Nicotinamide adenine dinucleotide
NDF	Neutral detergent fiber
NOAEL	No observed adverse effect level

NRL	National reference laboratory
PA	Pyrrolizidine alkaloids
PBTK	Physiologically-based toxicokinetic model
QA	Quinolizidine alkaloids
SPM	Secondary plant metabolites
TR	Transfer rate
VDLUFA	Association of German Agricultural Analytic and Research Institues
WHO	World Health Organization

Introduction

As the world's population continues to grow, so too does the demand for protein sources and, consequently, there is a concomitant increase in production of food of animal origin. Soybean extraction meal is a valuable protein feed source for both monogastric and ruminant animals due to its favorable amino acid composition and low content of antinutritional substances. However, there are many drawbacks to growing, processing and importing soybeans, including habitat destruction, increased greenhouse gas emissions, deforestation, and land use change. As a result, there has been a broader reconsideration of agricultural practices, advocating for the adoption of local protein sources, sustainable grazing systems, and agroforestry. Alongside the many benefits of this shift in thinking, there is also a potential risk from the presence of secondary plant metabolites (SPM) in both local protein sources and among pasture crops, which have not yet been sufficiently investigated. SPM are components that occur in plants as a protection against herbivores to ensure their reproduction and survival (Van Egmond 2004). This should prevent the plant from being ingested by herbivores, but ingestion can still occur, either through use as feed and associated processing (e.g. hay and silage), or through direct ingestion of the plant on pasture elevating the overall likelihood of food-producing animals consuming potentially toxic SPM. The transfer of individual SPM with toxic effects via feed into food of animal origin, primarily milk has been observed in the past and examples among others are pyrrolizidine alkaloids, cannabinoids and ptaquiloside from bracken fern (Hoogenboom et al. 2011; Virgilio et al. 2015; Wagner et al. 2022). Some plant parts are therefore already listed as undesirable substances in animal feed according to RL 2002/32 EG of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed (EU 2002). Still, for numerous plants species, there is limited or insufficient data available on concentrations of potentially toxic SPM and their transfer into milk. Examples for secondary plant constituents with a suspected but not conclusively proven transfer are Quinolizidine alkaloids (QA) from lupins (*Fabaceae*), and Hypoglycin A (HGA) and Methylenecyclopropylglycine (MCPrG) from sycamore maple trees (*Sapindaceae*). Lupins have been increasingly used in animal feed in recent years as an alternative to traditional protein sources. However, there are no data on whether their most prominent SPM, QA, may possibly pass into the milk. In general, QA effect acetylcholine receptors leading to a so-called anticholinergic syndrome including among others tachyarrhythmia and gastrointestinal symptoms. Sycamore maple trees are widespread deciduous trees in meadows and field margins. Active metabolites of their SPM, HGA and MCPrG, have caused severe morbidity in horses and other various herbivore species by inhibiting different enzymes in fatty acid metabolism. Recently, traces of the protoxins HGA and MCPrG have been detected in mares' milk as well as in bulk tank milk in northern Germany. To provide recommendations for both livestock management (feeding and grazing) and consumer safety, it's crucial to understand

the potential risks associated with the utilization of these plants in the context of livestock farming. The work presented in this thesis should help to shed light into the transfer potential of these two particular groups of SPM from feed into the milk of dairy cows.

Chapter I

Literature review

Livestock is facing different challenges due to the strong influence of some farming systems on the environment. The increased demand on protein-rich food due to rising populations on the one hand and the desire to reduce possible negative impact on the environment on the other demand changed measures. Apart from enteric fermentation processes in ruminants, manure management and energy consumption, feed production, processing, and transport account for a large share of total greenhouse gas emissions in livestock farming (Cheng et al. 2022). As 70% of the world's agricultural land is already used to produce animal feed, the creation of new land to meet increased demand is limited (FAO 2009). For this reason, various solutions are required to reduce greenhouse gas emissions and contribute positively to climate change in this sector, while at the same time guaranteeing food availability and safety. In the future, the use of terrestrial animal source food will continue to be crucial for sustainable and healthy nutrition due to its diverse ingredients including essential nutrients like fatty acids and amino acids as well as vitamins or carbohydrates (FAO 2023).

Dairy cow husbandry and the process of milk production hold a unique significance in this context. Through coevolution with the rumen microbiome, cows possess a sophisticated forestomach system that enables them to transform fibrous substances like grass and other coarse forage into valuable, nutrient-rich products like milk and meat, rendering them suitable for human consumption, unlike monogastric animals (Tabacco et al. 2018). Therefore, dairy farming positively contributes to the sustainable development goals of the United Nations stated in the 2030 Agenda for sustainable development, e.g. good health and wellbeing or zero hunger but nevertheless contributes negatively to other sustainable development goals like climate action or life on land to a large extent as dairy cattle and livestock produce a large share of greenhouse gas emissions (UN 2015; Rojas-Downing et al. 2017; Segerkvist at al. 2020).

Initial considerations have therefore been made to combine needs-based feeding with sustainability to reduce greenhouse gas emissions and contribute positively to climate change. Significant progress has been made in the field of dairy cow nutrition in recent times, such as improving enteric fermentation management through feed additives and supplements as secondary phenolic compounds like tannins or essential oils resulting in possible CH_4 reductions between 2.5 and 15% (Knapp et al. 2014; Caro et al. 2016). In addition, as reviewed by Peterson and Mittloehner (Peterson and Mittloehner 2021), promising supplements include, for example, nitrooxypropanol, which increases CH_4 emissions by 84% in vivo (Vyas et al.

2016). The use of nitrates in the diet is also a promising option for reducing enteral emissions, as nitrates are non-protein N sources that act as electron receptors in the rumen (Peterson and Mittloehner 2021).

According to the literature, another relevant strategy to reduce impacts is to intensify sustainable agriculture, e.g. milk production per cow or feed efficiency, through the correct choice of crops and crop rotations and the use of perennial or permanent grassland combined with agroforestry (Liebmann et al. 2008; Bava et al. 2014; Tabacco et al. 2018; Gislon et al. 2020). This is also in line with the sustainability goals formulated at national level as summarized in the report of the commission on the Future of Agriculture which includes the expansion of agroforestry and the reduction of land sealing as well as the cultivation of balanced crop rotations including domestic grain legumes (Commission on the Future of Agriculture 2021).

Sustainable grazing and agroforestry systems

Another step towards sustainable agronomic systems in dairy cow nutrition is the implementation of grazing systems and agroforestry. Depending on grazing management, climate and ecosystem, pastureland may significantly contribute to GHG emissions by not exceeding pastureland carrying capacity with an effective stocking rate, rotational grazing strategies and excluding degraded pastureland from livestock grazing (IFAD 2010). However, due to the intensification of agriculture, the proportion of grassland allocated for feeding dairy cows has tended to decline in recent years, despite the numerous benefits it offers (van den Pool-van Dasselaar et al. 2008; Hennessy 2020). Improved grazing comes with several advantages, such as enhanced animal health, better animal welfare, reduced feed costs and promotion of biodiversity (Washburn et al. 2002; White 2002; Burow et al. 2013; Kok et al. 2020; Angerer et al. 2021). Grazing may also have a positive effect on the composition of milk, such as increased levels of unsaturated fatty acids, conjugated linoleic acids and omega-3 fatty acids (Hennessy 2020).

The intensification of agriculture in recent years has led to a homogenization of land (Landis 2017). Agroforestry combined with grazing systems is therefore an effective approach to increasing biodiversity again (England 2020). Agroforestry is defined as the inclusion of trees different kinds integrated with crops or animals, providing positive properties on carbon storage capacity, soil conservation and water retention, therefore having positive influence on climate change (Pan et al. 2011; Torres-Cardona et al. 2020). In addition to potentially generating extra income for the farmer through the potential sale of timber, this practice also benefits animal husbandry by creating effective shade and protection within the pasture, consequently mitigating heat stress (Schütz et al. 2014; Kamal et al. 2018; Veissier et al. 2018; Quandt et al. 2023).

Domestic grain legumes

An effective approach towards a sustainable dairy cow nutrition involves exploring and supporting alternative protein sources to soybean such as domestic, home-grown grain legumes. These legumes can be cultivated in environmentally sustainable conditions, making them a viable substitute for soybean meal (Abraham et al. 2019). Only 5% of plant proteins used in the EU are produced in the EU (EU 2018). The Federal Government's protein crop strategy is therefore intended to help promote the cultivation of domestic grain legumes like lupins in Germany (Federal Ministry for Food and Agriculture 2020). In addition, organic livestock farming relies on locally produced feed while consumers are also demanding local home-grown protein alternatives (Naspetti et al. 2021). Therefore, the importance of domestic grain legumes will likely increase in the future.

The most growing legumes in Europe appear to be Faba bean (*Vicia faba L.*), pea (*Pisum sativum L.*) and soybean (*Glycine max L. Merr.*) with increasing tendency (Van Loon et al. 2023). Grain legumes are overall rich in protein (16-50%), dietary fibre (10-23%) and vitamins (Mophasa and Jideani 2017). Apart from their nutritional value, legumes are suitable as natural fertilizers in crop rotation systems due to their symbiosis with *Rhizobium* bacteria (Reganold and Wachter 2016; Abraham et al. 2019). Atmospheric nitrogen is converted into biologically usable nitrogen through the exchange of organic compounds and nitrogen between legume, bacterium and nitrogenases in legume root nodules. Due to this ability of nitrogen fixation, there is a reducing need for minerals and nitrogen fertilizers in agriculture (White et al. 2007). Thereby, the amount of fixed nitrogen depends on temperature and water availability as well as mineral N (Watson et al. 2017). With their strong and branched roots, they also improve loose structure and provide a higher water absorption capacity (Jensen et al. 2001). Thus, legumes create overall positive properties in relation to the nature of soils as well as in relation to climate change (EU 2018).

Numerous investigations have indicated that substituting soybeans with domestic grain legumes does not adversely affect the quality and quantity of animal products, especially in the diet of dairy cows (Guillaume et al. 1987; Watson et al. 2017; Abraham et al. 2019). Replacing cereal grains with lupins as grain legumes even resulted in increased milk production due to a higher metabolizable energy content than in cereal grains (White et al. 2007; Watson et al. 2017). In addition, their reduced fibre content, faster passage rate and their content of condensed tannins and saponins lead to reduced methanogenesis in the rumens of dairy cows and thus to a reduction in CH_4 excretion making them a viable protein substitute (Eckard et al. 2010). Therefore, the dairy industry, being less dependent on amino acid supply, is increasingly embracing these valuable alternatives (Jeroch et al. 2020).

Secondary plant metabolites

Plants originally produce SPM as a defense against herbivore predators and constantly changing abiotic and biotic influences thereby being essential for survival and reproduction (Böttger et al. 2018; Khare et al. 2020). Overall, there is a great diversity of chemical structures among SPM including terpenes, amino acids, alkaloids, glucosinolates, cyanogenic glucosides, phenolics or peptides (Jamwal et al. 2018; Khare et al. 2020). Primary compounds, in contrast, play an important role as precursors in metabolism, growth and development of plants fulfilling essential cellular functions (Böttger et al. 2018). The specific roles of SPM depend on the environment, growth and development of the respective plant species. The quality and quantity of SPM developed are influenced by various abiotic and biotic factors and the survival of plants under different conditions, resulting in overall high variability in SPM content (Meena et al. 2017).

In the past, whole plants or plant extracts were (and are still) used in medicine, nutrition, or pharmacy due to the manifold positive effects of SPM (Wallace 2004; Tiwari and Rana 2015). In ruminants for example, essential oils, like oregano oil, exhibit antimicrobial characteristics, including their effectiveness against *Escherichia coli* (Elgayyar et al. 2001). Condensed tannins have been attributed an anthelminthic property with a yet unexplained mode of action while saponins show protozoan suppression in ruminants (Athanasiadou and Kyriazakis 2004; Wallace 2004).

However, since they were originally designed to ensure survival of plants their mode of action is significantly dependent on the amount ingested by producing chemicals like for example bitter compounds that act on taste receptors in predators, or toxins that act at the cellular level to limit or prevent the uptake of the plant by animals (Böttger et al. 2018; Khare et al. 2020). Nonetheless, intake of these plants and their SPM by animals still occur. Reasons for this may be changes in shape, color, and taste, during e.g. feed processing and conservation, but also certain animal resistance mechanisms to individual SPM. As a result of ingestion, there may be significant adverse effects on animal health or, in the case of food-producing animals, the transfer of SPM through metabolic processes or accumulation in food of animal origin, such as meat and milk depending on doses, consumption and detoxification mechanisms (Freeland and Janzen 1974; Foley and Moore 2005).

The negative effects of some SPM on animal health have already been investigated. Well-known examples are SPM of the genus *Senecio spp*. *Senecio* plants are widespread on pastures in Europe (Cortinovis and Caloni 2013). They contain pyrrolizidine alkaloids like jacobine, jacoline, senecionine and their N-oxids with hepatotoxic and teratogenic properties in humans and animals (Wiedenfeld and Edgar 2010). Meadow saffron (*Colchicum autumnale*) is another plant that can be found in meadows and fields, especially in autumn. Its SPM

colchicine is a gastrointestinal poison that can cause poisoning in cows (Schrader et al. 2001; Kupper et al. 2010). However, SPM do not only occur in seasonal or sporadically growing plants but also in ubiquitous and evergreen trees such as e.g. european yew (*Taxus baccata*). Apart from the red aril of its fruits, all parts of the tree contain the cardiotoxic taxines, which can lead to poisoning and death in all animal species (Anadón et al. 2018). *Datura stramonium*, commonly known as jimson weed, contains tropane alkaloids in all parts of the plant with anticholinergic properties. Thereby, the plant is often found in hay or silage and therefore poses a potential risk for all animal species causing tachycardia, incoordination, and coma (Soler-Rodriguez et al. 2006).

Overall, due to their toxic properties some plant parts are already listed as undesirable substances in animal feed (EU 2002). Mentioned are e.g., for *Datura spp.* maximum contents in seeds and unground or crushed fruits containing alkaloids, glucosides or other toxic substances, alone or together. The regulations shall ensure that undesirable substances enter the food chain with negative impact on animal or human health.

The entry of SPM with toxic effects into milk, as food of animal origin, was already described in 1841 after the occurrence of the so-called „milk sickness“ in humans. The reason for this was the inclusion of snakeroot (*Eupatorium rugosum*) in the diet of dairy cows (Drake 1841). Thereby, intoxication in humans appeared before the intoxication was apparent in animals and the most prominent person affected by this poisoning was Abraham Lincolns mother. However, many SPM have not been fully investigated, or have not been investigated at all, in terms of their potential risk of transfer into food of animal origin. This problem has long been a concern, as the World Health Organization (WHO) and Food and Agriculture Organization of the United Nation (FAO) stated in its global strategy for food safety that the appearance of natural plant toxins in food and feed needs to be monitored to assure food safety, even though it seems to have a rather low priority in European food safety research (Van Egmond 2004; Liener 2009; FAO 2022).

Milk is one of the most important foods of animal origin. It was already consumed in 4000 b.c. and today the consumption of milk varies worldwide averaging 10 - 212 kg per person per year (Evershed et al. 2008; Vasileska and Rechkoska 2012; Zhang et al. 2021). Overall, due to its chemical properties as complex emulsion of lipids suspended in aqueous protein solution milk may be considered as minor route of excretion of SPM. Nevertheless, there are several factors that can influence the diffusion of undesirable substances like SPM across cell membranes into milk. There are various routes for the toxins to enter the milk: freely circulating, bound to proteins or in solution in circulating lipids. Several substances may have an affinity for milk constituents, so different proportions of the diverse milk constituents may influence the appearance of undesirable substances in milk. Diffusion may be dependent on concentrations in blood, efficiency of detoxification, lipophilicity, and basicity of the toxins (Panter and James

1990; Lopes et al. 2019). Furthermore, the elimination of the substances depends on their half-life in the body.

The transfer of SPM into milk has been proven to occur e.g. for *Brassica spp*. as there is a transfer of goitrogenic substances into milk. The so-called epidemic of goitre in Finland was attributed to the consumption of milk from cows fed rapeseed (Arstila et al. 1969). There is also a known transfer of several pyrrolizidine alkaloids from *Senecio* spp. (*Senecio jacobaea*) with hepatotoxic effects (Dickinson et al. 1976; Mulder et al. 2020). Transfer rates for SPM into milk (defined as the amount of SPM excreted with milk daily in relation to the daily oral intake of the SPM) may overall differ between 0.007% to 0.037% for tropane alkaloids, to 0.20% for Δ9-tetrahydrocannabinol in hemp to 0.1% for PA from *Senecio* spp. (Hoogenboom et al. 2011; Lamp et al. 2021; Wagner et al. 2022).
Nevertheless, there are still several plant toxins with a suspected but not yet proven transfer into milk. This applies, on the one hand, to individual representatives of the *Fabaceae*, as domestic grain legumes like lupins. On the other hand, cases in horses have indicated that grazing in combination with representatives of the *Sapindaceae*, such as sycamore maple trees, may pose a risk to animals and food, with a yet unexplored risk in dairy cows.

Quinolizidine alkaloids in lupins

Over 500 lupin species are described worldwide including domesticated as well as wildly appearing types (Wink et al. 1995). In Europe and the Mediterranean region, 12 lupin species occur with four species playing an important role in agriculture: *Lupinus albus*, *Lupinus luteus*, *Lupinus mutabilis* and *Lupinus angustifolius* (blue sweet lupin). The latter one being most widely used with more than 1.3 million t worldwide (Gresta et al. 2017).

Lupin seeds are quite large compared to other *Leguminosae* with up to 40% proteins (albumin, globulin fractions) and up to 20% lipid and fibre (Gresta et al. 2017). Lupins contain almost no starch contrary to other *Leguminosae* such as field peas. The amino acid profile is characterized by a low content of lysine and methionine compared to other legumes, while the crude fibre content and the contents of neutral and acid detergent-fibres (NDF and ADF) are higher (Böhme et al. 2016; Petterson 2016). In addition, lupins have a high fat content with a high proportion of unsaturated fatty acids, which can have a positive effect on the fatty acid pattern in milk (Böhme et al. 2016).
Lupins are used as protein-rich feed by dairy farmers, being less expensive than soja, with minimal processing, easy to store and handle and with high contents in metabolizable energy (ME) (White et al. 2007). Reported ME values for ruminants range from 11 to > 14 MJ/kg DM, with a mean of 13.3 (White et al. 2007). To make nutrients most available for ruminants, lupin seeds may be processed by hammer milling to increase the surface area for digestion. Since all parts can be digested in the rumen there is no need to dehull the seeds for ruminant feeding.

For monogastric animals, the cotyledons are more nutritious. Seeds are therefore dehulled by repeated abrasion of the testa in a flow mill. To produce flour, the cotyledons are then ground (Petterson 2016). Recommendations for the use in ruminant rations are up to 30% in complete feeding mixtures (Daenicke et al. 2023). However, if the lupin is not thermally pretreated, a protein source with a high proportion of non-degradable crude protein should be supplemented for high-yielding cows (Böhme et al. 2016).

Blue sweet lupins (*Lupinus angustifolius*) are often used in agriculture because of their higher resistance to *Colletotrichum lupini* (anthracnose) compared to yellow and white lupins (Böhme et al. 2016). Globally, Australia (603 941 ha area harvested) appears to be the biggest lupin crop producer followed by Poland (139 200 ha area harvested) (FAOSTAT 2023). In 2020 20 300 ha area were harvested with lupins in Germany, while 29 000 ha area were harvested in 2021 (FAOSTAT 2023). Overall, land use for lupin cropping in Europe is increasing. While in 2018 243 775 ha where harvested, already 251 253 ha were harvested in 2021 (FAOSTAT 2023).

Apart from their primary compounds and nutritional benefits, lupins contain several SPM (e.g. flavonoids, isoflavones, tannins, saponins, oligosaccharides and quinolizidine alkaloids (QA)). Some of them have negative effects while others have been shown to be beneficial in humans e.g. by preventing osteoporosis or posing antidiabetic and hypocholestaemic effects (Duranti et al. 2008; Petterson 2016; Escudero-Feliu et al. 2023). Despite from e.g. trypsin inhibitors, chymotrypsin inhibitors, tannins, saponins and α-galactosides, the main antinutritional factors are QA (Abraham et al. 2019).

The QA represent bicylic, tricylic or tetracylic derivatives of quinolizidine. Based on their content in lupins a discrimination is made between sweet (less than 500 mg/kg total QA) and bitter varieties (more than 10’000 mg/kg total QAs) (Pilegaard and Gry 2008; Böhme et al. 2016). However, semi-sweet and semi-bitter varieties are also common (Boschin et al. 2008). Lupin seeds with higher levels of bitter QA are unsuitable for human consumption and animal feed without pre-treatment (debittering) (BfR 2017). There are several methods for debittering available. Due to water-solubility of QA soaking of lupins in water for a longer period of 5 to 7 days leads to debittering resulting in differences in colors, nutritional value, and grain size (Erbas 2010; Carvajal-Larenas et al. 2013; Villacres et al. 2020). Other methods include boiling in water, soaking, germination or even microbial methods (Santana and Empis 2001; Boschin and Resta 2013; Mohammed et al. 2017). Previously, a new method has been developed making seeds edible in 26h with an aqueous debittering process and the use of ionic or adsorption resin as a promising approach for the future (Madelou et al. 2024). However, the process of debittering is not currently considered an economical or practical solution due to the high level of effort and cost involved (Villacres et al. 2020).

In general, sweet lupins may contain 0.01 – 0.08% QA per DM (Gessner & Orzechowksi 1974). Nevertheless, several food authorities recommended < 0.02% (200 mg/kg DM) for lupin seeds used for food production (ACNFP 1996; Direction générale de la santé 1998; Board of Food Standards Australia New Zealand 2016).

The biosynthesis of QA starts in the aerial tissue of the plants (Wink and Witte 1984). Initially, the parent substance lysine is first decarboxylated to form cadaverine (Bunsupa et al. 2012a). After an oxidative deamination of cadaverine to 5-aminopentanal, it gets spontaneously cyclized to piperidine Schiff Base (Bunsupa et al. 2012b). Additional chemical reactions, i.e. oxidative deamination, are forming the following major QA (e.g. lupanine and others) (Dewick 2002). Right after synthesis, QA are translocated to the reproductive organs via the phloem (Wink and Witte 1984; Lee et al. 2007a). At the end of synthesis, major structural classes can be distinguished: lupanine, angustifoline, lupinine, sparteine, multiflorine, aphylline, anagyrine and cytisine. The last two, however, are mostly found in *Thermopsis*, *Sophora*, *Echinosophora* and *Genista* (Wink 1987; Boschin and Resta 2013). Each species of lupin contains so called major alkaloids (> 1% of total alkaloids) and minor alkaloids (< 1% of total alkaloids) (Wink et al. 1995). As a result, the individual lupin varieties can also be assigned specific proportions of alkaloids. For narrow-leafed sweet lupins Petterson (Petterson 1998) reported for total QA a proportion of 42-59% of lupanine, 24-45% of hydroxylupanine, 7-15% of angustifoline and 1-1.5% of isolupanine in seeds while in *L. albus* reported proportions are 70% lupanine, 15% albine, 8% 13α-hydroxylupanine and 3% multiflorine (Wink et al. 1995).

Despite *L. angustifolius*, *L. albus, L. luteus,* and *L. mutabilis* are low in alkaloids and therefore suitable for agricultural practice (Boschin and Resta 2013). Pilegaard and Gry reviewed that there are different QA contents in mentioned lupin species used in agricultural practice (Pilegaard and Gry 2008). Total alkaloid contents in *L. albus* varied from approximately 100 to 4’200 mg/kg DM for sweet cultivars (Muzquiz et al. 1994), whereas in *L. angustifolius* total alkaloid contents ranged from 20 to 1’918 mg/kg DM (Harris 1994; Christiansen et al. 1997).

Nevertheless, several studies show that the content of QA depends not only on the variety, species, or plant part but also on other factors including harvest year, region or temperature and several abiotic factors like light or drought (Boschin et al. 2008; Frick et al. 2017; Cely-Veloza et al. 2022). Generally, QA accumulate in the seeds at the end of vegetation, while the maximum content of QA in the plant is reached during flowering (Boschin and Resta 2013). The QA contents can be influenced by light through a light mediated shift in chloroplast pH from 7 to 8 (Wink and Hartmann 1981). The incidence of light causes the pH to change from 7 to 8 so that enzymes involved in the synthesis can work at their pH optimum (Wink and Hartmann 1981). Therefore, QA contents are lower at night than in the afternoon where maximum QA contents are reached (Wink and Witte 1984). So, the time of harvest also shows an influence on the QA content.

There is also a variation in QA levels from year to year mainly depending on different environmental conditions (Jansen et al. 2005). Increased temperatures may lead to increased alkaloid contents as already reported by Jansen et al. (Jansen et al. 2009) and Boschin et al. (Boschin et al. 2008) as in mediterranean climates QA content is higher compared to continental climate in *L. albus* and *L. angustifolius*. Drought stress leads to increased contents of QA and is therefore considered as a great challenge in lupin production in Germany (Frick et al. 2017; Notz and Reckling 2022). For optimal growth, lupins need a slightly acidic soil, while growth as well as protein content is limited at neutral or alkaline pH (Jansen et al. 2010). Low soil pH (between 5.3 to 5.8) also leads to increases in QA content in *L. angustifolius* compared to high soil pH (between 6.7 and 7.1) (Jansen et al. 2012). Apart from external influences, the tillage of the soil with fertilizers also has a significant effect on the QA contents. Nitrogen in form of NO_3, NH_4 or N_2 leads to higher alkaloids contents as reported for *L. albus* (Ciesiolka et al. 2005).

Since lupins are partly cross-pollinated and the alkaloid content is dominantly inherited, crossbreeding or mutations can lead to the development of lupins rich in bitter substances from lupins originally poor in bitter substances (Böhme et al. 2016). The low alkaloid content may be influenced by different genes, so that lupins with a low alkaloid content can also develop into lupins with a high alkaloid content. This poses a major problem in the cultivation of own seeds (Böhme et al. 2016).

It has been hypothesized that growing systems (conventional vs. organic) may have an influence on the number of alkaloids produced, which may be related to different amounts of N-contents after fertilization in conventional growing systems and therefore a higher production of alkaloids compared to organic growing systems (Jansen et al. 2005; Gholamhoss et al. 2011). Lowest QA contents were described for bitter and sweet cultivars of *L. albus* during N-deficiency (Ciesiolka et al. 2005).

Only sweet lupins can be used for animal feeding as specified in the Catalogue of feed materials (EU 2013). With Commission Regulation (EC) No 1121/2009 of 29 October 2009 only those lupins which produce seed containing not more than 5% bitter seeds are defined as sweet lupins as it can be calculated in accordance with the testset n Annex II of this Regulation (EU 2009, EFSA 2019).

In Germany, the regulation on the marketing of seed of agricultural and vegetable species (SaatV), in the version published on 8 February 2006 (BGB I p. 344), last amended by Article 1 of the regulation of 13 July 2022 (BGB I p. 1186), specifies the conditions that must be met for the tested lupin to be considered as sweet lupins. For the lupin to be considered low in relation to bitter substances, only 1 bitter grain may be contained in 100 grains (German Federal Ministry of Justice 1986).

Grains may be examined using the grain cutting method according to Eggebrecht for *L. albus*, *L. angustifolius* and *L. luteus* (Eggebrecht 1949). Dry or swollen grains are cut crosswise, placed on a sieve, and immersed in an iodine solution for 10 seconds. After that they are rinsed with water for five seconds and then examined. The cut surface of grains rich in bitter substances turns brown in contrast to grains poor in bitter substances. The bitter grain content must not exceed 5%. According to EU Regulation (EC) 1221/2009 the protein crop premium for eligible sweet lupins is determined using these methods (EU 2009).

In general, methods for the determination of the total alkaloid content are distinguished from methods for the determination of individual alkaloids in the examination of lupins.
There are photometric and titrimetric methods for determination of total alkaloid content (Ruiz et al. 1977; Resta et al. 2008). To determine the concentrations of individual alkaloids thin layer chromatography, gas chromatography or LC/MS-MS methods may be used (Hwang et al. 2020; Eugelio et al. 2023; Keuth et al. 2023; Madelou et al. 2024).

Risk for human and animal health from QA and metabolites

The typical mode of action of QA involves the expression of the so-called anticholinergic syndrome affecting the central nervous system, the gastrointestinal system as well as the cardiovascular system (EFSA 2019; Petterson et al. 1987; Pothier et al. 1998). Thereby, mostly nicotinerg and muscarinerg acetylcholine receptors in the central and peripheric autonomic nervous system as well as voltage dependent ion channels are affected leading to typical symptoms like mydriasis, tachycardia or arrythmia, confusion and even respiratory paralyses (Galvan et al. 1984; Honerjager et al. 1986; EFSA 2019). Already 20 minutes after consumption first symptoms appear with a peak at 4-5 h after consumption (BfR 2017; EFSA 2019). While mild cases are characterized, for example, by coordination disorders, vomiting or diarrhea, severe poisoning shows respiratory paralysis, convulsions, tachyarrhythmia or cardiac arrest (Forrester 2006). Until now, there is still lack of information on chronic exposure to QA in humans and animals. However, due to their water solubility, they are believed to be eliminated before cumulative toxic effects are expressed (Boschin and Resta 2013). Overall, there is only little information on toxicological effects of individual QA except for sparteine, which was used for treatment of cardiac arrythmias in the past. In the form of sparteine sulfate, it was also used as a uterine contraceptive (Garg et al. 1973).

Sparteine and lupanine appear to be the most toxic QA in humans and laboratory animals (Petterson 1998). Intoxication with sparteine, lupanine or 13α-hydroxylupanine in rats, mice and guinea pigs showed similar clinical symptoms leading to the assumption that all QA may develop a similar mode of action (EFSA 2019). Investigations on acute exposure scenarios revealed after intraperitoneal injection of D-lupanine a (lethal dosis) LD_{50} for mice at 80 mg/kg

bodyweight (bw) (Carl Gordon and Henderson 1951). Occurring symptoms were paralysis, convulsive movements, exhaustion, and respiratory failure.
In elderly humans, poisoning cases are reported for estimated total alkaloid content ranging from about 10 to 50 mg/kg bw while for children lethal cases have been described for 10 mg/kg bw. These known cases are often related to insufficient debittering of lupin seed (EFSA 2019).

In farm animals most studies deal with information on lupins from non-European countries (i.e. *L. caudatus, L. leucophyllus*) containing teratogenic QA (e.g. anagyrine). The ingestion of these lupins leads to so-called crooked calf syndrome due to the intake of angayrine and piperidine (Shupe et al. 1967; Keeler 1976; Lee et al. 2007b).
For other lupins like *L. albus* or *L. angustifolius*, feedings trials mostly focus on the course of performance parameters (milk yield, feed intake, milk composition) during lupin feeding. Nevertheless, there are several reports on poisoning in pigs, poultry and cattle.
In pigs, reduced feed intakes and growing rates as well as vomiting and abortion have been reported after ingestion of *L. albus*, *L. angustifolius* and *L. luteus* (Godfrey et al. 1985; Casper et al. 1991; Boschin et al. 2022). Studies with poultry report similar effects, also affecting feed intake and live weight gain (Vogt et al. 1987; Olver and Jonker 1997).
In ruminants, Gardner and Panther reported measurable blood plasma alkaloid levels in cow, goat and sheep after oral gavage with *L. caudatus* 0.5 h after ingestion (Gardner and Panter 1993). Levels in blood peaked after 3 h and remained high until 8 h. Clinical signs only appeared in cows at 3.0 g QA/kg dry plant material in form of depression and incoordination as already reported by Keeler, while there were no signs in sheep and goats (Keeler 1976).
Gay et al. investigated the plasma disposition of *L. leucophyllus* lupin alkaloids in cattle with and without calves born having arthrogryposis (Gay et al. 2004). After gavage of 2 g dried plant material per day with a total alkaloid concentration of 16’700 mg/kg consisting of 5,6-dehydrolupanine (3’900 mg/kg), lupanine (1’000 mg/kg), 12-seco-12,13-didehydromultiflorine (2’500 mg/kg), anagyrine (3’300 mg/kg) as well as two unidentified alkaloids, after 10 (anagyrine, 5,6-dehydrolupanine) and 24 h (lupanine) maximum plasma concentrations were reached. After 1 to 4 h after the first dosage mild signs of depression, incoordination, and muscle tremors appeared in cows (Gay et al. 2004).
A study conducted by Green et al. investigated the toxicokinetic properties of several alkaloids. Four Holstein steers were orally dosed (2.5 g dried plant material per kg bw) with *L. leucophyllus* (17.2 mg/kg lupanine, 9.9 mg/kg 5,6-dehydrolupanine, 12.9 mg/kg anagyrine, 6.7 mg/kg unidentified alkaloids) (Green et al. 2015a). Serum alkaloid concentrations were monitored over 96 h. Already 15 minutes after dosing all four alkaloids were measured in serum. Maximum serum alkaloid concentrations were reached after 3.5 h for 5,6-dehydrolupanine, 4.5 h for anagyrine, and 15.5 h for lupanine. Additionally, Green et al. also investigated the differences between Angus and Holstein pregnant heifers regarding fetal

activity of fetuses after oral intake of 1.1 g/kg dried ground *L. leucophyllus* (5.7 mg/kg anagyrine, 7.6 mg/kg lupanine, 3.0 mg/kg unidentified alkaloid and 4.0 mg/kg 5,6-dehydrolupanine) (Green et al. 2015b). Thereby, in Holstein heifers' lower maximum serum concentrations and more fetal movement than Angus heifers were found suggesting breed dependent differences in toxicokinetic and genotoxic effects (Green et al. 2015b).
In vitro incubation in ruminal fluids of sheep, showed no degradation of sparteine after 24 h of incubation (Lanca et al. 1994). Aguiar et al. additionally investigated the fate of specific alkaloids (sparteine, lupanine, cytisine, atropine, quinidine, lobeline, harmaline, arecoline, nicotine, caffeine, pilocarpine, gramine, senecionine, and monocrotaline) in ruminal fluids of naive cow and sheep and the possible influence of ruminal microorganisms. The study found no degradation of lupanine and sparteine through rumen microbiota after 36 h of *in vitro* incubation and suggested that possible metabolizations may only occur in the liver or kidney of ruminants (Aguiar and Wink 2005).

Until now a full toxicologic assessment of the effects of QA and therefore the determination of a health-based guidance value (HBGV) is not possible due to lack of data, as the EFSA concluded in their opinion in 2019 (EFSA 2019). In general, there are four steps sufficient for risk assessment: hazard identification (to identify adverse effects of e.g. toxins or pollutants), dose-response assessment in relation to carcinogenic and non-carcinogenic effects, exposure assessment (intensity or frequency of exposure to several agents in humans) and risk characterization (potential for adverse effects to occur in exposed population) (BfR 2022). Thereby, establishing reference points based on dose-response studies is central. Possible RP may be the NOAEL (no-observed-adverse effect-level) defined as the highest dose without adverse effects on animal health. With the help of reference points HBGVs are then derived such as acceptable daily intakes (ADI) or tolerable daily intakes (TDI). In risk assessment, an HBGV is conducted by applying a 100-fold safety factor on the No-Observed-Adverse-Effect-Level (NOAEL) of an animal study, thereby the 100-fold safety factor of HBGV takes variabilities in between humans and animals into account (EFSA 2022).
However, in some cases, the data may not be sufficient to determine an HGBV, even though a reference point could be established. In this case, the margin of exposure (MoE) approach is applied. The MoE describes the ratio of the reference point of an adverse effect dose-response curve to the predicted or theoretical human intake (EFSA 2022).

Due to insufficient data, the MoE approach was applied for QA. For the characterization of the human hazard mainly the anticholinergic effects and the cardiac electrical conductivity following acute exposure were considered. Due to lack of data on individual QA, there is group approach for all QA. Thereby, the MoE approach uses the lowest single oral antiarrhythmic dose of sparteine 0.16 mg/kg bw as a risk following acute exposure as a reference point. An MoE ≥ 1 thereby indicates that there is no health concern (EFSA 2019).

Several studies have already investigated the QA content in lupin-based flour, seeds, meat products or coffee (Keuth et al. 2023). For non-debittered lupin seeds and lupin-based meat products MoE value < 1 were found while for chronic exposure there is no reference point.
Up to now there are no studies investigating the intake of QA via food of animal origin.
There has only been one published case report of possible QA intoxication in a human infant after its mother's consumption of goat's milk in early pregnancy. Lambs from the same goats showed skeletal deformities as described for crooked calf disease (Ortega and Lazerson 1987). This was indicative of QA intoxication (Shupe et al. 1967; Shupe et al. 1968).

Hypoglycin A and Methylenecyclopropylglycine in *Sapindaceae*

Acer pseudoplatanus, a tree belonging to the *Sapindaceae* family, is naturally found in several European countries, primarily in Germany, Italy, France, and other regions with a sub-Atlantic climate tendency (Weidema 2010; Caron et al. 2015). Due to its high growth rate as well as its economic value, this species is particularly attractive in agroforestry (Straigyte and Baliuckas 2015). Their seeds may fly up to 200 m and are therefore invasive on pastures and adjacent land (Straigyte and Baliuckas 2015).

In 1918, Kling proposed sycamore seeds from *Acer pseudoplatanus* as possible feed during war due to shortage of fodder (Kling 1918). Even though it was known for its high content of strongly astringent tannins which makes the feed almost inedible, there was a production of maple seed feed by threshing dried seeds to free them from the hull and then grinding them in a grist mill. Kling analyzed the seeds on their nutritional value and recognized 92% water, 21.3% crude protein, 8.9% crude fiber, 39.8% nitrogen-free extracts and 13.4% so-called crude wheat and thus showing similar conditions to distiller’s grains or dried brewer’s grains. He concluded, that due to its composition it is most suitable for horses as well as cattle even though feeding to horses resulted in colic-like symptoms (Kling 1918).

At the beginning of the 20th century Harold Scott (Scott 1917) recognized the vomiting sickness of Jamaica due to poisoning through Akee fruit (*Blighia sapida*) also belonging to the family of *Sapindacea*, domestic in West Africa, West Indies and the Caribbean area (Henry et al. 1998; Barceloux 2009; Emanuel and Benkeblia 2011). The Akee is known as a bright oval fruit in the size of a small pear which breaks open when ripe. The rough outer rind thereby splits from one end to three sometimes four segments. The ripe seeds are black and the base of each being clasped by the aril or arillus, a cream-colored or yellow substance (Jordan and Burrows 1937; Brown et al. 1991). Scott also recognized that conditions that caused poisoning where a) unopened ackees, b) Ackees picked from a decayed, bruised, or broken branch, c) Ackees which had not been opened naturally, but which have been forced open, d) Ackees with a soft spot in an otherwise apparently sound fruit concluding that immaturity or over-ripeness may be the cause for poisoning (Scott 1917).

Afterwards, in 1954 Hassall, Reyle and Feng investigated the toxic components of Akee and discovered two toxic polypeptides named Hypoglycin A (HGA; L-(methylencyclopropyl)-alanine), a neutral component with the molecular formular $C_7H_{11}N_1O_2$ and a molecular weight of 141 g/mol (Von Holt et al. 1964), and Hypoglycin B, an acidic component, (HGB; y-Glutamylhypoglycin), consisting of HGA and glutaric acid (Hassall et al. 1954). Gray and Fowden additionally found methylencyclopropylglycine (MCPrG) as a homologue of HGA in litchi seeds with the same toxic effects at different doses, while γ-glutamyl-MCPrG was found in *Acer pseudoplatanus* (Gray and Fowden 1962; Melde et al. 1989; Fowden and Pratt 1973).

Since the 1980s there has been an increased incidence of an atypical muscle disease, known in Europe as atypical myopathy (AM), for pastured horses. In the U.S., the disease is known as seasonal pasture myopathy, due to its increased incidence in spring and fall (Sponseller et al. 2012; Votion 2012). These intoxications in horses have been attributed to the ingestion of the seeds and seedlings of the sycamore maple tree (*Acer pseudoplatanus)* (Van Der Kolk et al. 2010; Unger et al. 2014; Bochnia et al. 2015).
In both cases, there is an increased degeneration of muscle fibers (mainly type I) and accumulation of triacylglycerols in skeletal and cardiac muscle cells due to disturbances in mitochondrial metabolism (Cassart et al. 2007). Affected horses exhibit muscle tremors, increased sweating, increasing weakness, recumbency, as well as muscle cramps. Atypical myopathy is usually fatal for horses (Votion 2016). In 1995 approximately 99 horses died due to the fatal course in Northern Germany (Brandt et al. 1997).
Following studies showed that several maple trees, including *Acer pseudoplatanus* and *Box elder trees*, produce HGA, as well as HGB and are therefore considered as an environmental hazard since sycamore is known to be a common plant in meadows and fields (Fowden et al. 1972; Van Der Kolk et al. 2010; Votion 2012; Valberg et al. 2013).
Sycamore seeds and seedlings, and therefore sycamore toxins, can also be introduced into feed via hay and silage, in addition to ingestion of seeds and seedlings during the grazing period.

Once ingested, HGA gets metabolized to methylenecyclopropylacetyl (MCPA) in the liver (Von Holt et al. 1964). MCPA is known as a strong inhibitor of fatty acid oxidations (ß-oxidation). In its active form, as MCPA-Coenzym A (MCPA-CoA), it inhibits short and medium chain Acyl-CoA-dehydrogenases involved in ß-oxidation (e.g. isovaleryl-CoA-deyhdrogenasis or glutaryl-CoA-dehydrogenasis) as well as the transport of fatty acids into the mitochondria (Von Holt et al. 1964; Tanaka 1972; Wenz et al. 1981; Bochnia et al. 2019). As a result, fatty acids conjugated to carnitine that cannot be broken down are found in the blood and urine of affected individuals (Sander et al. 2017). Furthermore, due to the inhibition of short-chain ß-oxidation, free fatty acids (e.g. propionic or isovaleric acid) are also found in the serum of affected individuals (Barceloux 2009). Fatty acids are stored in muscle cells of the respiratory muscles,

deeper layers and in the myocardium leading to fatty muscle cell infiltration and hyaline degeneration (Cassart et al. 2007). The disturbances in fat metabolism further lead to increased consumption of glucose and nicotinamide adenine dinucleotide (NADH) and the subsequent breakdown of glycogen (Lai et al. 1991; Lai et al. 1993; Barceloux 2009). The result of this disturbance in energy metabolism is hypoglycemia in humans, rats, mice, rabbits and monkeys whereas hyperglycemia is frequently diagnosed in horses for reasons that are still ambiguous (Feng and Patrick 1958; Melde et al. 1989; Meda et al. 1999; Blake et al. 2006). The active metabolite of MCPrG, on the other hand, is methylenecyclopropylformyl (MCPF), transformed to a CoA-Thioester resulting in MCPF-CoA (Gishla et al. 1990). MCPF-CoA blocks the second step of ß-oxidation and, in particular, enoyl-coA-hydratases (Li et al. 1999; Dakoji et al. 2001).

Overall, the formation as well as the mode of action of both metabolites leads to a reduction of several cofactors including CoA and carnitine which results in turbulences in transport systems of long chain fatty acids and ß-oxidation in general (Bressler et al. 1969; Wenz et al. 1981). It is assumed that HGA and MCPrG thus complement each other's toxic effects simultaneously. Conjugated metabolites of MCPA and MCPF, MCPA-Glycine, MCPA-Carnitine as well as MCPF-Glycine and MCPF-Carnitine were found as a form of excretion product in the urine and described as detoxified forms of HGA and MCPrG (Tanaka et al. 1972; Sander et al. 2017; Bochnia et al. 2019; Renaud et al. 2022). So far, however, the toxicological effect of these metabolites is not known.

Risk for human and animal health from HGA and metabolites

Investigations on the toxic effects of HGA showed that it is lethal to kittens, guinea pigs and rats at LD_{50} of 90 mg/kg bw (Hassall et al. 1954). The toxins are water soluble and toxic effects are related to the nutritional status of the animals, as the effects are stronger in restrained animals (Hassall et al. 1954). Feng and Kean (Feng and Kean 1955) therefore hypothesized that toxicological effects might me depend on the carbohydrate: protein ratio ingested, as presented diets increased in carbohydrate and low in protein led to increased effects after HGA ingestion. When 5 groups of 5 mice each were injected intravenously with HGB (100 mg per kg bw) and sacrificed at hourly intervals for determination of liver glycogen, the liver of mice given lethal doses of HGB showed fatty metamorphosis (Chen et al. 1957). Overall HGA was more potent than HGB judged from the lethal doses (Chen et al. 1957; Feng and Patrick 1958). Intraperitoneal injection of 30 mg/kg HGA per bw resulted in brain and limb formation in fetuses of rats during the last 5 days of gestation (Persaud 1972). The acute toxic dose, determined through the oral administration of a crude aqueous HGA extract was 100 mg/kg bw as reported by Feng and Patrick (Feng and Patrick 1958). The maximum tolerated daily intake (MTD) was set at 1.50 ± 0.07 mg HGA/kg body weight following a 30-day study in male and female rats

(Blake et al. 2006). Still, there is overall insufficient data to adequately determine risk after ingestion.

Clinical manifestations present in humans associated with the consumption of fruits containing HGA are known as Jamaican Vomiting Sickness or Acute Encephalopathy (Scott 1917; Meda et al. 1999). Clinical symptoms often develop within 6-48 h. Thereby, the severity of symptoms depends on the ingested dose. Symptoms include severe vomiting, followed by a clinically unremarkable phase, which is followed by again severe vomiting, epileptic seizures, and coma. Other symptoms include hypoglycemia, liver damage, and aciduria (Meda et al. 1999; Katibi et al. 2015). Children are particularly affected by intoxications (Scott 1917). Clinical cases of severe and fatal intoxication in children after ingestion of ackee fruit and therefore HGA have been reported from several countries (Barennes et al. 2004; Katibi et al. 2015). Deaths in these cases occur up to 48 h after ingestion of the toxins (Barennes et al. 1998; Katibi et al. 2015). Studies on unexplained deaths and poisoning cases in India in 2017 showed that intoxication with HGA and MCPrG also led to increased mortality in children and the development of neurological symptoms. These poisoning cases were due to ingestion of the Litchi fruit (Litchi Chinensis) (Shrivastava et al. 2017). The occurrence and severity of Jamaican vomiting disease are, similarly, to poisoning in animals, believed to be linked to malnutrition (Feng and Kean 1955). This is particularly true in children (Henry et al. 1998; Meda et al. 1999).

Sycamore is known to be a common plant in meadows and fields. The use of hay and silage in feeding therefore poses a risk of potential introduction of the toxin into the feed via hay and silage. In addition, there is a risk of ingestion of the seeds and seedlings during the grazing period. Overall, the seeds, seedlings and leaves of maple trees show different concentrations of HGA and MCPrG depending on the season and stage of development. The concentrations in the seedlings decrease with further development (March-June) (97 mg/kg DM to 4508 mg/kg DM) (Westermann et al. 2016; Votion et al. 2019; El-Khatib et al. 2022). Seeds of sycamore maple collected in spring contained 0 mg/kg to 3683 mg/kg HGA (Westermann et al. 2016; Votion et al. 2019), while HGA concentrations in seeds of *Acer pseudoplatanus* collected in autumn range between 266–2962 mg/kg DM (El-Khatib et al. 2022). Collected leaves contained on average 0 to 2303 mg HGA/kg DM (Westermann et al. 2016; El-Khatib et al. 2022).

Different strategies (pesticides, mowing, ensiling) have already been studied in terms of their influence on HGA occurrence and toxin degradation (Gonzalez-Medina et al. 2019). No significant degradation could be detected by mowing down the seedlings in mid-May to mid-June (time 0 d: 272.2 µg HGA/g, time 15 d: 181.8 µg HGA/g) as well as after herbicide treatment (time 0 d: 243 µg HGA/g, time 15 d: 206 µg HGA/g). In contrast, 48 h after mowing down, a significant increase in HGA concentration was detected in dying seedlings (time point 48 h: 801.9 µg/g). Thereby, the increase in younger vegetation stages of the seedlings was

higher than in older vegetation stages. The analysis of seeds stored for 8 months in hay and silage bales also showed still acceptable levels of HGA (105 and 256 µg/g). Another study tested the effects of mowing and herbicidal spraying with different herbicides during early spring (Ghislain et al. 2022). Thereby, all methods significantly increased the amount of sycamore seedlings compared to the control. However, a decline of 78-86% in the number of seedlings was also found in the control plots after three to four weeks, probably due to displacement by a compact grassland vegetation (Ghislain et al. 2022). Even though the number of seedlings decreased due to herbicide treatment, the content of HGA in the remaining seedlings remained unchanged. Therefore, tillage is not sufficient to completely remove the risk of poisoning. Due to its rapidly advancing reproduction capacity (Krabel and Wolf 2013), the growth of *Acer pseudoplatanus* is despite its natural distribution in Europe, e.g. in Germany, France and Austria, already expanding to the Scandinavian area (e.g. Norway and Sweden) which indicates an increasing risk (Weidema and Buchwald 2010; Caron et al. 2015). In summary, an input of the toxin using silages, hay and fresh grass is therefore given and cannot be avoided by standardized methods.

Observations made in horses could also be found in milu deer (*Elaphurus davidianus*). In a case report by Bunert et al., a form of atypical myopathy was identified in milu deer (25 milu deer from Duisburg Zoo), which could also be traced back to maple toxin ingestion by analyzing HGA and MCPA (Bunert et al. 2018). Only those animals that showed symptoms had elevated levels of MCPA conjugates in their blood and urine. In another study, both HGA and MCPrG as well as their metabolites were detected in 3 diseased milus (Bochnia et al. 2020). Clinically affected animals showed muscle weakness and stiffness and developed brown coloration of the urine (myoglobinuria) as well as hyperextension within two days (Bunert et al. 2018; Bochnia et al. 2020). Both horses and milu deer are showing overall increased mortality (Van Galen et al. 2012; Bunert et al. 2018; Bochnia et al. 2020).

In addition, a case report from 2016 described two camels (*Camelus bactrianus*) suffering from intoxication (Hirz et al. 2021). The camels developed typical clinical symptoms described, such as muscle weakness, recumbency, teeth grinding and increased sensitivity of affected muscle groups. The animals were euthanized due to the severity of their symptoms. HGA, MCPrG as well as their metabolites were detected in serum of affected camels. Recently, HGA and MPCA-Carnitine were detected in gnus (*Connochaetes taurinus taurinus*) from a Zoo in France with severe symptoms (depression, tremor, decubitus) in two out of three animals after removal of access to pasture, clinical signs disappeared within 3 d (Renaud et al. 2022).

Despite the occurrence of intoxications in wild ruminants, no cases have been described in domesticated ruminants. Even though unusual outbreaks of severe rhabdomyolysis where also reported in cattle in the United Kingdom in the 1970s none of these cases could be attributed to intoxication with sycamore toxins (Barton and Allen 1973; Johnston 1975;

Linklater 1977). Barton and Allen thereby reported, that 4 out of 26 heifers were affected with paralytic conditions after being pastured in spring. Two of these heifers showed elevated temperatures, difficulties in standing as well as hard to touch and firm dorso-lumbal and glutear muscle masses. The urine was red brown colored and serum samples showed elevated creatin kinase (CK) levels (Barton and Allen 1973). Linklater described two recumbent and five ataxic 18-month-old heifers with elevated CK proportional to their clinical manifestations (Linklater 1977). While in a case report by Johnston (Johnston 1975) two ten-month-old heifers showed firm gluteal and shoulder muscles, myoglobinuria and worsening in condition 6 d after accessing a pasture.

In a case report by Gonzales-Medina et al. (Gonzalez-Medina et al. 2021) serum samples from ewes and their lambs were examined after 0, 2 and 7 d, after two group of sheep had been moved to a pasture with sycamore maple trees. Both HGA and, in the case of one ewe, MCPA conjugates could be detected in the serum of the animals without any adverse health effects. After 48 h HGA was detected in all sheep of group 1 (32.2 ng/mL; 5.6 – 124.4 ng/mL) and declined significantly at day 7 (16.3 ng/mL; 5.2-22.0 ng/mL). MCPA-carnitine was detected in one ewe, with the highest levels on HGA in serum) on day 2 at 11.75 ng/mL and in traces in 4 other ewes. In group 2 Group 2 HGA was detectable in 3 ewes and 2 corresponding lambs at 48 h (13.5 ng/mL ± 5.9) with no traces of MCPA-carnitine. HGA levels of 8.82 - 23.71 ng/ml were also detected in the serum of the lambs without any clinical symptoms. If one compares the blood concentrations between the milu deer as wild ruminants and sheep as domesticated ruminants, it is striking that despite higher serum concentrations of HGA and MCPA-carnitine (126.4 ng/mL and 11.75 ng/mL), in contrast to the toxins found in wild ruminants (35 and 1.64 ng/mL), there was no expression of clinical symptoms in sheep and the animals did not show any impairment at any time (Bunert et al. 2018; Gonzalez-Medina et al. 2021).

This reinforces the hypothesis that in domesticated ruminants there are underlying mechanisms that prevent intoxication with associated expression of clinical signs.

A known form of detoxification in ruminants is the metabolism of SPM by rumen microbes. In a recently published *in vitro* study, the behavior of the toxin in ruminal fluid of sheep was investigated (Gonzalez-Medina et al. 2021). The aim was to prove whether ruminants already degrade HGA with the help of their microbiome in the rumen, thus preventing systemic uptake. For this purpose, maple seeds were incubated in ruminal fluid of 5 sheep *in vitro* and then examined after a short period of either one or two hours for the content of HGA, MCPA and their respective metabolites. After this time, a release of the toxins from the seeds was detected, but neither degradation nor metabolization to MCPA conjugates took place. According to the study, the rumen microbiome does not play a decisive role in the detoxification of HGA and the MCPA metabolites.

A transfer of HGA and MCPrG was first suspected when a study described a foal developing atypical myopathy immediately after birth after its dam had developed atypical myopathy during pregnancy by grazing on a pasture with sycamore maple (Karlikova et al. 2018). Elevated levels of acylcarnitine were detected in the blood of the foal (exanoylcarnitine - C6, octanoylcarnitine - C8, decanoylcarnitine - C10, octenoylcarnitine - C8-1, and decadienylcarnitine - C10-2). In addition, MCPA-carnitine was detected in serum at concentrations as low as 0.01 µmol/L and compared with levels in horses suffering from atypical myopathy (0.10-0.42 µmol/L). No MCPA-carnitine could be detected in control samples from healthy horses. It is unclear whether the toxin entered the foal's organism via the placenta or the milk (Karlikova et al. 2018).

To study transfer in mares, from fall 2018 to spring 2019, blood samples were collected from all AM affected mares at the Liege University Equine Clinic (Renaud et al. 2021). In the presence of foals not yet weaned, milk and serum samples of the mares were sampled. In total, samples were collected from 4 mares and their foals. In this process, foals 1, 3, and 4 were taken to the clinic because of suspected AM, but a diagnosis of atypical myopathy was made only in foal 4 because of elevated creatine kinase levels (27 712 IU/L) and pigmenturia. Foal 1 developed colitis, whereas foal 3 was diagnosed with *Rhodoccocus equi* infection (Renaud et al. 2021). However, given that the foals were 1-5 months old, ingesting maple seeds and seedlings cannot be excluded. HGA could be detected in the milk of mare 1, 3, and 4 (0.01 µmol/L, 0.06 µmol/L, 0.02 µmol/L). MCPA-carnitine could be detected at concentrations > 2 nmol/L in all milk samples except that of mare 2. Mares 2, 3, and 4 had detectable HGA concentration in serum, thus confirming the intake of sycamore maple toxins. In addition, all 4 mares had detectable MCPA-carnitine in their blood (0.35 nmol/L - 8.16 nmol/L). All 4 foals had HGA and MCPA-carnitine in their blood. Foal 4, which was diagnosed with AM, demonstrated the highest level of MCPA-carnitine in her blood (39.80 nmol/L) (Renaud et al. 2021).

Sander et al. examined the milk of a mare grazing with her foal near a pasture where some cases of AM had recently been diagnosed (Sander et al. 2020). A milk sample was collected after the expression of symptoms. Due to the severity of the symptoms, the foal had to be euthanized subsequently. Analysis of milk samples revealed an HGA level of 0.4 µg/L. MCPA-glycine and MCPA-carnitine were detectable at concentrations of 18.5 and 24.6 µg/L, respectively. MCPG was not detected in the milk sample, but the metabolites MCPF-glycine and MCPF-carnitine were identified at concentrations of 0.8 and 60 µg/L, respectively. In addition, 6 commercial mare milk samples from 6 traders from different regions of Germany were analyzed for their contents of HGA, MCPrG and their metabolites. In one of these 6 commercial milk samples a HGA content of 2.4 µg/L and a MCPrG content of 1.3 µg/L was

found. The glycine derivatives were absent, but MCPA-carnitine and MCPF-carnitine were detected at levels of 0.4 and 2.7 µg/L, respectively (Sander et al. 2020).

To study transfer into the milk of sheep, serum and urine samples from 3 North Country Mule groups (Group 1: 10 ewes and 10 lambs (4 days old), Groups 2 and 3: 5 ewes and 5 lambs (4 days old) each) were analyzed for MCPA-carnitine and HGA (Gonzalez-Medina et al. 2021). Groups 1 and 2 were moved to a pasture contaminated with sycamore seedlings for grazing, while group 3 served as a control group. Samples were collected from group 1 one day before moving to the pasture and 2 and 7 days after moving to the pasture, while animals from groups 2 and 3 were sampled one day before moving to the pasture and 2, 5 and 7 days after moving to the pasture. No samples from the lambs were available from group 1. HGA was detected in the serum of 3 ewes from group 2 and 2 associated lambs after 48 h (13.5 ng/ml ± 5.9) (Gonzalez-Medina et al. 2021).

In a recently published study by Bochnia et al., bulk milk samples from 4 randomly selected dairy farms from northern Germany (Schleswig-Holstein and North Friesland) keeping their cows on pastures were investigated (Bochnia et al. 2021). Only one pasture was found to have sycamore maple trees. In one sample (1), a HGA content of 69 µg/L in 1a and 17 µg/L in 1b could be detected in duplicate determination (same tank, same time) (Bochnia et al. 2021).

Nevertheless, the data available are not sufficient to confirm a statement about a potential transfer into milk, as ingestion has not been confirmed in dairy cows and the methods listed for the analysis of HGA have not been validated. In the run-up to this study, a method was therefore validated at the German Federal Institute for Risk Assessment to examine milk samples for their content of HGA, HGB, MCPrG and their metabolites (El-Khatib et al. 2023). The method was implemented in 68 milk samples from 35 commercial dairy farms without information on access to a pasture with sycamore maple trees or sycamore maple toxins or the possible introduction of maple toxins via feed. Even though samples contained no quantifiable amounts on sycamore maple toxins, data from previous studies indicate that a transfer may occur in dairy cows due to the presence of maple trees on the pasture, seasonal differences, or the introduction of maple toxins via the feed.

Chapter II

Aims and objectives of the thesis

These alterations including the use of domestic grain legumes as well as the implantation of grazing and agroforestry, may pose potential hazards that necessitate further examination to guarantee food safety while having a favorable impact on climate change. Food-producing animals hold a unique role amid these transformations. Despite the impacts of climate change, preserving food safety remains crucial. It is imperative to thoroughly examine potential risks associated with both new feed sources like agroforestry and traditional feed sources like grain legumes that may have gained newfound significance in this context. It is plausible that certain plants within these feed sources produce secondary plant metabolites with harmful effects that could be transmitted to milk after consumption by dairy cows, yet this risk has not been thoroughly assessed and there is only limited research available.

Therefore, the first section of the thesis will examine the transfer of QA from domestic lupins (*Lupinus angustifolius*), used as an alternative protein source in dairy cow feeding, into milk.

The objective of this investigation is to identify the alkaloid composition of the lupins used and compare it with established alkaloid compositions found in sweet lupins. Subsequently, the transfer of the individual alkaloids into the milk of cows will be examined. Special attention will also be paid to the profile of transferred QA. With the help of these results, individual information on the transfer for the individual QAs, such as transfer rates or half-lives, can then be determined by means of toxicokinetic modelling. The development of the model should serve as a basis for predicting transfer processes in the future. These results will then be used to identify and assess the potential risk to consumers.

The second part attempts to show the possible intake of sycamore seedlings from *Acer pseudoplatanus* and their SPM HGA and MCPrG by dairy cows on a pasture and the possible transfer of these SPM into milk.

If sycamore seedlings and therefore HGA and MCPrG are ingested by cows, there could be a possible transfer of the protoxins or their metabolites into the milk. The aim of this study was therefore to investigate this possible transfer and evaluate the potential risk to consumers. The study seeks to obtain data on the effects of sycamore maple toxin intake on animal health as they are highly toxic in several herbivoral species. We furthermore investigated the metabolism and possible transfer of HGA, HGB, MCPrG and their metabolites into the milk of dairy cows.

Overall, this thesis attempts to fill the knowledge gap of these plant toxins entering the food chain. The data collected will help to explore the risks associated with the use of these plants in the context of animal husbandry to make recommendations for sustainable agriculture and consumer safety.

Chapter III

Investigations on the transfer of quinolizidine alkaloids from blue sweet lupins (*Lupinus angustifolius*) into the milk of dairy cows

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Abstract

Lupin varieties with a low content of quinolizidine alkaloids (QAs) like blue sweet lupin (BSL) have long been used as a protein source for dairy cows. A health concern for humans may arise from the transfer of acute toxic QAs from feed into cow's milk. This study is the first to quantify the transfer of QAs from BSL into cow's milk with experimental and modeling methods. Four lactating dairy cows were subjected to two 7 day feeding periods with 1 and 2 kg/d BSL, respectively, each followed by a depuration period. BSL contained 1774 mg/kg dry matter total QAs. Individual milk samples were taken twice daily and QA contents in feed and milk determined with liquid chromatography−tandem mass spectrometry. Transfer of QAs into the milk was already seen with the administration of 1 kg/d BSL, with differences in transfer rates (TRs) between individual QAs. A toxicokinetic model was derived to quantify and predict QA feed-to-food transfer. For the four most prominent QAs, our model shows an α-half-life of around 0.27 d. TRs were obtained for six QAs and were between 0.13 (sparteine) and 3.74% (multiflorine). A toxicological assessment of milk containing QAs as measured in this study indicated a potential health concern.

Introduction

Lupins have a long tradition as a protein source in animal nutrition because of their high crude protein content (up to 40% in dry matter, DM) and they are further gaining importance in Europe, especially in organic animal husbandry. While several secondary plant metabolites in lupins have been shown to have beneficial effects (e.g. antidiabetic or antioxidant activity) [1], some alkaloids are known to have detrimental effects on human and animal health. The latter is the case for quinolizidine alkaloids (QAs), which constitute the main secondary plant metabolites occurring in lupins, offering protection against insects and herbivores.[2] To date, more than 300 lupin species are known, with varying QA contents. Depending on their alkaloid content, lupins are commonly classified into bitter lupins (with a total QA content of up to 8% in DM) and sweet lupins with a low alkaloid content.[3] This low alkaloid content should not exceed 0.05% in DM (500 mg/kg DM) in agricultural practice, while levels < 0.02% in DM (<200 mg/kg DM) are recommended by health authorities for lupin seeds used for food production.[4–7] The synthesis of QAs occurs mainly in the leaves, but they are distributed via the phloem into other parts of the plant including the seeds, causing a bitter taste as a protection against herbivores.[8] More than 170 QAs have been identified among lupin species, with lupanine, 13α-hydroxylupanine and sparteine being the most abundant ones.[9] Depending on their chemical structure, QAs can be chemically divided into e.g. sparteine and its derivatives, lupanine and its derivatives, angustifoline and its derivatives, multiflorine and its derivatives , lupinine and anagyrine. (Figure 1).

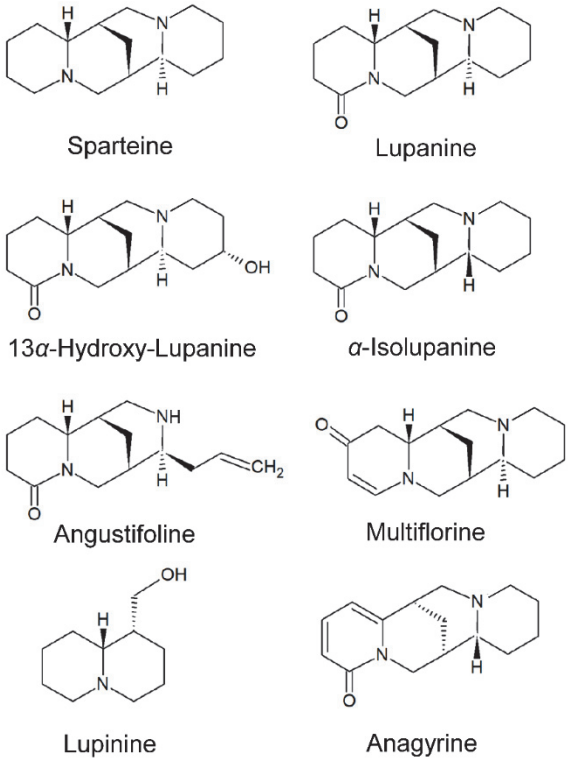

Figure 1. Chemical structures of selected Quinolizidine Alkaloids (QAs).

The QAs exert their toxicity by inhibiting acetylcholine receptors and voltage-dependent ion channels in the central nervous system, on motor endplates and the peripheral autonomic nervous system, where the individual QAs appear to have different levels of toxicity.[10] Common acute toxic exposure symptoms in humans and mammals include respiratory depression, vomiting and tachycardia.[10,11] Some QAs, such as anagyrine, also show teratogenic properties and have been associated with congenital skeletal malformations (crooked calf disease) in calves.[12] Thus, to minimize the risk of QA intoxication in livestock animals, only sweet lupins are listed as feed for livestock species in the catalogue of feed materials.[13]

However, mutations, crossbreeding or recombination can result in descendants with higher QA contents despite their original classification as sweet lupins.[14,15]

Most of the toxicological data originate from research on lupanine and sparteine, the latter compound being used as a pharmaceutical in the past.[10] The European Food Safety Authority (EFSA) stated that ‘anticholinergic effects and changes in cardiac electric conductivity’ are the relevant endpoints for risk assessment. A dose of 0.16 mg sparteine/kg bodyweight (bw) was identified as the ‘lowest single oral effective dose’ in humans for such acute effects, while no reference point could be identified for risks potentially resulting from chronic exposure. Due to similar modes of action of QAs, EFSA assumed dose additivity for all derivatives. Furthermore, due to the limited overall data basis and the associated uncertainties, no health-based

guidance value could be derived. Therefore, EFSA applied the margin of exposure (MoE) approach for a preliminary risk characterization, using the dose of 0.16 mg sparteine/kg bw as an appropriate reference point. The authority concluded that an MoE > 1 would not indicate a health concern. However, the assessment revealed the possibility of exposures for some consumers groups, resulting in MoE values < 1, indicating a potential risk for these consumers. Additionally, EFSA stated that there is indirect evidence of a possible transfer of QAs from feed into milk, due to the QAs' weak basic nature, which makes milk a possible additional exposure source.[10] However, until now there has only been one published case report of possible QA intoxication in a human infant after their mother drank goat milk in early pregnancy.[16] Lambs from the same goats showed skeletal deformations as described for crooked calf disease, indicating QA intoxication.[17] In the present study, we tested the hypothesis that QAs from lupin in the diet of dairy cows are transferred into cow's milk. We determined the profiles of six QA in milk and quantified the transfer rates of the four most prominent QAs from lupin seeds into the milk of four lactating dairy cows fed with increasing amounts of QA-containing sweet lupin seeds. We conducted a toxicological assessment in order to evaluate the potential risk resulting from the sole exposure to QAs via milk containing QA levels as measured in the present study.

Materials and methods

Ethics approval statement

All experimental procedures involving animals were approved by the local authority (Regional Office for Health and Social Affairs, Berlin - LAGESO, Germany) under registration number StN010/19.

Animals, housing and sampling

Four Holstein-Friesian dairy cows (3 primiparous, 1 multiparous, 58 ± 11 days in milk) with an average milk yield of 30.4 ± 4.12 kg/day were housed in one group in an open barn stable with free access to water. During the experiment, which lasted 46 days in total, cows were milked twice daily at 6.00 a.m. and 4.30 p.m. in a tandem milking parlor (Lemmer Fullwood). Milk samples were taken during each milking and stored at -20°C until being analyzed for QAs contents.

Lupin seeds and diets

Lupin seeds (whole grain, untoasted, *Lupinus angustifolius var. Boregine* (blue sweet lupine, BSL)) harvested in Brandenburg, Germany, approximately 52°6'N 12°7'E, in August 2019 were milled in a common hammer mill (Siemens) to pass a screen of 3 mm, divided into four subsamples of 25 kg each and stored in a container under dry, cool and dark conditions prior

to use. Forages, beet pulp and minerals were offered as a partial mixed ration (27.7% grass silage, 29.5% maize silage, 6.0% straw, 30.1% hay, 6.0% beet pulp, 0.61% minerals) *ad libitum* in feeding troughs. A concentrate mixture was provided in separate feeding troughs, transponder-controlled one for each cow, to meet the energy requirements for a milk yield of 25 kg/d energy-corrected milk (Table 1).

Table 1. Composition of experimental diets

	Experimental diets[a]		
	BSL-free	BSL-1	BSL-2
Ingredients (g/kg DM)			
Concentrate mixture	569.6	569.6	569.6
Rapeseed meal	430.4	289.6	140.8
BSL	0	140.8	289.6
Chemical composition (g/kg DM)			
Crude protein	288	275	262
Crude ash	69.6	63.7	57.4
NDF[b]	274	271	267

[a]Blue sweet lupin seeds (BSL), BSL-free, blue sweet lupin free feeding; BSL-1, blue sweet lupin seeds 1 kg; BSL-2, blue sweet lupin seeds 2 kg.
[b]Neutral Detergent Fiber (NDF)

The feeding trial, carried out in July to September 2020, started with a 7-day adaptation period without lupin seed meal (BSL-free (AP)). Afterwards 1 kg of rapeseed meal was replaced by 1 kg BSL for seven days (BSL-1). Therefore, a corresponding mixture of rapeseed meal, BSL and dairy concentrate was prepared and offered in two equal portions daily at 7 am after the morning milking and 2 pm before the evening milking to ensure total uptake. The period was followed by a 10-day depuration period (BSL-free (DP1)), without BSL in the diet. Afterwards, 2 kg rapeseed meal was replaced by 2 kg BSL (BSL-2). Therefore, again a corresponding mixture of rapeseed meal and BSL was prepared and fed twice daily for 7 days, which was followed again by a 10-day depuration period (BSL-free (DP2)).

Analysis of feed ingredients

Feed components were analyzed for DM, crude ash, crude protein and neutral detergent fiber (NDF) according to VDLUFA (Association of German Agricultural Analytic and Research Institutes) standard methods.[18,19,20]

Analysis of milk ingredients

Milk yield was recorded daily. Milk samples were taken twice daily during each milking and stored at -20°C for analysis of QAs. In regular intervals, milk samples were taken for proximate

analysis of milk protein, fat and lactose according to § 64 L01.00-78 of the German Food and Feed Code (LFGB) and milk urea according to directive 1.13 of the German Association for Performance and Quality Testing e.V. (DLQ).[21,22]

Solvents and chemicals

All organic solvents used in this work were at least analytical grade. Solvents used for LC-MS/MS analysis were LC-MS grade.

Analytical standards

For identification and quantification the following analytical standards were used (+)-13α-hydroxylupanine (purity 97%, TRC), (+)-lupanine perchlorate (purity 97%, TRC), (+)-α-iso-lupanine perchlorate (purity 97%, TRC), (-)-angustifoline (purity 97%, CfmOT), (-)-lupinine (purity 96%, sigma-aldrich), multiflorine (purity 99%, CfmOT) and (-)-sparteine-sulfate · 5 H_2O (purity, 98%; Targetmol), respectively.

QAs in BSL and milk

For determination of the QAs in ground BSL, representative samples of about 100 g each were collected (samples from 4 storage containers of 25 kg). Subsequently the samples were ground with an Ultra Centrifugal Mill passing a sieve of 1 mm. QA analyses were performed at the National Reference Laboratory (NRL) for Feed Additives at the German Federal Institute for Risk Assessment (BfR). Samples were analyzed for nine QAs (anagyrine, cytisine, angustifoline, 13α-hydroxylupanine, isolupanine, lupanine, lupinine, multiflorine, sparteine), which were also used to calculate the sum of the QAs. Analysis and quantification of all samples was done using high-performance liquid-chromatography-tandem mass spectrometry with electrospray ionization in positive ion mode (LC-ESI-MS/MS; API 6500 Sciex®). Each measurement was performed in duplicate.

Two in-house validated sample preparation methods were utilized, one for solid (feed) and the other for liquid matrices (milk). Briefly, BSL or milk samples were mixed and the QAs extracted with an acidified acetonitrile/water solution. For this purpose, 5 g of BSL was extracted with 5 ml extraction solution (0.1% formic acid, acetonitrile/water, 50:50, v/v) or 2 ml milk was extracted with 25 ml extraction solution (0.1% formic acid, acetonitrile/water, 90:10, v/v). After 15 minutes extraction time in an overhead-shaker, the samples were frozen (-80°C) to precipitate proteins. After thawing, samples were centrifuged (4000 × *g*) for 5 minutes to separate precipitated proteins from the solution.

For milk samples, additionally a degreasing step of the supernatant was included by using n-hexane. The n-hexane layer was discarded.

The sample extracts must be diluted with ultrapure water and injection solution. The dilution factor depends on the concentration of the analytes in the respective sample and must be

within the concentration range of the standard curve used. The concentration range of the standard curves are between 0.5 mg/kg and 5.5 mg/kg for BSL and between 34 and 370 µg/kg for milk samples. After centrifugation (4000 × *g* for 5 minutes), the final supernatant was decanted into a 2 mL crimp vial for injection into the LC-ESI-/MS-MS. Measurement results were evaluated with the software Analyst 1.6.

For identification (examples of chromatograms are given in figures S6-S10 in the supporting information pages S1-S4), a retention time window of ± 0.1 min around the expected retention time of the corresponding QA was set. Furthermore, the QAs were identified by using two multiple reaction monitoring (MRM) transitions (at least 1 precursor and 2 product ions detected) and calculating the relative ion ratio between both MRM transitions according to regulation (EU) 2021/808.[23] Quantification was performed by preparing a matrix-matched external calibration curve using the analytical standards mentioned before. Briefly, the obtained validation parameters of both methods (milk and BSL), are summarized here for the assessment of the transfer study.

For the analysis of QA in BSL, the recovery was determined by analyzing soybean meal fortified at two different QA concentrations 5 mg/kg and 50 mg/kg (n = 6), respectively. The recoveries ranged for all determined QAs between 80 and 110%. The coefficient of variation (CV) as measure for the repeatability of the applied methods was below 10%. The inter-laboratory reproducibility determined by analyzing samples on different days, by different operators and with different LC-MS/MS instruments was below 10%.

For the BSL, the LOD ranged between 0.01 mg/kg (lupanine) and 0.36 mg/kg (multiflorine), the LOQ between 0.03 mg/kg (lupanine) and 1.19 mg/kg (multiflorine).

For the analysis of milk, the recovery was determined by analyzing milk samples fortified at two different QA concentrations 6 µg/kg and 60 µg/kg (n = 6), respectively. The mean recovery for the determined QAs ranged between 85 and 105 %. The CV as measure for the repeatability is below 10 %. The inter-laboratory reproducibility determined by analysing samples on different days, by different operators and with different LC-MS/MS instruments was below 8 %. For milk, the LOD ranged between 0.02 µg/kg (13α-hydroxylupanine) and 0.41 µg/kg (multiflorine), the LOQ between 0.06 µg/kg (13α-hydroxylupanine) and 1.36 µg/kg (multiflorine).

Statistical analysis

Statistical analyses were carried out using the MIXED procedure of SAS (version 9.4, 2016, SAS Institute Inc., Cary, NC, USA). Days and periods were included as fixed effects in the model for milk yield, fat, protein, urea and lactose concentration. Measurements taken on the same cow but at different times were considered as repeated measures. Multiple comparisons among periods were evaluated by Tukey's post hoc test. A *p*-value of < 0.05 was considered as indicative for significant difference between periods.

Toxicokinetic modelling of QA transfer into milk

To derive transfer parameters relevant for risk assessment and to allow the prediction of the transfer of QAs from feed into cow´s milk, a mathematical model was developed based on the data, specifically a 3-compartment physiologically-based toxicokinetic (PBTK) model (Fig. 2 and eqs 1-2). The model was fitted for the four most prevalent QAs, for which enough data was available: lupanine, 13α-hydroxylupanine, isolupanine and angustifoline.

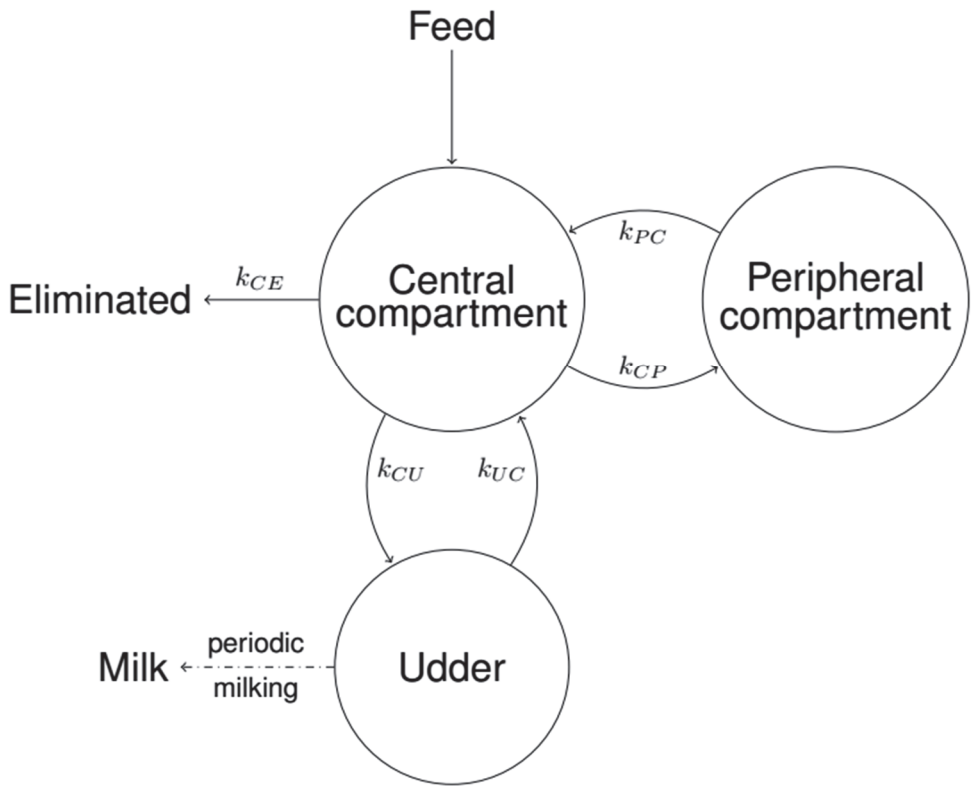

Figure 2. Schema of the 3-compartment model of QA toxicokinetics in dairy cows. The central compartment represents the entry point for QAs into the cow and the output site for elimination (grouping unabsorbed as well as putatively metabolized and/or excreted QAs). The peripheral compartment acts as a small storage. The udder compartment is where the milk is produced, stored and periodically emptied at milking events (together with the QAs contained). The parameters k_{ij} represent the transition rates from compartment i to compartment j for the compartments: i,j = C, Central; P, Peripheral; U, Udder and E, Elimination.

The PBTK model in Fig. 2 was compared to other similar models (with different arrangements of compartments) using the Bayesian information criterion (BIC), where the chosen model performed best (data not shown). The chosen model consists of 3 compartments. The first one is the central compartment, the entry point for QAs with feed into the cow, as well as the place where QAs are eliminated. This elimination groups together unabsorbed as well as putatively metabolized and/or excreted QAs. The central compartment represents both blood plasma and

a biological component (e.g., groups of cells, proteins or lipids) that is in rapid equilibrium with plasma regarding QAs. The second compartment is the peripheral compartment, which acts as a small storage for QAs; it is a biological component that more slowly exchanges QAs with the central compartment. The third and last is the udder compartment, which can also exchange QAs with the central compartment, while producing and storing milk and, critically, excreting QAs with that milk at periodic milking events. Since only milk data were available, the exact biological nature of all the components of each compartment could not be established, which doesn't undermine the predictive ability of the model. The PBTK model (Fig. 2) is described by the following differential equations between milking events:

$$\dot{\boldsymbol{A}}(t)=\boldsymbol{M}\boldsymbol{A}(t)+\boldsymbol{I}(t)\ , \qquad (1)$$

where A(t)=$(A_C(t), A_U(t), A_P(t))^T$ is the amount vector containing the amount of the respective compartment at time t; I(t) is the input vector at time t and **M** is the transition matrix given by

$$\mathbf{M} = \begin{pmatrix} -(k_{CP}+k_{CU}+k_{CE}) & k_{UC} & k_{PC} \\ k_{CU} & -k_{UC} & 0 \\ k_{CP} & 0 & -k_{PC} \end{pmatrix} \qquad (2)$$

Here the model parameters k_{ij} represent the transition rates from compartment i to compartment j for the following compartments: i,j = C, Central; i,j = P, Peripheral; i,j = U Udder and i,j = E, Eliminated (conceptually lumping any metabolization and excretion). Here, the complete emptying of the udder compartment occurs twice daily during the periodic morning and evening milking events.

A peripheral compartment was included based on the shape of the data from the depuration period (Fig. S11, Days 14-17 and 31-34), where a bi-phasic behavior (two half-lives) was apparent. A very dominant short *α*-half-life, reflecting elimination of QAs from the central compartment, and a second less prevalent longer *β*-half-life, reflecting elimination of QAs from the peripheral compartment, were identified. The model mechanics assume complete and uniform absorption of QAs into the central compartment distributed uniformly across five hours after feeding; this does not imply that the effective physiological absorption is 100%; the effective absorption from feed and bioavailability for milk excretion is included via the interplay of rate constants $k_{ij.}$ The last piece of the model is the implementation of the periodic emptying of the udder at each milking time, which is performed algorithmically as detailed in the Supporting Section Complete Toxicokinetic Model.

An optimization approach was used to obtain model parameters k_{ij} by minimizing the log squared error for the best fit.[24] In addition, the tails of the depuration (ten days after start of feeding for each feeding period) was weighted with only 25% in order not to overvalue the more irrelevant *β*-phase of elimination. Data below limit of quantification (LOQ) or limit of detection (LOD) were also considered for the fit by interpreting them as an interval in which the true values lie, so that the error function does not penalize values within that interval. A

permutation test was applied to check the hypothesis of a dose-dependent transfer into the milk.[25] Confidence intervals were derived using the delete-two jackknife method.[26] In addition, the optimized model for each QA was used to estimate transfer parameters: the *α*- and *β*-half-lives of the respective elimination phases as well as the steady-state transfer rate (TR) from feed to milk, defined as

$$TR = \frac{\text{amount in milk[ng/d]}}{\text{amount in feed[ng/d]}} 100\%. \quad (3)$$

Lastly, the relative transition amount (RTA) was determined for each QA. RTA is helpful to understand at what point there is a transition from the *α*- to the *β*-elimination phase. Specifically, RTA tells us at what amount in milk (as a percentage of steady state or maximum) the slope of the depuration is better approximated by the *β*-half-life rather than the *α*-half-life. A more detailed description of the derivation of transfer parameters can be found in the Supporting Sections.

Assessment of consumer exposure to QAs using EFSA RACE tool

The EFSA Rapid Assessment of Contaminant Exposure (RACE) software tool was used to estimate the exposure to QAs resulting from milk consumption, considering the determined QA levels.[27] With the help of food consumption information from the EFSA Comprehensive European Food Consumption Database, RACE provides an estimate of acute and chronic exposure from single foods. These values can then be compared with relevant toxicological reference points. For the assessment, maximum QA levels in milk during the exposure phases were used. As in the EFSA opinion on QAs, risk characterization was performed by applying the MoE approach, using the dose of 0.16 mg sparteine/kg bodyweight as reference point.

Results

Feed intake and milk yield

Throughout the experiment, the whole concentrate proportion was ingested, indicating no obvious adverse effect of BSL on concentrate intake. The forage mixture was provided *ad libitum* and individual intake was not recorded. Milk yield slightly declined over the course of the experiment from 31.6±4.7 kg/d to 29.1±4.5 kg/d ($p < 0.001$) (Table 2).

Table 2. Milk yield and milk composition of the cows.

	experimental periods[a]						
	BSL-free (AP1)	BSL-1	BSL-free (DP1)	BSL-2	BSL-free (DP2)	SEM	*p*-value period
Milk yield (kg)	31.6[b]	31.4[b,c]	30.3[b,c,d]	29.9[c,d]	29.1[d]	1.04	0.002
Fat (%)	3.85[b,c]	4.13[b]	3.91[b,c]	3.50[c]	3.77[b,c]	0.23	0.039
Protein (%)	3.01	2.90	2.85	2.86	2.96	0.07	0.144
Lactose (%)	4.83	4.84	4.84	4.81	4.66	0.08	0.136
Urea (mg/L)	668	626	632	675	520	62.1	0.128

SEM, standard error of the mean

[a]BSL-free (AP1), Adaptation period, blue sweet lupin free feeding; BSL-1, experimental period 1, blue sweet lupin seeds 1 kg; BSL-free (DP1), depuration period 1, blue sweet lupin free feeding; BSL-2, experimental period 2, blue sweet lupin seeds 2 kg; BSL-free (DP2), depuration period 2, blue sweet lupin free feeding.

[b-d]Means in the same row with different letters differ significantly.

The period had a significant effect on fat content in milk ($p = 0.039$), with highest contents found in BSL-1 with 4.13% and lowest contents in BSL-2 with 3.5%. Contents of protein, lactose and urea in milk did not differ between periods ($p > 0.05$). The lactation stage of the individual cows and external influences, such as the outside temperature, which exceeded 25°C throughout the present experiment, can have an impact on the performance parameters like milk production.[28] Additionally, other authors previously reported decreases in milk yield due to the feeding of lupin seeds in comparison to feeding rapeseed meal or soybean meal based concentrates, which might be related to the lower crude protein (CP) content in lupin seeds (Table 1).[29–31] In addition to a generally lower CP content in lupin seeds, the CP of unprocessed lupin seeds is known to be extensively degraded in the rumen, causing a reduction in amino acid flux to the duodenum.[30,32] Joch suggested that decreases in milk yield may be due to the lower methionine content of lupin protein, although the addition of ruminally protected methionine did not increase milk yield in that study.[31]

Milk fat represents the most variable component in milk and can be influenced by nutritional as well as physiological aspects.[33] Froidmont showed increased levels of milk fat after protein replacement of soybean meal with lupin and attributed increased milk fat to the higher fiber content in lupin seeds, with a concomitant increase in acetate liberation in the rumen as a precursor for milk fat.[34] This was not observed in the present study, and may depend on other

dietary effects and lactation stage of the cows. A reduced milk fat content due to the feeding of lupin seeds has also been observed by others.[35,36]

Quinolizidine alkaloids in BSL and transfer into milk

The sum of determined QAs in the present BSL ranged between 0.17–0.19% in DM and was higher than the commonly reported <0.05% for "sweet" lupins.[4] Higher levels of QAs in *L. angustifolius* have been reported before and are most likely due to abiotic influences, cross mutation and backcrossing with wild varieties. Higher outdoor temperatures or lower soil pH values during the growing season can also lead to higher levels of QAs in sweet lupins.[37,38] The literature reports as main alkaloids for *L. angustifolius*: lupanine, 13α-hydroxylupanine, isolupanine, angustifoline, 13-angeloyloxylupanine and 13-tigloyloxylupanine.[39,40] We found a slightly different set in the present study, where levels of 13α-hydroxylupanine, lupanine, angustifoline and isolupanine in BSL where higher than levels of multiflorine and sparteine resulting in high intakes of 13α-hydroxylupanine and lupanine (Table 3). Intake of total QAs was 1774 mg/d during the BSL-1 feeding period and 3549 mg/d during the BSL-2 feeding period (Table 3).

Table 3. Intake of Quinolizidine alkaloids (QAs) with BSL in mg/d

	Experimental diets[b]	
QA intake with BSL (mg/d)	BSL-1 kg	BSL-2 kg
Total[a]	1774	3549
Angustifoline	223	446
13α-Hydroxylupanine	702	1404
Isolupanine	129	257
Lupanine	715	1430
Multiflorine	2.45	4.89
Sparteine	3.03	6.06

[a]Quinolizidine alkaloids (QAs) as analyzed in BSL
[b]Blue sweet lupine (BSL), BSL-1, blue sweet lupin seeds 1 kg/d; BSL-2, blue sweet lupin seeds 2 kg/d

Toxicity of QAs has been more thoroughly studied for sparteine and lupanine in humans and rats, while effects of other QAs have not yet been systematically investigated.[10,12,41,42] In rat studies, a lower toxicity was observed for lupanine and 13α-hydroxylupanine than for sparteine.[42,43] Until now there are only few studies evaluating the toxicity of QAs in cattle. For

instance, cattle showed reduced voluntary feed intake when intact lupin seeds were fed in contrast to lupin seeds that were previously detoxified by boiling and soaking in water.[44] However, QA intake with *L. albus* used in that study was considerably higher than in the present one, reaching estimated levels of 60 g/d of lupanine and 21g/d 13α-hydroxylupanine with *L. albus*. Increased levels of QAs therefore appear to result in decreased appetite, confirming observations made by others.[30,45] No negative effects on animal health were seen in the present study with intakes of 1.27–2.54 mg lupanine/kg bw, and 1.24–2.49 mg 13α-hydroxylupanine/kg bw. However, other studies with cattle observed symptoms like reduced general condition, frothing at the mouth and protrusion of the nictating membrane with higher total QA intake levels of 57.6 g QAs/kg bw.[46] Severe toxic effects of QA in cattle have been described only for the teratogenic QA anagyrine.[47,48,49] During critical times of gestation, the ingestion of several lupin species by pregnant cattle has been associated with the so-called crooked calf syndrome.[12,50,51] Anagyrine has been identified as a main causative QA, but anagyrine was not detected in BSL used in the present study.[50,51] So far, possible intoxication of calves by anagyrine or other QA in milk has not been reported, but according to the present results has to be taken into consideration.

There are currently no maximum levels of QAs for animal or human nutrition in the EU. Nevertheless, gathering knowledge concerning the transfer of QAs from feed to animal food is vital. Although animals showed no adverse health effects in the present study, it was demonstrated that with the administration of only 1 kg sweet lupin seeds, a transfer of QAs into the milk occurs, resulting in a total QA concentration of 2.81 mg/kg milk. Although the quantities of QAs excreted via milk differed slightly between the cows, the QA excretion pattern was similar (Figure 3).

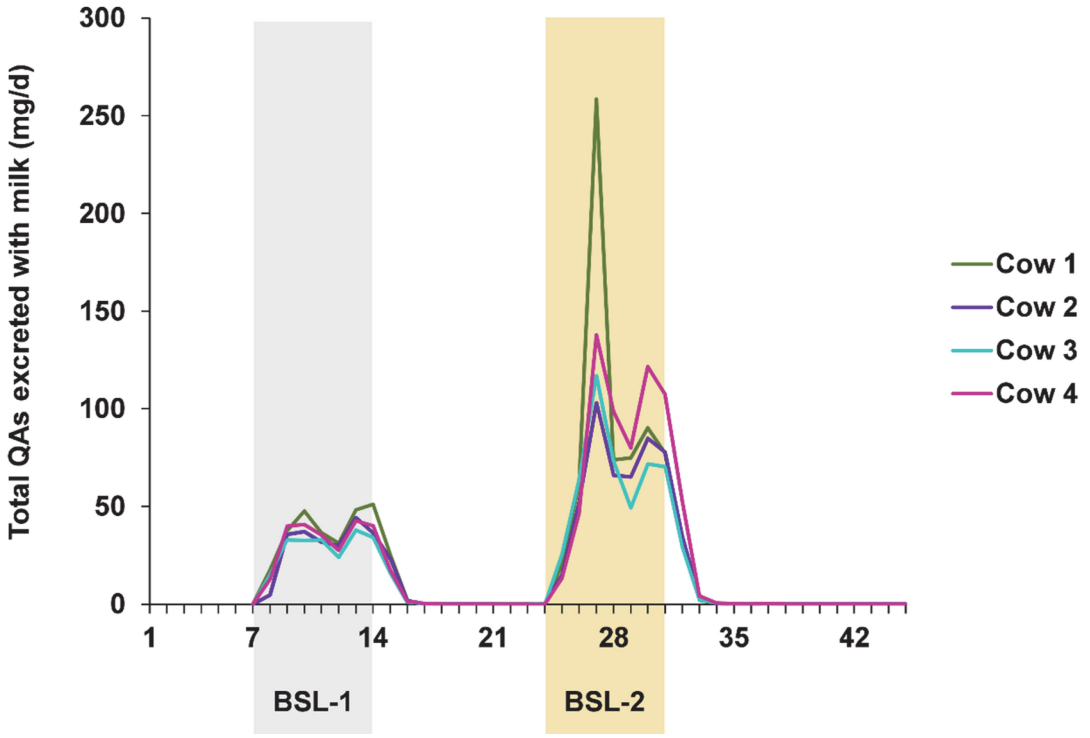

Figure 3. Total QAs excreted with milk daily. Shaded are the feeding periods BSL–1 (blue sweet lupin seeds 1 kg/d) and BSL–2 (blue sweet lupin seeds 2 kg). Unshaded are periods with no QA feeding: the adaptation periods before BSL-1 as well as the depuration periods following BSL-1 and BSL-2.

The concentrations of individual QAs quantified in morning and evening milk during steady state are shown in Figure 4.

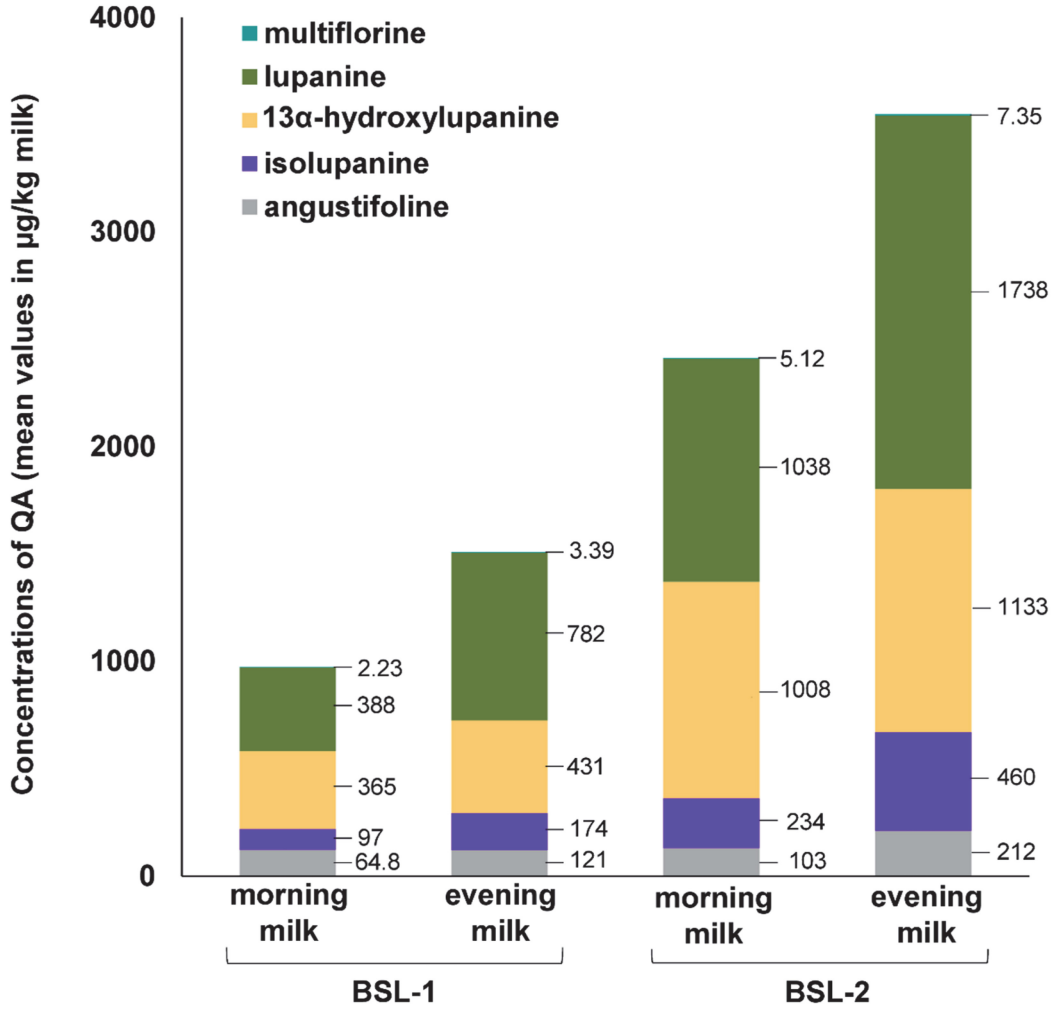

Figure 4. Quinolizidine alkaloid (QA) contents in morning and evening milk during BSL-1 and BSL-2 feeding (mean values in steady state in µg/kg). Feeding periods, BSL–1 (blue sweet lupin seeds 1 kg/d) and BSL–2 (blue sweet lupin seeds 2 kg/d)

As in BSL, 13α-hydroxylupanine and lupanine were found to be the most abundant QA in milk. Despite of average contents in BSL of 3.03 mg/kg, concentrations in milk of sparteine were near or below the LOQ of < 0.10 µg/kg milk.

Concentrations of multiflorine, angustifoline and especially lupanine were noticeably higher in the evening milk than in the morning milk (Figure 4). This effect was also reflected in the higher TRs of multiforine, angustifoline and lupanine for evening milk (Table 4). In the evening, cows were fed with lupin seeds two hours before milking, while in the morning cows were milked before feeding. It follows that QAs both from morning feeding and in part from evening feeding were excreted in the evening milk, while in the morning milk only the remainder was found. Interestingly and in contrast to the other QAs, the TR of 13α-hydroxylupanine was higher in the morning than in the evening milk (Table 4). An explanatory hypothesis is the possible biotransformation of QAs in the cow. So far, metabolism of individual QAs has been investigated only in rats, pigs, rabbits and humans.[10,52,53] Studies in rats showed that sparteine

was oxidized to lupanine, which was found in the urine of orally dosed rats *in vivo* (suspected microsomal metabolization), while lupanine was found to be presumably transformed to a hydroxyl derivative through a yet unknown pathway.[10,54] Until now, there exists no information regarding the possible metabolization of lupanine into 13α-hydroxylupanine in cows. However, conversion could explain its higher values in the morning milk.

It is known that ruminants can render certain plant toxins harmless via microbial metabolization in the rumen. However, an *in vitro* rumen fermentation study conducted in our department (data not shown) did not find ruminal degradation of lupanine, which confirms previous results of Aguiar.[55] Accordingly, metabolization of QAs in the liver might be the cause for the observed differences in QA excretion but further research is needed in this regard. Other metabolites were not investigated with the current analytical method, therefore, an occurrence of possible metabolites in milk cannot be excluded.

Toxicokinetic modeling and transfer rates for QAs

As a first step, the hypothesis of dose-dependent QA transfer into milk[24] was tested. A permutation test was applied to verify whether the experimental data allow rejection of the hypothesis. With the exception of angustifoline, the permutation test provided no indication of a non-linear dose dependent transfer rate for the QAs studied. The apparent non-linearity for angustifoline was neglected because it can be attributed to the small sample size. Therefore all QAs were fitted to the 3-compartment PBTK model (eqs 1-2, Figure 2) using the data for all cows and all experimental periods (both doses BSL-1 and BSL-2) simultaneously to obtain the optimized model parameters (Table S9). Results of the PBTK model for QA excretion via milk are shown in Figure 5.

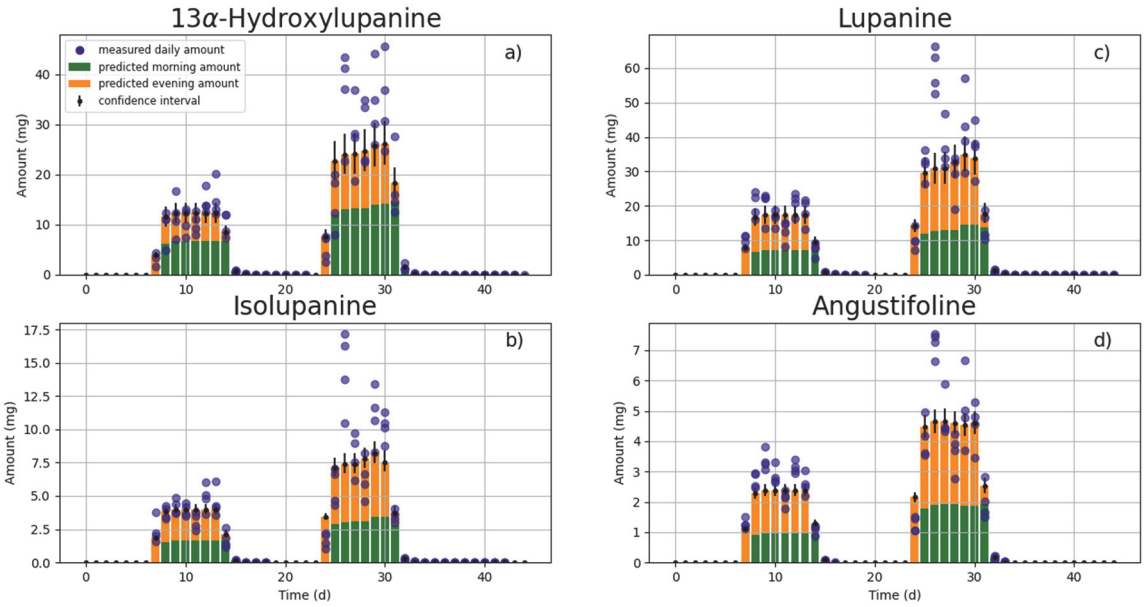

Figure 5. Daily amounts excreted via milk for four QAs. Bars denote the toxicokinetic model results plotted together with their confidence intervals across animals (divided into morning – yellow – and evening excretion –green). Blue dots represent the daily amount excreted obtained from the feeding experiment.

In Fig. 5, QA excretion from morning and evening milk was lumped together as daily excretion (total bar for model and dots for experiment). During the first BSL feeding period (BSL-1), the concentration profiles of QAs could be adequately predicted. Concerning the second feeding period (BSL-2), the model was only able to reproduce the average behavior, as the measured QA contents in milk displayed higher variability. In particular, the model was unable to reproduce the apparent peak (Figure 5, day 27) in the analyzed QA contents in milk at the beginning of BSL-2 feeding, which might indicate more complex underlying kinetics. Since the PBTK model could nevertheless reproduce the average behavior, it was used to calculate transfer parameters, namely transfer rates (TRs, Table 4), milk excretion *α*- and *β*-half-lives (Tables S6-S7).

Table 4. Estimated transfer rates (TRs) of QAs from feed into milk, which is made up out of morning+evening milk. * Marks the QAs for which no model was developed but nevertheless a rough approximation of the TRs from the data was made.

	Mean [%] =(morning+evening)	**95% Confidence Interval** [%]
13α-Hydroxylupanine	**1.74** (0.95+0.79)	1.34 - 2.16
Lupanine	**2.31** (0.96+1.35)	1.85 – 2.77
Isolupanine	**2.92** (1.21+1.71)	2.57 – 3.35
Angustifoline	**1.05** (0.43+0.62)	0.93 – 1.18
Multiflorine*	**3.74** (1.79+1.95)	-
Sparteine*	**0.13** (0.06+0.06)	-

All four investigated QAs showed fast and dominant milk excretion *α*-half-lives of around 0.27 d (Table S6), which are similar to the literature plasma half-lives for lupanine of 0.29 and 0.23 d in cows.[48,49] In contrast, the half-life of lupanine in beef cattle reported in another study was 0.48 days with a mean residence time of 50 to 61 h, equivalent to a half-life of 1.44 d and 1.76 d, respectively, in a 1-compartment setting.[49,56,57] Those values are considerably higher than the derived values of the present study (Table S6), suggesting that there are differences in the kinetic behavior of lupanine between different breeds or production purposes and may be attributable to the lack of excretion with milk.[49]

The shape of the data profile from the depuration period (Fig. S11, Days 14–17 and 31–34) shows a bi-phasic behavior (two half-lives). The chosen model (Figure 2) reproduces this behavior; from it, *β*-half-lives of 2.48–5.18 d for the four QAs were estimated (Table S7). The intake of QAs from sources other than measured feed can be excluded. Additionally, the QA analysis showed values above the LOQ in the depuration periods contrary to the adaptation period. This suggests that small amounts of QAs remained in the peripheral compartment after exposure, resulting in an extended ß-half-life during the depuration period. But how relevant are these *β*-half-lives for risk analysis? The answer comes from the postulated parameter relative transition amount (RTA) (eq S17, Table S8) that quantifies the relative importance of the *α*- and *β*-half-lives. RTA indicates when the system moves from the *α*-phase to the *β*-phase of depuration. The RTAs found for QAs (Table S8) range from 0.11% to 0.33% of the steady state amounts, which means that more than 99.67% of the depuration occurs in the *α*-phase. Therefore, the ß-phase of depuration is practically irrelevant, provided it happens at amounts that are toxicologically of no concern. Combined with knowledge of a very short α-half-life

(Table S6), we conclude that in most cases it is possible to rely on a simple multiplicative Transfer Rate (TR) calculation. The TR into morning + evening milk of individual modeled QAs (Table 4) range from 1.05% for angustifoline to 2.92% for isolupanine. Furthermore, although the data did not allow the development of a PBTK model for sparteine, its transfer rate can be roughly estimated directly from the data by averaging

$$TR = \frac{\text{Daily excretion}}{\text{Daily feed}} \quad (4)$$

for all days in apparent steady state with measurements above LOQ, resulting in a TR of 0.12%. The same method for multiflorine yields a TR of 3.74%. These results may partly be explained by the fact, that so far, it cannot be ruled out that individual QAs are metabolized to other QAs in resulting in higher TR for individual QA. Although the use of simple multiplicative calculations using TR should suffice for most cases of risk analysis, the full predictive toxicokinetic model code is included as part of the Supporting Information as it can help understanding how theses contaminants are transported into the milk.

Assessment of consumer exposure to QAs using EFSA RACE tool

A preliminary estimation of the dietary acute exposure by using the EFSA RACE tool showed that the sole consumption of milk containing QAs at a level as measured in the present study might result in intakes above 0.16 mg/kg bw for high milk consumers.[8] This, in turn, means that the corresponding MoE is < 1, reflecting an exposure in the effect level and consequently a health concern (Table 5).

Table 5. Comparison of the exposure of high (P95) milk consumers to the lowest single oral effective dose for QA. The EFSA Rapid Assessment of Contaminant Exposure (RACE) tool was used for calculation of different exposure scenarios. Maximum QA in the milk during BSL-1 and BSL-2 of the feeding experiment and a lowest single oral effective dose of 0.16 mg sparteine/kg bodyweight/d were taken as a basis. Bold numbers: exceedance of MoE 1.

Population group	High consumer (P95)	
	QA content in cows milk (µg/kg)	
	BSL-1 max 19607.3	BSL-2 max 90186.5
	Comparison of exposure to toxicological reference point expressed as MoE	
Infants	**0.04**	**0.01**
Toddlers	**0.11**	**0.02**
Other children	**0.18**	**0.04**
Adolescents	**0.41**	**0.09**
Adults	**0.72**	**0.16**
Elderly	**0.93**	**0.20**
Very elderly	**0.96**	**0.21**
Pregnant woman	**0.77**	**0.17**
Lactating woman	**0.92**	**0.20**

Already in BSL-1 MoEs < 1 were measured for all population group. Additionally, in BSL-2, maximum levels in milk also represent an exposure in the effect level for all population groups. The calculated MoE values refer only to the sole consumption of raw milk containing QA levels as measured in the present study. Therefore, it cannot be excluded that dilution, processing of milk, as well as the production of dairy products may have consequences for the content of QA and, consequently, for the exposure level and the resulting MoE values.

However, QA contents and QA profiles differ considerably between lupin breeds and even within the same variety. The different excretion patterns of individual QAs also show that further investigations are necessary to understand the metabolism of QAs within dairy cows.

In conclusion, the present study proves the transfer of QAs from BSL into milk of dairy cows already at low inclusion levels of lupin seeds in the ruminant diet.

Abbreviations used

AP, adaptation period; BIC, Bayesian information criterion; BfR, German Federal Institute for Risk Assessment; BSL, blue sweet lupin; DLQ, German Association for Performance and Quality Testing e.V.; DM, dry matter; DP, depuration period; EFSA, European Food Safety Authority; LC/MS-MS, high-performance liquid-chromatography-tandem mass spectrometry; LFGB, German Food and Feed Code; LOQ, limit of quantification; MoE, margin of exposure; MRM, multiple reaction monitoring; NDF, neutral detergent fiber; NRL, national reference laboratory; PBTK, physiologically-based toxicokinetic model; QAs, quinolizidine alkaloids; TR, transfer rate; VDLUFA, Association of German Agricultural Analytic and Research Institutes

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Notes

The authors declare no competing financial interest.

Supplementary material

LC/MS-MS chromatograms

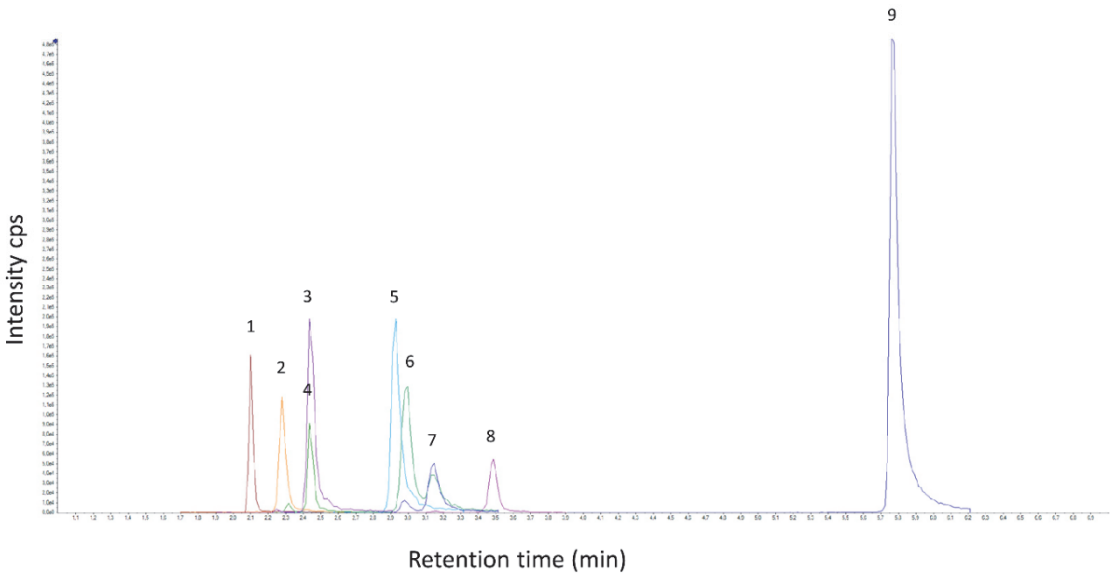

Figure S6. Overlay LC-MS/MS chromatogram of the quantifier MRM transitions of nine QAs in a standard solution with a concentration of 2.5 ng/ml each (1. cytisine (Rt = 2.1 min), 2. lupinine (Rt = 2.3 min), 3. thermopsine (Rt = 2.45 min), 4. 13-hydroxylupanine (Rt = 2.45 min), 5. multiflorine (Rt = 2.95 min), 6. lupanine (Rt = 6.0 min), 7. iso-lupanine (Rt = 3.15 min), 8. angustifoline (Rt = 3.5 min), 9. sparteine (Rt = 5.8 min).

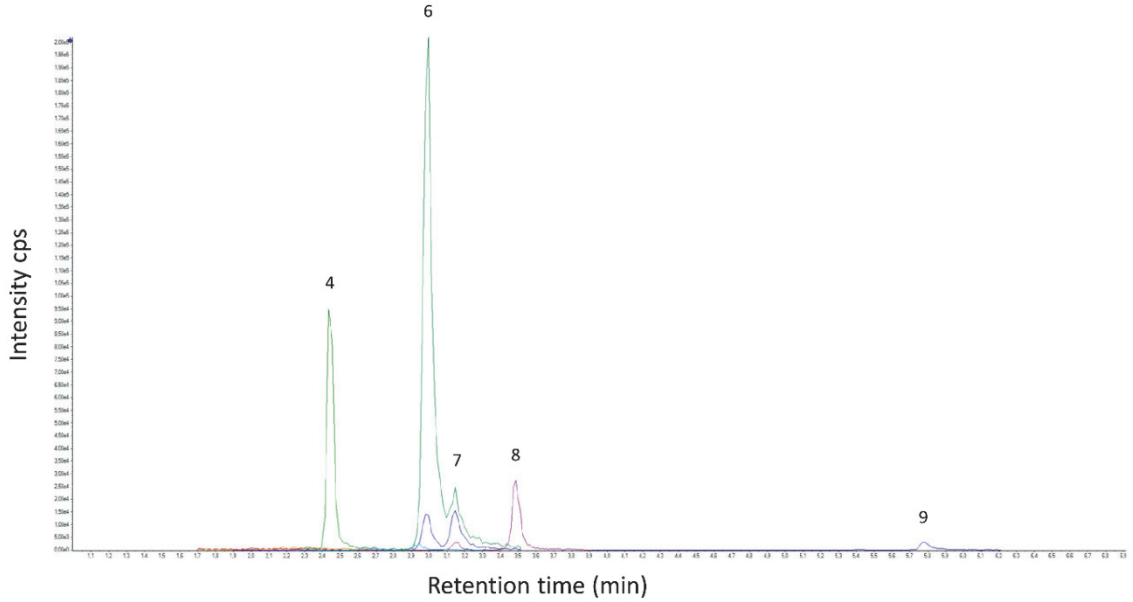

Figure S7. Overlay LC-MS/MS chromatogram of the quantifier MRM transitions of five QAs analysed in lupin seeds (whole grain, untoasted) used for feeding (4. 13-hydroxylupanine 3.6 ng/ml (715 mg/kg), 6. lupanine 3.8 ng/ml (765 mg/kg), 7. iso-lupanine 0.7 ng/ml (140 mg/kg), 8. angustifoline 156 ng/ml (156 mg/kg), 9. sparteine < LOD; Dilution 1:8000).

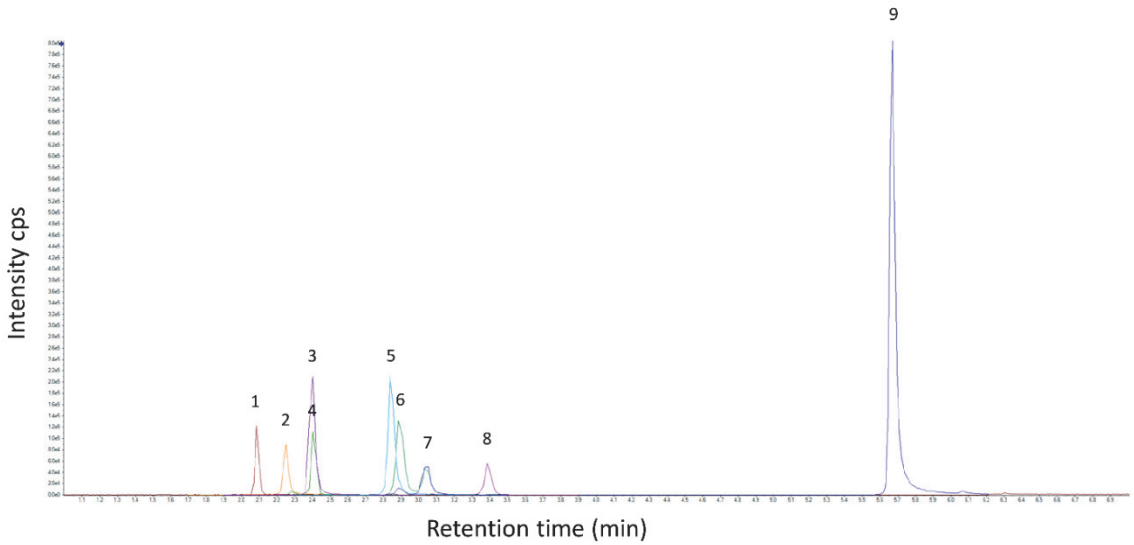

Figure S8. Overlay LC-MS/MS chromatogram of the quantifier MRM transitions of nine QAs in a matrix matched calibration by utilizing cow milk (dilution 1:20) fortified at a level of 2.5 ng/ml (substances and Rt see figure 1).

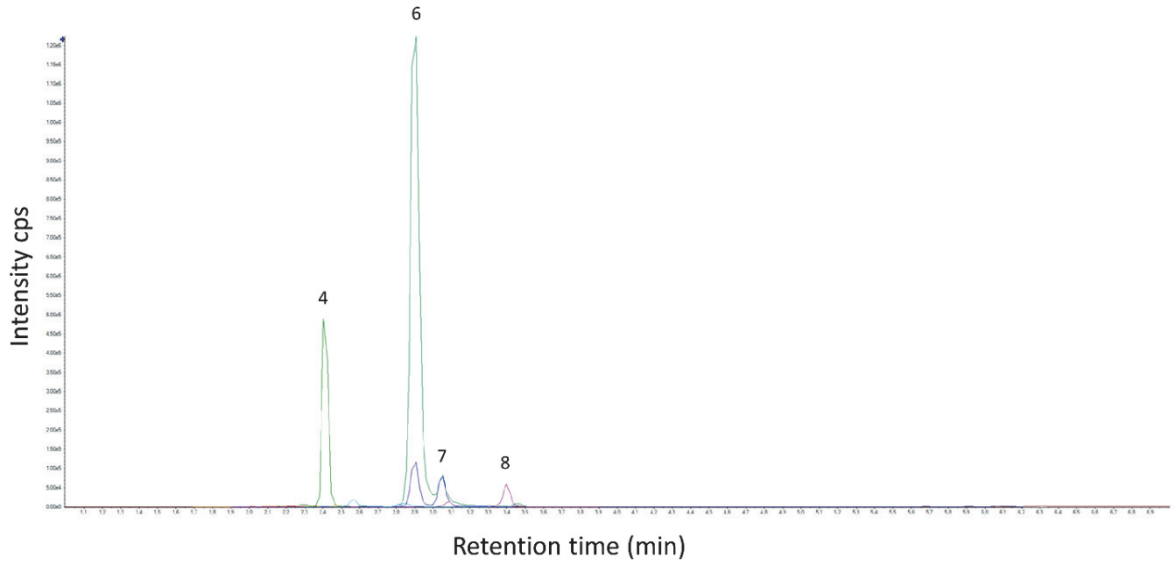

Figure S9. Overlay LC-MS/MS chromatogram of the quantifier MRM transitions of four QAs analysed in a cow milk sample (dilution 1:20) 4. 13-hydroxylupanine and 6. lupanine (both shown QAs are outside of the linear range), 7. iso-lupanine 3.5 ng/ml (117 µg/kg), 8. angustifoline 2.9 ng/ml (97 µg/kg).

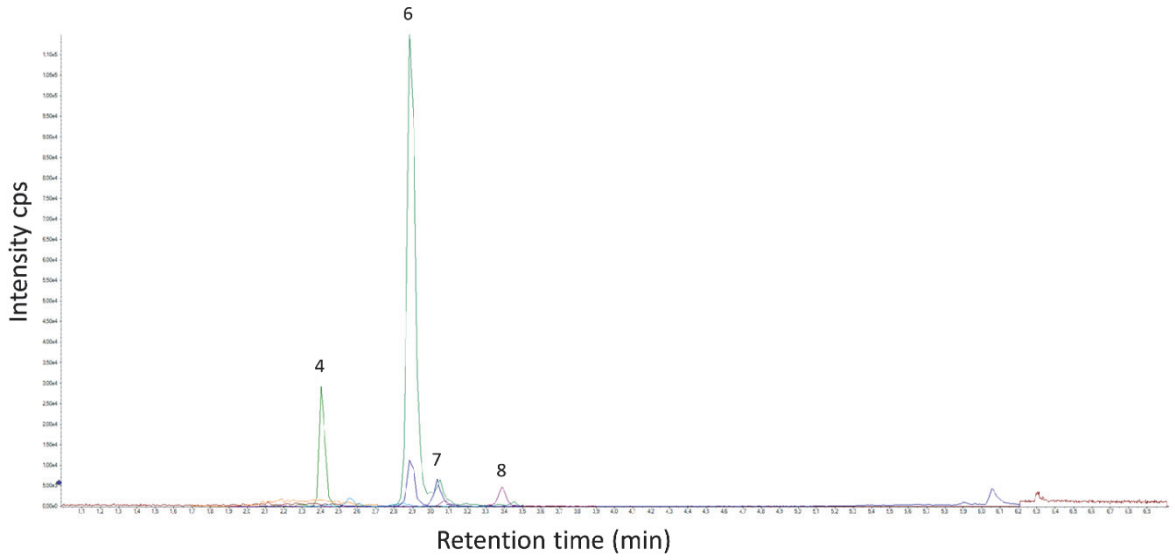

Figure S10. Overlay LC-MS/MS chromatogram of the quantifier MRM transitions of four QAs analysed in a cow milk sample (dilution 1:200) 4. 13-hydroxylupanine 1.2 ng/ml (404 µg/kg), 6. lupanine 1.9 ng/ml (642 µg/kg).

Calculation of transfer parameters

Transfer rates

The steady state transfer rates were approximated by assuming that a constant feeding period with the same daily intake *D*[ng/d] (for simplicity D=1 ng/d). Then, the total output via milk at the 100th day M_{100}[ng] was derived via simulating the system until the 100th day, whereupon the transfer rate is given by

$$TR = \frac{M_{100}}{D} 100\% \tag{S1}$$

Half-lives

The half-lives of the model are derived analytically. The amount of QA excreted at the n'th morning milk can be described by the following equation

$$X_{n,mor} = (\underbrace{e^{M14/24} I_l e^{M10/24} I_l}_{=:A})^n X_{0;mor} \tag{S2}$$

for a given starting vector $X_{0;\,mor}$ at the 0'th morning milk. Here, M is the transition matrix of the PBTK model and I_l is the matrix describing the milking process, i.e.

$$I_l = \begin{pmatrix} 1 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 1 \end{pmatrix} \tag{S3}$$

Note that I_l only induces two non-zero eigenvalues (1 with multiplicity 2) and $e^{M14/24}$; $e^{M10/24}$ are both invertible, which is why A induces two non-zero eigenvalues λ_1, λ_2. Assuming $\lambda_1 \neq \lambda_2$, then A can be expressed as

$$A = D \begin{pmatrix} \lambda_1 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & \lambda_2 \end{pmatrix} D^{-1} \tag{S4}$$

for some invertible matrix D. Furthermore, it follows

$$A^n = D \begin{pmatrix} \lambda_1 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & \lambda_2 \end{pmatrix}^n D^{-1} \tag{S5}$$

$$= D \begin{pmatrix} e^{ln(\lambda_1)} & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & e^{ln(\lambda_2)} \end{pmatrix}^n D^{-1} \tag{S6}$$

Therefore, the half-lives induced by *A* are given by

$$\tau_\alpha = \frac{\ln(2)}{-\ln(\lambda_1)} \quad \text{(S7)}$$

$$\tau_\beta = \frac{\ln(2)}{-\ln(\lambda_2)} \quad \text{(S8)}$$

Finally, note that the amount in each compartment at the evening milking time of the n'th day can be expressed as follows

$$X_{n,\,eve} = e^{M10/24} I_n X_{n,mor} \quad \text{(S9)}$$

Therefore, morning and evening milk have the same half-lives and

so does the whole milk of the day as

$$X_{n,\,tot} = X_{n,\,mor} + X_{n.\,eve} \quad \text{(S10)}$$

Relative transition amount (RTA)

The relative transition amount describes the amount relative to a steady state at which the decay (starting from steady state) of the amount of QA excreted with milk is better described by the τ_β rather than the τ_α . The decay is described by a biexponential function (except for the first day), i.e.,

$$A(t) = C_1 e^{\lambda_1 t} + C_2 e^{\lambda_2 t} \quad \text{(S11)}$$

Note that A(t) is the continuous expansion of the QA excretion function, as this only makes sense in a discrete setting, i.e., $A|_{\mathbb{N}}(t) = \pi_{Udder}(X_{t;tot})$ with π_{Udder} being the projection onto the udder compartment. The time point at which this happens can be expressed by $\tilde{t}$

$$\frac{d}{dt} C_1 e^{\lambda_1 t}|_{t=\tilde{t}} = \frac{d}{dt} C_2 e^{\lambda_2 t}|_{t=\tilde{t}} \quad \text{(S12)}$$

$$\Leftrightarrow \lambda_1 C_1 e^{\lambda_1 \tilde{t}} = \lambda_2 C_2 e^{\lambda_2 \tilde{t}} \quad \text{(S13)}$$

$$\Leftrightarrow \tilde{t} = \frac{ln\left(\frac{\lambda_1 C_1}{\lambda_2 C_2}\right)}{\lambda_2 - \lambda_1} \quad \text{(S14)}$$

Thus, knowing the half-lives (section 1.2), only C_1; C_2 are unknown. To derive these, the function A(t) is solved for two different time points, i.e. for simplicity t0 = 0 and t10=10. This can be done by simulating the 101th and the 111th day assuming a 100 day feeding period. Note that the 101st day is chosen as the start of A instead of the 100th, due to the partial influence of the feeding on the decay of the first day of the depuration phase. Then C_1 and C_2 can be calculated as follows

$$C_1 = A(0) - C_2 \quad \text{(S15)}$$

$$C_2 = \frac{A(10) - A(0) e^{\lambda_1 10}}{e^{\lambda_2 10} - e^{\lambda_1 10}} \quad \text{(S16)}$$

Together with equations (S11) and (S14), the amounts at the transition time can now be calculated. Then the relative transition amount (RTA) is given by

$$RTA = \frac{A(\tilde{t})}{A_{ss}} 100\% \quad \text{(S17)}$$

where A_{ss} are the amounts excreted during steady state.

Transfer parameters

Table S6. *α*-half-lives τ_α of the simulated QA. The mean value was derived via fitting the model to the four experimental cows and the confidence interval (α=0.05) was derived using the delete-one jackknife method.

	Mean (d)	95% confidence interval (d)
Hydroxylupanine	0.28	0.26 - 0.31
Lupanine	0.26	0.25 - 0.28
Isolupanine	0.26	0.23 - 0.29
Angustifoline	0.27	0.24 - 0.29

Table S7. *ß*-half-lives τ_β of the simulated QAs. The mean value was derived via fitting the model to all four cows and the confidence interval (α=0.05) was derived using the delete-one jackknife method.

	Mean (d)	95% confidence interval (d)
Hydroxylupanine	3.51	2.66 – 5.41
Lupanine	3.04	2.00 – 5.93
Isolupanine	2.48	2.17 – 2.95
Angustifoline	5.18	2.85 – 25.79

Table S8. The relative transition amount from alpha into beta phase of the simulated QA. The mean value was derived via fitting the model to all four cows and the confidence interval (α=0.05) was derived using the delete-one jackknife method.

	Mean (%)	95% confidence Interval (%)
Hydroxylupanine	0.14	0.11 - 0.17
Lupanine	0.11	0.01 - 0.17
Isolupanine	0.34	0.19 - 048
Angustifoline	0.14	0.10 – 0.18

Complete toxicokinetic model

The PBTK model (Fig 2) between milking events can be described by a linear equation system of the form

$$\dot{\boldsymbol{A}}(t)=\boldsymbol{M}\boldsymbol{A}(t)+\boldsymbol{I}(t) \quad \text{(S18)}$$

where M is the transition Matrix given by

$$\mathbf{M} = \begin{pmatrix} -(k_{CP}+k_{CU}+k_{CE}) & k_{UC} & k_{PC} \\ k_{CU} & -k_{UC} & 0 \\ k_{CP} & 0 & -k_{PC} \end{pmatrix}. \quad \text{(S19)}$$

Here the model parameters k_{ij} represent the transition rates from compartment i to compartment j for the following compartments: i,j=C, Central; i,j=P, Peripheral; i,j=U , Milk and i,j=E, Eliminated (conceptually lumping any metabolization and excretion). Alternatively, the same model can be written as the system of differential equations

$$\dot{A}_C(t) = -(k_{CP}+k_{CU}+k_{CE})A_C(t) + k_{UC}A_U(t) + k_{PC}A_P(t) \quad \text{(S20)}$$

$$\dot{A}_U(t) = k_{CU}A_C(t) - k_{UC}A_U(t) \quad \text{(S21)}$$

$$\dot{A}_P(t) = k_{CP}A_C(t) - k_{PC}A_P(t) \quad \text{(S22)}$$

Periodic milking

The last piece of the model is the implementation of the periodic milking or emptying of the udder at each milking time, which is calculated algorithmically as follows:

$X = (0,0,0)^T$ #Initialization
MilkList=[] #Intialzing the array containing the milk data
for i=0:numberOfExperimentHours-1:
    if [i,i+1] is feeding time:
        I=$(\frac{daily\ dose}{10}, 0,0)^T$
    else:
        I=$(0,0,0)^T$
    $x^* = -M^{-1}I$
    $X = x^* + e^M(X - x^*)$
    if i+1 is milking time:
        MilkList.append(X["Udder"])
        $X = I_l X$

Here MilkList contains the QA amount excreted at each milking time, thereby alternating between morning and evening milk. The feeding times and milking times follow the experimental schedule for fitting the data and can be fixed for a predictive model for the general case. The total QA amount excreted per day can be calculated by adding the amounts for morning and evening milking, as is done for the predictive model included as code. The best-fit values for the model parameters in eq S19 are reproduced in Table S9.

Table S9. Optimized model parameters k_{ij} for each of the modeled QAs.

	k_{CP} (1/d)	k_{PC} (1/d)	k_{CU} (1/d)	k_{UC} (1/d)	k_{CE} (1/d)
Hydroxylupanine	$5.40*10^{-3}$	$2.00*10^{-1}$	$6.57*10^{-2}$	1.69	2.41
Lupanine	$4.87*10^{-3}$	$2.28*10^{-1}$	$2.24*10^{-1}$	6.25	2.61
Isolupanine	$1.44*10^{-2}$	$2.81*10^{-1}$	$2.87*10^{-1}$	6.12	2.67
Angustifoline	$4.65*10^{-3}$	$1.34*10^{-1}$	$1.05*10^{-1}$	6.59	2.59

Semilogarithmic plot to show biphasic behavior during depuration

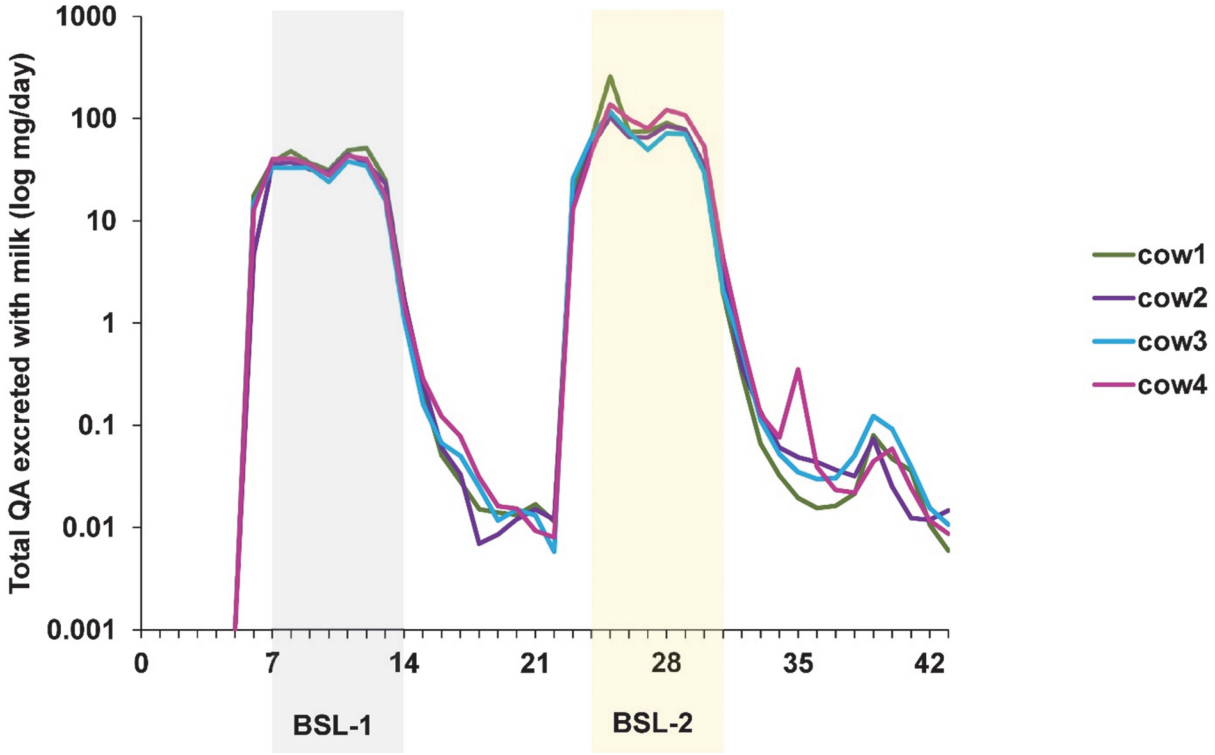

Figure S11. Logarithmic plot of total QA excreted with milk daily in mg/d. The depuration periods following BSL–1 (blue sweet lupine 1 kg/d) and BSL–2 (blue sweet lupine 2 kg/d) show a biphasic behavior: an initial fast α-phase and a later slow β-phase.

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Chapter IV

Detection of hypoglycin A and MCPrG metabolites in milk and urine of pasture dairy cows after intake of sycamore seedlings

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Abstract

Hypoglycin A (HGA), methylenecyclopropylglycine (MCPrG), hypoglycin B (HGB) and γ-glutamyl-α-(methylenecyclopropyl) glycine (γ-glutamyl-MCPrG) are secondary plant metabolites occurring in sycamore maple (*Acer pseudoplatanus*) as well as several other *Sapindaceae* (e.g*., Blighia sapida)*. By interfering with energy metabolism they may cause severe intoxication in humans and other species. However, to date, there is not enough data available concerning intake, metabolism or excretion of sycamore maple toxins in dairy cows. In May 2022, five cows were observed over four days, when they had first access to a pasture with two sycamore maples. Grazing of their seedlings that grew numerously in between the pasture plants was monitored by direct observation. Milk samples were drawn both from individual cows and from the bulk tank. Spontaneous urine samples were collected from all cows on day 3 after access to the pasture. Seedlings (100 g) were sampled on the pasture and analyzed, together with milk and urine samples, for sycamore toxins and their metabolites using LC/MS-MS and LC-HR-MS. Cows ingested sycamore seedlings while grazing. Values of HGA in milk were below the limit of quantification. However, metabolites of HGA and MCPrG were detected in individual milk samples already at the end of the first day of grazing. Urine samples of all 5 cows showed higher concentrations of conjugated HGA and MCPrG metabolites than in milk. Observations suggest that dairy cows may have a low susceptibility towards sycamore maple toxins. However, whether this could be attributed to foregut fermenting species in general, requires further elucidation.

Keywords: secondary plant metabolites, food safety, ruminants, observational, milk, LC/MS-MS, plant toxin, poisoning

Introduction

Exposure to the non-proteinogenic amino acids hypoglycin A (HGA) and methylenecyclpropylglycine (MCPrG) can lead to severe intoxication in several species, including humans[1, 2], horses[3, 4], deer[5, 6], gnus[7] and camels[8]. As secondary plant constituents of the soap tree family (*Sapindaceae*), these substances have been found in fruits of litchi (*Litchi chinensis*)[9], akee (*Bligha sapida*)[1, 10, 11] and various maple trees including sycamore maple (*Acer pseudoplatanus*).[3, 12-14] Ingestion of seedlings and seeds of sycamore maple trees is known to cause poisoning in grazing horses resulting in the so-called atypical pasture myopathy (AM) characterized by muscle stiffness, myoglobinuria, frequently found hyperglycaemia and mortalities.[4, 15, 16] Contrary to horses, studies conducted in humans or laboratory animals reveal hypoglycaemia following the ingestion of maple toxins.[2, 17-19]

Additionally, hypoglycin B (HGB) and γ-glutamyl-MCPrG haven been detected in seeds[13] and seedlings[20] of sycamore maple trees. However, data in rabbits, monkey or rats indicate a lower

hypoglycaemic activity of HGB compared to HGA.[21] Noteworthy, HGB and γ-glutamyl-MCPrG are barely addressed in scientific publications dealing with sycamore intoxications in farm animals, albeit their co-occurrence is relatively likely.

Regarding the mode of action of HGA and MCPrG it is known, that metabolites of both compounds disrupt fatty acid metabolism and, thus, cause an interference with energy metabolism.[22-25] Following the formation of the intermediates methylenecyclopropylpyruvate and methylenecyclopropylglyoxalate from HGA and MCPrG, respectively, and conversion to methylenecyclopropylacetate (MCPA) or methylencyclopropylformiate (MCPF), conjugation with coenzyme A (CoA) occurs, leading to the formation of the metabolically active forms methylencyclopropylacetyl – CoA (MCPA-CoA) and methylencyclopropylformyl – CoA (MCPF-CoA) for HGA and MCPrG, respectively.[26] These compounds are potent inhibitors of acyl-CoA dehydrogenases and enoyl-CoA hydratases enzymes involved in ß-oxidation - resulting in excretion of incomplete degradation products of fatty acids in urine and an altered acylcarnitine profile in blood.[26-28] The metabolites MCPA and MCPF are conjugated with carnitine or glycine and may be excreted via the renal system. The presence of these metabolites in urine and blood of affected horses and humans is considered a biomarker of exposure to HGA and MCPrG.[4, 9]

Recently, the detection of HGA traces in duplicate samples from one bulk milk tank of a dairy farm raised concerns that ingestion of sycamore seeds by dairy cows may pose a risk for animals and consumers.[29] However, to our knowledge there is neither a direct proof that dairy cows ingest sycamore seedlings nor are there data on metabolism and excretion of maple toxins by dairy cows available. For ruminants in general, toxic effects caused by HGA/MCPrG have only been demonstrated in the browsing Père David's deer, gnus, and Bactrian camels[5-8] while there are no known cases of poisoning in grazers like sheep or intermediate types like goats.[30, 31] Gonzales-Medina et al. [32] detected HGA in serum samples from ewes and their lambs and also MCPA conjugates in one ewe at 0, 2 and 7 days after grazing on a pasture with sycamore seedlings. No animal in this case showed any adverse effects suggesting a low (or even no) sensitivity for the toxins in sheep. This observation supports the assumptions of differences in toxicological susceptibility between various ruminant species. Additionally, the detection of HGA traces in the serum of lambs of the above mentioned ewes, suggests that the compound may also be transferred into the milk of ewes as it has already been reported for mare's milk.[33] Physiological and gastrointestinal peculiarities in ruminants could result in low sensitivity to the toxic effects of certain plant toxins.[34] For example, due to the large size of the rumen as well as the long retention time, ruminal transformation of HGA might occur before entering the proximal small intestine as the site for HGA absorption.[7, 35] First insights into the effects of the rumen microbiome on detoxification, though, showed no significant

decrease in HGA concentrations but rather an increase of yet unknown cause over a short period of 2 hours of *in vitro* incubation in ruminal fluid of adult sheep.[32]

Therefore, the purpose of this study was to investigate whether (1) dairy cows voluntarily ingest sycamore seedlings, and if so, (2) ingestion would result in excretion of HGA, MCPrG, HGB, γ-glutamyl-MCPrG or metabolites in milk or urine or (3) cows develop certain clinical signs such as those described in AM horses.

Materials and Methods

Ethics statement

All procedures were in accordance with national and international guidelines for animal welfare. Owners gave informed consent for their cows' inclusion in the study and were asked if they agreed to systematic observation of the animals on their habitual pasture. Cows were physically evaluated by a veterinarian daily. Collection of milk and urine samples followed routine milk performance checks and on the basis of routine veterinary diagnostics by a veterinarian. Owners agreed to maple toxin analysis in samples. The study was permitted by the institute's animal welfare officer. Therefore, approval was not required for this observational study as treatments were not applied to animals as confirmed by the animal welfare officer of the German Federal Institute for Risk Assessment.

Animals and video recording of cows

In May 2022, five (Holstein-Frisian × Jersey × Norwegian Red) cows (2 primiparous, 3 multiparous, Cow 1 – Cow 5(C1-C5)), approximately 207 ± 7 days in milk, with an average milk yield of 21.3 ± 6 kg, out of a herd of 87 cows, were kept on a pasture (1800 m^2) with two sycamore maple trees and numerous seedlings growing among the grass. This was the first time in 2022 that the cows were allowed to graze on that pasture. Therefore, an earlier contact with sycamore seedlings from that pasture was unlikely. The pasture was exclusively available for the five cows from 11 a.m. to 3 p.m. over four consecutive days, while the remaining 82 cows were grazed on a neighboring pasture with visual contact. Cows had *ad libitum* access to water, which is delivered by a groundwater pump, and received a partial mixed ration (71% grass silage, 10.1% maize silage, 10.1 % beet pulp, 1.3% wheat, 1.3% grain maize, 0.8% straw, 0.5% protein press cake, 0.15% minerals, 0.15% bicarbonate, 0.1% fermented cereals) *ad libitum* on the feed ally in the barn apart from grazing times. Additionally, a concentrate mixture was provided in the barn in a separate feeding trough, transponder-controlled for each cow, to meet the energy requirements for individual milk yields. During the time on pasture animals were continuously observed by two independent observers. If spotted, uptake of seedlings was documented by video recording (2 × SONY Handycam HDR-CX240, Apple iPhone 8).

Collection of seedling and vegetation samples

Samples of *Acer pseudoplatanus* seedlings were collected on the pasture located in North Rhine-Westphalia, Germany, on the last day (day 4) of the trial. Seedlings of both two-leaf as well as four-leaf stage were sampled representatively in the open area directly under the trees as well as among the grasses (Figure 1).

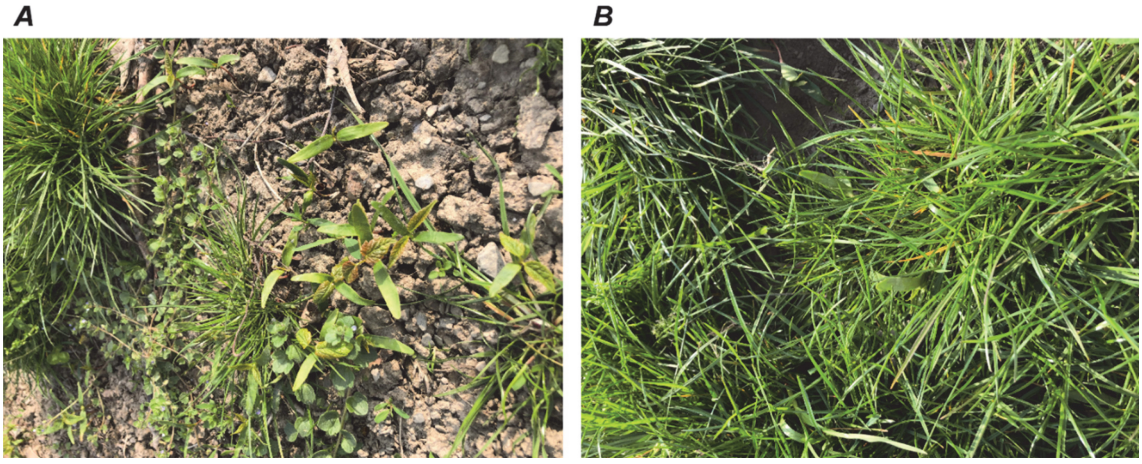

Figure 1. Sycamore maple seedlings with cotyledons and the first pair of leaves on the pasture. A: Open area, B: Among grasses.

Altogether, 100 g of seedlings were air-dried at room temperature, homogenized (approx. 500 µm) using knife/ball mills (Retsch, Haan, Germany) and stored under dry conditions at room temperature for subsequent sycamore maple toxin analysis. Additionally, 500 g of seedlings and remaining vegetation, respectively, were collected and stored at –20 °C until nutrient and chemical analysis.

Milk sampling

Cows were milked twice daily at 6:30 a.m. and 6:30 p.m. Milk samples were obtained uniformly to represent milk composition from milk letdown to emptying following routine milk performance checks with milk meters (TRU-Test datamars, Auckland, New Zealand*)* in a fishbone milking parlor (Westfalia/Gea Germany, Düsseldorf, Germany). Samples were available from all 5 animals from the first morning before their release on the pasture (day 1), the first evening milking after their first release on the pasture (day 1), as well as on day 2 and 3 from morning and evening milking, along with morning milk of day 4. Therefore, milk from day 1 of the study that was milked in the morning, served as T0, as cows had no access to the pasture beforehand. Additionally, the tank milk of the entire herd (n=87) was sampled in the morning and evening. Samples were stored at -20 °C and analyzed for HGA, MCPrG and their respective metabolites.

Botanical survey on experimental plots

Before the onset of grazing seven 50×50 cm plots were established that represented density and species composition of the pasture vegetation. Four plots contained sycamore seedlings, while the remaining three plots served as control with no seedlings. Plots with seedlings represented areas with numerous seedlings (n=33/m^2) as well as areas with few seedlings (n=4/m^2). Dominating plants were identified as *Taraxacum officinale*, *Trifolium repens* as well as various Pocaceae. Daily at 10:30 am, plots were photographed (Nikon D90, Apple iPhone 8). Seedlings were counted before cows were allowed to graze.

The proportions of the grazed area were determined for each plot. “Grazed” in the context of this study is defined as visible signs of missing, *i.e.* ingested plant parts. Other traces of activities on the grass as footprints or damage by cows resting on the ground, were ignored. For estimation of the grazed parts of the plots (50×50 cm) they were divided into 10×10 cm sub-plots. In each sub-plot, remaining vegetation, expressed as percentage (%) of original 100%-coverage as well as remaining seedlings were checked from day 1 to day 4 in order to examine whether seedlings were eaten by the cows unintentionally along with grasses. To obtain the extent of ingestion of feed plants on the total area, *i.e.*, the utilization of vegetation by the animals, the remaining amount of untouched plants on day 2 to day 4 was compared to the original amount on day 1 (100%).

Proximate analyses of seedlings and grassland growth

Biomass of both seedlings and remaining vegetation were analyzed for dry matter (DM), crude ash, crude protein, crude fat and crude fiber according to VDLUFA (Association of German Agricultural Analytic and Research Institutes) standard methods.[36]

Toxin analysis in seedlings

(S)-Hypoglycin A (HGA, purity 85%), HGB (γ-glutamyl-hypoglycin,, 98%), MCPrG (, 97%) and (MCPA)-C (97%) standards were purchased from Toronto Research Chemicals (Toronto, Canada). MCPA-G (97%) and MCPF-G (97%) standards were purchased from IsoSciences (Ambler, PA, USA).

Seedlings were analyzed according to El-Khatib et al.[20] Additionally, qualitative detection of HGB was achieved by comparison of retention times and spectra in samples with HGB reference substance. The reference substance was not available at the time of method development and validation. Thus, quantification of HGB in seedlings was not possible at the time of analysis and only a qualitative detection was carried out once the substance was available. All solvents used in this study were at least analytical grade. Solvents used for liquid chromatography–tandem mass spectrometry (LC–MS/MS) and high resolution-tandem mass spectrometry (HR-MS/MS) analysis were of LC–MS grade.

Briefly, 5 mL of deionized water were added to 0.5 g of homogenized plant material (plants with roots), the mixture was pre-vortexed and placed in the ultrasonic bath for 10 minutes at room temperature (Sonorex Super, Bendelin, Berlin, Germany). The samples were centrifuged for 10 minutes at 4000 rpm (Heraeus Megafuge 16, Thermo Fisher Scientific, Waltham, USA). Afterwards, the supernatant was filtered (Ahlstrom Folded filters, NeoLab Heidelberg, Germany) and transferred to a new 15 mL tube. The residue was extracted again with 5 mL deionized water, centrifuged, filtered and combined with the first extract. The samples were then measured (undiluted or diluted 1 in 25 with 5% methanol/water) by LC-MS/MS (Q-Trap 6500+, AB Sciex Germany GmbH, Darmstadt, Germany) or LC-HR-MS (QExactive Focus, Thermo Fisher, Dreieich, Germany).

Toxin analysis in milk

Milk samples were analyzed according to El-Khatib et al. [37] Briefly, 10 mL of milk samples were mixed with 10 mL 1% formic acid in methanol (v/v). Additionally, 100 µL formic acid and 1 mL of EDTA solution were added. After shaking the samples in an overhead shaker for 20 minutes, they were refrigerated at -20°C for 2 hours. Afterwards, samples were centrifuged at 4000 rpm and 4 °C for 10 minutes. The supernatant of the sample was then transferred to a tube containing 0.1 g of C18 material (Polygoprep 300-30C_{18}, Macherey–Nagel, Düren, Germany) and 2 mL of acetonitrile (ACN) and shaken in an overhead shaker for 15 minutes. Subsequently, samples were centrifuged at 4000 rpm and 20 °C for 10 minutes. In case of MCPF-Carnitine, the unequivocal confirmation and quantification was not possible due to the lack of a reference standard. However, there is sufficient evidence from mass spectrometric data that MCPF-Carnitine was present in samples. Within a mass tolerance of 3 ppm, the accurate mass of MCPF-Carnitine was detected by HR-MS. In addition, 3 probable mass transitions for MCPF-Carnitine have been monitored and showed the same retention time. The retention time of the tentative MCPF-Carnitine signal (3.37 min) lies within the predicted range considering the elution profiles of MCPF-Glycine (3.47), MCPA-Glycine (3.83) and MCPA-Carnitine (3.66). Thus, a qualitative approach was used. To this end, the chromatographic peak areas of tentatively identified MCPF-Carnitine were compared to investigate if any trends in levels can be seen in the samples.

Urine sampling and sycamore maple toxin analysis in urine

Urine samples were obtained from all 5 cows on the basis of routine veterinary diagnostics upon spontaneous micturition on day 3 at 4.30 pm and afterwards analyzed for their levels of HGA, MCPrG and their respective metabolites by applying the method of El-Khatib et al.[37] Briefly, the urinary creatinine concentration was measured at an accredited medical analytics laboratory (Labor 28 GmbH, Berlin, Germany). The urine samples were subsequently diluted with 5% MeOH to a creatinine concentration of 0.1 mg/dL and then analyzed by LC-MS/MS.

Urine samples of dairy cows kept and taken care of at the research farm of the German Federal Insitute for Risk Assessment (BfR) served as controls.

Statistical analysis

Statistical analysis was carried out using R Version 4.2.1 (2022-06-23)[38] package MCMCglmm.[39] A linear mixed effect model was perfomed using MCMCglmm(MCPA ~ day + time, random=~cow) as code, where time represents the time point (morning or evening) at which samples were taken. Cows were included as random effects in order to take into account that the animals were repeatedly sampled. Significance was assumed, if *p*-values were below 0.05.

Results and Discussion

Here we report, for the first time, the ingestion of sycamore seedlings with measurable concentrations of HGA and MCPrG by dairy cows during grazing on pasture. We detected metabolites of these substances in urine as well as in milk samples. The cows did not show clinical signs such as visible manifestations of illness or discomfort. Nevertheless, possible subclinical changes in acylcarnitine profiles in urine or blood cannot be conclusively excluded.

Seedling intake and nutritional values

Already during the first day of the experiment, an ingestion of sycamore seedlings by the cows was observed visually and also captured on video (Figure 2).

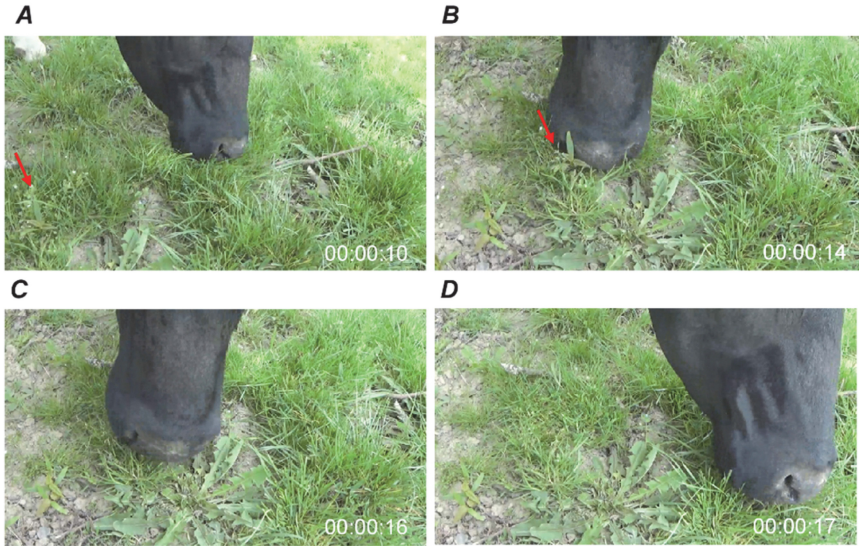

Figure 2. Ingestion of Acer pseudoplatanus seedling by study cow. A: Seedling appears in the picture while the cow is grazing. B: Cow touches the seedling with the mouth. C: Mouth of the cow is located above the seedling. The seedling is ingested. D: The seedling is no longer visible.

In addition, over the 4-day period, number of seedlings decreased in all experimental plots containing seedlings (plot 1 – plot 4), while the relative amount of ingested plant parts of the remaining vegetation increased (plot 1- plot 7) (Figure 3).

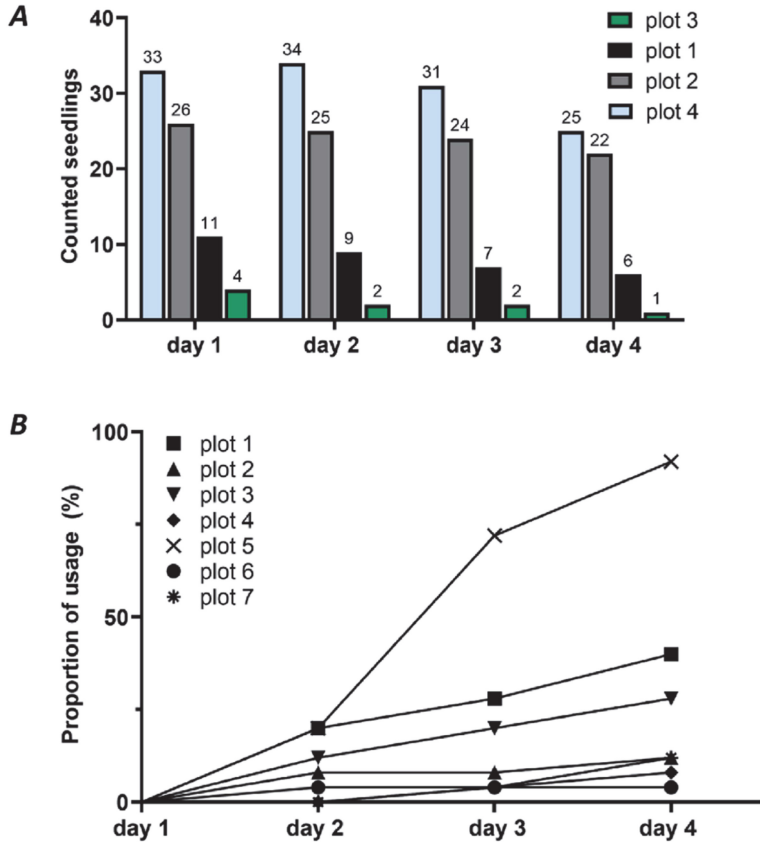

Figure 3. Overview of experimental plots. A: Counted seedlings (*Acer pseudoplatanus*) per plot (50x50cm) per day. Plots were checked and photographed daily for remaining seedlings before moving animals to pasture. B: Proportion of usage of experimental plot 1 to 7.

There was no difference in the number of missing seedlings of two- and four leaves stage indicating that cows, contrary to horses, may not discriminate between seedlings of two- and four-leaves stage.[40] Since the seedlings were not selected but always eaten along grasses and forbs, we observed their ingestion as by-product of grazing. Ghislain et al.[41] reported, that 78-86% of seedlings disappear naturally on a pasture within three to four weeks. Nevertheless, as there is not only direct proof of consumption on field level by various distinct field methods, but also on chemical level as metabolites of HGA and MCPrG are further detected in urine and milk samples, the decrease in seedlings in experimental plots likely goes back to ingestion by the cows.

Seedlings contained on average 2.6 ± 0.05 g HGA/kg DM and 0.2 ± 0.002 g MCPrG/kg DM (Table 1).

Table 1. Composition of sycamore maple seedlings and pasture grass.

		Seedlings	Pasture grass
		g/kg	g/kg
Dry matter		285	273
	HGA[a]	2.6	
	MCPrG[b]	0.2	
	HGB[c]†	present	
Dry matter	Crude protein	11.6	12.3
	Crude ash	29.9*	7.6
	Crude fiber	6.2	10
	Ether extract	1.6	2.5

[a]Hypoglycin A (HGA)
[b]Methylenecyclopropylglycine (MCPrG)
[c]Hypoglycin B (HGB)
*Data can only be assessed to a limited extent, as contamination with soil is possible due to the root content.
† Qualitative detection

Comparable contents of HGA in sycamore seedlings, 2.1-3.4 and 0.3-2.7 g/kg, have been reported by Baise et al. and Gonzales Medina et al., respectively.[14, 42]

Hypoglycin B was detected in all plant samples. Crude fiber contents in seedlings (21.6 g/kg FW) were lower than in the rest of the herbaceaous vegetation (36.7 g/kg FW) (Table 1). Nevertheless, crude fiber contents in seedlings were in agreement with contents for seedlings previously reported by Aboling et al.[40] Fiber content of the herbaceaous vegetation, in Table 1, was expected as sampling was done on the onset of the grazing season with low crude fiber content and increased crude protein content before shooting.[43] The seedlings in this study showed lower fat and protein content compared to the pasture grass as already described in the literature.[28]

Several reports have shown that herbivores may select their diets either due to nutritional needs or as a strategy to reduce toxin intake.[44, 45]

Here, there were no major differences at least between the nutritional value of the seedlings and that of the remaining grass. Since cows were able to meet their energy and nutrient needs through the partial mixed ration and the addition of concentrates offered in the barn, increased intake of either seedlings or grass caused by reduced availability of nutritious feed is not likely in the present study.

Overall, the selective grazing behavior of cows, due to genetic selection for high yielding patterns, is no longer as distinct as in non-domesticated herbivores and is predominantly seen in heterogeneous areas[46, 47], leading to the assumption that cows in this study could simply not discriminate between seedlings and grass. Freeland and Janzen[48] reported that cows may include a variety of plant species in their diet up to high everness so that secondary plant components derived from a single plant variety enter the body below harmful levels.

Referring to studies reporting that Père David's deer from two Zoo's in Germany picked up either seedlings and seeds [5] or actively ingested leaves and seeds of sycamore maple trees[6], there is no information on the nutrient supply of the diseased Père David's deer. Even if species-specific requirements are met, active submission of the plant may result in increased uptake due to inevitably overgrazed areas, boredom of animals or attractiveness of presented plant parts. In addition, there is no information on whether the animals could have avoided harmful levels of secondary compounds due to complementary feed intake as described by Freeland and Janzen.[33] This is in contrast to the study conducted here as well as to the study conducted in ewes and their lambs by Gonzales Medina et al.[32] Nevertheless, since clinical cases rarely occur in enclosures, it must be assumed that the susceptibility of the individual animal species still varies.

Our results prove the hypothesis that cows ingest maple seedlings with high levels of maple toxins when seedlings are present on the pasture. It is however not clear whether animals could simply not discriminate seedlings and pasture plants by taste or smell or if there is indeed an avoidance strategy up to a certain tolerance level by taking up a variety of grasses and plants as postulated by Freeland and Janzen.[48]

Observations related to effects on animal health

After ingestion of the seedlings, the studied cows showed neither visible signs of illness or discomfort nor decline in milk yield throughout the observation period and thereafter, as we were informed by the owners. Nevertheless, based on the available data, subclinical changes in the organism of the animals cannot be excluded. Subsequent studies should therefore examine the defined intake of toxins, the course of concentrations of toxins in the blood, and clinical parameters indicative of a subclinical disturbance of metabolism.

The outcome is contrary to that in Père David`s deer in two Zoo´s in Germany as well as gnus in a Zoo in France which developed clinical signs with a rapid progression comparable to those also observed in horses.[5-7] Several publications report that already relatively small amounts of maple toxins may be sufficient to poison equids.[4, 14, 27] Maple toxin poisoning in horses results in muscular weakness and stiffness following respiratory depression and recumbency leading to death within 72 hours.[15] Complementary myoglobinuria is also a common clinical sign in horses and was also seen in poisoned deer with fatal course.[6]

On the other hand, similar to the findings in the present study with cows, no clinical signs of poisoning were observed in studies with pastured ewes and their lambs as well as with goats exposed to sycamore seedlings. This may suggest that there might be differences in the susceptibility to toxic effects of HGA and MCPrG in some ruminant species as compared to horses.[7, 32] The susceptibility to maple toxins of species beyond horses has not yet been evaluated systematically.

After ingestion and further metabolization of HGA and MCPrG as mentioned before, MCPA-CoA causes toxicity by inhibiting acyl-CoA dehydrogenases, isovaleryl-CoA dehydrogenases and 2-methyl-branched chain acyl-CoA dehydrogenases and thus blocking the first step of ß-oxidation. MCPF-CoA inhibits enoyl-CoA hydratases in mitochondria and peroxisomes. Therefore, it has been hypothesized, that both amino acids simultaneously strengthen the inhibition of ß-oxidation leading to disruption of energy metabolism.[6] As a result of the disturbances in ß-oxidation, acyl residues that cannot be broken down further will be excreted, among others, via urine. However, MCPA and MCPF may also be further metabolized by conjugation with glycine or carnitine and excreted with urine.[9] Therefore, the occurrence of metabolites in serum and urine has been used to confirm diagnosis of AM in horses.[4, 49]

In this study, neither HGA nor MCPrG could be detected in the urine samples. Individual levels of MCPA-Glycine (15160 to 66228 nmol/mmol creatinine) and MCPF-Glycine (561 to 1705 nmol/mmol creatinine) detected in urine samples of all five cows on day 3 are present in Table 2. No carnitine adducts were found in the urine.

Table 2. The concentration of MCPrG, HGA and their metabolites in urine samples of study cows (C1-C5) on day 3.

item	urine (nmol/mmol creatinine)				
	C1	C2	C3	C4	C5
HGA[a]	-	-	-	-	-
MCPA-G[b]	15160	66228	15192	45091	53623
MCPA-C[c]	-	-	-	-	-
MCPrG[d]	-	-	-	-	-
MCPF-G[e]	947	561	1391	1065	1705

[a]Hypoglycin A (HGA) (LOD and LOQ are 166 and 546 nmol/mmol creatinine, respectively)
[b]Methylenecyclopropylacetyl-Glycine (MCPA-G) (LOD and LOQ are 160 and 527 nmol/mmol creatinine, respectively)
[c]Methylenecyclopropylacetyl-Carnitine (MCPA-C) (LOD and LOQ are 70 and 232 nmol/mmol creatinine, respectively)
[d]Methylenecyclopropylglycine (MCPrG) (LOD and LOQ are 295 and 974 nmol/mmol creatinine, respectively)
[e]Methylenecyclopropylformyl-Glycine (MCPF-G) (LOD and LOQ are 92 and 303 nmol/mmol creatinine, respectively)
-, not detected

The level of MCPA-Glycine was consistently higher than that of MCPF-Glycine. This corresponds with the higher concentrations of HGA compared to MCPrG in the seedlings. However, the ratio between MCPA-Glycine and MCPF-Glycine was not consistent between the cows and ranged between approximately 11 and 118.

The concentrations of MCPA-Glycine as a metabolite of HGA in this study (Table 2) were higher than the levels that have been observed in poisoned deer (4600 und 16800 nmol/mmol creatinine), whereas values of MCPF-Glycine (Table 2), as a metabolite of MCPrG, were lower in cows than in deer (1800 und 7500 nmol/mmol creatinine) even though higher contents of MCPrG were detected in the seedlings of the present study (200 mg/kg) in contrast to the contents in seeds (42.9 mg/g) and leaves (0.1 mg/g) ingested by the deer.[6] MCPA-Glycine levels found in urine of poisoned horses (280 - 1970 nmol/mmol creatinine) were lower than concentrations in urine of cows in the present study.[4]

Contrary to the findings in deer and horses, HGA, MCPA-Carnitine and MCPF-Carnitine were not detected in urine samples of our study, which could indicate differences in absorption and/or metabolism. The fact that neither HGA nor MCPrG was detected in urine samples in this study strongly suggests that there was a rapid and complete modification in cattle contrary to the findings in deer. It has been hypothesized that the development of clinical signs of poisoning in horses and Père David's deer may be more related to the amount of MCPrG

ingested rather than with HGA due to high concentrations of MCPrG metabolites in urine samples and increasing toxic effects of AM in horses and deers.[6] However, the toxicological relevance and role of individual maple toxins and metabolites is still not clarified.

There are different hypotheses that may explain the varying susceptibility of cattle and other species to plant toxins. Due to intense ruminal fermentation by a complex microbiome transformation of toxins into various metabolites before absorption into the blood might occur. HGA and MCPrG represent, as amino acids, hydrophilic and soluble substances that can already be utilized by microbes in the rumen. Additionally, due to a high ruminal retention time in large ruminants, it is therefore reasonable to assume that HGA and MCPrG probably do not reach the site of their absorption, the proximal small intestine, in sufficiently high concentrations.[7, 30, 50] A shorter retention time, as suspected in camelids and sheep, therefore might result in HGA absorption depending on feed availability or exposure to the toxins as proved by the detection of HGA in serum of sheep and goats.[7, 32] In contrast, however, the toxins could have a short retention time in the rumen due to their hydrophilicity and a following quick transition to the liquid phase if they are quickly released from the plant matrix.

Still, *in vitro* incubation of HGA in ruminal fluid for 2h intended to test the microbial influence on the fate of toxins showed no degradation but rather a significant increase in concentrations.[32] Because of longer *in vivo* retention times in the rumen[51], investigations with long incubation-periods are necessary to fully understand and evaluate the impact of rumen microbes on the fate of maple toxins, which has not been considered in the past. The results of this study may indicate that partial conversion of protoxins to their active forms already occurs in the rumen or post-absorptive resulting in high concentrations on MCPA-G.

Findings of HGA in bulk tank milk samples from a farm in northern Germany led to the assumption that absorption of HGA may also occur in large ruminants [29] depending on the extent of transformation of toxins in the foregut system. However, the detection of HGA in cows' milk has not yet been confirmed by other studies.

Renaud et al. [7] observed recently that some of the exposed co-grazing animals may have low concentrations of MCPA-Carnitine or MCPA-Glycine in serum or urine but do not show any clinical signs of poisoning. This observation of subclinical poisoning cases is in agreement with findings of Bochnia et al.[4]. Compared with diseased animals subclinically poisoned cases had lower levels of free carnitine and acylcarnitines in serum.[7, 27]

Although cows in the current study did not show any clinical signs, the influence of maple toxin intake on the energy metabolism of cows should be examined more closely, e.g. by investigating fatty acid metabolism alterations and free fatty acids in urine and serum.

By adapting to high performance patterns regarding glucose metabolism, cattle developed a high efficiency in energy utilization in contrast to other ruminant species.[52] It may be

hypothesized that the low susceptibility of cows to the toxic effects of HGA and MCPrG may result from increased gluconeogenesis in dairy cows.[52, 53]

Milk samples

An initial objective of the study was to identify whether there is a transfer of sycamore maple toxins into milk of dairy cows after ingestion of the seedlings. Here we describe for the first time the occurence of HGA and MCPrG metabolites in milk of dairy cows, demonstrating that at least metabolites may be transferred into the milk following the ingestion of HGA-/MCPrG-containing plant material.

The levels of HGA, MCPrG and their respective metabolites in individual milk samples are depicted in table 3.

Table 3. The concentration (µg/L) of HGA and the metabolites MCPA-G/MCPA-C, MCPF-G and peak areas of MCPF-C in individual milk samples of cows (C1-C5).

		HGA[a] µg/L					MCPA-G[b] µg/L					MCPA-C[c] µg/L					MCPF-G[d] µg/L					MCPF-C[e] Peak area †				
day	**time**	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5
1	**am**	-	-	-	-	-	3.7	-	<LOQ	-	-	<LOQ	-	-	-	-	-	-	-	-	-	++	+	+	+	+
	pm	-	-	-	-	-	8.6	20	12	8.2	18	2.1	9.2	1.9	-	6.8	-	-	-	-	-	++	++	++	++	+++
2	**am**	-	-	-	-	-	1.4	5.2	2.1	<LOQ	2.5	-	<LOQ	-	-	<LOQ	<LOQ	<LOQ	-	-	-	++	++	++	++	+++
	pm	-	-	-	-	-	39	44	35	14	44	11	7.9	3.0	<LOQ	14	-	<LOQ	-	-	-	++	+++	++	++	+++
3	**am**	-	<LOQ	-	-	-	9.5	15	4.7	2.9	8.8	1.2	0.78	<LOQ	-	2.4	1.2	1.4	<LOQ	<LOQ	<LOQ	+++	+++	++	++	+++
	pm	-	-	-	-	-	34	99	50	74	51	2.9	11	2.5	4.1	7.9	<LOQ	-	-	-	-	+++	+++	+++	+++	++++
4	**am**	-	<LOQ	-	-	-	4.3	17	6.0	7.2	8.3	-	<LOQ	-	-	1.3	<LOQ	1.6	<LOQ	<LOQ	<LOQ	+++	+++	+++	+++	++++

[a]Hypoglycin A (HGA) LOQ 1.12 µg/L; [b]Methylenecyclopropylacetyl-Glycine (MCPA-G) LOQ 0.99 µg/L; [c]Methylenecyclopropylacetyl-Carnitine (MCPA-C) LOQ 0.75 µg/L; [d]Methylenecyclopropylformyl-Clycine (MCFP-G) LOQ 1.09 µg/L, [e]Methylenecyclopropylformyl-Carnitine (MCPF-C).

<LOQ: Values below LOQ but above LOD (Hypoglycin A (HGA) LOD 0.34 µg/L, Methylenecyclopropylacetyl-Glycine (MCPA-G) LOD 0.30 µg/L, Methylenecyclopropylacetyl-Carnitine (MCPA-C) LOD 0.23 µg/L, Methylenecyclopropylformyl-Glycine (MCFP-G) LOD 0.33 µg/L

-, not detected

† Peak areas in milk samples. +, > 1.00E+04; ++, > 1.00E+05; +++, > 5.00E+05; ++++, > 2.00E+06

Values of MCPrG in milk were below the limit of detection (LOD) (LOD = 2.63 µg/L). Likewise values of HGA were either below the LOD (LOD = 0.34 µg/L), or between LOD and the limit of quantification (LOQ) (LOQ = 1.12 µg/L) (henceforward referred to as "< LOQ").

Unexpectedly, traces of MCPA-Glycine have already been detected in the milk of two cows (C1 and C3) before they were moved to the pasture with sycamore seedlings indicating an exposure to maple toxins already beforehand.

Since the cows entered the pasture for the first time of the year, previous grazing on the area with sycamore seedlings can be excluded. Prior studies have noted that HGA may not be completely degraded during storage of hay and silage over a period of 8 months.[42] However, in the present study, the grass used for feed production (e.g. silage or hay), derived from the farm's own land, was harvested from a location far apart from the pasture with sycamore maple trees. Nevertheless, it has to be considered that the feed indeed did contain traces of HGA, e.g. from seedlings stemming from seeds that were transported over a longer distance by the wind or that a contamination of the samples occurred. Another study reported that rain water collected from seedlings may also contain measurable amounts on HGA, nevertheless, the source of the animals' drinking water was groundwater, so water contamination is unlikely in this case.[54]

Already on day 1 of the study, milk samples from the evening milking contained quantifiable amounts of MCPA-Glycine (5/5 cows) and MCPA-Carnitine (4/5) in individual milk samples.

No quantifiable amounts of HGA and MCPrG as well as MCPF-Glycine were found. HGB was not detected in the milk despite its presence in maple seedlings.

Therefore, we hypothesize, that there may be a quick absorption and metabolism of maple toxins in dairy cows after ingestion, followed by a transfer into the milk. This could be related to a direct absorption of the non-proteinogenic amino acids and their metabolites in anterior parts of the digestive tract. There is some evidence that certain amino acids may also be absorbed prior the small intestine in the rumen.[55] Another explanation could be a faster passage rate through the rumen to the small intestine, since the uptake of spring feed may lead to waterier, liquid chyme washing through the rumen avoiding degradation by ruminal microbes.[7] Future studies should therefore elucidate in more detail the possibilities of gastrointestinal absorption of maple toxins.

Despite high contents of HGA in the seedlings, only the associated metabolites could be found in milk samples at low levels. On the following days of the study, MCPA-Glycine was detected in individual milk samples in morning and evening milk of all 5 cows (only C4 morning milk of day 2 was < LOQ) while MCPA-Carnitine was detected in morning (2/5, both < LOQ) and evening (5/5, C4 < LOQ) milk of day 2, morning (4/5, C3 < LOQ) and evening (5/5) milk of day 3 and morning milk (2/5, C2 < LOQ) of day 4 in individual milk samples. Values measured in

evening milk where higher than in morning milk for MCPA-Glycine and MCPA-Carnitine. In case of MCPA-Glycine, concentrations in evening milk were on average 12.5-fold higher (36.45 µg/L) compared to morning milk ($p < 0.004$). This supports the hypothesis that there is a rapid absorption after maple toxin intake with a subsequent transfer into milk. Since cows did not graze seedlings at night, there were lower contents in morning milk compared to evening milk.

In contrast, MCPF-Glycine was only detected in quantifiable amounts in morning milk of day 3 (2/5) and 4 (1/5), while there were values below LOQ in only 1/5 cows in evening milk (i.e. not detected in 4/5 cows) of day 2 and 3 suggesting differences in MCPrG metabolism or kinetics in comparison with HGA.

Additionally, what stands out in Table 3 is that there is a trend of increasing levels of MCPA-Glycine over the study days. On average, MCPA-Glycine concentrations in milk increased by approximately 9 µg/L per day ($p < 0.004$).

This may indicate that despite a quick metabolism of maple toxins, slight accumulation may have occurred for MCPA-Glycine over the days of the experiment contrary to protoxins HGA and MCPrG. Moreover, a repeated or even increased intake with incomplete elimination of metabolites could lead to increasing levels in milk in the course of the study. Further development of clinical signs cannot be excluded in this case. Nevertheless, supplementary studies are necessary to conclusively assess the kinetics of the individual toxins, especially protoxins, including defined uptake.

Even though the direct estimation of the concentrations of MCPF-Carnitine was not possible due to lack of a reference standard, peak areas could still be used to get an impression on the increase of that metabolite over the course of the experiment. In general, the concentrations of the tentatively identified MCPF-Carnitine seemed to increase in all the subsequent milk samples. In all cows, the maximum MCPF-Carnitine peak areas were in samples of day 3.

The fact that HGA was measured in only two samples below LOQ in cows´ milk is contrary to the findings of Sander et al. [33] in mare milk samples with concentrations of 0.4 µg/L HGA in a milk sample of an AM affected horse as well as 2.4 µg/L HGA in one out of five commercial milk samples. On the other hand, elimination patterns of metabolites of the former study by Sander et al., who detected 18.5 µg/L MCPA-Glycine, 24.6 µg/L MCPA-Carnitine, 0.8 µg/L MCPF-Glycine and 60 µg/L MCPF-Carnitine in a milk sample of an AM affected mare as well as 1.3 µg/L MCPrG, 0.4 µg/L MCPA-Carnitine and 2.7 µg/L MCPF-Glycine in one out of five commercial mares` mik samples, available in store, are in line with our results.

It is interesting to note, that values of HGA were below LOQ of 1.1 ug/L in all milk samples contrary to the findings of Bochnia et al. 2021 (17 and 69 ug/L). In our study, HGA was detected above LOD but below LOQ in two samples from only one cow with relatively high contents on detected metabolites compared to the other cows suggesting that concentrations of HGA in

milk may be related to exposure depending on seedling intake or diet preferences of individual cows. However, the small sample size limits such hypothesis that might be verified by long-term research in the future.

The samples from the bulk tank in our study contained only MCPA-Glycine and traces of MCPA-Carnitine (Table 4).

Table 4. The concentrations of HGA, MCPrG and respective metabolites in bulk milk tank samples of cows (herd of 87 cows, 5 cows included in the study). Dotted lines show the time of emptying of the bulk tank.

		HGA[a] (µg/L)	MCPA-G[b] (µg/L)	MCPA-C[c] (µg/L)	MCPrG[d] (µg/L)	MCPF-G[e] (µg/L)
day	**time**					
1	**pm**	-	5.2	< LOQ	-	-
2	**am**	-	3.0	-	-	-
	pm	-	4.7	< LOQ	-	-
3	**am**	-	3.9	-	-	-
	pm	-	5.0	-	-	-
4	**am**	-	2.8	-	-	-

[a]Hypoglycin A (HGA)
[b]Methylenecyclopropylacetyl-Glycine (MCPA-G)
[c]Methylenecyclopropylacetyl-Carnitine (MCPA-C)
[d]Methylenecyclopropylglycine (MCPrG)
[e]Methylenecyclopropylformyl-Glycine (MCPF-G)
-, not detected
< LOQ: Values below LOQ but above LOD (Methylenecyclopropylacetyl-Glycine (MCPA-G) LOD 0.30 µg/L, Methylenecyclopropylacetyl-carnitine (MCPA-C) LOD 0.23 µg/L.

Even though the rest of the herd did not have access to the pasture with maple seedlings, low levels of MCPA-Glycine were detected in the bulk tank milk on all days of the experiment in the morning and in the evening with lower levels in morning than in evening milk agreeing with the findings in individual milk samples. However, in contrast to individual milk samples from study cows, MCPA-Carnitine in bulk tank milk samples was measured on day 1 and 2 in the evening above LOD but below LOQ. Of note, the bulk tank was emptied only every second days.

Still, it is important to emphasize here that the inclusion of 5 study cows was sufficient to achieve measurable concentrations of conjugated metabolites in the total tank milk that included milk of 87 lactating cows.

The conjugated metabolites should be considered as biomarkers of exposure to HGA and MCPrG. However, to date, there is no data available whether the metabolites MCPA-Glycin/Carnitine and MCPF-Glycin/Carnitine themselves have any toxicological relevance.
The observations proved that dairy cows in this study did not avoid maple seedlings during grazing. After ingestion of sycamore seedlings, metabolites appeared in milk samples of the five study cows as well as in tank milk of the whole herd in less than 12 hours. Metabolites were also detectable in the urine of cows. These findings were accompanied by the absence of any clinical signs or discomfort in the animals. Therefore, it seems that cows quickly metabolized and excreted HGA and MCPrG and have a low or completely missing susceptibility to HGA poisoning as it is also known for small ruminants compared with the highly susceptible horses. Moreover the study provides a basis for following investigations on suspected ruminal transformations. Despite its exploratory nature, this study offers some insight into the transfer behavior of maple toxins into the milk. It was shown that under a typical setting for maple uptake on a pasture the transfer of the toxins HGA and MCPrG themselves into milk may be negligible. However, conjugated metabolites are transferred to the milk in less than 12 hours. To quantitatively evaluate this transfer of maple toxin metabolites into milk, further data are required.

Abbreviations used

AM, Atypical Pasture Myopathy; BfR, German Federal Institute for Risk Assessment; CoA, Coenzym A; DM, dry matter; FW; fresh weight;HGA, Hypoglycin A; HGB, Hypoglycin B; MCPA, Methylenecyclopropylacetyl; MCPrG, Methylenecyclopropylglycine ; NRL, National Reference Laboratory; LC/MS-MS, Liquid Chromatography - Mass Spectrometry; LC-HR-MS, Liquid Chromatography – High Resolution Mass Spectrometry; VDLUFA, Association of German Agricultural Analytic and Research Institutes

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Notes

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Chapter V

Discussion

The global population is set to increase to 9.6 billion by 2050 (UN 2013), thereby triggering a surge in demand for livestock products, which currently accounts for 17% of total kilocalories and 33% of global protein consumed (Rosegrant 2002). In this regard, agriculture encounters significant challenges in the face of climate change, encompassing the competition for water and land and ensuring the security of food availability (Thornton and Gerber 2010). Agriculture is a significant contributor to global greenhouse gas emissions, both via direct methane emissions and indirect emissions from fertilizer use, feed transportation and processing (IPCC 2006) emphasizing the need for a more sustainable future. The global climate report's significant approach is evident in its integration of livestock systems with forestry and crop production (IPCC 2014).

The feeding of imported soy is an increasing problem both economically and ecologically by changing costs and the far-reaching consequences in terms of climate change. Domestic grain legumes as lupins provide a high variability in relation to their growth conditions and have a sufficiently high protein content for milk synthesis (Nanas et al. 2023). In addition, the cultivation of lupins is financially supported by the government to promote the cultivation of domestic grain legumes (BMEL 2020). Therefore, they are a viable substitute for conventional protein sources but also pose a risk due to the presence of their SPM, QA, that could be harmful and may be passed on to the milk along with their otherwise advantageous primary plant compounds as reviewed in **Chapter I**.

Another step towards achieving a more sustainable agriculture is the growing integration of grazing into dairy systems, alongside utilizing agroforestry as a land management technique (O'Mara 2012; Zurek 2022). Rotational grazing, effective stocking rates and not exceeding the carrying capacity of pastureland by maintaining an efficient stocking rate represent valuable measures to increase carbon sequestration (FAO 2009). The incorporation of agroforestry, which entails strategically planting trees in fields and meadows to encourage reforestation, provides benefits in terms of protecting livestock from excessive heat. This prevents a decrease in feed consumption and related impacts on production efficiency in dairy cows while protecting the environment by improving the quality of air, water and biodiversity and helping carbon sequestration as reviewed in **Chapter I** (Jose 2009; Nardone et al. 2010; Smith et al. 2012).

However, these alterations in management and feeding also require ensuring safe food production. Livestock and ruminant-based systems involve inevitable contact between animals and SPM. Therefore, it is essential to investigate the effects of these compounds on animal health and their transfer to animal-derived food.

In general, SPM provide protection against various herbivores and ensure the survival of plants. However, ingestion can occur and the transfer of potentially toxic SPM into milk is already a known risk for several plant families. For other plants, however, both the effect on animal health and the transfer of their SPM into food of animal origin have not yet been fully investigated. Still, due to their chemical properties, transfer into milk is still indicated as reviewed in **Chapter I**. The ingestion of these toxins by dairy cows through feed or on pasture poses a potential risk, as milk remains one of the most important foods of animal origin worldwide and its processing plays an important role in the food chain. Some plant parts are therefore already listed as undesirable substances in animal feed (EU 2002) (**see Chapter I**).

Within the present thesis, two studies were conducted to investigate the transfer of SPM into the milk of dairy cows via feed. In the first project of the thesis (**Chapter III**) we therefore aimed to determine the transfer of quinolizidine alkaloids (QA) from blue sweet lupins (*Lupinus angustifolius*) into the milk of dairy cows. From the data obtained, the individual transfer rates and half-lives were determined, and the transfer behavior of individual QA was modelled toxicokinetically. In addition, the results allowed a first preliminary risk assessment to be carried out, showing a potential risk for individual population groups in the exposure scenario presented here. (**Chapter III**).

Chapter IV has been dedicated to investigating whether pastured dairy cows ingest maple seedlings from sycamore maple trees (*Acer pseudoplatanus*) with detectable levels of HGA, MCPrG, and HGB and if so, if there is a transfer of these substances and their respective metabolites into milk (**Chapter IV**). In addition, we tested whether the toxicological effect after ingestion develops in a similar way as in horses.

Quinolizidine alkaloids

Lupins are suitable as an alternative source of protein in animal feed. Overall, lupins withstand varying site conditions to a significant extent (White et al. 2007). The effects of climate change are becoming increasingly evident through extended and frequent occurrences of droughts and floods. Lupin seeds are resilient to various abiotic stressors, such as polluted or acidic soils and are simple to store and process (White et al. 2007; Coba de la Peña et al. 2012). Besides the supply of sulfur-containing amino acids, such as methionine, they provide good nutritional properties for both monogastric and polygastric animals (Jeroch et al. 2020).
However, due to their content on QA, their use has so far been lower than that of other legumes (Rodes-Bachs and Van Der Fels-Klerx 2023). Still, given the national protein strategy and the financial support provided by the state, as well as their favorable suitability as protein feed, there is an incentive to promote and anticipate a future increase in cultivation (BMEL 2020). A high variability in QA content of individual lupin species represents a possible risk, as the EFSA stated in 2019 that there could be a possible transfer of QA from feed to milk due to their chemical structure. However, no data were available (Pilegaard and Gry 2008; EFSA 2019).
The results of the present study show that narrow-leafed lupins (*L. angustifolius*) used in this study, have QA levels (0.17-0.19%) above the reported limit for sweet lupins in agricultural practice (0.05%) as well as levels recommended by health authorities (0.02%) (ACNFP 1996; Direction générale de la santé 1998; Board of Food Standards Australia New Zealand 2016). Nevertheless, levels range in between reported QA levels for *L. angustifolius* between 20 – 1’918 mg/kg (DM) und 950 – 14’000 mg/kg DM (Pilegaard and Gry 2008; Carvajal-Larenas et al. 2016). The alkaloid profile corresponded to the reported alkaloid profiles in *L. angustifolius* as the main QA reported for *L. angustifolius* are lupanine, 13α-hydroxylupanine, isolupanine, angustifoline, 13α -angeloyloxylupanine and 13α -tigloyloxylupanine (Wink et al. 1995; Boschin et al. 2008). To date, there is no evidence of teratogenic QA (anagyrine, cytisine or ammodendrine) in European lupins. However, due to their teratogenic effects, testing for these in feed is generally indicated (Ortega and Lazerson 1987).

Currently, there are not enough data to adequately assess the occurrence and levels of QA in different agriculturally used lupin varieties. Hence, it seems imperative to conduct a systematic investigation into the presence of alkaloids in different lupin varieties in Germany in the future. Consequently, following this study, the analysis of QA in freshly harvested lupins is an integral part of the national monitoring program from 2022 onwards. First results focusing on lupin seeds and lupin products from the German retail market reveal high QA contents in bitter lupin seeds (21’000 and 20’000 mg/kg DM) and rather low QA contents in investigated sweet lupin seeds (152 mg/kg DM), while QA contents in lupin-based products range from 1’192 mg/kg DM in coffee substitutes to 519 mg/kg in lupin flours (Keuth et al. 2023). It is important to note

that there is considerable variability in these results, highlighting the need for further data collection.

Remarkably, even the inclusion of 1 kg of lupin in the cows' diet resulted in the transfer of QAs into the milk with no detrimental effects on animal health (**Chapter III, Figure 3**). As detected in BSL, highest contents of QA in milk were found for lupanine and 13α -hydroxylupanine, but also angustifoline, multiflorine, isolupanine and sparteine were found in milk in varying amounts (**Chapter III, Figure 4**). The transfer of these alkaloids into milk has been demonstrated for the first time. This finding generally reinforces EFSA's assertion that transfer could occur due to their chemical composition (EFSA 2019).

Despite detectable levels of sparteine in feed (3.03 mg/kg DM), it was only found in very low concentrations near or below LOQ in milk with an average transfer rate of 0.13% as the overall lowest transfer rate (**Chapter III, Table 2**). Sparteine, due to its pharmaceutical applications, possesses the most extensive amount of available data. It has been found to have several beneficial effects, such as anti-hypertensive, anti-pyretic or anti-arrhythmic properties (Yovo et al.1984). However, it also exhibits adverse effects, including anti-muscarinerg, depressive and oxytocic effects (Flores-Soto et al. 2006; Tsiodras et al. 1999). Overall, a higher toxicity is suspected of sparteine than of lupanine (Yovo et al. 1984). Consequently, a lower transfer rate of sparteine into milk is considered beneficial in terms of consumer protection.

Overall transfer rates ranged from 1.05% for isolupanine to 3.74% for multiflorine meaning that the transfer rate is comparatively high compared to the transfer behavior of other SPM, which range from 0.037% for tropane alkaloids to 0.20% for Δ9-tetrahydrocannabinol in hemp (Lamp et al. 2021; Wagner et al. 2022). Overall, significant differences were observed in the transfer rates of individual QA into the milk, which may be attributed to both their chemical structure and possibly metabolic processes in the intermediary metabolism. The significance, thereby, of different transfer rates in terms of consumer safety has not yet been clarified and should be the focus of future studies.

Preliminary risk assessment revealed that in the given scenario with maximum QA content in milk there might be a risk for high end consumers (P95) in all population groups (**Chapter III, Table 5**). Following this transfer, it is necessary to further investigate whether the alkaloids remain stable during each processing step and thus continue to be present in heat-treated dairy products since we only tested for raw milk. To achieve this, market products should be systematically analyzed in the future to determine their content of QA. Similarly, just as there have been studies examining the QA content in solid foods (Keuth et al. 2023), it is essential to systematically investigate cow's milk products as well. Due to that, a national monitoring will be conducted by the individual federal states in Germany to determine QA levels in milk products on the German retail market from autumn 2023.

Given their diverse toxic effects, the relatively limited understanding of their impact on farm animals, and the potential for transfer into milk, it is essential to maintain minimal levels of QA concentration and conduct regular monitoring. To minimize the content of QA, there are various strategies that can be employed. One approach is to focus on cultivating lupin varieties that naturally have low QA levels, allowing them to be sold without the need for debittering. This is crucial because debittering lupins may result in economic losses. In addition to the financial impact, debittering also results in the loss of valuable components in animal feed, such as proteins, minerals, and monosaccharides (Gulisano et al. 2019; Otterbach et al. 2019; Rodes-Bachs and Van Der Fels-Klerx 2023).

Consequently, to address the issue of higher alkaloid contents and to eliminate the need for additional debittering, targeted breeding programs should be implemented. These programs should aim to develop lupin varieties with naturally reduced levels of alkaloids. It is important to emphasize that unregulated independent breeding should be avoided to ensure the effectiveness and reliability of the breeding efforts. Noteworthy, the role of QA in benefiting plant growth, reproduction and overall survival should be considered. Plants low in alkaloids are inherently more vulnerable to various environmental factors (Jamwal et al. 2018). Thus, while implementing these measures, it is crucial to strike a balance that maintains the positive aspects of QA while mitigating its potential negative effects. Farmers want varieties that are more resistant to disease and extreme weather conditions, according to a survey conducted in Germany in 2022 which is overall in contrast to the reduction of secondary plant constituents and requires the development of other resistance strategies against the various environmental influences (Notz and Reckling 2022). One approach may be to reduce or even avoid transport of QA from QA producing plant parts into the seeds. This would then result in a high alkaloid content of the plant and a lack of alkaloids in the seeds. Initial investigations into the enzymes and transport systems responsible have already been carried out (Frick et al. 2017; Mancinotti et al. 2021; Mancinotti et al. 2022).

The enzymes responsible for the formation of lupanine play a significant role in determining the concentration of QA in lupin. Thus, targeting the synthesis process at this stage presents a potential opportunity for reducing QA levels (Rodes-Bachs and Van Der Fels-Klerx 2023).

Recommendations for lowering QA contents were also reviewed by Rodes-Bachs and Van der Fels-Klerx (Rodes-Bachs and Van Der Fels-Klerx 2023). According to this, lupin varieties should contain high levels of 13α -hydroxylupanine to protect against herbivores, although the effects of hydroxylupanine on animal and human health have not been sufficiently investigated. Further opportunities to lower the alkaloid content are presented by relatively high soil pH, cold environment with diurnal cycles as well as organic growing systems (Rodes-Bachs and Van Der Fels-Klerx 2023).

Obtaining additional data on the occurrence and toxicological effects of QA is crucial not only for consumer protection but also for ensuring the safe utilization of lupins as feed. Currently, the kinetics and metabolism of QA have primarily been investigated in laboratory animals, revealing intermediate metabolism. For instance, sparteine has been found to undergo metabolism to lupanine in the livers of rats and rabbits at low concentrations, as observed in *in vivo* studies (Ohnhaus 1985; Chaudhuri 1990).

The findings of the present study also indicate the possibility of individual QA metabolization, as evidenced by the differing transfer rates observed in the morning and evening for 13α - hydroxylupanine compared to the other QAs (**Chapter III, as shown in Figure 4 and Table 2**). Our unpublished studies have indicated that ruminal metabolism, which often affects the toxicity of secondary plant compounds (Loh et al. 2020), does not have any impact on the concentration of toxins in this case. This finding aligns with the research conducted by Aguiar and Wink (Aguiar and Wink 2005), where similar conclusions were drawn regarding the influence of ruminal metabolism on toxin levels. Until now, toxicological effects are largely only known for sparteine and all other QAs are assessed by EFSA in a cumulative approach like sparteine. Effects of individual QA should therefore be further investigated in terms of toxicology and toxicokinetic. There are already suspicions regarding their varying affinities for binding to muscarinic and nicotinic acetylcholine receptors, which have been studied using porcine brain membrane preparations (Schmeller et al. 1994; Green et al. 2013).

The model calculations performed in this study aid in predicting the transfer of QA into milk based on the determined QA contents in feed samples. This predictive capability is valuable for anticipating and monitoring the presence of QA in milk, facilitating proactive measures and appropriate control strategies. Further studies in this field have the potential to accomplish two key objectives. Firstly, they can help establish toxicological guideline values for QA, which would provide crucial information for assessing the safety of these compounds. This knowledge would enable researchers and regulators to establish appropriate limits for QA content in various products. Secondly, these studies can provide valuable insights and recommendations regarding the utilization of lupin products with different QA contents. By understanding the variations in QA levels, experts can make informed suggestions on the appropriate use of lupin-based products in different contexts, considering factors such as dietary requirements, health considerations, and potential risks associated with QA exposure

Hypoglycin A and MCPrG

Hypoglycin A and MCPrG are found in *Sapindaceae* as SPM in the form of toxically active non-proteinogenic amino acids. Although their toxic effects have been well known since the beginning of the 20th century, there has been very little research into their transfer to food of animal origin or even their intake by dairy cows. There is only evidence that transfer to mare's milk occurs and that transfer to cow's milk may be possible (Sander et al. 2020; Bochnia et al. 2021).

The concentrations of maple toxins in sycamore seedlings as detected in the present study with 2.6 g/kg HGA and 0.2 g/kg MCPrG (**Chapter IV, Table 1**) correspond to those already reported for maple seedlings ranging from 97 mg/kg DM to 4508 mg/kg DM (Westermann et al. 2016; Votion et al. 2019; El-Khatib et al. 2022). Overall, however, a high variability is found, depending on season and vegetation stage with decreasing concentrations during further development in seedlings (Votion et al. 2019). This, in turn, may lead to increased levels of ingested HGA and MCPrG, as well as the transfer of HGA and MCPrG into the milk depending on season. If contamination is present, moving the cows to the pasture at a certain time could reduce milk contamination.

The initial aim of this study was to determine whether dairy cows ingest sycamore maple seedlings when grazing, despite the presence of detectable levels of maple toxins. Herbivores are overall considered to be specialists in terms of their feed selection (Hofmann 1989; Provenza 1995). Physiological, biochemical, and anatomical features distinguish ruminants from the rest of the herbivores. Several reports have shown that herbivores, especially cattle, may develop a selective grazing behavior due to their nutritional needs or as a strategy to reduce toxin intake depending on breed and season (Provenza 1995; Hessle et al. 2008). In this context, highly domesticated breeds are often considered to be less selective, as energy is spent on production rather than foraging, in contrast to less domesticated breeds (Schütz et al. 2001; Schütz and Jensen 2001). This hypothesis would be consistent with the results shown, as no selective grazing behavior of high-yielding cows could be demonstrated as seedlings were ingested (**Chapter IV, Figure 3**). In the reported cases of poisoning in ruminants, ingestion of maple toxins occurred through active presentation of plant parts (Bochnia et al. 2020) or through an area that was less covered with vegetation (Bunert et al. 2018). All cases of intoxication with HGA and MCPrG known so far refer to ruminants that were held in captivity. An increase in intake could therefore be explained by an active presentation of the plant parts or by insufficient additional intake of herbage mass due to overgrazed areas (Bochnia et al. 2020; Hirz et al. 2021; Renaud et al. 2022). Grazing sheep on a pasture with sycamore seedlings in fact did not show any symptomatology despite detectable

concentrations in blood and thus proven intake agreeing with the current findings and supporting our hypothesis (Gonzalez-Medina et al. 2021).

Nutritional status may influence susceptibility to different SPM (Illius and Jessop 1995; Foley and Moore 2005). This is also true for known cases of HGA intoxication in humans and especially in children. A dose-dependent toxicity has been reported in children with malnutrition and poor socio-economic background after consumption of akee or litchi (Shrivastava et al. 2017; Ajayi et al. 2019; Sarkar et al. 2020). Missing the evening meal and therefore low levels of blood glucose led to fatal cases of poisoning in children after litchi and therefore HGA and MCPrG ingestion (Ponnaiah et al. 2023). In horses, too, different courses of disease have been reported in terms of their expression and time course despite simultaneous access to maple seedlings and seeds (Bochnia et al. 2015). The authors thereby concluded that expression of symptoms might be related to the carbohydrate/protein ratio in their diet (Bochnia et al. 2015).

Both by the reduced number of sprouts and by the detection of the metabolites in milk, the intake of seedlings by cows has been proven (**Chapter IV, Figure 2, Figure 3, Table 3, Table 4**). Based on the observations, it seems reasonable to assume that the seedlings were ingested as a by-product of grazing. Interestingly, cows did not exhibit any clinical symptoms despite having higher levels of HGA and MCPrG metabolites in their urine compared to diseased milus (Bochnia et al. 2020) (**Chapter IV, Table 2**). This observation raises the question of what metabolic differences exist in the ruminal and intermediate metabolism between different animal species. It is reasonable to assume that the altered physiology in the forestomach system of ruminants could lead to a metabolization or detoxification of maple toxins, which is supported by the results of Gonzales-Medina et al. (Gonzalez-Medina et al. 2021) and the study presented here. However, the hypothesis might be doubtful when case reports of non-domesticated ruminants are included (Bunert et al. 2018). Based on this, it has been hypothesized that the manifestation of toxic effects could be associated with variations in retention times and digesta flow (Renaud et al. 2022). This is particularly relevant for large ruminants such as cattle, which possess a relatively larger rumen size and longer retention times compared to small ruminants while they exhibit less liquid chymus and a reduced ventral groove, which further differentiates their digestive processes (Hofmann 1984; Hofmann et al. 2008; Renaud et al. 2022). An *in vitro* study revealed that there was a significant increase in HGA concentrations after 2 hours of incubation in ruminal fluid (Gonzalez-Medina et al. 2021). Typically, amino acids are directly utilized in microbial metabolism within the rumen for microbial protein synthesis or small peptides. Beyond microbial needs, amino acids are deaminated to ammonia and carboxylic acids (Van Soest 1994). Only undegradable amino acids, as well as those derived from microbial protein synthesis, are reaching the lower digestive tract. This means that most amino acids are metabolized by rumen microbes rather than being absorbed intact by the ruminant (Kung and Rode 1996). Due to their structural

similarity, it has been hypothesized that non-proteinogenic amino acids may undergo the same metabolic pathways as proteinogenic amino acids (Rodrigues-Corrêa and Fett-Neto 2020). However, their overall transport also depends on their solubility and whether it moves with liquid or moves within the fibre mat (Van Soest 1994). The findings of Gonzales-Medina et al. *in vitro* indicate that there is no evident degradation in the rumen (Gonzales-Medina et al. 2021). However, it is still uncertain whether the formation of simple or conjugated metabolites could have occurred during incubation, which could potentially account for the outcomes of the present study. Overall, the findings of the current study reveal a swift metabolization process, which could suggest two possible scenarios. Firstly, it could indicate a rapid passage rate through the rumen into the small intestine followed by absorption (Krägeloh et al. 2017). Secondly, it could imply absorption taking place directly within the rumen, either as protoxins or already metabolized.

The results of the current study suggest that the protoxins HGA and MCPrG are somehow metabolized, because only their respective conjugated metabolites MCPA-G and MCPF-G were detected in both urine and milk (**Chapter IV**). Hence, apart from partial degradation in the rumen the livers metabolization in the intermediary metabolism could potentially serve as an underlying factor.

Studies conducted in mice revealed that HGA could potentially undergo transformation into its active metabolites within the liver (Von Holt 1966). It is plausible that varying susceptibility to HGA among different ruminant species could also be attributed to disparities in liver function and energy metabolism. Notably, variations have been observed in the composition of cytochrome P450 enzymes across different ruminant species highlighting the need for additional toxicological investigations involving diverse animal species (Machala et al. 2003; Szotakova et al. 2004).

In contrast to the study conducted by Bochnia et al. (Bochnia et al. 2021), only conjugated metabolites MCPA-G, MCPA-C as well as MCPF-G and MCPF-C could be detected in the milk in the current study (**Chapter IV**). The detection of metabolites in blood or urine is commonly used as a diagnostic indicator for ingestion of maple toxins in both humans and horses (Bochnia et al. 2015; Sander et al. 2019). In contrast to the results reported by Bochnia et al. (Bochnia et al. 2021), in the current study only MCPA-G was detected in bulk tank milk samples, but no protoxins (**Chapter IV**). The precise impacts of those metabolites found in the milk remain uncertain. However, the conjugation of metabolites often suggests that the substances have undergone detoxification processes. Conjugation is a common metabolic pathway in which substances undergo chemical modifications to reduce their toxicity or enhance their elimination from the body (Chen 2020; Mulder et al. 2020). Therefore, the presence of conjugated metabolites in the milk may suggest a diminished level of toxicity in comparison to the original substances. Indeed, the existing literature on metabolites and their

effects can be contradictory, with some studies discussing the presence as toxic metabolites (Renaud et al. 2022), while others suggest the existence of detoxification mechanisms (Sander et al. 2017). Overall, this highlights the importance of complementary studies to elucidate the toxicology of individual metabolites of HGA and MCPrG. The analysis of milk tank samples revealed that the dilution of individual milk samples with tank milk resulted in noticeably decreased levels (**Chapter IV, Table 4**). Nevertheless, even with the dilution, traceable quantities of MCPA conjugates could still be detected in the milk.

As there is insufficient data to assess the associated risk, the introduction of maple toxins through feed should be avoided. The most straightforward approach is to actively avoid sycamore trees in pastures, near pastures or at the edge of fields. However, it should be noted that there is still a potential risk of additional contamination through airborne seeds, which could introduce the substances into the cows' environment. Based on the findings of this study, it is recommended to further investigate the following key aspects. Firstly, to understand the reduced susceptibility in cows, it is important to investigate both the ruminal metabolism and hepatic metabolism of maple toxins. Secondly, conducting toxicological tests can provide valuable insights into whether the conjugated metabolites present in milk have a similar toxic effect as the protoxins HGA and MCPrG. In addition, it should also be investigated whether an intestinal deconjugation of the metabolites could possibly occur after reuptake as known for certain xenobiotics (Haiser and Turnbaugh 2013). Overall, these studies are necessary to characterize the risk identified, both in terms of the effects of the individual metabolites or possible re-conversions to their protoxins, and in terms of the influence of nutritional status on the expression of symptomatology. Only then can the risk in this case can be fully assessed.

Secondary plant compounds in the context of climate change

Given the pressing issue of climate change, it is imperative to implement changes in agriculture to reduce greenhouse gas emissions, restore biodiversity, and ensure food safety. To achieve a change, it is necessary to use land more sustainably and promote domestic plant species to reduce imports. Both conducted studies in this thesis provide evidence that investigating the transfer of secondary plant constituents from feed into the milk of farm animals is a crucial aspect of ensuring food safety. Given their chemical structures, it is evident that the transfer of numerous secondary plant constituents is likely, thus offering a broad scope for additional complementary research.

In addition to the plants and their components that are already native to Germany and Europe, it is worth noting that climate change is causing noticeable and significant impacts on their distribution, on animal poisoning and potential incorporation into the food chain (Nagy et al. 2023). In addition to the transmission of microbial organisms, there is already significant knowledge regarding the dissemination of mycotoxins and PCBs (polychlorinated biphenyls) (Tirado et al. 2010). Higher temperatures in water and reduced flow rates are known factors that lead to the accumulation of contaminants in the water (Tirado et al. 2010). Apart from increased temperatures, increased CO_2 levels also have an impact on plant growth through an indirect effect on primary plant constituents (Marchi et al. 2004; Kim and Kang 2010). CO_2 influences not only photosynthesis, but also the photosynthetic carbon sequestration phenomenon as a mechanism to convert primary plant compounds into secondary plant compounds (Jia et al. 2014; Jamloki et al. 2021). Therefore, as has been shown for gossypol and tannins, higher CO_2 levels may also lead to higher concentrations of other secondary plant metabolites (Reddy et al. 2004). Furthermore, rising temperatures as expected (IPCC 2022) have also contributed to two major aspects including the development of higher levels on SPM as well as the enhanced growth of certain plant species that previously did not thrive in the respective regions. The latter one is thereby true for *Acer pseudoplatanus,* as it is experiencing increased growth in Scandinavian areas due to improved growing conditions (Weidema and Buchwald 2020, Caron et al. 2015). That an increase in secondary plant compounds can occur in connection with rising temperatures is also shown by the results of the present QA contents in blue sweet lupins (**Chapter III**).

Due to nutritional properties, milk is likely to remain an essential product despite the advent of alternatives. The OECD and FAO even concluded in their Agricultural Outlook 2022-2031 that dairy will remain the fastest expanding livestock in the world, with production expanding from 910 964 kt pw in 2023 to approximately 1 039 320 kt pw in 2032 (OECD/FAO 2023). Therefore, ensuring safe consumption and thus research into the transfer behavior of different SPM from feed to milk will remain of great importance in the future. To ensure the safety of milk and dairy

products while promoting sustainable agriculture, it is imperative to thoroughly examine the potential risks associated with alternative protein sources and pasture farming in the context of climate change. This will enable the development of feeding and management recommendations in the future.

Study limitations

For both plant families and plant toxins analyzed in this thesis, a transfer into milk could be detected, which could pose a potential risk to consumer safety and animal health. Nevertheless, it is important so address certain constraints present in the current research.
Both studies included a limited number of animals, deliberately chosen to be statistically significant without posing unnecessary risk. Therefore, they provide a solid foundation for future larger-scale studies. Both studies focused on the adverse effects of the SPM used. However, it is important to note that this research project did not investigate the various positive effects of individual SPM.

In the experiment conducted on the transfer of QA from blue sweet lupins into cow's milk, only one variety of blue sweet lupin with a defined concentration of QA was used instead of several varieties and comparative QA contents. However, the QA content found aligns with the values presented in the literature, indicating a representative approach. Moreover, this study only analyzed the transfer from feed to milk in one breed of cow which, however, is highly relevant in dairy cow husbandry. The risk assessment only applies to the highest levels of QA found in milk. Lower levels were not thoroughly analyzed, which is a common practice in risk assessment known as a worst-case scenario.

The study on the transfer of HGA and MCPrG into cow's milk did not measure the exact amount of toxins ingested. Hence, the quantity of seedlings that the individual cows might have ingested remains uncertain. The study design aimed to ensure the well-being and health of the animals while observing their natural behavior on pasture. The nutrient content, mineral supply, and composition of the ration may significantly influence feed intake and potentially the intake of toxins from pasture. In the current study, we ensured the dairy cows' ration was ruminant-friendly and met their energy and nutrient requirements. The study design of the conducted research differs from a classic animal experiment. Due to the high toxicity of HGA and MCPrG in horses and other herbivores, an observational study was carried out to obtain realistic results and reduce the risk to animal health.

Conclusion and perspectives

The objectives of the studies carried out were 1) to investigate the possible transfer of quinolizidine alkaloids from lupins into the milk of cows and 2) to investigate the possible intake of sycamore seedlings from *Acer pseudoplatanus* and their SPM HGA and MCPrG by dairy cows on a pasture and the possible transfer of these SPM into milk. In both cases, a potential risk to consumer safety was identified.

In the first study (**Chapter III**), the data obtained revealed a consumer risk even with a quantity of 1 kg of the selected lupin used in dairy cow feed. In the second study, both the ingestion of supposedly toxic plant parts of *Acer pseudoplatanus* and a transfer of respective metabolites of HGA and MCPrG into the milk were proven (**Chapter IV**). However, due to the lack of data to date, the risk to the consumer cannot be conclusively assessed.

The subsequent steps may involve a more in-depth analysis of the following aspects. To gain a comprehensive understanding of QA content in lupins, a structured analysis of the QA content of the varieties available in Germany is necessary. This data will enable the establishment of maximum QA content recommendations for lupins, ensuring the safety of milk.

Additional studies are necessary to comprehensively assess the risk associated with the intake of HGA and MCPrG through sycamore tree plant parts. These studies should include a defined intake quantity of toxins to shed more light on the transfer behavior of the toxins into milk. Further studies on the metabolism in animals and humans are also of great importance.

Chapter VI

Summary/Zusammenfassung

Transfer of plant toxins from feed into milk – a challenge for future sustainable milk production systems?

Climate change presents significant challenges for agriculture. The emission of greenhouse gases calls for a reassessment that influences the manufacture and processing of animal feed. Sustainable solutions, such as cultivating domestic grains as protein sources and adopting techniques like grazing livestock on grasslands and agroforestry, will gain importance. However, certain plants that are increasing in significance possess SPM. SPM serve various significant functions and have diverse effects on their surroundings. In addition to benefiting the plant and its environment, the primary motivation for producing these compounds is to provide a growth advantage and protection against herbivore predators, ensuring plant survival and reproduction. Nevertheless, ingestion of plants with SPM still occurs resulting in a possible risk to both animals and consumers if there is a transfer of SPM via feed into food of animal origin, especially into milk.

For some SPM transfer into the milk is already investigated while for others there is still lack of data as summarized in **Chapter I**.

Chapter I reviews the current literature on two secondary plant metabolites for which there is still a lack of data on their occurrence and possible transfer to milk, although there are indications that transfer may be possible.

The QA are naturally occurring alkaloids in *Fabaceae.* Their best-known representative with a wide range of applications in animal nutrition, due to their beneficial protein content, are lupins, which are subdivided into sweet and bitter lupins depending on their QA content. QAs have multiple toxicological effects that result in a so-called anticholinergic syndrome causing among other coordination disorders, respiratory paralysis, tachyarrhythmia or cardiac arrest. Due to its chemical structure, a transfer into the milk of cows was suspected, but there are no data so far.

Sapindaceae, a different botanical family, possesses recognized toxic properties. Within this family, certain SPM, namely HGA, MCPrG, and HGB, have led to significant toxic effects in humans, horses, and wild ruminants. Important representatives of the *Sapindaceae* are sycamore maple trees, which can be found in meadows and fields with contents on HGA, MCPrG and HGB in their seeds and seedlings. Initial studies suggest that these SPM may be transferred into the milk of mares or cows after ingestion of seeds or seedlings. Nonetheless,

thorough research on the effects of these substances and their excretion in milk has not been conducted in dairy cows.

Chapter II therefore explains the aims and hypothesis of the current thesis. The main part of this thesis consists of two published manuscripts summarized in **Chapter III and IV**. The primary objective was on increasing knowledge on the transfer QA into the milk of dairy cows as well as on the intake of maple toxins HGA, MCPrG and HGB and their subsequent transfer into the milk of dairy cows. These efforts aimed to provide a more comprehensive assessment of the risks posed to both animals and consumers.
Furthermore, a toxicokinetic model was derived to predict the feed to food transfer for QAs (published in Engel et al. 2022, **Chapter III**).

The first study was conducted as a feeding trial in four Holstein-Friesian dairy cows. During the trial rapeseed meal was switched for either one or two kg of narrow-leafed lupins (*L. angustifolius* variety *Boregine*) for seven days as experimental periods with respective depuration periods. During these periods milk was sampled twice daily and analyzed on their respective QA content with an in-house validated novel LC/MS-MS method. Furthermore, milk ingredients were monitored regularly. Based on the data three-compartment toxicokinetic model was derived to predict feed to food transfer.
The results reveal that an intake of 1’774 mg QA per cow per day had no effects on animal health. Thereby, the pattern of the used lupin was like those already reported for narrow-leafed lupins even though total QA content was in the upper range of reported QA contents. Already the administration of 1 kg of lupins resulted in a transfer of QA into milk with different transfer rates for all QAs. Administration of twice the number of lupins (2 kg) showed a significant dose-dependent transfer of QA into milk. Calculation of individual transfer rates revealed transfer rates differing from 1.05% for isolupanine to 3.74% for multiflorine (**Chapter III).**
With maximum QA contents in milk a preliminary risk assessment was made for high consumers (P95) indicating a potential risk for consumers in this scenario (**Chapter III**). Nevertheless, data on toxicokinetic and occurrence of QA in feed and food is lacking.

Chapter IV aimed to investigate if there is an intake of sycamore seedlings by dairy cows while grazing and if so, if there is a transfer of their SPM into milk without the occurrence of clinical signs as known for other herbivores like horses after SPM ingestion. For that, five cows were subjected to an observational study over 4 days. Cows had access to a pasture with numerous seedlings growing between grass over a defined period. Additionally, they received a partial mixed ration in the barn *ad libitum* and concentrate feed suitable for their respective milk yields. Milk of individual cows was sampled twice daily as well as bulk tank milk of the whole herd (n=87) and analyzed on their content of HGA, MCPrG, HGB and their respective metabolites with a novel validated LC/MS-MS method. Experimental plots were placed on the pasture and seedlings were counted and photographed daily before cows were allowed to graze. Additionally, cows were observed by two independent observers and intake was captured if

possible. Already on the first day, intake of sycamore maple seedlings was observed in dairy cows as a by-product of grazing. Noteworthily, only respective conjugated metabolites of HGA and MCPrG were measured in milk samples already on day 1 after grazing. Urine samples revealed MCPA-G contents above contents measured in diseased Peré David's deer without the appearance of clinical symptoms. Statistical analysis revealed an increasing trend in MCPA-G contents in milk. There is still lack of data on toxicological effects of conjugated metabolites, but cows may be in general less susceptible to maple toxin intoxication.

In conclusion, the present thesis highlights that a transfer of the investigated secondary plant metabolites into milk is possible. Current developments in relation to climate change call for a fundamental rethink of the agricultural sector. The significance of local forage and agroforestry methods is increasingly acknowledged. Further investigations are imperative to appraise the potential risk to consumers and to provide suggestions for farm management, feeding, and grazing practices. In the case of lupins, preliminary risk assessment revealed a possible risk for certain consumer groups. However, the risk to the consumer can be further investigated and reduced by testing the lupins available on the market for their QA content, adapting the recommendations for use and carrying out additional toxicological studies. For SPM found in *Sapindaceae* including *A. pseudoplatanus* is remains uncertain whether the conjugated metabolites of HGA and MCPrG found in the milk of individual cows as well as bulk tank milk represent a potential risk to consumers. Nevertheless, both uptake by cows with apparently reduced susceptibility and the possibility of transfer were demonstrated which emphasizes the necessity to produce additional data.

To promote sustainable agriculture, it is necessary to enhance the use of indigenous legumes like lupins and pasture farming. This progression necessitates a comprehensive analysis of the linked hazards. By establishing these potential threats, we can advance our knowledge of farming and agriculture and create positive environmental impacts that counteract climate change.

Transfer von Pflanzentoxinen aus Futtermitteln in die Milch - eine Herausforderung für zukünftige nachhaltige Milcherzeugungssysteme?

Der Klimawandel stellt die Landwirtschaft und Milchkuhhaltung vor große Herausforderungen. Der Ausstoß von Treibhausgasen erfordert einen Wandel, der sich auf die Herstellung und Verarbeitung von Futtermitteln auswirkt. Nachhaltige Lösungen, wie der Anbau einheimischer Körnerleguminosen als Eiweißquellen und die Anwendung von Techniken wie der Weidehaltung und der Agroforstwirtschaft, werden zukünftig an Bedeutung gewinnen. Einige Pflanzenfamilien, die in diesen Systemen eine wichtige Rolle spielen, besitzen jedoch sekundäre Pflanzeninhaltsstoffe (SPM). Die SPM erfüllen verschiedene wichtige Funktionen und haben vielfältige Auswirkungen auf ihre Umgebung. Neben dem Nutzen für die Pflanze und ihrer Umwelt besteht der Hauptgrund für die Produktion dieser Verbindungen darin, einen Wachstumsvorteil und Schutz gegen Pflanzenfresser zu bieten und so das Überleben und die Fortpflanzung der Pflanze zu sichern. Dennoch werden SPM-haltige Pflanzen auch von Lebensmittel-liefernden Tieren aufgenommen, was ein mögliches Risiko für Tiere und Verbraucher darstellt, wenn SPM über Futtermittel in Lebensmittel tierischen Ursprungs, insbesondere in die Milch, gelangen.

Für einige sekundäre Pflanzeninhaltsstoffe ist der Übergang in die Milch bereits untersucht worden, während für andere noch keine Daten vorliegen, wie in **Kapitel I** zusammengefasst ist.

Kapitel I gibt einen Überblick über die aktuelle Literatur zu zwei sekundären Pflanzeninhaltsstoffen, über deren Vorkommen und möglichen Transfer in die Milch noch keine Daten vorliegen. Es gibt jedoch Hinweise, dass ein Transfer möglich ist. Die QA sind natürlich vorkommende Alkaloide in *Fabaceaen*. Ihre bekanntesten Vertreter mit einem breiten Einsatzspektrum in der Tierernährung, aufgrund ihres vorteilhaften Proteingehaltes, sind Lupinen, die auf Grundlage ihres QA-Gehaltes in Süß- und Bitterlupinen eingeteilt werden. Die QA weisen multiple toxische Effekte auf, die in einem sogenannten anticholinergen Syndrom resultieren. Ausprägungen resultieren in koordinatorischer Dysfunktionen, respiratorischer Paralyse, Tachyarrythmien oder Herzstillständen. Aufgrund ihrer chemischen Struktur, wurde ein Transfer der QA in die Milch bereits vermutet, jedoch nicht nachgewiesen.
Die Familie der *Sapindaceae* weist ebenfalls durch ihre Inhaltsstoffe toxische Merkmale auf. Innerhalb dieser Familie führen die sekundären Pflanzeninhaltsstoffe HGA, MCPrG und HGB zu signifikanten toxischen Effekten bei Menschen, Pferden und Wildwiederkäuern. Wichtige Vertreter der *Sapindaceae* sind Bergahornbäume (*Acer pseudoplatanus*), die häufig auf Wiesen und an Feldrändern aufzufinden sind. Sie enthalten HGA, MCPrG und HGB in ihren Samen und Keimlingen. Erste Studien zeigen, dass es zu einem Transfer der genannten sekundären Pflanzeninhaltsstoffe in die Milch von Stuten und Kühen kommen könnte. Bis jetzt wurden jedoch sowohl die Auswirkungen der Toxine auf die Tiergesundheit von Kühen als auch der mögliche Transfer in die Milch nicht hinreichend untersucht.

In **Kapitel II** werden die Ziele und Hypothesen der vorliegenden Arbeit erläutert. Der Hauptteil dieser Arbeit besteht aus zwei veröffentlichten Manuskripten, die in **Kapitel III** und **IV** zusammengefasst sind. Das Hauptziel bestand darin, das Wissen über den Transfer von QA in die Milch von Milchkühen sowie über die Aufnahme der Ahorntoxine HGA, MCPrG und HGB und deren nachfolgenden Transfer in die Milch von Milchkühen zu erweitern. Diese Untersuchungen zielten darauf ab, die möglichen Risiken für Tiere und Verbraucher zu erforschen.
Darüber hinaus wurde ein toxikokinetisches Modell zur Vorhersage des Transfers von QAs in die Milch abgeleitet (veröffentlicht in Engel et al. 2022, **Kapitel III**).

Die erste Studie wurde als Fütterungsversuch an vier Holstein-Friesian Milchkühen durchgeführt. Während des Experiments wurde über jeweils sieben Tage mit entsprechenden Absetzphasen Rapsschrot gegen ein oder zwei Kilogramm schmalblättrige Lupinen (*L. angustifolius*, Sorte Boregine) ausgetauscht. Während dieser Zeiträume wurden zweimal täglich Milchproben entnommen und mit einer intern validierten neuen LC/MS-MS-Methode auf ihren jeweiligen QA-Gehalt analysiert. Darüber hinaus wurden die Inhaltsstoffe der Milch regelmäßig analysiert. Auf der Grundlage der Daten wurde ein toxikokinetisches Drei-Kompartiment-Modell abgeleitet, um den Übergang vom Futter in das Lebensmittel Milch vorherzusagen. Die Ergebnisse zeigen, dass eine Aufnahme von 1‘774 mg QA pro Kuh und Tag keine Auswirkungen auf die Tiergesundheit hatte. Dabei war das QA-Muster der verwendeten Lupine ähnlich dem, welches bereits für schmalblättrige Lupinen berichtet wurde, während der Gesamt-QA-Gehalt eher im oberen Bereich der berichteten QA-Gehalte lag. Bereits die Verabreichung von 1 kg Lupinen führte zu einem Transfer von QA in die Milch mit unterschiedlichen Transferraten für alle QAs. Die Verabreichung der doppelten Menge an Lupinen (2 kg) zeigte einen signifikanten dosisabhängigen Transfer von QA in die Milch. Die Berechnung der einzelnen Transferraten ergab Transferraten, die zwischen 1,05% für Isolupanin und 3,74% für Multiflorin lagen (**Kapitel III**).
Für die maximalen QA-Gehalten in der Milch wurde eine vorläufige Risikobewertung für Vielverzehrer (P95) vorgenommen, die auf ein potenzielles Risiko für Verbraucher in diesem Szenario hinweist. Dennoch fehlen Daten zur Toxikokinetik und zum Vorkommen von QA in Futter- und Lebensmitteln.

In **Kapitel IV** sollte untersucht werden, ob Milchkühe auf der Weide Bergahornkeimlinge aufnehmen und ob es dabei zu einer Übertragung ihrer SPM in die Milch kommt, ohne dass klinische Symptome auftreten, wie sie bei anderen Pflanzenfressern wie Pferden nach der Aufnahme dieser SPM bekannt sind. Zu diesem Zweck wurden fünf Kühe einer Beobachtungsstudie über 4 Tage unterzogen. Die Kühe hatten über einen definierten Zeitraum Zugang zu einer Weide mit zahlreichen Bergahorn-Keimlingen, die zwischen dem Gras wuchsen. Zusätzlich erhielten sie im Stall eine partielle Mischration *ad libitum* und ein für ihre jeweilige Milchleistung geeignetes Kraftfutter. Die Milch der einzelnen Kühe wurde zweimal

täglich, sowie die Tankmilch der gesamten Herde (n=87), beprobt und mit einer neuen validierten LC/MS-MS-Methode auf ihren Gehalt an HGA, MCPrG, HGB und ihren jeweiligen Metaboliten hin analysiert. Ergänzend wurden Versuchsflächen auf der Weide angelegt, und die darauf befindlichen Keimlinge täglich gezählt und fotografiert, bevor die Kühe Zugang zur Weide hatten. Zusätzlich wurden die Kühe von zwei unabhängigen Beobachtern beobachtet und die Aufnahme der Keimlinge nach Möglichkeit videographisch festgehalten. Bereits am ersten Tag wurde bei den Milchkühen die Aufnahme von Bergahornkeimlingen während des Weidegangs beobachtet. Anschließend konnten bereits am Tag 1 nach dem Weidegang die entsprechenden konjugierten Metaboliten von HGA und MCPrG in Milchproben gemessen werden. Urinproben wiesen MCPA-G Gehalte auf, die über den bei erkrankten Davidshirschen gemessenen Werten lagen, ohne dass klinische Symptome bei den Milchkühen auftraten. Die statistische Analyse ergab eine steigende Tendenz des MCPA-G Gehalts in der Milch. Es gibt noch keine Daten über die toxikologischen Auswirkungen konjugierter Metaboliten, aber Kühe könnten im Allgemeinen weniger anfällig für Vergiftungen durch diese Pflanzentoxine sein.

Zusammenfassend zeigt die vorliegende Arbeit, dass ein Transfer der untersuchten sekundären Pflanzenstoffe in die Milch möglich ist. Die aktuellen Entwicklungen im Zusammenhang mit dem Klimawandel erfordern ein grundlegendes Umdenken in der Landwirtschaft. Lokale Futtermittel und agroforstwirtschaftliche Methoden werden in Zukunft immer mehr an Bedeutung gewinnen. Weitere Untersuchungen sind unerlässlich, um das potenzielle Risiko für die Verbraucher abzuschätzen und Vorschläge für Betriebsführung, Fütterung und Weidehaltung zu machen. Im Fall der Lupine ergab die vorläufige Risikobewertung ein mögliches Risiko für bestimmte Verbrauchergruppen. Das Risiko für den Verbraucher kann jedoch weiter untersucht und verringert werden, indem die auf dem Markt erhältlichen Lupinen auf ihren QA-Gehalt untersucht, die Verwendungsempfehlungen angepasst und zusätzliche toxikologische Studien durchgeführt werden. Bei SPM, die in *Sapindaceae* einschließlich *A. pseudoplatanus* vorkommen, ist nach wie vor ungewiss, ob die konjugierten Metaboliten von HGA und MCPrG, die in der Milch einzelner Kühe sowie in der Tankmilch gefunden wurden, ein potenzielles Risiko für die Verbraucher darstellen. Dennoch wurde sowohl die Aufnahme durch Kühe mit offensichtlich verminderter Suszeptibilität als auch die Möglichkeit einer Übertragung nachgewiesen, was die Notwendigkeit zusätzlicher Daten unterstreicht.

Um eine nachhaltige Landwirtschaft zu fördern, müssen einheimische Leguminosen wie Lupinen und die Weide- und Forstwirtschaft verstärkt genutzt werden. Diese Entwicklung setzt eine umfassende Analyse der damit verbundenen Gefahren voraus. Durch die weitergehende Untersuchung dieser potenziellen Gefahren, kann das Wissen über die nachhaltige Landwirtschaft erweitert und eine positive Auswirkung auf die Umwelt im Hinblick auf den Klimawandel erzielt werden.

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Selbstständigkeitserklärung

Hiermit erkläre ich, Anna Maria Engel, die vorliegende Arbeit selbstständig verfasst zu haben und keine anderen als die hier aufgeführten Quellen und Hilfsmittel verwendet zu haben. Die Arbeit ist in dieser Form noch keiner anderen Prüfungsbehörde vorgelegt worden.

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