Transfer and Metabolism of Pyrrolizidine Alkaloids from *Jacobaea vulgaris* Gaertn. in Ruminants

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Justus Liebig University Giessen

presented by
Julian Tänzer

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Declaration of Authenticity

I hereby declare that I have prepared the submitted thesis independently and without unauthorized external assistance, using only the aids mentioned in the thesis. All passages that are quoted verbatim or in meaning from published works, as well as all information based on oral statements, are clearly identified as such. In the investigations I conducted and mentioned in the thesis, I adhered to the principles of good scientific practice as laid out in the "Satzung der Justus-Liebig-Universität zur Sicherung guter wissenschaftlicher Praxis". In accordance with § 22 para. 2 of the general regulations for modularized study programs, I consent to a review of the thesis using plagiarism detection software.

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This work was conducted from november 2019 to august 2024 in the unit "Plant- and Mycotoxins" within the department "Safety in the Food Chain" at the German Federal Institute for Risk Assessment in Berlin.

1st Referee:
Prof. Dr. Gerd Hamscher
Institute of Food Chemistry and Food Biotechnology, Justus Liebig University, Giessen, Germany
2nd Referee:
PD Dr. Robert Pieper
Department Safety in the Food Chain, German Federal Institute for Risk Assessment, Berlin, Germany

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List of Abbreviations

ADI	Acceptable daily intake
Adm.	Administration
BMDL	Benchmark dose level
bw	Body weight
DHP	Dehydropyrrole
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
HRMS	High resolution mass spectrometry
JKK	*Jacobaea vulgaris* Gartn.
MOE	Margin of exposure
ND	No data
NMR	Nuclear magnetic resonance spectrometry
PA/PANO	Pyrrolizidine alkaloid (free base / *N*-oxide)
PA	Free base pyrrolizidine alkaloid
PANO	*N*-oxide pyrrolizidine alkaloid
PA-SAFE-FEED	Research project "Studies on the transfer of pyrrolizidine alkaloids into livestock"
UHPLC	Ultra high performance liquid chromatography

1. Abstract

Pyrrolizidine alkaloids (PA/PANOs) are compounds found in over 6000 plant species, including *Jacobaea vulgaris* Gaertn. (common ragwort). Upon hepatic activation, these protoxins exhibit hepatotoxic and carcinogenic properties. This study aimed to investigate the ruminal metabolism and tissue transfer of PA/PANOs found in *Jacobaea vulgaris* Gaertn. in ruminants to better assess the uptake of PA/PANOs by grazing livestock.

This study investigated the ruminal metabolism of cyclic diesters. The nine major free base pyrrolizidine alkaloids (PA) and corresponding *N*-oxides (PANO) from *Jacobaea vulgaris* Gaertn. were examined *in vitro* with rumen liquid from cattle. The results confirmed that all PANOs were rapidly reduced to the corresponding PAs, and most PAs were swiftly metabolized. Compounds such as jacobine, jaconine, and senkirkine exhibited slow elimination, while jacoline remained almost stable. For the first time, it was shown that cyclic diesters are reduced in the rumen to 1,2-saturated metabolites. This reduction is crucial, as the 1,2-double bond is structurally necessary for the toxification of PAs in the liver.

Analysis of ruminal fluids from *in vivo* feeding studies with cattle, sheep, and goats confirmed the *in vitro* results. Samples from these *in vivo* feeding studies also revealed a low transfer of PA/PANOs into the muscle tissue of the animals, with mostly jacoline, jacobine, and jaconine being detectable, consistent with the elimination rate observed *in vitro* in the rumen. Therefore, the risk of exposure to PA/PANOs through the consumption of meat from ruminants exposed to doses of PA/PANO similar to those in this study appears to be low. Moreover, the reported average levels of PA/PANO contamination in feed used in Europe are significantly lower than the doses used in this study.

Interestingly, the metabolites identified in the rumen were not detected in the muscle tissue. A huge amount of these metabolites was detected in the feces of the animals, suggesting that these metabolites may not pass the intestinal barrier or are further transformed in other processes in the body of the animals.

This study demonstrates that the rumen plays a crucial role in detoxifying PA/PANOs. This filtering function substantially reduces the uptake of PA/PANOs within the animal's body, presumably decreasing the health threat posed by these substances to ruminants. Additionally, ruminal activities result in a low transfer of PA/PANOs into animal products such as meat, thereby lowering the risk of exposure through meat consumption.

2. Zusammenfassung

Pyrrolizidinalkaloide (PA/PANOs) sind Verbindungen, die in über 6000 Pflanzenarten vorkommen, darunter *Jacobaea vulgaris* Gaertn. (Gemeines Jakobskreuzkraut). Nach hepatischer Aktivierung zeigen diese Protoxine hepatotoxische und kanzerogene Eigenschaften. Ziel dieser Studie war es, den Pansenmetabolismus und den Transfer ins Gewebe von PA/PANOs aus *Jacobaea vulgaris* Gaertn. bei Wiederkäuern zu untersuchen, um die Aufnahme von PA/PANOs besser bewerten zu können.

In dieser Studie wurde der Pansenmetabolismus zyklischer Diester untersucht. Neun freie Basen (PA) und deren korrespondierenden N-Oxide (PANO), die vor allem in *Jacobaea vulgaris* Gaertn. zu finden sind, wurden *in vitro* mit Panseninhalt von Rindern untersucht. Die Ergebnisse bestätigten, dass alle PANOs schnell zu den entsprechenden PAs reduziert werden und die meisten PAs rasch weiter metabolisiert werden. Verbindungen wie Jacobin, Jaconin und Senkirkine zeigten eine langsame Eliminierung, während Jacolin nahezu stabil blieb. Zum ersten Mal konnte gezeigt werden, dass zyklische Diester im Pansen zu 1,2-gesättigten Metaboliten reduziert werden. Diese Reduktion ist von entscheidender Bedeutung, da die 1,2-Doppelbindung strukturell für die Toxifizierung der PAs in der Leber notwendig ist.

Analysen von Panseninhalt aus *in vivo* Fütterungsstudien mit Rindern, Schafen und Ziegen bestätigten die *in vitro* Ergebnisse. Proben aus diesen *in vivo* Fütterungsstudien zeigten auch einen geringen Transfer von PA/PANOs in das Muskelgewebe der Tiere, wobei hauptsächlich Jacolin, Jacobin und Jaconin nachgewiesen wurden, was mit den *in vitro* beobachteten Eliminierungsraten im Pansen übereinstimmt. Daher scheint das Risiko einer Exposition gegenüber PA/PANOs durch den Verzehr von Fleisch von Wiederkäuern, die ähnlichen PA/PANO-Dosen wie in dieser Studie ausgesetzt sind, gering zu sein. Darüber hinaus liegen die durchschnittlichen PA/PANO-Gehalte in Futtermitteln, die in Europa verwendet werden, signifikant unter den in dieser Studie verwendeten Dosen.

Interessanterweise wurden die im Pansen identifizierten Metabolite nicht im Muskelgewebe nachgewiesen. Eine große Menge dieser Metabolite wurde im Kot der Tiere gefunden, was darauf hindeutet, dass die Metabolite möglicherweise die Darmbarriere nicht passieren oder in anderen Prozessen des Körpers der Tiere weiter umgewandelt werden.

Die vorliegende Studie zeigt, dass der Pansen eine entscheidende Rolle bei der Entgiftung von PA/PANOs spielt. Diese Filterfunktion reduziert die Aufnahme von PA/PANOs erheblich, wodurch vermutlich das Risiko durch diese Substanzen für Wiederkäuer verringert wird. Darüber hinaus führt die Aktivität des Pansens zu einem geringen Transfer von PA/PANOs in tierische Produkte wie Fleisch, wodurch das Risiko einer toxischen Exposition durch Fleischkonsum verringert wird.

3. Introduction

3.1. The PA-SAFE-FEED project

This doctoral thesis was conducted within the collaborative project "Studies on the Transfer of Pyrrolizidine Alkaloids into Livestock" (PA-SAFE-FEED). Therefore, the project will be briefly introduced in the following.

3.1.1. Background

Grasslands are essential for providing forage to grazing livestock. Biodiverse grasslands, in particular, are highly valuable for supporting biodiversity, yet this diversity can also include potentially toxic plants. Pyrrolizidine alkaloid (PA/PANO) producing species, such as *Jacobaea vulgaris* Gaertn. (JKK, tansy ragwort) and various *Senecio* species, are such ecological valuable species in pastures, offering pollen and nectar for insects. However, the presence of ragwort in these ecosystems poses a challenge, especially for extensive and organic farming, where restrictions on weed control, fertilization, and mowing increase the risk to livestock being exposed to these plants containing toxic PA/PANOs. In northern Germany, *Jacobaea vulgaris* Gaertn. poses a crucial issue. These toxic plants not only potentially threaten animal health, with being on field and in feed, but also pose risks to consumers, if PA/PANOs enter the food chain via animal products.

Differences in the sensitivity to PA/PANO toxicity have been published among ruminants with sheep being relatively resistant and no avoidance of PA/PANO plants during grazing has been observed. While cattle tend to avoid fresh ragwort if alternative vegetation is available, contaminated hay or silage is not rejected, increasing the risk of PA/PANO ingestion.

Thus, reliable data for suitable recommendations and risk management strategies in Germany and the EU is requires.

3.1.2. Project objective

The PA-SAFE-FEED project aims to determine acceptable PA/PANO levels in feed from a consumer protection and livestock health perspective. The project focused on ruminants such as cows, sheep, and goats. Feeding studies were conducted to assess the health impacts of various PA/PANO levels in feed. The project also evaluated the transfer of PA/PANOs into milk and muscle tissues. Transfer was determined for both the total PA/PANO content and each individual PA/PANO. This enables conclusions about PA/PANO transfer into animal tissues when feed is contaminated with ragwort species other than *Jacobaea vulgaris* Gaertn. The project data should support organic farming goals, such as biodiversity conservation, biological production, and regional feed use.

3.2. Pyrrolizidine alkaloids

Pyrrolizidine alkaloids are secondary plant metabolites produced by 3% of all flowering plants (Wexler and Anderson, 2005). Notable representatives include plants primarily from the families *Asteraceae*, *Boraginaceae*, and *Fabaceae* (Hartmann, 1999). It is presumed that plants synthesize PA/PANOs as a defense mechanism against herbivores, particularly insects (Hartmann, 1999; Wink, 2019). However, these substances are also toxic to livestock and humans, and are among the most abundant plant toxins (Stegelmeier *et al.*, 1999). In plants, pyrrolizidine alkaloids are predominantly present in their *N*-oxide form (Molyneux *et al.*, 2011).

3.2.1. Structure

Depending on the genus, plants contain various types of PA/PANOs. All PA/PANOs possess a pyrrolizidine ring system, consisting of two five carbon rings with a nitrogen atom at position 4 (Figure 1) (Häseler *et al.*, 2016). Pyrrolizidine alkaloids are composed of a necine base and necic acid. Structurally, the pyrrolizidine ring system is present in the necine base, which features a hydroxymethyl group at position 1 and a hydroxyl group at position 7. These two groups are connected to necic acids via an ester bond (Wiedenfeld *et al.*, 2008).

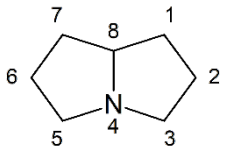

Figure 1: Pyrrolizidine ring system: basic structure of pyrrolizidine alkaloids.

Depending on the nitrogen configuration, PA/PANOs exist as free bases (PA) or *N*-oxides (PANO) (red box in Figure 2). Necine bases are further categorized based on whether they have a 1,2-double bond or a single bond. Additionally, the stereochemistry at position 7 and other characteristics play a role in determining the type of necine base (blue box in Figure 2) (Hartmann and Witte, 1995; Häseler *et al.*, 2016; Roeder, 1995).

Based on the esterification type of the necine base and necic acid, PA/PANOs are classified into monoesters, open-chain diesters, and cyclic diesters. In monoesters, one of the two hydroxyl groups is esterified with a carboxylic acid. In open-chain diesters, both hydroxyl groups are esterified with separate carboxylic acids. In cyclic diesters, also known as macrocycles, the two hydroxyl groups are esterified with the same dicarboxylic acid (yellow box in Figure 2) (Hartmann, 1999; Hartmann and Witte, 1995; Roeder, 2000; Wiedenfeld *et al.*, 2008).

The esterified necic acids are carboxylic acids with up to ten carbon atoms, which can be branched and vary in their degree of saturation and attached functional groups (Hartmann, 1999; Rizk, 1991; Robins, 1989; Wiedenfeld *et al.*, 2008). This structural diversity leads to a large number of different PA/PANOs

– approximately 650 pyrrolizidine alkaloids are known (Hartmann and Witte, 1995; Mattocks, 1986; Rizk, 1991; Roeder, 1995).

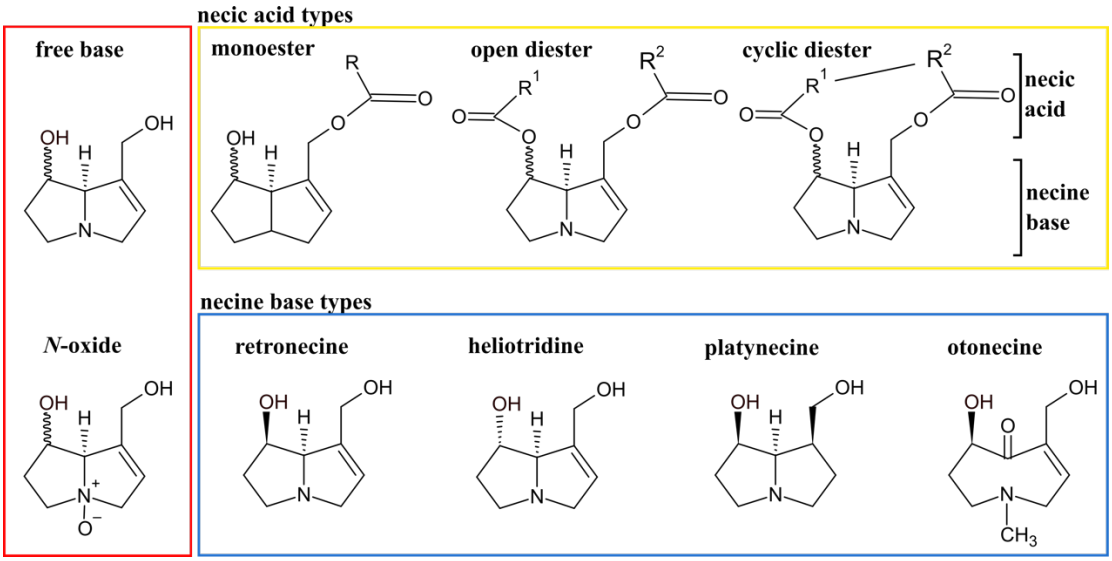

Figure 2: Classification of pyrrolizidine alkaloids based on various characteristics such as the oxidation status of the nitrogen (red), the degree of esterification (yellow), and the structure and stereochemistry of the necine base (blue).

3.2.2. Pyrrolizidine alkaloids from *Jacobaea vulgaris* Gaertn.

Jacobaea vulgaris Gaertn. (syn. *Senecio jacobaea* L.) is a biennial plant that becomes perennial when damaged (Harper and Wood, 1957). As a pioneer plant, it quickly colonizes open areas and has low requirements (Cameron, 1935; McEvoy, 1984). It is one of the most common toxic plants on pastures (Fu *et al.*, 2004; Stegelmeier *et al.*, 1999; Wiedenfeld and Edgar, 2011). Originally from Great Britain, it has spread across Eurasia and has been introduced to America, Africa, Australia, and New Zealand (Harper and Wood, 1957; Mclaren *et al.*, 2000). In these regions, it causes economic damage through losses of livestock, unusable forage, and control measures (Bull *et al.*, 1968; Culvenor, 1985; Naranjo, 1987).

In the genera *Jacobaea* (and also *Senecio*), only PAs that are cyclic diesters with a necine base of the retronecine or otonecine type are present (Kalač and Kaltner, 2021; Lu *et al.*, 2021). Studies by Joosten *et al.* (2011) and Jung *et al.* (2020) identified up to 27 PA/PANOs in JKK, though only about ten occur in relevant quantities. Most of the PA/PANOs in JKK are of the retronecine type, with senkirkine being an exception as it is a otonecine type (Macel *et al.*, 2004; Witte *et al.*, 1992). The PA/PANOs can be further divided into two groups: those with and without angelic acid as a structural element. Jacoline, jaconine, and jacobine are PA/PANOs without angelic acid, while most other PA/PANOs in JKK contain this structure (Jung *et al.*, 2020; Macel *et al.*, 2004; Witte *et al.*, 1992). Individually, the PAs can be differentiated by the positions of hydroxyl groups, epoxide groups (jacobine, erucifoline), and chlorine atoms (jaconine) (Figure 3) (Macel *et al.*, 2004; Witte *et al.*, 1992).

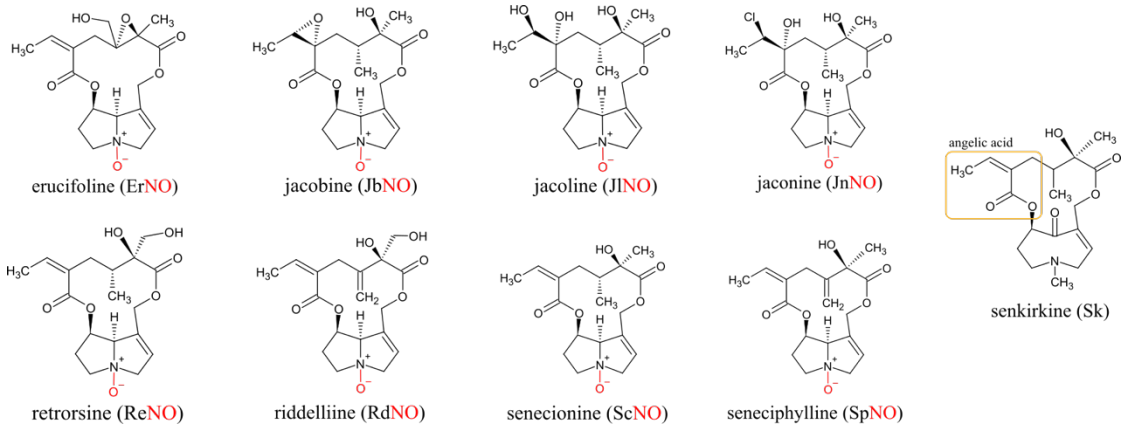

Figure 3: Chemical structures from PA/PANOs in *Jacobaea vulgaris* Gaertn.

3.3. Metabolism and Toxicology

3.3.1. Absorption, distribution and excretion

The majority of studies conducted on this topic are performed on rats and other rodents. Orally ingested pyrrolizidine alkaloids are rapidly absorbed in rats and mice. Within less than 30 minutes PA/PANO concentrations were found in the plasma of the animals (Brauchli *et al.*, 1982; Wang *et al.*, 2011; Williams *et al.*, 2002). Once ingested, the PANOs are reduced to the free base form, which is absorbed in the animal's intestine. This is known for rats and mice and rabbits (Mattocks, 1971; Powis *et al.*, 1979; Yang *et al.*, 2020). Via the portal vein the PAs are transported to the liver. There, among other metabolic reactions, the PAs are re-oxidized to PANOs (Wang *et al.*, 2011; Williams *et al.*, 2002). In section 3.3.3. a detailed discussion of the liver metabolism is provided. PA/PANOs also have been detected in the blood, kidneys, and lungs of rats and mice (Eastman *et al.*, 1982; Estep *et al.*, 1991). PA/PANOs are excreted from the body of rats via urine, bile, and feces (Estep *et al.*, 1990, 1991). However, the PA/PANOs are also partially reabsorbed and thus enter the enterohepatic circulation, as observed in rats and mice (Candrian *et al.*, 1985; Eastman *et al.*, 1982; Estep *et al.*, 1990). Excretion into the milk in general is only minimal (Eastman *et al.*, 1982; Hoogenboom *et al.*, 2011; Mulder *et al.*, 2020).

3.3.2. Ruminal metabolism

The primary function of the rumen is the breakdown of plant material like cellulose and the *de novo* synthesis of proteins by the microbiome (Hungate, 1966; Van Soest, 1994). However, during grazing, animals are also exposed to a variety of toxic xenobiotics. The rumen microbiome can detoxify some of these (Aguiar and Wink, 2005; Craig *et al.*, 1992; Loh *et al.*, 2020).

In most plants, PANOs constitute the largest proportion of the PA/PANO load and therefore enter the rumen of ruminants in extensive quantities. It has been demonstrated *in vitro* that the *N*-oxides (all configurations) are converted fast into the corresponding free bases in rumen liquid of sheep and cattle (Dick *et al.*, 1963; Lanigan, 1970; Mulder *et al.*, 2020). Several studies indicate the further elimination of PAs like heliotrine, supinine, intermedine, lycopsamine (monoesters) and lasiocarpine, echimidine

(open diesters) in *in vitro* incubation with rumen liquid of sheep (Culvenor *et al.*, 1984; Dick *et al.*, 1963; Lanigan and Smith, 1970; Russell and Smith, 1968). The evidence regarding cyclic diesters is less clear. Mulder *et al.* (2020) demonstrated a metabolic elimination of PAs from *Jacobaea vulgaris* Gaertn. plant material (cyclic diesters) incubated with rumen liquids of cattle. Wachenheim *et al.* (1992) showed such an elimination for jacobine and seneciphylline (*in vitro* studies using rumen liquid of cattle and sheep). Contrary, Aguiar *et al.* (2005) showed *in vitro* that senecionine is metabolically not eliminated in rumen liquid of sheep and cattle, while observing elimination for monocrotaline (both cyclic diesters). Also, Swick *et al.* (1983) concluded from a not observed detoxification of *Jacobaea vulgaris* Gaertn. plant material treated with rumen liquid from sheep, that no reductive metabolization of the free bases occurred.

Apparently, open-chain diesters and monoesters are metabolized by the bacterium *Peptostreptococcus heliotrinreducens*, which, however, cannot metabolize cyclic diesters (Hovermale and Craig, 2002; Lanigan, 1976). To date, no microorganism involved in the metabolism of cyclic diester has been isolated (Lodge-Ivey *et al.*, 2005).

Some authors suggest that the microorganisms required for the metabolization of free bases are not ubiquitous, and therefore, animals do not naturally harbor these microorganisms in their rumen (Aguiar and Wink, 2005; Shull *et al.*, 1976; Swick *et al.*, 1983). Others believe PAs can be metabolized without prior exposure. Lanigan *et al.* (1970) and Culvenor *et al.* (1984) showed in their experiments that animals fed large amounts of PA/PANO-containing plants exhibited increased PA metabolism. This suggests that the microorganisms required for PA metabolization are present in most animals, but metabolism is increased upon exposure (Aguiar and Wink, 2005). The described differences in the ability to metabolize PAs are also reflected in documented feeding trials (see section 3.5.1).

The first rumen metabolite of PAs was described by Dick *et al.* (1963). In an *in vitro* experiment with rumen liqiuid from sheep, 7α-hydroxy-1-methylene-8α-pyrrolizidine was produced from heliotrine. Other studies confirmed the formation of this 1-methylene metabolite for heliotrine, lasiocarpine, echimidine, and the cyclic diester monocrotaline (Hovermale and Craig, 2002; Lanigan and Smith, 1970; Russell and Smith, 1968). For PAs like those in *Jacobaea vulgaris* Gaertn., only a low formation of these metabolites is reported (Hovermale and Craig, 2002). Mulder *et al.* (2020) suggest that cyclic diester PAs are eliminated by cleavage of the ester bonds.

In general, the described metabolites lack a 1,2-double bond, which is the prerequisite to express toxicity (see section 3.3.3). Culvenor *et al.* (1976) showed that the 1-methylene metabolite of lasiocarpine did not form pyrrolic esters in rat microsomes, and did not lead to signs of toxic effects in rats. Shull *et al.* (1976) also showed a reduced toxicity in rats for *Jacobaea vulgaris* Gaertn. incubated in rumen liquid of cattle. At the same time Shull *et al.* (1976) and Swick *et al.* (1983) did not see such a reduction when using rumen liquid from sheep.

Depending on the study, authors offer different explanations for the varying sensitivity of ruminants to PA/PANO toxicity (see section 3.5.1). Authors who did not observe PA elimination in the rumen attribute

the different sensitivities to varying enzyme activities in the liver (Aguiar and Wink, 2005; Shull *et al.*, 1976; Swick *et al.*, 1983). Others believe the reason lies in the ability to metabolite PAs in the rumen to non-toxic metabolites (Craig *et al.*, 1992; Lanigan, 1976; Wiedenfeld and Edgar, 2011). It is likely that both organs contribute to this effect. Wachenheim *et al.* (1992) demonstrated that sheep rumen fluid contains more PA-metabolizing microorganisms and that PAs are eliminated faster compared to cattle. Simultaneously, the study by Craig *et al.* (1986) implies that liver metabolism also plays a crucial role: when the same dose of PAs was administered intravenously, cattle showed higher sensitivity than sheep.

3.3.3. Liver metabolism

The fundamental mechanism of PA/PANO biotransformation is similar in animals and humans (IPCS, 1988; Wiedenfeld and Edgar, 2011). Pyrrolizidine alkaloids absorbed in the intestine enter the liver via the portal vein and are bioactivated by liver enzymes. 1,2-unsaturated pyrrolizidine alkaloids are primarily metabolized through three pathways: hydrolysis by esterases (detoxification), oxidation to the corresponding *N*-oxide (detoxification), and oxidation to dehydropyrrolizidine (toxification) (Chen *et al.*, 2010; Fu *et al.*, 2004). The toxification process requires activation and relies on certain structural features of the PAs. The necine base must be unsaturated at the 1,2-position and esterified with a carboxylic acid at position C9 or C7. The esterified carboxylic acid must be at least singly branched and consist of a minimum of five carbon atoms (Allgaier and Franz, 2015; Roeder, 1995).

The hydrolysis by esterases represents a crucial step in the detoxification of PAs. This nonspecific esterase activity primarily occurs in the liver, but also in other tissues. The resulting necine bases and necic acids are considered non-toxic *(Chen* et al*., 2010; Mattocks, 1986; Roeder, 1995).*

The formation of pyrrolizidine alkaloid *N*-oxides (PANOs) in the liver represents a second detoxification pathway (Mattocks, 1971). Their polarity and ability to conjugate facilitate their excretion. However, it is possible that these *N*-oxides are reduced back to their corresponding PAs in the liver, thereby becoming available for toxification processes again (Chen *et al.*, 2010; Mattocks, 1986). Otonecine-type PAs cannot be detoxified through the formation of *N*-oxides because a methyl group is conjugated to the nitrogen of the necine base (Lin *et al.*, 2000).

Pyrrolizidine alkaloids of the retronecine and heliotridine types are hydroxylated at position C3 or C8 by cytochrome P450 monooxygenases and spontaneously react to dehydropyrrolizidine alkaloids through dehydration (Allgaier and Franz, 2015; Chen *et al.*, 2010; Cooper and Huxtable, 1996; EFSA, 2011). These unstable dehydropyrrolizidines can further react in three ways. 1) The cleavage of the ester at position C7 leads to the formation of an aromatic pyrrole, which reacts with nucleophiles such as DNA or proteins. This is crucial for the toxicity of PAs, as the adducts with cellular components explain their genotoxic, mutagenic, and cytotoxic effects (Allgaier and Franz, 2015; Fu *et al.*, 2004; Roeder, 1995). These bound pyrrolizidines can also be re-released and become reactive again (Allgaier and Franz, 2015). The aromatic pyrroles can also be scavenged by nucleophilic glutathione. 2) The formed aromatic pyrrole reacts with water leading to the formation of dehydropyrroles (DHPs). They

also react with nucleophiles such as DNA, proteins, or glutathione. Due to their higher stability, dehydropyrroles can migrate to other tissues (Allgaier and Franz, 2015; Yang *et al.*, 2016). 3) Dehydropyrrolizidines can also directly react with glutathione, leading to detoxification (Yang *et al.*, 2016).

Otonecine types are converted to dehydropyrrolizidines by cytochrome P450-dependent monooxygenases through oxidative *N*-demethylation. After demethylation, a ring closure occurs, forming a hydroxylated PA that can also react to dehydropyrrolizidines (Chen *et al.*, 2010; Roeder, 1995). 1,2-saturated pyrrolizidine alkaloids can also form dehydropyrroles; however, these are stable and do not react with nucleophiles (Mattocks and White, 1971).

3.3.4. Toxicology for ruminants

In ruminants, primarily sub-acute and chronic effects are observed for PA/PANOs (Kalač and Kaltner, 2021; Wiedenfeld and Edgar, 2011). The following symptoms and effects are derived from studies involving various animal species, breeds, PA/PANO plants, and administration methods, which influence the absorption, metabolism, and mode of action of PA/PANOs. However, the symptoms are comparable, thus a general overview is provided here.

Liver and other organ damage result in typical clinical manifestations of PA/PANO poisoning. This syndrome, known as "Winton Disease" or "Pictou Cattle Disease" is characterized by symptoms such as loss of appetite, depression, ataxia, diarrhea, and wandering behavior (Anjos *et al.*, 2010; Barri *et al.*, 1984; Damir *et al.*, 1982; Johnson *et al.*, 1985; Molyneux *et al.*, 2011; Wiedenfeld and Edgar, 2011). Additional symptoms include weight loss, reduced milk production, lethargy, hunched back, irritability, unpredictable behavior, tenesmus, abdominal distension, and dyspnea (Anjos *et al.*, 2010; Barri *et al.*, 1984; Damir *et al.*, 1982; Dickinson *et al.*, 1976; Johnson *et al.*, 1985; Molyneux *et al.*, 2011; Wiedenfeld and Edgar, 2011). A few hours before death, jaundice and hemoglobinuria have been described (Anjos *et al.*, 2010).

Liver damage can be assessed by measuring enzyme parameters in the blood of the animals. Liver injury leads to the release of enzymes into the blood, indicating the action of hepatotoxins in general and thus also PA/PANOs (Ford *et al.*, 1968). However, the specific enzyme parameters depend on the overall condition of the animal, including health, age and sex (Johnson and Smart, 1983). Commonly examined enzymes related to PA/PANOs include aspartate aminotransferase (AST) and γ-glutamyl transferase (GGT), which are general indicators of liver damage. Elevated levels of these parameters in the blood can indicate PA/PANO-induced toxicity (Anjos *et al.*, 2010; Baker *et al.*, 1991; Craig *et al.*, 1986; Culvenor *et al.*, 1984; Goeger *et al.*, 1982a; Johnson, 1982; Johnson *et al.*, 1985; Johnson and Molyneux, 1984; Johnson and Smart, 1983; Maia *et al.*, 2013; Molyneux *et al.*, 1991; Mortimer and White, 1975; Nobre *et al.*, 2005; Ohlsen *et al.*, 2022). Some studies also consider other enzymes (Damir *et al.*, 1982; Dickinson, 1980; Dickinson *et al.*, 1976; Ford *et al.*, 1968).

Pathological examinations of animals reveal primarily liver damages. Fibrosis (Anjos *et al.*, 2010; Damir *et al.*, 1982; Dickinson *et al.*, 1976; Jago, 1969; Johnson *et al.*, 1985; Johnson and Molyneux, 1984; Molyneux *et al.*, 1991), necrosis (Anjos *et al.*, 2010; Dickinson *et al.*, 1976; Johnson *et al.*, 1985; Johnson and Molyneux, 1984; Mattocks, 1986; Thorpe and Ford, 1968), hyperplasia (Jago, 1969), and hemorrhages in the liver are observed (Johnson *et al.*, 1985; Mattocks, 1986). The liver appears swollen and discolored (Johnson and Molyneux, 1984; Molyneux *et al.*, 1991) and sometimes fatty (Damir *et al.*, 1982). Overall, liver cirrhosis (Jago, 1969) or veno-occlusive disease (VOD) can occur (Damir *et al.*, 1982; Johnson *et al.*, 1985; Thorpe and Ford, 1968). At the cellular level, megalocytosis (Damir *et al.*, 1982; Dickinson *et al.*, 1976; Jago, 1969; Thorpe and Ford, 1968) and karyomegaly (Jago, 1969) are observed.

Other organs also show hemorrhages, hyperplasia, and obstructions (Damir *et al.*, 1982; Johnson *et al.*, 1985; Johnson and Molyneux, 1984). Copper poisoning (Anjos *et al.*, 2010; Damir *et al.*, 1982), changes in the central nervous system (Anjos *et al.*, 2010; Johnson *et al.*, 1985), and pulmonary edema (Damir *et al.*, 1982) are also associated with PA/PANO poisoning in ruminants.

3.3.5. Toxicology for humans

The toxic effects of pyrrolizidine alkaloids in humans are similar to those observed in ruminants and occur in acute, subacute, and chronic forms (Kalač and Kaltner, 2021). Acute poisonings manifest through symptoms such as abdominal pain, bloody necrosis, hepatomegaly, ascites, and diarrhea (Koleva *et al.*, 2012; Wiedenfeld *et al.*, 2008; Wiedenfeld and Edgar, 2011). Subacute exposure often leads to veno-occlusive disease (VOD) of the liver, which damages the sinusoidal endothelial cells and hepatocytes around the central vein (Wiedenfeld *et al.*, 2008). Chronic exposure to PAs can result in chronic VOD, causing necrosis and fibrosis, eventually leading to liver cirrhosis (Chen and Huo, 2010; Wiedenfeld *et al.*, 2008).

Unlike in ruminants, the genotoxic and carcinogenic effects of chronic PA exposure are concerns in humans. Studies in rats have demonstrated that PAs can induce tumors in the liver and other organs (Fu *et al.*, 2004; IARC, 1983, 1987; NCI, 1978; NTP, 2003).

3.4. Exposure

3.4.1. Exposure of ruminants to *Jacobaea vulgaris* Gaertn.

Livestock is confronted with *Jacobaea vulgaris* Gaertn. through its growth on pastures used for grazing or the production of forage. Whether grazing animals selectively avoid or consume ragwort is a topic of ongoing debate. Generally, it is assumed that the plant is not selectively avoided in hay and silage and is thus ingested (Kalač and Kaltner, 2021). Thorpe and Ford (1968) observed that cattle rejected pellets with a high content of ragwort, a finding also noted by Goeger *et al.* (1982b) in sheep. Cattle are believed to graze on ragwort in the field under certain conditions, e.g. feed shortages, but otherwise consciously avoid it (Brumme, 2015; Gilruth, 1905; Johnson and Molyneux, 1984; Naranjo, 1987). However, young

plants in the seedling or rosette stage cannot selectively be avoided and are consumed as part of the forage (Brumme, 2015; Stegelmeier *et al.*, 1999). Numerous studies indicate that sheep graze on ragwort in large quantities voluntarily (Brumme, 2015; Cameron, 1935; Gilruth, 1905; Goeger *et al.*, 1982a; Harper and Wood, 1957; Naranjo, 1987; Ohlsen *et al.*, 2022; Schmidl, 1972; Sharrow and Mosher, 1982). Although no studies have confirmed that goats graze on *Jacobaea vulgaris* Gaertn., reports of poisoning incidents indicate that they come into contact with the plant (Anholt and Britton, 2017). Also they are known to actively consume *Senecio inaequidens* (Sánchez Valdés *et al.*, 2022).

In the field, the abundance of ragwort and thus the exposure to PA/PANO can be controlled through management practices. After harvesting, ensiling forage provides an effective method for reducing PA/PANO levels in feed (Gottschalk *et al.*, 2015; Kalač and Kaltner, 2021; Klevenhusen *et al.*, 2019). Jimenez *et al.* (2013) found that during the ensiling of ragwort, primarily jacoline, jacobine, and jaconine remain while the other PAs were eliminated. No reduction in the PA/PANO content is reported during the production of hay or pellets (Kalač and Kaltner, 2021; Kaltner *et al.*, 2018; Wiedenfeld and Edgar, 2011). A European survey found average levels of 0.29 mg/kg of PA/PANOS in roughage and forage (EFSA, 2011).

3.4.2. Exposure of humans

Humans are exposed to PA/PANOs through a variety of foods, particularly plant-based products such as grains, salads, and spices. Documented cases of human intoxication by PA/PANOs are largely associated with these foods. An overview of such incidents is available in several publications (EFSA, 2011; Wiedenfeld and Edgar, 2011). Additionally, honey, eggs, herbal medicines, and dietary supplements contribute to human PA/PANO exposure (BfR, 2007, 2020; Gottschalk *et al.*, 2020; Kaltner *et al.*, 2020, 2020a; Mulder *et al.*, 2015, 2016, 2018; Roeder, 1995; Wiedenfeld and Edgar, 2011). The exposure to PA/PANOs through milk is considered low or negligible (Dusemund *et al.*, 2018; Klein *et al.*, 2024; Mulder *et al.*, 2015, p. 201, 2018). Studies with extensive sampling of commercially available milk have detected only trace amounts of PA/PANOs (0.03 – 0.30 µg/L). No PA/PANOs have been detected in meat samples from retail markets (Huybrechts and Callebaut, 2015; Klein *et al.*, 2024; Mulder *et al.*, 2015, 2018).

Since PA/PANOs are genotoxic and carcinogenic substances, no acceptable daily intake (ADI) can be established. To assess potential risks, the margin of exposure (MOE) is used for such substances. The European Food Safety Authority (EFSA) has established a benchmark dose lower confidence limit (BMDL10) of 237 µg/kg bw/day as a reference point for calculating the MOE from observed concentration data and consumption data (EFSA *et al.*, 2017). Klein *et al.* (2024) determined MOEs for milk based on their data, which exceed 10,000 notably, suggesting no need for regulatory action.

3.5. Feeding studies and carry-over

As early as the beginning of the 20th century, certain cases of intoxication in ruminants were linked to the consumption of *Jacobaea vulgaris* Gaertn. (Gilruth, 1903). Since then, numerous cases have been documented. An overview can be found in various sources (Bull *et al.*, 1968, p. 198; Mattocks, 1986; Molyneux *et al.*, 2011; Panziera *et al.*, 2018). Given that these cases often lack precise information on the amount of plant material ingested by the animals and the PA/PANO content in the plants, these reports only allow for rough estimates of the toxicity of the plant and their contained PA/PANOs. However, the emergence of this PA/PANO issue prompted targeted feeding studies to better understand the toxicity (see section 3.5.1). Over the course of the century, in addition to cases of poisoning in ruminants, human intoxications by PAs were also observed (Wiedenfeld and Edgar, 2011). This led to investigations into whether and to what extent PA/PANOs are transferred to animal-derived foods (see section 3.5.2).

3.5.1. Feeding studies

Table 1 and 2 summarize feeding studies with cattle, goat and sheep involving *Jacobaea vulgaris* Gaertn. and *Senecio* spp. plants. There are considerable differences and missing information regarding the following aspects: animal species, breed, number, sex, and age of the animals, health status (including pregnancy and body weight), administered plant species, PA/PANO content of the plant, mode of administration, duration and frequency of administration, and measured endpoints. Generally, the cited studies tend to use female animals.

Despite the differences in the study designs and the resulting difficulties in directly comparing the studies, similar effects can be observed in the studies. Some effects, however, are inconsistently reported or have only been investigated in individual studies. To enable a rough comparison, table 1 and 2 display the different trials with key information. For the reported doses, several major assumptions had to be made in some cases, as relevant data is missing in these studies.

Within each study and under similar conditions, it becomes evident that the dose of administered PA/PANOs influences the animals' health. This is manifested in stronger symptoms and increased mortality rates at higher doses (Ford *et al.*, 1968; Goeger *et al.*, 1982a; Johnson *et al.*, 1985; Johnson and Molyneux, 1984; Maia *et al.*, 2013). However, dose-dependence is not clearly observed in all studies (Mortimer and White, 1975; Nobre *et al.*, 2005), and in one case, even an inverse relationship was reported (Johnson and Smart, 1983).

The method of PA/PANO administration and the duration of exposure also shows effects across various studies. Generally, animals demonstrate higher tolerance to PA/PANOs when plants are incorporated into their feed, mimicking realistic consumption conditions. For instance, cattle that received the same dose throughout the day exhibited no symptoms compared to those given a single bolus (Johnson *et al.*, 1985; Johnson and Molyneux, 1984). The form of administration (pure or within plants) seems to play a minor role in single-dose studies (Molyneux *et al.*, 1991). Additionally, animals that

ingested PA/PANOs continuously throughout the day could tolerate the amounts longer without symptoms or fatalities compared to those given a single dose per day (Johnson and Molyneux, 1984).

There can also be delays between the last PA/PANO intake and the onset of symptoms. Molyneux *et al.* (1988) and Johnson and Smart (1983) observed this in cattle, while Dickinson *et al.* (1980) reported similar findings in goats. New pregnancies and heat stress were triggers for the symptoms in these cases (Dickinson, 1980; Johnson and Smart, 1983).

Also, animal weight, age, and overall health play a role. Johnson and Molyneux (1984) demonstrated that lighter cattle exhibited problems at a certain dose, while heavier animals did not. This study also indicated that older laboratory animals coped better with PA/PANOs, however, this was not observed in sheep (Johnson and Molyneux, 1984; Mortimer and White, 1975). Johnson and Molyneux (1984) assume increased cellular activity in younger animals leading to enhanced formation of PA-toxins.

Although pregnant animals may suffer from prior or current PA/PANO intoxication, Johnson and Smart (1983) concluded from complication-free births that the fetuses are not harmed. This suggests either detoxification by the mother or that reactive metabolites do not cross the placenta. Calves and kids that consumed milk from mothers exposed to doses of 9.4 ± 5.6 mg/kg bw/day or 16.0 mg/kg bw/day of PA/PANOs also showed no adverse effects (Dickinson, 1980; Dickinson *et al.*, 1976). Liver damage caused by PA/PANOs appears to be potentially reversible (Goeger *et al.*, 1982a).

In Section 3.3.2, the adaptation of animals to PA/PANOs was discussed. For example, Culvenor *et al.* (1984) demonstrated this adaptation in the rumen fluid of animals from feeding trials with *Echium plantagineum*. Anjos *et al.* (2010) showed adaptation to monocrotaline in *Crotalaria* seeds in a feeding trial with sheep. Animals initially receiving lower doses of monocrotaline over an extended period coped better with a single dose of 342.0 mg/kg bw compared to animals that had not previously been exposed to monocrotaline and received a single dose of 205.2 or 273.6 mg/kg bw.

Table 1: Feeding studies with cattle using *Jacobaea vulgaris* and *Senecio* species.

Source	Plant	Species	Animal #	Adm. type	Duration adm. [d]	Dose [mg/kg bw/d]	Absolut dose [mg/kg bw]	Dead animals	Time to death [d]**	Changes			Assumptions
										Clinical	Enzyme activity	Pathologic/ histologic	
Thorpe *et al.* (1968), Ford *et al.* (1968)	*J. vulgaris*	cattle	1	A3	38	6.5 ± 2.6 *	248 ± 100 *	1	49	++	++	++	250 ± 25 kg bw; 0.2-0.4% PA/PANO amount in DM plant
			2		33	4.3 ± 1.8 *	144 ± 58 *	2	60	++	++	++	
			1		38	2.2 ± 0.9 *	83 ± 33 *	1	55	++	++	++	
			1		128	2.2 ± 0.9 *	279 ± 112 *	1	166	+	++	++	
Mortimer and White (1975)	*J. vulgaris*	calf	3	B1	77	3.6 ± 1.5 *	182 ± 88 *	3	50	++	ND	++	specified bw ± 10%; 0.2-0.4% PA/PANO amount in DM plant
			3		77	2.1 ± 0.9 *	88 ± 47 *	3	42	++	ND	++	
Dickinson *et al.* (1976)	*J. vulgaris*	cattle	4	B3	35	9.4 ± 5.6	178 ± 29	4	35	++	++	++	
Johnson *et al.* (1976)	*J. vulgaris*	cattle	2	B2	57/30	9.9 ± 3.9	386 ± 149	2	74 ± 35	ND	++	++	0.2-0.4% PA/PANO amount in DM plant
			2		75	3.9 ± 1.8	292 ± 138	2	75 ± 1	ND	++	++	
			2		50/84	3.0 ± 1.4	159 ± 143	2	90 ± 51	ND	++	++	
Johnson *et al.* (1982)	*J. vulgaris*	calf	1	B1	18	3.0	54	0	150	++	+	++	
Johnson *et al.* (1983)	*J. vulgaris*	cattle	7	B2	15	2.3	33,8	4	188 ± 101	++	+	++	
			7	B2	15	2.0	29,7	0		-	-	-	
Johnson and Molyneux (1984)	*S. douglasii*	cattle	2	B1	16	5.0	80	0		+	+	+	
			4		18	8.0	144	1	449	+	+	+	
			2		16	10.0	160	0		+	+	+	
			3		20	10.0	200	2	46	++	+	++	
			4		10/10	10.0	200	3	220	++	+	++	
			8		15	13.0	195	8	61	++	++	++	
			2		14	15.0	210	2	38	++	++	++	
			2		15	18.0	285	1	45	++	++	++	
			2		2	40.0	80	2	3	++	++	++	
			5	A2	100	2.0	200	0		ND	-	-	
			5		100	4.0	400	0		ND	-	-	
			5		100	6.0	600	0		ND	-	-	
			3		20	10.0	200	0		ND	-	-	
			4		20	20.0	400	0		ND	+	-	
			4		20	30.0	600	0		ND	+	-	
			4		12	30.0	360	0		ND	-	-	

Table 1 continued: Feeding studies with cattle using *Jacobaea vulgaris* Gaertn. and *Senecio* species.

Source	Plant	Species	Animal #	Adm. type	Duration adm. [d]	Dose [mg/kg bw/d]	Absolut dose [mg/kg bw]	Dead animals	Time to death [d]**	Changes			Assumptions
										Clinical	Enzyme activity	Pathologic/ histologic	
Johnson *et al.* (1985)	*S. riddellii*	cattle	4		20	20.0	400	0		-	-	-	
			4	A2	20	40.0	800	1	158	+	+	+	
			4		20/20/20	30.0	600	1	400	+	-	+	
			4		20	10.0	200	0		-	-	-	
			4	B4	20	20.0	400	0		-	+	-	
			4		20	20.0	400	3	138 ± 148	++	++	+	
			4		20	10.0	200	0		-	-	-	
			4		20	15.0	300	4	57 ± 18	++	++	++	
			4	B2	20	20.0	400	4	32 ± 6	++	++	++	
			4		20	25.0	500	4	54 ± 21	++	++	++	
			2		5	60.0	300	2	27 ± 4	++	++	++	
Mollyneux *et al.* (1991)	*S. riddellii*	calf	3	B1	20	45.0	900	3	29 ± 8	++	++	++	
			3	D1	20	4.5 Rd	90	0		-	+	-	
			3	D1	20	40.5 RdN	810	3	44 ± 5	++	++	++	
			3	D1	20	45.0 Rd+RdN	900	3	50 ± 14	++	++	++	
Fletcher *et al.* (2011)	*S. brigalowensis*	calf	ND	ND	42	2.5	105	0		-	-	-	

A1: Voluntary in the field; A2: Plant in feed; A3: Pellets made from plants and animal feed; B1: Oral administration of the plant; B2: Oral administration of the plant in slurry; B3: Administration of the plant via fistula; B4: Oral administration of plants in gelatin; C1: Oral administration of plant extract; D1: Administration of pure substance via fistula – /: Break during the Administration; *: Assumptions were made in order to be able to determine administered doses; **: After first administration; -: No changes; +: Small changes; ++: Severe changes; Adm.: Administration, bw: Body weight; DM: Dry matter; ND: No data.

Table 2: Feeding studies with sheep and goats using *Jacobaea vulgaris* Gaertn. and *Senecio* species.

Source	Plant	Species	Animal #	Adm. type	Duration adm. [d]	Dose [mg/kg bw/d]	Absolut dose [mg/kg bw]	Dead animals	Time to death [d]**	Changes			Assumptions
										Clinical	Enzyme activity	Pathologic/ histologic	
Gilruth (1905)	*J. vulgaris*	sheep	2	A2	168	14.1 ± 6.4 *	2363 ± 1076 **	0		-	ND	-	50 ± 10 kg bw; 0.2-0.4% PA/PANO-amount in DM plant
Mortimer and White (1975)	*J. vulgaris*	lamb	10	B1	112	9.0 ± 3.0 *	1008 ± 475 **	1	ND	++	+	+	0.2-0.4% PA/PANO amount in DM plant
		sheep	8	B1	112	9.0 ± 3.0 **	1008 ± 475 **	0		++	+	+	0.2-0.4% PA/PANO amount in DM plant
			4		140	9.3 ± 3.5 **	528 ± 1840 **	3	175	++	ND	++	
			4		28	9.3 ± 3.5 **	2399 ± 1277 **	0		+	ND	+	
			4		56	9.3 ± 3.5 **	4789 ± 2549 **	1	35	+	ND	+	
Craig *et al.* (1986)	*J. vulgaris*	sheep	3	C1	20	9.8	196	0		-	-	+	
Ohlsen *et al.* (2020)	*J. vulgaris*	sheep	63	A1	163	34.0 ± 14.0 **	2142 ± 882 **	0		-	-	-	40 ± 5 and 60 ± 5 kg bw; 20% DM; 0.2-0.4% PA/PANO amount in DM plant
					163	50.0 ± 19.7 **	3150 ± 1241 **	0		-	-	-	
Dickinson (1980)	*J. vulgaris*	goat	4	B3	125	16.0	2000	3	133 ± 30	++	+	++	
Goeger *et al.* (1982)	*J. vulgaris*	kid	2	A3	381	33.0 ± 13.7 **	12545 ± 4874 **	1	668	ND	-	+	0.2-0.4 % PA/PANO amount in DM plant
			1		155	8.8 ± 4.1 **	1363 ± 642 **	0		ND	-	-	
		goat	1	A3	388	10.9 ± 5.1 **	4224 ± 1991 **	1	739	ND	-	++	0.2-0.4 % PA/PANO amount in DM plant
			1		152	24.5 ± 11.5 **	3717 ± 1752 **	1	503	ND	-	++	
			2		162/114	13.0 ± 5.0 **	1790 ± 775 **	0		ND	-	-	
			1		43	5.7 ± 2.7 **	244 ± 115 **	0		ND	-	-	
Hippchen *et al.* (1986)	*S.vernalis*	goat	2	B3	107	15.3 ± 4.4 **	1638 ± 475 **	0		ND	ND	+	50 ± 10 kg bw; 0.5-0.7% PA/PANO amount in DM plant

A1: Voluntary in the field; A2: Plant in feed; A3: Pellets made from plants and animal feed; B1: Oral administration of the plant; B2: Oral administration of the plant in slurry; B3: Administration of the plant via fistula; B4: Oral administration of plants in gelatin; C1: Oral administration of plant extract; D1: Administration of pure substance via fistula – /: Break during the Administration; *: Assumptions were made in order to be able to determine administered doses; **: After first administration; -: No changes; +: Small changes; ++: Severe changes ; Adm.: Administration, bw: Body weight; DM: Dry matter; ND: No data.

Lethal doses

Many of the feeding studies presented in table 1 and 2 administered doses that led to the death of the animals. Modern feeding studies focus on more realistic doses and aim less to induce severe diseases. Studies that used higher dosages allow the estimation of the lethal dose of animals exposed to *Jacobaea vulgaris* Gaertn. In cattle, a total intake of 0.05-0.2 kg JKK/kg bw over several days is considered lethal, whereas for sheep and goats, it is estimated to be 1.25-4.04 kg JKK/kg bw (Goeger *et al.*, 1982a; IPCS, 1988). The varying lethal doses indicate that cattle, sheep, and goats exhibit different sensitivities to *Jacobaea vulgaris* Gaertn. Hereinafter these three species are categorized along with others according to their sensitivity (Goeger *et al.*, 1983; Hooper, 1978; Hooper and Scanlan, 1977; Maia *et al.*, 2013; Mortimer and White, 1975):

sheep = goat = rabbit > mice > rat > cattle > chicken > pig

The differences in sensitivity are likely due to variations in digestive systems and differing rates of PA/PANO metabolism in both the rumen and liver. This explains why non-ruminants can be less sensitive or similarly sensitive to PA/PANO compared to ruminants. (Duringer *et al.*, 2004; Lanigan, 1972; Peterson and Jago, 1984; Shull *et al.*, 1976; White *et al.*, 1973).

3.5.2. Carry-Over

Some feeding studies were conducted to investigate the transfer of PA/PANOs from *Jacobaea vulgaris* Gaertn. into tissues of animals used for food production. Studies in which milk from exposed cattle and goats was fed to calves, kids and rats showed that PA/PANOs partially transfer into the milk. However, because the toxicity of the milk was low, the transfer is considered minimal, even with high doses of *Jacobaea vulgaris* Gaertn. administered to the mother (Dickinson, 1980; Goeger *et al.*, 1982b; Johnson, 1976). Dickinson *et al.* (1976) confirmed in two studies that transfer of PA/PANOs into the milk from cattle and goats administered with *Jacobaea vulgaris* Gaertn. was low, with 0.1–0.2% and only jacoline detectable in the milk. The authors also showed, that calves, kids and rats fed with this milk were not harmed. Based on this, the authors assessed the toxicity of jacoline as low (Dickinson, 1980; Goeger *et al.*, 1982b). This low transfer rate into ruminant milk has been confirmed by many other studies (Candrian *et al.*, 1991; Deinzer *et al.*, 1982; Hoogenboom *et al.*, 2011; Mulder *et al.*, 2020; Panariti *et al.*, 1997). These studies also confirmed that when *Jacobaea vulgaris* Gaertn. is fed, the main PA in the milk is jacoline. Studies with further *Senecio* species that produce a jacoline free PA-profile could demonstrate that otonecines are predominantly detectable in the milk (Hoogenboom *et al.*, 2011; Mulder *et al.*, 2020). The observed low transfer into milk is consistent with concentrations measured in commercial samples (Klein *et al.*, 2024; Mulder *et al.*, 2015, 2018).

There are currently few studies on the transfer of PA/PANOs from feed into the meat of ruminants. Fletcher *et al.* (2011) reported that feeding *Senecio brigalowensis* to weaned calves resulted in a low

transfer, primarily of otonecine alkaloids, into the muscle tissue. However, concentrations or transfer rates were not specified (Fletcher *et al.*, 2011).

For evaluating the carry-over, it is important to note that both meat and milk are usually processed before consumption. De Nijs *et al.* (2017) for example showed that during microbial fermentation during cheese and yoghurt production PA/PANO concentration was reduced which can further alter the PA/PANO profile and content.

3.6. Objective

Pyrrolizidine alkaloids, naturally occurring toxins found in plants such as *Jacobaea vulgaris* Gaertn., possess hepatotoxic and carcinogenic properties that can affect both humans and animals. *Jacobaea vulgaris* Gaertn. spreads rapidly and extensively. Considering the health risks associated with contaminated grazing lands and feed, and known cases of livestock intoxication, authorities monitor the spread of this plant with concern.

Previous animal studies have primarily focused on high concentrations of PA/PANOs and their severe to lethal effects. However, studies on the carry-over into animal products like milk are scarce, and for meat, almost non-existent. This gap in data complicates the formulation of adequate recommendations for animal and consumer protection for the use of grassland that is infested with PA/PANO producing plants.

In this context, the PA-SAFE-FEED project was initiated to determine, through *in vivo* feeding studies, the dose-dependent health effects of PA/PANOs on cows, sheep, and goats, and to investigate the transfer of these substances and their metabolites into animal products. Additionally, *in vivo* and *in vitro* experiments aimed to elucidate the kinetics and metabolism of these substances. The goal was to collect data that can be used for risk assessment and competent authorities to manage grazing land with PA/PANO-plants and feed contaminated with PA/PANOs.

The tasks of this doctoral thesis included analytical and evaluative support for the *in vivo* studies as well as the conduct and analysis of *in vitro* experiments to identify PA/PANO metabolites. Other studies within the PA-SAFE-FEED project were conducted by project partners at Ludwig-Maximilians-Universität, the Max Rubner Institute, and the Friedrich-Loeffler-Institute.

Since pyrrolizidine alkaloids are metabolized in the rumen of animals, this work aimed to simulate the rumen *in vitro* using rumen fluid from fistulated cattle, to observe the behavior of PA/PANOs and to enable the identification of potential metabolites. To determine the extent to which PA/PANOs and their metabolites are resorbed by the animals in the gastrointestinal tract, Ussing chamber experiments with various bovine epithelial tissues from the gastrointestinal tract were planned. Given that PA/PANOs are converted into toxic compounds in the liver of humans and animals, the toxification of PA/PANOs from *Jacobaea vulgaris* Gaertn. and their ruminal metabolites should be investigated using microsomes from cattle, sheep, and goats.

High-resolution mass spectrometry should be used to analyze *in vitro* and *in vivo* samples, providing structural information about the metabolites and ensure the *in vivo* relevance of these metabolites. Analyzing PA/PANO and metabolite contents in various tissue types (blood, milk, muscle, liver, bile, urine, feces) from *in vivo* experiments should lead to a better understanding of the distribution and fate of PA/PANOs in the animals. Overall, the data generated in this dissertation aimed to elucidate the mechanisms leading to the transfer of PA/PANOs from *Jacobaea vulgaris* Gaertn. into the tissues of ruminants

4. Summarizing discussion

Pyrrolizidine alkaloids are plant toxins found in over 6000 plant species, including *Jacobaea vulgaris* Gaertn. After bioactivation in the liver, they exhibit hepatotoxic and carcinogenic properties harmful to both humans and animals. It is known that ruminants consume these plants either by choice or because they lack the ability to selectively avoid them. Leading to potential health problems for livestock and due to carry-over into animal products also for humans. Studies suggest low carry-over of PA/PANOs into milk and meat, but as the overall recovery for these substances within these studies is low, PA/PANOs fate in the animal body still is poorly understood. This raises questions about the absorption, distribution, metabolism, and excretion (ADME) of these compounds in ruminants and this study aimed to account for these topics. Findings of this doctoral thesis have been published and the two respective publications are accessible in chapter 6. The PA-SAFE-FEED project in which this thesis was conducted is still ongoing. Thus, many of the data and results have not yet been published or fully finalized. Therefore, the following discussion integrates, analyzes, and contextualizes the published as well as the unpublished results.

Previous studies showed that PA/PANOs are metabolically eliminated in the rumen. For mono- and open diesters also the identification of formed metabolites is described. The metabolism of cyclic diesters in rumen is poorly understood (Loh *et al.*, 2020). Therefore, the aim of this work was to investigate, the rumen metabolism of cyclic diesters, which are produced by *Jacobaea vulgaris* Gaertn. and *Senecio* species. Nine PAs and their corresponding PANOs were studied *in vitro* using bovine rumen content (publication 1). It was demonstrated that all PANOs being rapidly and quantitatively transformed into their corresponding free bases, consistent with the results of Mulder *et al.* (2020). We also confirmed further elimination for most of the free bases but observed different elimination rates, with jacobine, jaconine and senkirkine being eliminated more slowly, while jacoline even remained comparatively stable throughout the experiment.

For the first time, ruminal metabolites of cyclic diesters from *Jacobaea vulgaris* Gaertn. were identified in our study. The first transformation step included the reduction of the double bond in the necine base to 1,2-saturated structures. This molecular change was proposed by the high-resolution mass spectrometric determination of respective sum formulas and supported by fragmentation patterns characteristic for saturated PAs. This finding aligns with studies observing 1,2-saturated metabolites for mono- and open diesters in the rumen (Dick *et al.*, 1963; Hovermale and Craig, 2002; Lanigan, 1970; Russell and Smith, 1968). The reduction is a crucial step in the ruminan metabolism of pyrrolizidine alkaloids since the 1,2-double bond is structurally necessary for PA bioactivation in the liver. In a second step the metabolites were further reduced. The molecular formula changed by obtaining two hydrogens during these second reduction. Since the fragmentation pattern could not be used to determine the location of the reduction, the structure of these metabolites remains uncertain. Nevertheless, we found, that these second-step metabolites were primarily identified for PAs with an angelic acid structure in

their necic acid, such as erucifoline, retrorsine, riddelliine, senecionine, seneciphylline, and senkirkine. This suggests that the second reduction affects the carbon-carbon double bond in the necic acid part of the molecule.

Platyphylline, a commercially available 1,2-saturated PA, is theoretically identical to the first-step metabolite of senecionine that we have postulated (publication 1). Unfortunately, it was found to have a different retention time, suggesting additional structural modifications during metabolism. These changes do not appear to affect the molecular formula and, as a result, are not detected by HRMS (unpublished date). Mulder *et al.* (2020) predicts an ester cleavage for cyclic diesters, which we could neither confirm nor exclude on basis of fragmentation patterns of metabolites. Experiments with hepatic microsomes from cows, goat, human and rats have shown that the ruminal metabolites were not metabolized further by liver microsomes suggesting that a bioactivation during hepatic metabolism towards pyrrolic metabolites will not occur (unpublished data). In addition, we showed that the identified ruminal metabolites are not detectable in the blood, milk nor muscle. Our studies showed that most of the metabolites were excreted in the feces, with only trace amounts found in other tissues. This suggests that these metabolites are poorly absorbed in the intestines (unpublished data). A full structure elucidation, for example with nuclear magnetic resonance spectrometry, could help explain why PAs are adsorbed and their ruminal metabolites not.

Demonstrating a high recovery for the ruminal *in vitro* experiment it can be assumed that all quantitative relevant ruminal metabolites have been identified. The investigation of the bovine rumen fluids from the *in vivo* experiments of the PA-SAFE-FEED project confirmed the quantitative relevance of the found metabolites. Also, the other findings were verified, as no PANOs were detectable in the rumen fluids, whereas PAs, particularly jacoline, jacobine, and jaconine, were detectable (publication 1). In addition, rumen fluid samples from sheep and goats were analyzed for PA/PANOs and their metabolites. Despite general concentration differences attributed to different experimental designs and physiological differences, similar metabolic processes were observed (publication 2). The extensive and rapid metabolism of PA/PANOs in the rumen probably explains the low levels of these compounds in the animals' muscle tissues.

Only small amounts of PA/PANOs were detected in the muscle of the animals from the PA-SAFE-FEED experiments, consistent with earlier studies (publication 2) (Fletcher *et al.*, 2011). However, this work is the first that determined the transfer parameters for the individual PA/PANOs from *Jacobaea vulgaris* Gaertn. into muscle tissue of ruminants. Interestingly, mostly jacoline, jacobine, and jaconine were detectable in all muscle samples from the three studied animal species. This correlates with *in vitro* results showing that these three PAs were eliminated more slowly in the rumen. However, other PAs, like senkirkine, erucifoline, retrorsine and seneciphylline were also found in small amounts in the *in vivo* rumen fluids of the three species. Since these PAs were also detectable in the plasma (unpublished data) but were under the limit of detection in the meat from sheep and goat, rumen metabolism probably is not solely responsible for the selective PA transfer into muscle. A study on laying hens, fed with

Jacobaea vulgaris Gaertn., showing also transfer of selected PAs like jacoline into muscle, supports the hypothesis, that also the liver metabolism influences PA transfer into meat (Mulder *et al.*, 2016). It is extensively proven that pyrrolizidine alkaloids like the ones in *Jacobaea vulgaris* Gaertn. are metabolized during the biotransformation in the liver of species like huma, rats, cattle and sheep (IPCS, 1988; Wiedenfeld and Edgar, 2011). Geburek *et al.* (2020) demonstrated that individual PAs are metabolized at different rates in experiments with rat and human microsomes, with jacoline being barely eliminated. In own studies with bovine and sheep microsomes, we also observed such differences (unpublished data), also suggesting that the liver metabolism has an effect on the selective transfer from PAs into muscle meat of cattle, sheep and goat.

With PA concentrations in meat from sheep and goats that were prematurely withdrawn from the PA-SAFE-FEED feeding study due to severe health reactions, and incorporating data from the dose-finding study conducted prior to this feeding study, we were able to demonstrate that the PA content in the muscle meat of the animals decreased rapidly and that almost no PAs were detectable after 48 hours (publication 2). However, this data is not suitable for precise kinetic evaluations, and further investigations are required to provide a more detailed analysis.

Interestingly, PA metabolites identified in the rumen were found only in trace amounts in muscle, milk, and plasma samples, yet were present in extensive quantities in feces (unpublished data). This suggests that these metabolites are unable to cross the intestinal barrier, which is unexpected given their proposed structural similarity to PAs. Own Ussing chamber experiments with epithelia from different parts of the ruminal gastrointestinal tract, showed comparable permeability for PA/PANOs and their rumen metabolites (unpublished data). Buchmueller *et al.* (2022) showed that in human Caco-2 cells, representing the intestinal barrier, echimidine and intergerimine (open diester and monoester) passed the barrier less efficient compared to monocrotaline, senkirkine, senecionine or retrorsine (cyclic diesters) (Buchmueller *et al.*, 2022). As mentioned earlier we found indications that the cyclic diesters are structurally changed in the rumen of the animals. Although the fragmentation patterns of the metabolites do not indicate a cleavage in the necic acid, it cannot be ruled out that some kind of cleavage occurs leading to steric hindrance, probably leading to a different behavior in the intestinal tract. Further studies are needed to fully understand these processes.

The metabolic processes occurring in the rumen have a substantial impact on the toxicity of PA/PANOs in animals. When ruminants consume plants that produce PA/PANOs, their livers are exposed to a different PA load compared to monogastric animals. PAs that are rapidly metabolized in the rumen tend to be quickly processed in the liver as well (publication 1, Geburek *et al*. 2020). Therefore, with eliminating these reactive PAs the rumen metabolism results in a PA load that is less reactive during hepatic metabolism. As a result, ruminants encounter a PA load with reduced toxic potential, as PAs that are less metabolically active in the liver produce fewer pyrrolic metabolites, which are associated with genotoxic and carcinogenic effects. This might give an explanation for the lower sensitivity of ruminants to the toxicity of PA/PANOs. However, there are also differences in sensitivity among ruminant species.

Studies have shown that sheep and goats can tolerate higher amounts of *Jacobaea vulgaris* Gaertn., and therefore higher levels of PA/PANOs, compared to cattle ((Goeger *et al.*, 1982b; Mortimer and White, 1975)). In terms of ruminal metabolism, one could speculate a higher ruminal transformation rate of PA/PANOs for animals that are more robust, hence for sheep and goat. However, our studies did not provide data for a clear explanation for the varying sensitivities among these ruminants. In the PA-SAFE-FEED study, sheep and goats were given higher doses of PA/PANOs compared to cattle. Despite this, sheep showed lower concentrations of PA/PANOs and their metabolites in rumen fluid than cattle, whereas goats had higher concentrations (publication 2). It is important to note that comparing PA/PANO concentrations and their metabolites in the rumen is challenging due to anatomical and physiological differences, such as relative rumen volume, saliva production, and rumen passage rates of ingested material. An adjusted study design that accounts for these factors might help clarify the role of the rumen concerning the different sensitivities. Other studies have estimated that goats and sheep have two to three times the number of PA metabolizing bacteria compared to cattle, suggesting higher metabolic rates in the rumens of these more robust species (Wachenheim *et al.* 1992). Craig *et al.* (1986) showed that sheep cope better with intravenously administered PA/PANOs compared to cattle, indicating that different levels of liver activity in the animals may also play a role for their sensitivity. Own experiments showed that bovine microsomes barely eliminated the studied PA/PANOs, while sheep microsomes partially metabolized them (unpublished data). As increased elimination is associated with increased formation of toxic pyrrolic metabolites, it remains unclear why sheep showed a higher resistance during the study of Craig *et al.* (1986). Further research is needed to better understand the precise mechanisms and differences between species.

Our study identified jacoline, jaconine, and jacobine as the predominant pyrrolizidine alkaloids present in the meat samples. Similarly, Knoop *et al.* (2024) reported that these three PAs were the primary compounds transferred to cattle milk in the PA-SAFE-FEED feeding studies. Compared to the original plant material, the animal's body appears to reduce the overall PA/PANO burden, effectively filtering out PAs such as senecionine, seneciphylline, erucifoline, riddelliine, and retrorsine, since some of these filtered compounds are known to be activated in the liver, forming toxic pyrrolic metabolites. Even though jacobine, which is transferred to the muscle and meat of the animals, also was found to produce significant amounts of pyrrolic metabolites, while jacoline did not form these metabolites (Geburek *et al.*, 2020). Nevertheless, in general the toxic PA burden is reduced by the animal body raising the question of whether such PA burdens should be considered less concerning. Currently, the classifies all PAs as equally potent regarding their toxicity. However, there are studies showing different hepatic reactivity, that is why several studies propose to use potency factors for PAs (Allemang *et al.*, 2018; Frei *et al.*, 1992; Haas *et al.*, 2023; Lester *et al.*, 2019; Merz and Schrenk, 2016). Some feeding studies also have shown that calves or rats fed milk from cattle that consumed high levels of *Jacobaea vulgaris* Gaertn. experienced little to no adverse effects. This raises the question if jacoline, the primary PA transferred to milk, may be less toxic than other PAs in the plant (Johnson, 1976; Miranda *et al.*, 1981).

Contrary, Goeger *et al.* (1982b) observed adverse effects in rats fed contaminated milk and those fed equivalent amounts of *Jacobaea vulgaris* Gaertn., suggesting that the pyrrolizidine alkaloids PAs transferred to the milk are as potent as those found in the plant.

Beside filtering reactive PAs, we showed that the metabolic activities in the ruminants also lead to transfer of only small amounts of PAs into meat. The PA concentrations found in the meat of animals receiving the highest PA/PANO dosages in the PA-SAFE-FEED study were used to calculate a theoretical MOE (EFSA, 2005). This calculation utilized a $BMDL_{10}$ of 237 µg/kg bw/day as the toxicological reference point for PAs and considered estimated consumption of cattle and sheep meat (EFSA, 2011; EFSA *et al.*, 2017). Goat meat was not considered due to its low consumption. For cattle and sheep meat, MOEs were found to be around or above 10,000 (publication 2). It has to be considered that animals in the PA-FEED-SAFE trial received their PA/PANO administration once or twice a day, probably influencing the transfer. In a natural scenario, animals consume PA/PANOs gradually throughout the day with their feed, probably leading to more efficient metabolism. Therefore, even if the animals ingest PA/PANOs at levels as high as the highest dosage used in our study, the MOE should remain above 10,000. Moreover, with average PA/PANO concentrations of 0.29 mg/kg in forage and roughage, and even lower levels in silages, the PA/PANO contamination in feed across Europe theoretical results in lower doses than those used in our study (Bolechová *et al.*, 2015; EFSA, 2011; Gottschalk *et al.*, 2015; Mulder *et al.*, 2009). Consequently, margin of exposure values below 10,000 are questionable. According to the risk assessment concept, an MOE of 10,000 or higher is considered to be of low concern from a public health perspective and is deemed a low priority for risk management actions. Additionally, it is important to consider that muscle tissue measured in the PA-SAFE-FEED study may undergo processing or aging before being consumed, which could potentially lower the PA concentration further.

When evaluating our feeding study, it is crucial to consider several factors to accurately translate the findings to husbandry conditions. Research by Johnson *et al.* (1984, 1985) highlights that the toxic effects of PA/PANOs are influenced by the mode of administration. Their studies demonstrated that toxicity varies depending on whether PA/PANOs are given as a single bolus or distributed throughout the day. In natural settings, ruminants consume their feed gradually, allowing for continuous rumination and digestion over an extended period. This results in the gradual absorption of PA/PANOs, unlike the typical single-dose administration in feeding studies. Johnson *et al.* (1984, 1985) concluded that the rapid absorption from a one-shot bolus leads to more pronounced toxic effects. In contrast, the slow intake of PA/PANOs in the rumen likely facilitates more effective metabolism and detoxification. Therefore, studies involving ruminants must take these effects into account and design their experiments accordingly. Theoretically, the form in which the PA/PANO bolus is administered could also influence toxicity. For instance, administering the dose as intact plant parts might result in a slower release compared to using plant extracts, where the PA/PANOs are already dissolved. However, the literature does not provide clear evidence to support this hypothesis (Johnson and Smart, 1983).

In the PA-SAFE-FEED feeding studies, PA/PANOs were administered as extracts via a stomach tube once per day. Based on the observations of Johnson *et al.* (1984, 1985), we assume that the chosen method of administration allowed more PA/PANOs to pass unmetabolized through the rumen compared to ingestion of whole plants. Consequently, it is hypothesized that if the chosen doses had been administered under natural feeding conditions, they would have resulted in less health effects and transfer rates. Despite these considerations, feeding studies must balance multiple requirements. From an analytical perspective, using an extract is advantageous as it enables the creation of a homogeneous mixture, ensuring that all animals receive identical amounts and compositions of PA/PANOs during the feeding trial. Achieving such homogeneity with *Jacobaea vulgaris* Gaertn. plants would not have been feasible. Additionally, administering the dose as plant material could have posed challenges for small ruminants due to the volume required, potentially impacting their feeding behavior and acceptance. However, it was subsequently found that using extracts also presented problems. The extraction process likely altered the PA/PANO profile. The extract contained more free bases than are typically found in plants, which usually have a PA/PANO ratio of about 0.1, whereas the extract had a ratio of 2.0. Since PANOs are very quickly reduced to PAs in the rumen of animals, this difference can be neglected. However, the extract also contained higher concentrations of jaconine compared to plants, which could have potentially influenced the transfer and toxicological effects.

In general, animal studies are difficult to compare. Even under similar conditions, differences in observations can occur (Johnson and Smart, 1983). For example, two studies that used comparable PA/PANO doses over a similar period of time as the PA-SAFE-FEED study, reported much stronger symptoms, including death. This was observed even in one study where PA/PANOs were administered throughout the day as plant pellets (Johnson and Smart, 1983; Thorpe and Ford, 1968).

Currently, maximum levels of PA/PANOs in foodstuffs are regulated only for certain plant-based foods and dietary supplements. According to Regulation (EU) 2023/915, these foods must be tested for 35 specific PA/PANOs. This selection primarily includes PA/PANOs that are prevalent in various plant species from different families, aiming to cover the PA/PANO contamination in foods with a manageable number of individual substances. Contaminations with *Jacobaea vulgaris* Gaertn. and *Senecio* plants can be detected by monitoring PA/PANOs such as retrorsine, senecionine, and seneciphylline, along with their corresponding *N*-oxides. If efforts are made to protect consumers from PA/PANO contamination in animal-derived products, the existing scope would not be useful because the activities of the rumen and liver lead to an altered PA-profile.

In conclusion, it can be debated whether ruminants can be fed contaminated feed or grazed on pastures containing *Jacobaea vulgaris* Gaertn. Two aspects need to be considered: animal welfare and consumer protection. According to §3 of the animal welfare act, it is prohibited to feed an animal with substances that cause pain, suffering, or harm. In the highest dosage group of the PA-SAFE-FEED cattle experiment enzyme levels increased over the four weeks of the trial, indicating the beginning of liver damage (Knoop *et al.*, 2023). Knoop *et al.* (2023) suggest that liver damage could also occur in animals from

lower dosage groups when exposed to PA/PANOs over extended periods. As a result, the authors recommend that cattle exposure to PA/PANOs be avoided. In the PA-SAFE-FEED study, sheep and goats exhibited more pronounced reactions, with several animals needing to be excluded from PA/PANO dosing prematurely (unpublished data). Interestingly, Ohlsen *et al.* (2022) reported that all sheep remained healthy, despite potentially higher doses and longer exposure times. This raises the possibility that under real-world conditions, equivalent doses used in the PA-SAFE-FEED project may not lead to the same level of harm or suffering in animals. Additionally, it is important to consider that PA/PANO-producing plants, such as *Jacobaea vulgaris* Gaertn., are seasonal, raising questions about the feasibility of chronic exposure. For instance, the highest dosage in the PA-SAFE-FEED cattle trial corresponds to the consumption of 20-30 *Jacobaea vulgaris* Gaertn. plants per day per animal.

Evaluating the risk to ruminants from PA/PANO exposure is complex, given the multiple factors involved, such as animal health, ecological considerations, and other interests. It is crucial for decision-makers to carefully examine the data, including those generated in this doctoral thesis and the PA-SAFE-FEED project, and to assess it objectively. This will enable informed, beneficial decisions to be made that prioritize both animal health and consumer safety.

Most of the scientific research on pyrrolizidine alkaloids, particularly on ruminal metabolism and feeding studies, was conducted between 1960 and 1990. Several factors may explain why only a few groups continued to focus on this topic after that period. One likely reason might be, that with the knowledge gained during those decades, improved forage and grassland management practices were implemented, reducing the incidence of PA intoxication in ruminants. As a result, scientific attention shifted to other areas of interest. The decline in feeding studies can also be attributed to stricter ethical standards, which have made it more challenging to obtain approval for such experiments. However, it remains intriguing that research on ruminal metabolism was not fully extended to cyclic diesters PAs. These compounds are present in some of the plants crucially affecting the health of grazing animals, and thus, they should have been of considerable interest.

However, the present study, could elucidate the fate of these pyrrolizidine alkaloids from *Jacobaea vulgaris* Gaertn. ingested by ruminants. It was found that highly toxic PAs are primarily metabolized into non-toxic compounds in the rumen of these animals. Consequently, the transfer of these PAs in the tissues of the animals, such as meat, is low. Given the minimal carry-over, it can be assumed that, considering the observed contamination levels of feed with pyrrolizidine alkaloids in Europe, there is a low risk to consumers from PA/PANOs in animal-derived food products.

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6. Cumulative part

6.1. 1. Publication

Rumen Metabolism of Senecio Pyrrolizidine Alkaloids May Explain Why Cattle Tolerate Higher Doses Than Monogastric Species

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Rumen Metabolism of *Senecio* Pyrrolizidine Alkaloids May Explain Why Cattle Tolerate Higher Doses Than Monogastric Species

Julian Taenzer, Matthias Gehling, Fenja Klevenhusen, Janine Saltzmann, Sven Dänicke, and Anja These

Abstract: Rumen metabolism of *Senecio* pyrrolizidine alkaloids (PAs) and their *N*-oxide forms was studied by mass spectrometry in *in vitro* batch culture incubates and confirmed in *in vivo* samples. Most *N*-oxides were found to undergo rapid conversion to their corresponding free bases, followed by biotransformation to metabolites hydrogenated at both the necine base and the necic acid moiety. Therefore, rumen metabolism can be considered a detoxification step, as saturated necine base structures are known as the platyphylline type, which is regarded as less or nontoxic. Individual *Senecio* PAs, such as jacoline, are metabolized slowly during rumen fermentation. PAs that showed limited biotransformation in the rumen in this study also showed limited transformation and CYP-mediated bioactivation in the liver in other studies. This could not only explain why PAs that are comparatively metabolically stable can pass into milk but also suggest that such PAs might be considered compounds of lesser concern.

Keywords: *Jacobaea vulgaris, Senecio jacobaea, pyrrolizidine alkaloids, rumen fermentation, plant toxins, metabolism, detoxification, N-oxides, ragwort, mass spectrometry*

Introduction

In recent years, the spread or change in occurrence of plants belonging to the genus *Senecio* has caused increasing debate, but systematic surveys on this issue are rare. For example, an increasing spread of ragwort (*Jacobaea vulgaris*) has been observed in northern Germany, causing concern among farmers and consumers because of the potential risks to human and livestock health.[1−4] Reasons for the generally high spreading potential of ragwort are likely to be the high germination capacity of the seeds combined with the low demands on soil quality. The extent to which such observation of population dynamics was short-lived is not easy to clarify but must be taken into account. A comprehensive survey of ragwort population trends in the United Kingdom over a 30 year period found that *Senecio* abundance both significantly increased and decreased within that time period. Over the entire period, however, there were no changes in abundance or frequency, and it was concluded that no long-term trends in ragwort populations were evident.[5] *Senecio* plants contain hepatotoxic and carcinogenic pyrrolizidine alkaloids that occur in plants as a free tertiary base form (denoted as PAs in this article) and their corresponding *N*-oxides (denoted as PANOs in this article).[6] The *N*-oxides account for most of the total PA/PANO content in plants, approximately 90%.[7,8] *Senecio* plants form (macro)cyclic PA/PANO diesters that, like all toxicologically relevant PA/PANOs, bear a CC double bond in the 1,2-position of the necine base.[8−11] In Figure 1, the major alkaloids of the genus *Senecio* are shown. During hepatic metabolism,

bioactivation occurs through oxidation of the 1,2unsaturated pyrrolizidine ring to reactive intermediates like pyrrolic metabolites, which are considered to cause toxic effects to humans and animals.[6,12–16] In farm animals, poisonings related to *Senecio* spp. had already been known since the end of the 19th century and were described, for example, under the names “walking disease” (USA), “dunziekte” (South Africa), “Winton disease” (New Zealand), or “Schweinsberger disease” (Germany).[17]

Concerning PA susceptibility, marked differences between farm animal species were reported with a comparatively high susceptibility in pigs, followed by cattle, while goats and sheep appear to be almost resistant.[18,19] These observations could be explained by differences in enzymatic activities, resulting in different overall balances of the detoxification and activation pathways during metabolism. Ruminants as foregut fermenters seem to tolerate higher doses of harmful secondary plant metabolites, and rumen microbial activity has been discussed as a cause of relative resistance to PA poisoning compared to monogastric animals.[18–21] However, varying degrees of tolerance to PA were also found between ruminant species.[22,23] A study by Wachenheim et al. investigated *in vitro* the biotransformation of *Senecio* PA/PANOs in the rumen inoculum of goats, sheep, and cattle.[24] The authors found the highest transformation rate in goats, followed by sheep in a comparable range and cows with an order of magnitude difference. They also showed that rumen bacteria play an important role in the detoxification of PA/PANOs, but identification of the metabolites was hampered by the limited

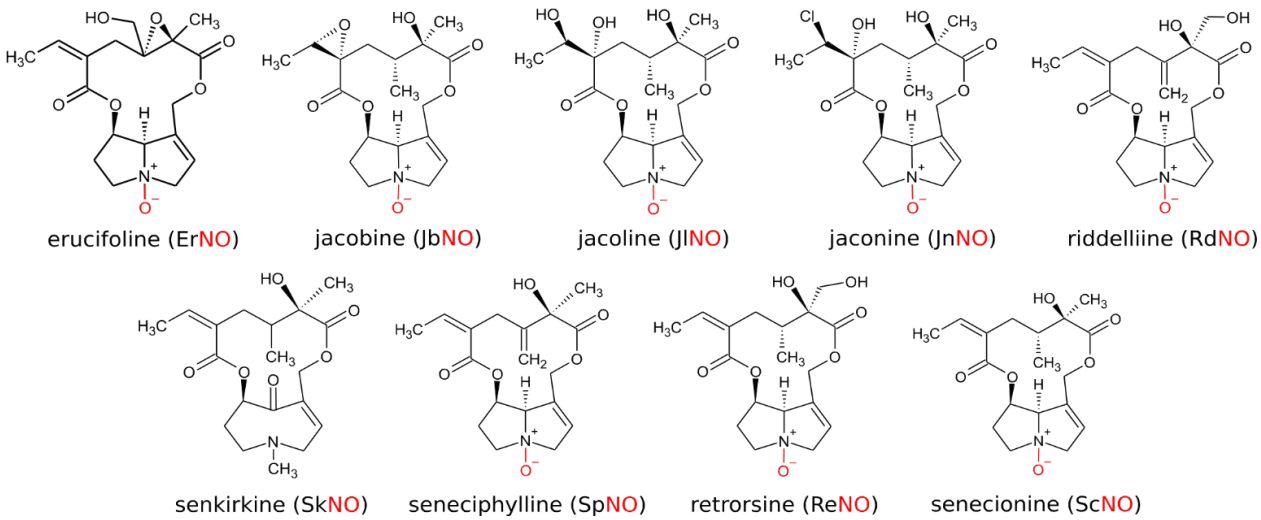

***Figure 1**: Structures of the major pyrrolizidine alkaloids produced by Senecio plants. Each compound occurs both as free base form and corresponding N-oxide (marked in red).*

technical capabilities available at the time.[24] Mulder et al. demonstrated a transfer of PANOs into their corresponding tertiary base form *in vivo* and *in vitro*, but no further identification of rumen metabolites was established.[25]

However, rumen metabolism of PAs not only appears to play a critical role in relative resistance to PA poisoning.

Rather, rumen metabolism should also be considered in terms of whether it could explain why individual PAs have higher transfer rates to milk than others. Carry-over studies conducted so far have shown limited transfer of PAs into milk. Dickinson et al. conducted a two-week feeding study with lactating cows applying a dose of 1% plant material related to bodyweight (16 mg/kg bw *Jacobaea vulgaris* PAs) for a period of 5−7 days and then gradually decreased the dose by 50−75%.[26] The specific alkaloids determined in the fed plant material were jacobine, seneciphylline, jacoline, jaconine, and jacozine (detection of PAs and PANOs as sum), although subsequently only jacoline was identifiable in milk. The concentrations in milk ranged from 94 to 167 μg/L, or 470−835 μg/L when corrected for a reported recovery rate of 20%; that is about 0.1% of PAs was estimated to transfer into the milk. During the feeding period, 25−100 mL/kg bw/day of the obtained milk was given to respective calves. As no changes or lesions were observed in the calf liver, the authors concluded that the specific toxicity of jacoline would have to be tentatively evaluated as low. They further concluded that due to the selected transfer of jacoline, a biological transformation of the other alkaloids took place prior to secretion into the milk or the preferred adsorption of jacoline from the gastrointestinal tract might be a decisive factor. Hoogenboom et al. conducted a carry-over study that was accompanied by a comprehensive analysis of all relevant matrices for a broad set of PA compounds.[27] Dairy cows were administered for three weeks with increasing amounts (50− 200 g/day) of dried ragwort, which had a PA content of 2.3 g/ kg. These ragwort dosages were 20−100 times lower than those applied by Dickinson et al., but comparable results were obtained with estimated carry-over rates of PAs of about 0.1%. Higher rates were found for jacoline with 4% and otonecine type PAs, such as senkirkine. Besides other pharmacokinetic parameters, extensive metabolism of these compounds in cattle may be the reason for the comparatively low carry-over.

Analysis of data from several studies shows effective biotransformation of PA/PANOs in cattle.[25,27] Mulder et al. determined an overall balance of 2.9 to 4.5% depending on the *Senecio* species administered, indicating that only a small portion of the doses administered can be quantified and the fate of these substances is more or less unknown.[25] In particular, valid exposure and dose response data for toxicologically relevant analytes are needed for different livestock species. Data available to date indicate that ruminants can tolerate higher doses of ragwort. Therefore, the aim of this study was to investigate rumen metabolism including identification of ruminal metabolites of *Senecio* PA/PANOs, which could explain the lowered susceptibility of ruminants toward PA toxicity.

Material and Methods

PAs such as senecionine, senecionine *N*-oxide, retrorsine, retrorsine *N*-oxide, seneciphylline, seneciphylline *N*-oxide, jacoline, jacoline *N*-oxide, jaconine, merenskine *N*-oxide (isomer of jaconine *N*-oxide), jacobine, jacobine *N*-oxide, erucifoline, and erucifoline *N*-oxide were purchased from Phytoplan (Heidelberg, Germany) or in the case of riddelliine and riddelliine *N*-oxide from Oskar Tropitzsch (Marktredwitz, Germany). Methanol and water (both LC−MS grade) were purchased from

Merck KGA (Darmstadt, Germany). Ingredients of the *in vitro* incubation buffer were obtained from Carl Roth (Karlsruhe, Germany). All chemicals obtained were of the highest purity that was commercially available.

***In vitro* Batch Culture System.** *Source of Rumen Fluid.* The collection of rumen content to conduct the *in vitro* studies was approved by the Berlin State Office for Health and Social Affairs (LaGeSo, number G 0319/18). Rumen content (liquid and solids) was collected from three multiparous fistulated lactating and nonlactating Holstein cows (between 3 and 6 years old) 3 h after the morning feeding. The cows were kept according to the German Animal Welfare Act and were fed with a partial mixed ration containing 230 g of grass silage, 245 g of maize silage, 50 g of straw, 250 g of hay, 170 g of rape seed meal, 50 g of beet pulp, and 5 g of vitamin−mineral mixture per kg dry matter (DM). A milk performance concentrate mixture (containing barley, wheat, rapeseed meal, molasses, calcium carbonate, sodium chloride, magnesium oxide, Ca/Na phosphate, and monocalcium phosphate) was individually provided according to their energy requirements for milk yield. The components of the daily ration were virtually free from PA. Additionally blank samples in the *in vitro* studies were tested by LC−MS and did not contain PAs (LOQ). Equal amounts from the liquid and solid phase from the rumen content of all three cows were pooled and deoxygenated to maintain anaerobic conditions. One part of this merged rumen inoculum was mixed with four parts of a simplified phosphate−bicarbonate buffer as described by Mould et al.[28] This solution was homogenized (Ultraturrax TP 8/10, Janke & Kunkel (IKA), Staufen, Germany), rinsed with gaseous nitrogen, and kept at 39 °C.

Composition of PA/PANO Mixtures. Subsequently, 10 ± 0.5 g of the rumen mixture was filled into 25 mL Hungate tubes and spiked with (1) a PANO mixture (containing the same proportion of erucifoline *N*-oxide, jacobine *N*-oxide, jacoline *N*-oxide, merenskine *N*-oxide [isomer of jaconine *N*-oxide], retrorsine *N*-oxide, riddelliine *N*-oxide, senecionine *N*-oxide, and seneciphylline *N*-oxide), resulting in a final concentration of 14.7 μg per PANO/mL, or (2) a PA mixture (containing the same proportion of erucifoline, jacobine, jacoline, retrorsine, riddelliine, senecionine, and seneciphylline) with a final concentration of 14.7 μg per PA/mL, or (3) single PA dissolved in MeOH/H_2O (5/95, v/v). Incubation with PANO and PA mixtures was repeated three times over the course of two months. Each incubation was performed in duplicate, resulting in six replicates.

Experimental Protocol for Incubations. After flushing with nitrogen once again, the tubes were sealed with rubber stoppers and aluminum crimp caps. The tubes were incubated for various time periods (0, 0.5, 1, 2, 4, 6, and 20 h) at 39 °C while being shaken at 250 rpm (simulation of 1−2 rumen contraction per minute). The incubation was stopped by adding 14 mL of 0.05 M H_2SO_4. Samples were centrifuged at 363 g for 15 min (Thermo Fisher Scientific Multifuge X1R Pro with a TX-400 rotor, Waltham, USA), and 500 μL of the supernatant was filtered through a centrifugal filter (modified Nylon 0.2 μm, VWR, Radnor, USA) at 23,500 g for 10 min (Eppendorf 5424 R centrifuge with an FA-45-24-11 rotor, Hamburg, Germany). Samples were stored at 5 °C until mass spectrometric analysis.

The storage period was not more than 14 days. The redox potential and the pH were measured before incubation and after each sampling to ensure rumen physiological conditions. Gas pressure was measured hourly with a gas transducer to confirm fermentation (GMH 3161-07-EX, GHM Messtechnik GmbH, Regenstauf,
Germany). Gas production was calculated according to Mauricio et al. To distinguish between nonenzymatic and enzymatic reactions, controls were included in each run.[29] The control approach was performed in the same way as the incubated samples, but the ruminal inoculum had been autoclaved at 121 °C for 3 h prior to incubation.

***In vivo* Samples.** *In vivo* samples were obtained from a feeding study with dairy cows, which was conducted in June and July 2020 by the Friedrich-Loeffler Institute within the framework of the carry over-project "PA-SAFE-FEED".[30] The feeding study was performed in agreement with the German Animal Welfare Act accepted by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Germany (protocol number 33.19-42502-04-19/3191). The study was conducted with 20 lactating cows, which were subdivided in five groups (n = 4 per group). The cows housed in group pens, which were equipped with slatted floors and high bed cubicles. Water and a total mixed ration, which consisted of maize silage (30%), grass silage (30%), and concentrate feed (40%) on DM basis, were offered ad libitum. Two control groups were treated with water or molasses, respectively, while three groups were administered a *Jacobaea vulgaris* extract for 28 days. The extract was obtained through multiple extractions of dried *Jacobaea vulgaris* (harvested in summer 2019) with MeOH/H_2O (90/10, v/v). After extraction, MeOH was removed by evaporation. PA/PANO concentration in the extract was determined by means of LC−MS/MS after dilution with MeOH/H_2O (5/95, v/v).
With regards to the individual body weight and dose group of the cows, a certain amount of the extract was weighed and made up with molasses so that all cows received a similar amount of carbohydrates.
This mixture was dissolved in 800 mL of water and administered through a gavage directly to the rumen of the cows. Extract amounts were chosen to meet the respective PA doses of 0.45, 0.9, and 1.8 mg/ kg bw/day.
Samples of ruminal fluid were taken with a gavage directly before 1.5 and 24 h after PA bolus administration at days 0, 7, 14, and 28 of the trial. Metabolic activity in the *in vivo* samples was stopped by freezing them, and the samples were shipped to the BfR. Reactivation after thawing was prevented by adding 75 μL of MeOH to 425 μL of the sample. Finally, the samples were filtered through a centrifugal filter (modified Nylon 0.2 μm, VWR, Radnor, Pennsylvania) at 23,500 g for 10 min (Eppendorf 5424 R centrifuge with an FA-45-24-11 rotor, Hamburg, Germany). Without further preparation, the samples were stored at 5 °C until mass spectrometric analysis. The storage period was not more than 14 days.

Detection Using Liquid Chromatography Combined with High-Resolution Mass Spectrometry. Chromatographic separation was achieved using an UltiMate 3000 ultrahigh-performance liquid chromatography system (Thermo Fisher Scientific, Waltham, USA) in combination with a 150 mm × 2.1 mm 1.9 μm C18 Hypersil Gold column with guard protection (Thermo Fisher Scientific, Waltham, USA). The column temperature was maintained at 40 °C, and the injection volume was 2 μL. The solvent consisted of H_2O (A) and MeOH (B) containing 0.1% formic acid and 5 mM ammonium formate. Samples were eluted at a flow rate of 0.3 mL/min with a gradient as follows: 0−0.5 min A: 95%/B: 5%, 7.0 min A: 50%/B: 50%, 7.5 min A: 20%/B: 80%, 7.6 min A: 0%/B: 100%, and 10.1−15 min A: 95%/B: 5%.

The LC system was coupled to a Q-Exactive Focus high-resolution hybrid quadrupole−Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, USA). All samples were measured in positive ionization mode using the variable data-independent acquisition acquiring a full scan in the range of *m*/*z* 100−1500 with a resolution of 70,000 for quantitation as well as MS2 data by fragmentation of three mass range windows (*m*/*z*: 100−500; 500−1000; and 1000− 1500) applying a resolution of 17,500 for confirmation using a collision energy of 36 eV for all three mass range windows. As source parameters, the following values were applied: ion spray voltage: 5000 V, capillary temperature: 270 °C, vaporizer temperature 300 °C, sheath gas pressure 45 psi, aux valve flow 10 psi, and ion sweep gas pressure 10 psi. High-resolution product ion scans (ddMS2) were acquired to confirm rumen metabolites of PAs applying a collision energy of 35 eV and a resolution of 17,500 using a precursor ion width of 1 amu.

To ensure the validity of quantitative data, the following measurements have been performed. First, at the beginning of each sequence, a PA mix is injected to test the performance of the LC−MS system in terms of sensitivity of MS response and stability of retention time. Second, a spiked matrix blank is included in each sequence to verify sample preparation. The acceptance criteria for routine recovery should range between 60 and 140%. Quantitation of the PANO/PA was achieved with a 11-point matrix-matched standard calibration (0.25, 0.5, 1.0, 2.5, 5.0, 15, 30, 60, 120, 240, and 360 ng/mL). A weighted calibration is used, and it is checked whether the accuracy of the back-calculated concentration of the respective calibration level using the calibration curve is in the range of 80−120%. Since no metabolite was available as the standard for metabolite quantification, their concentrations were semiquantitatively estimated using retrorsine as the calibrant, assuming the same mass spectrometric response.

Identification of Metabolites. Metabolites were identified using the untargeted workflow of the compound discoverer software (Thermo Fisher Scientific, Waltham, Massachusetts) in combination with mass spectrometric screening tools such as the precursor ion scan. To be identified as a metabolite, candidates had to meet the following conditions: (1) metabolites were not allowed to be in the blank or control samples and (2) the fragmentation of the metabolites had to show fragments characteristic for PAs. The sum formula of the metabolites was predicted based on their accurate mass. The deviation of the measured accurate mass and the sum formula derived for the metabolites had to be below 1 ppm

including the necessity of a matching isotopic pattern. Structures were suggested based on the fragmentation pattern of product ion spectra. Product ion spectra for selected ruminal metabolites are provided as a supplementary material to illustrate the fragment ions and neutral losses that were used for interpretation and creation of structure proposals.

Calculation of Half-Lives. The PA concentration over time was taken to calculate individual half-lives. Using R (version 4.0.2 from cran.r-project.org) with RStudio (version 1.3.1093), a local regression (LOESS) was derived for each PA and used to estimate half-lives.

Software and Statistics. Variance analysis was performed via one- or two-way analysis of variance (ANOVA) with SPSS 26.0.0.1 (IBM, Armonk, New York). The significance level was set to $p \leq 0.05$. Only data from PAs and PANOs were tested.

Results and Discussion

***In vitro* Incubation of *Senecio* PANOs and PAs.** Individual PA and PANO standards were incubated with inocula from the rumen in order to identify metabolites of ruminal metabolism based on mass spectrometry.

PANO Mixture. To study and compare the behavior of *N*oxide forms, a PANO mixture was subsequently incubated, since they are the most important fractions in plants in terms of quantity. For all PANOs tested, the concentration decreased rapidly. After 30 minutes of incubation, on average only 5 ± 1% of the initial concentration was present, and after 1 h, only traces were measurable (Figure 2). No differences in the rate of degradation were detectable between individual PANOs. Almost to the same extent as the *N*-oxide (PANO) concentration decreased, the concentration of the corresponding tertiary base (PA) increased. On average, after 1 h, 85 ± 10% of the initial PANO content could be quantified as the corresponding PA. Lower formation rates were only found for jaconine and jacobine, which were 30 ± 6 and 48 ± 13%, respectively (Figure 2).

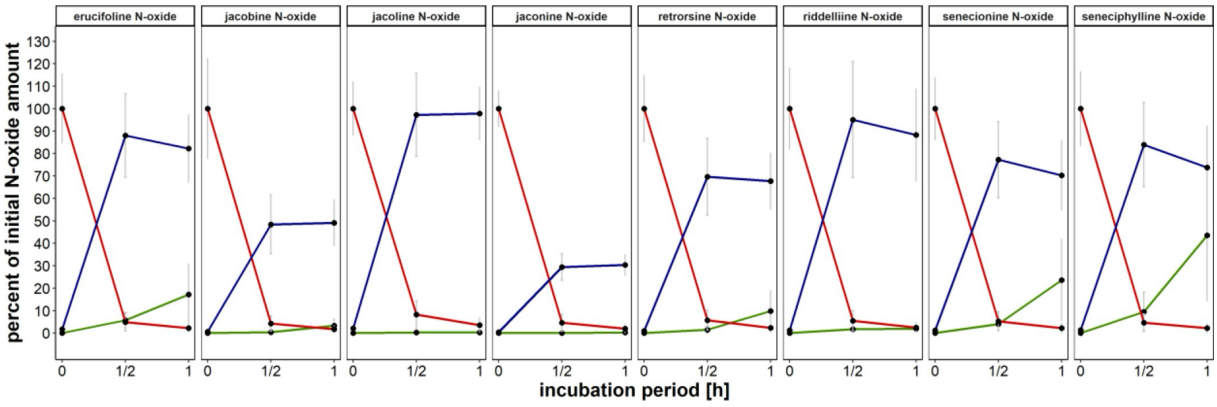

Figure 2*: Incubation of a mixture of Senecio PANO standards (structures shown in Figure 1) with rumen inoculum from fistulated cows (n = 6). The concentration plots during the first hour of incubation show that the PANO concentration decreases (red lines), while at the same time the concentration of the corresponding PA increases (dark blue lines). The subsequent degradation of PAs is denoted by the formation of further metabolites, which are shown as green line. Error bars show the standard deviation of the measurements.*

PA Mixture. Further degradation of PAs was investigated by incubation of a PA mixture. As shown in Figure 3 after 20 h, the majority of *Senecio* PAs tested was on average degraded to below 1% of the initial level, but differences in the kinetics were observed. This can be expressed by determining the halflives of the respective PAs in the incubation experiments (Figure 4). While seneciphylline and senecionine showed the fastest degradation, riddelliine, erucifoline, and retrorsine ranged in the middle, and slower degradation rates and thus higher resistance to rumen metabolization were observed for jacobine, jaconine, senkirkine, and especially jacoline of which 73 ± 8% was still detectable after 20 h (Figure 4).

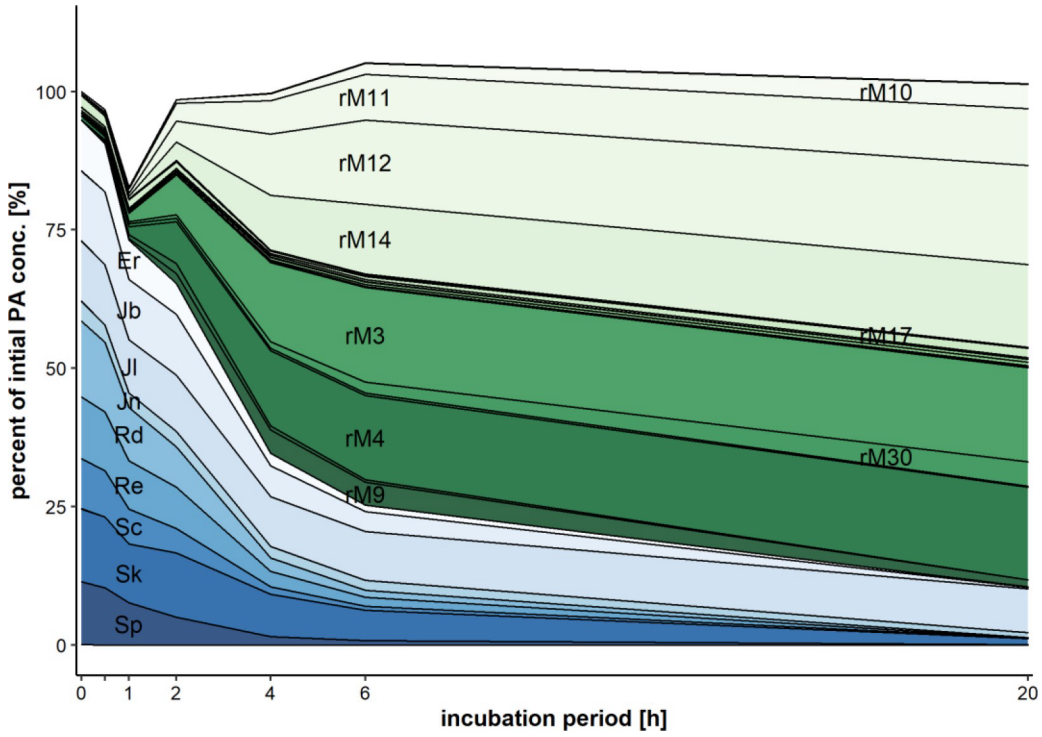

***Figure 3**: Incubation of a mixture of Senecio PA standards (structures shown in Figure 1) with inocula from rumen-fistulated cows. Samples were taken at t = 0, 0.5, 1, 2, 4, 6 and 20 h. Shown are the concentrations of the PAs and their ruminant metabolites (rM). Analytes in blue represent 1,2unsaturated PAs while metabolites are shown in green which are mostly 1,2-saturated. Data present the average of three experiments, each incubated in duplicates. With Er for erucifoline, Jb for jacobine, Jl for jacoline, Jn for jaconine, Rd for riddelliine, Re for retrorsine, Sc for senecionine, Sk for senkirkine and Sp for seneciphylline.*

These data are in line with the study of Mulder et al. in which the authors already demonstrated a conversion of the *N*-oxides into the free base.[25] Nevertheless, they reported much slower transformation rates. This could be because Mulder et al. incubated plant material, implying that some delay in metabolism could result from the additional time required for digestion of the plant material, including PA/PANO extraction, prior to rumen degradation. In addition, Mulder et al. used filtered rumen liquids, while in our study, solid parts from the rumen were also used for incubation. A study by Wachenheim et al. demonstrated that ruminal solids increased the degradation rate of macrocyclic *Senecio* PA.[24] Therefore, it is possible that microorganisms relevant for the degradation rather adhere to the solid parts, resulting in a higher microbial density and consequently higher degradation rates of PAs. We verified these findings by our experiments and confirmed a slower degradation when applying filtered ruminal fluid only for incubations (data not shown).

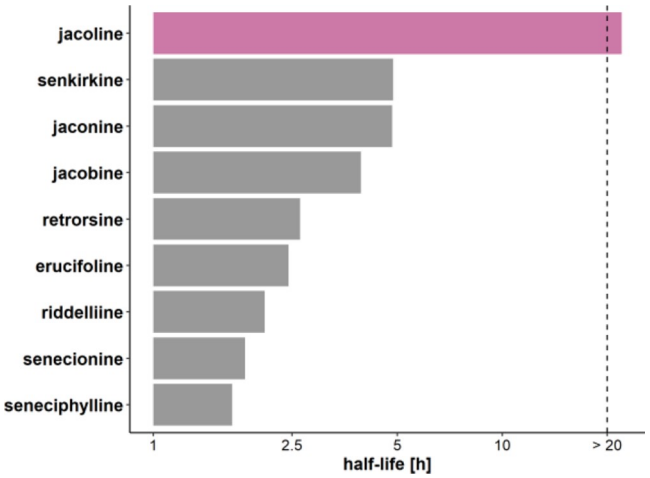

***Figure 4**: Half-lifes [h] of PAs determined by incubation with rumen inoculum from fistulated cows. Shown are the mean values resulting from incubations of Senecio PA and PANO mixtures performed in three biological replicates each, where each replicate was performed as a technical duplicate (in total n = 12). No value could be estimated for jacoline because 73% of jacoline was still detectable after 20 h (shown in purple).*

Identification of Rumen Metabolites and Balancing of Overall Recovery. As shown before, all tested *Senecio N*oxides (PANOs) were reduced to their corresponding PAs, which in turn undergo further degradation. For congeners with short half-lives, like senecionine, even during short incubation times, a further degradation of the free base form (PA) was already detectable (Figure 2). Based on mass spectrometric fragmentation, it was found that the ruminal metabolites formed were saturated in the necine base; i.e., the double bond in the 1,2-position of the ring system was hydrated by the ruminal microbes. This reaction could be observed as a common principle for all *Senecio* PAs (Figure 5). Such 1,2saturated necine base structures formed during ruminal

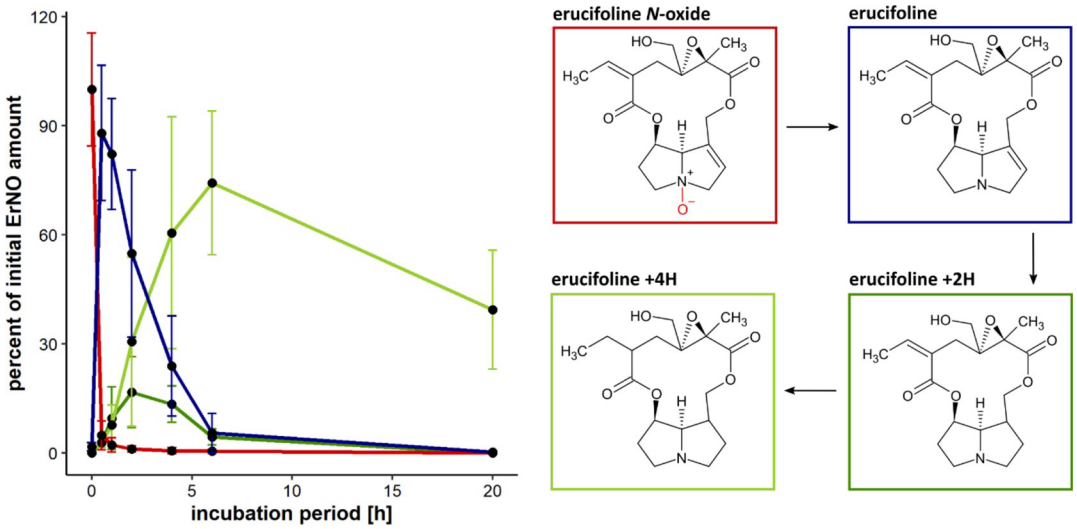

***Figure 5**: Overview of structural changes of pyrrolizidine alkaloids during rumen metabolism using erucifoline N-oxide (ErNO) as an example. All N-oxides present in Senecio plants are transformed into the corresponding PA, followed by the reduction of double bonds present in the necine base and necic acid.*

metabolism are also produced by plants and are known as platyphylline type PA. This structure type is described as less or nontoxic as the double bond is generally considered as the precondition for PAs to exert their liver toxicity.[12,31] Consequently, the transformation of 1,2-unsaturated ring PAs into their saturated forms can be considered as the detoxification step. Further transformation steps could be elucidated and described as reduction of the double bonds present in the necic acid moieties (Figure 5). In addition, several minor metabolites resulting from acetylation (rM24, rM25), propionylation (rM27), and epoxide opening (rM16) could be detected. A total of 36 metabolites were identified after *in vitro* incubation, most of which were 1,2-saturated (Table 1).

***Table 1**: Identified Rumen Metabolites of Senecio PANO/PA Including Mass Spectrometric Information Relevant for Detection[a].*

category [b]	ruminal metabolite	sum formula	monoisotopic mass	$(M+H)^+$	confirming-ion 1 (*m/z*)	confirming-ion 2 (*m/z*)
A, B, C, D, E	rM3	$C_{18}H_{27}NO_5$	337.1887	338.1960	122.0965	140.1072
A, B, C, D, E	rM4	$C_{18}H_{29}NO_5$	339.2043	340.2119	122.0964	140.1070
A, B, C, D, E	rM10	$C_{18}H_{27}NO_6$	353.1836	354.1910	140.1071	122.0964
A, B, C, D, E	rM11	$C_{18}H_{27}NO_6$	353.1840	354.1912	140.1070	122.0964
A, B, C, D, E	rM12	$C_{18}H_{27}NO_6$	353.1833	354.1928	122.0964	140.1070
A, B, C, D, E	rM14	$C_{18}H_{29}NO_6$	355.1993	356.2062	140.1071	122.0966
B, C, D, E	rM9	$C_{18}H_{27}NO_6$	353.1836	354.1907	140.1071	122.0964
A, C, E	rM32	$C_{18}H_{25}NO_5$	335.1727	336.1806	140.1071	122.0965
A, C, E	rM3b	$C_{18}H_{27}NO_5$	337.1884	338.1960	140.1072	122.0965
A, C, E	rM8	$C_{18}H_{25}NO_6$	351.1679	352.1758	140.1069	122.0964
B, D, E	rM30	$C_{18}H_{27}NO_6$	353.1833	354.1911	140.1071	122.0965
C, D, E	rM7	$C_{18}H_{25}NO_6$	351.1679	352.1755	122.0964	140.1070
C, D, E	rM17	$C_{18}H_{29}NO_7$	371.1944	372.2018	140.1070	122.0964
C, D, E	rM18	$C_{18}H_{29}NO_7$	371.1946	372.2021	140.1071	122.0965
C, E	rM6	$C_{18}H_{25}NO_6$	351.1682	352.1752	120.0808	138.0914
D, E	rM1	$C_{16}H_{27}ClN_2O_2$	314.1761	315.1532	140.1070	96.0808
D, E	rM2	$C_{14}H_{27}NO_8$	337.1731	338.1864	120.0809	138.0915
D, E	rM29	$C_{19}H_{29}NO_5$	351.2040	352.2115	122.0964	140.1070
D, E	rM15	$C_{19}H_{27}NO_6$	365.1833	366.1910	120.0808	138.0914
D, E	rM36	$C_{19}H_{27}NO_6$	365.1839	366.1912	122.0602	150.0915
D, E	rM33	$C_{18}H_{25}NO_7$	367.1628	368.1701	138.0913	120.0807
D, E	rM16	$C_{18}H_{25}NO_7$	367.1635	368.1705	138.0913	94.0551
D, E	rM19	$C_{19}H_{27}NO_7$	381.1782	382.1858	120.0808	138.0914
D, E	rM20	$C_{18}H_{25}NO_8$	383.1576	384.1658	138.0913	118.0652
D, E	rM22	$C_{18}H_{27}O_6NS$	385.1560	386.1632	138.0913	120.0808
D, E	rM23	$C_{18}H_{28}ClNO_6$	389.1606	390.1677	140.1070	122.0965
D, E	rM25	$C_{20}H_{31}NO_7$	397.2101	398.2182	140.1072	122.0965
D, E	rM27	$C_{21}H_{33}NO_7$	411.2258	412.2316	122.0966	140.1068
E	rM5	$C_{18}H_{25}NO_6$	351.1683	352.1755	120.0808	155.1066
E	rM31	$C_{19}H_{29}NO_6$	367.1989	368.2068	140.1071	
E	rM34	$C_{18}H_{27}NO_7$	369.1789	370.1862	138.0914	120.0809
E	rM21	$C_{19}H_{29}NO_7$	383.1939	384.2015	120.0809	138.0915
E	rM24	$C_{20}H_{25}NO_7$	391.1630	392.1701	120.0807	138.0912
E	rM26	$C_{18}H_{29}NO_9$	403.1837	404.1837	140.1070	122.0964
E	rM28	$C_{22}H_{35}NO_7$	425.2414	426.2503	122.0965	140.1071
E	rM35	$C_{27}H3_6N_2O_6$	484.2571	485.2642	122.0964	294.2067

a Metabolites with an 1,2-unsaturated necine base are highlighted in gray. [b] A: major *in vivo* metabolite; B: major *in vitro* metabolite; C: measured *in vivo*; D: measured *in vitro*; E: only identified in individual standard incubations.

To assess the quantitative relevance of the identified metabolites, the overall recovery was determined, compared to the initial PA amount. For this approach, an equiconcentrated mixture of *Senecio* PAs shown in Figure 1 was incubated and analyzed for the 36 identified rumen metabolites. The results are shown in Figure 3 and indicate a sufficient overall recovery, ranging from 80% at t = 0.5 h and 105% at 6 h. Out of the analyzed 36 metabolites, only 24 were detected in the incubated PA mixtures above their limit of quantitation, of which only 8 reached concentrations of at least 2.5% of the concentration of the initial PA at t = 0 (median 0.5%). These eight metabolites are formed in relatively high concentrations. They account for about 90% of the total recovery and thus have a significant impact on the overall recovery and are consequently of relevance for quantitative description of ruminal metabolism of *Senecio* PAs. Since the main principle of ruminal metabolism involves the hydration of double bonds, the structural diversity of naturally occurring *Senecio* PAs, which differ mainly in their degree of saturation or in the position of the double bonds, is reduced. Rumen metabolism ends up with a limited number of 1,2-saturated metabolites of quantitative importance, as is illustrated in Figure 6, which shows the marker PAs of *Senecio* plants (black header) and their fate in ruminal metabolism. For example, senecionine and seneciphylline or riddelliine and retrorsine differ only in the degree of saturation of the necine acid moiety. Enzymatic hydration of these double bonds produces ruminal metabolites that have the same molecular formula but may differ in stereochemistry. Since chromatographic separation of stereoisomers is generally poor and other LC conditions that might be more suitable for separation of PA isomers have not been tested, this question cannot be answered.[32]

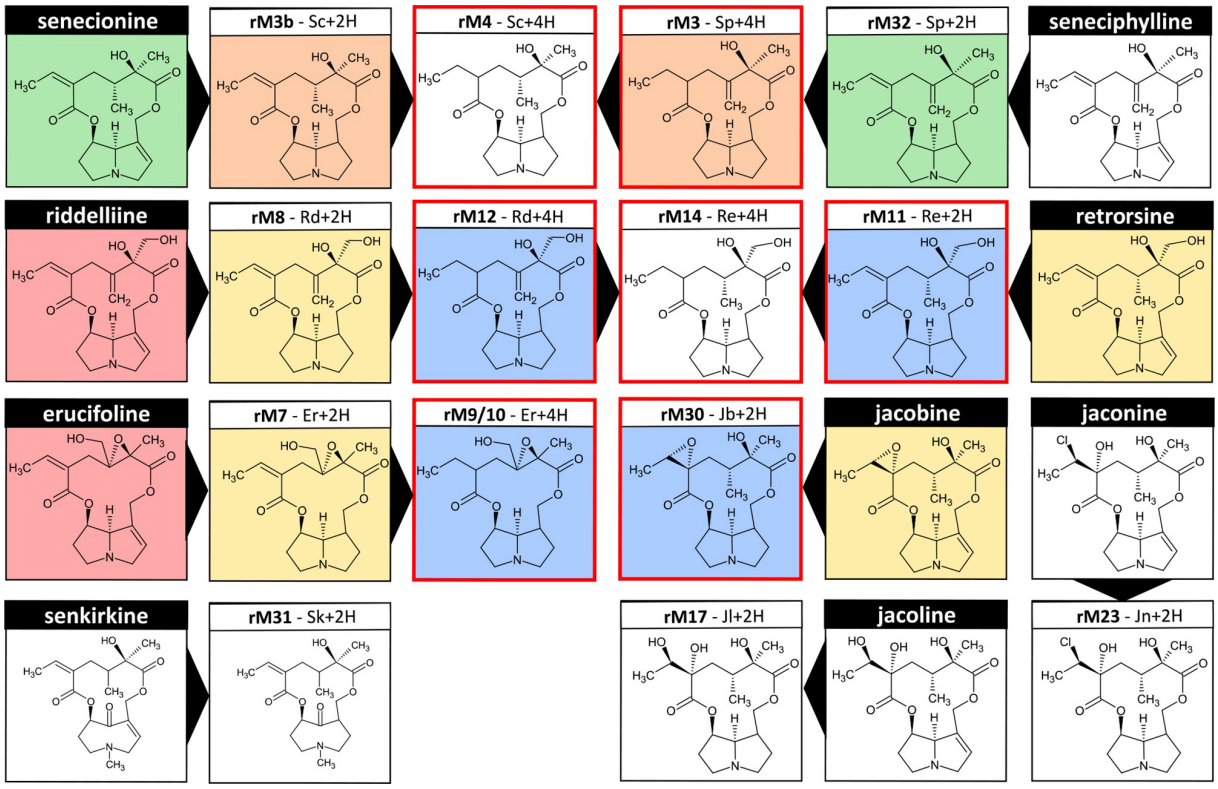

***Figure 6**: Structure proposals for identified main metabolites of rumen metabolism from Senecio PA (black headline). Metabolites with the same sum formula (molecular mass) are highlighted with the same color. Some metabolites can result from various PAs, such as rM3 or rM4, which can be formed by both senecionine and seneciphylline. Metabolites, which accounted for about 90% of the total recovery in in vitro experiments, are outlined in red.*

Determination of Ruminal Metabolites in *In vivo* Samples from Feeding Experiments. The transferability of data obtained in batch culture experiments to rumen metabolism *in vivo* was investigated by analyzing rumen fluids from a 28-day feeding trial with cows. Therein, three different bolus doses of PA/PANOs were administered orally and samples were collected 1.5 and 24 h after bolus administration on days 0, 7, 14, and 28 of the experiment. Mean concentrations of PA/PANOs and their ruminal metabolites in rumen fluids per dose group (n = 4 per dose) are shown in Figure 7. Comparing the metabolite profile of the batch culture experiments (Figure 3) with the *in vivo* data (Figure 7), ruminal degradation appeared to be faster *in vivo*. For example, the metabolite profile of 1.5 h sampling time in rumen liquids *in vivo* rather resembled those data of 20 h *in vitro* incubation. After 1.5 h *in vivo*, almost no 1,2-unsaturated PA is present in the ruminal fluid samples and the majority of detectable compounds represent ruminal metabolites with an 1,2saturated pyrrolizidine ring (blue vs green bars in Figure 7). In *in vitro*, this situation is reached only after an incubation period of several hours (Figure 3). The slower *in vitro* degradation could likely be due to a depletion of metabolic capacity and a reduction in microbial activity *in vitro*. It should also be taken into account that the variations in pH *in vivo* are likely

to be different from those *in vitro*. In *in vitro*, the fermentation products cannot be eliminated; moreover, buffering by saliva and ruminating is missing.

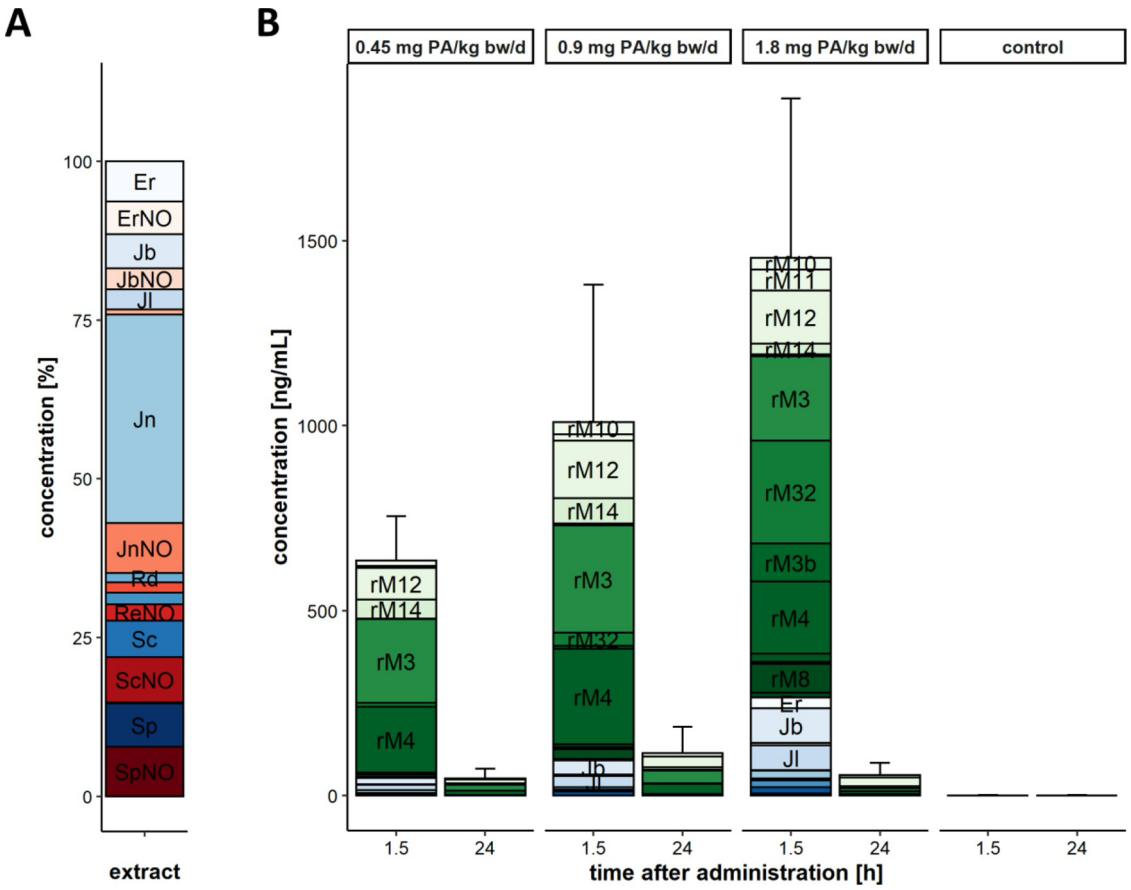

***Figure 7**: (A) PA (blue) and PANO (red) profile of a Jacobaea vulgaris extract administered to dairy cows via gavage in a 28-day feeding study and (B) Determined concentrations of 1,2-unsaturated PAs (blue) and PANOs (red) and their rumen metabolites (green) in ruminal liquids of tested cows. Three different doses were orally administered in comparison to a control without PAs. Samples were taken 1.5 and 24 h after gavage on day 7, 14, and 28 of the study. The mean values per dose group (n = 4) and days are shown with error bars indicating the standard deviation of the summed amount. With Er for erucifoline, Jb for jacobine, Jl for jacoline, Jn for jaconine, Rd for riddelliine, Sc for senecionine, Sp for seneciphylline (NO indicates the respective N-oxide).*

As expected from the results of the *in vitro* incubation experiments, the *in vivo* data confirm a complete reduction of PANOs to the corresponding PAs followed by their metabolization toward saturated structures. Exceptions were those 1,2-unsaturated PAs that showed slow degradation rates, i.e., high half-lives in batch culture experiments (Figure 4). They could also be detected *in vivo* in ruminal fluid samples of the tested cows 1.5 h after gavage. These PAs were jacoline, jacobine, jaconine, and senkirkine, which account for about 85% of the 1,2-unsaturated PAs detectable in the rumen. Since *in vitro* incubation of individual standards provided no evidence that these PAs like jacoline were

metabolically formed from other PAs, their pending presence in ruminal fluid samples results from slow degradation. The differences in ruminal degradation kinetics between individual PAs observed in this study have also been reported in other studies in which the hepatic degradation of PAs was investigated.[33] In those previous studies, PA degradation by incubation with rat and human liver microsomes was investigated. Interestingly, a high congruence in terms of reactivity can be observed for both rumen and hepatic metabolism. In addition, for *Senecio* PAs, a low hepatic degradation was accompanied by a low formation potential of reactive metabolites.[33,34] If the human and rat hepatic metabolism results would also apply for cattle, the observed higher transfer rates of certain compounds into milk, such as for jacoline, could be explained by their generally higher metabolic stability.[35] PAs with a fast degradation in batch culture experiments (Figure 4) were effectively metabolized, and only their 1,2-saturated rumen metabolites were detected instead (Figure 7). This significantly reduces the PA/PANO load in the digestive tract and thus the dose that can enter the liver after absorption, where CYP-mediated metabolism to reactive metabolites occurs.[8−15] Moreover, this means that the liver of ruminants is flooded by a *Senecio* PA/ PANO mixture with a completely different chemical composition compared to monogastric species. In ruminants, this mixture is depleted in structures with a high formation potential of reactive metabolites. 1,2-Saturated necine base PAs, such as the platyphylline type formed during ruminal metabolism, are not converted to reactive or toxic metabolites during incubation with rat or human liver microsomes.[31] Therefore, rumen metabolism could be an explanation for the lower susceptibility of ruminants, compared to monogastric species, to PA toxicity.[18,19] The qualitative composition of 1,2-unsaturated PAs in ruminal fluid in this study is in agreement with the results of Mulder et al., but differences were found in their quantity of conversion.[25] The administered doses in the present study and in the study by Mulder et al. can be directly compared. While Mulder et al. administered a dose of 1 mg PA/kg body weight, 0.9 mg PA/kg body weight was administered as the medium dose in the present study. In addition, the body weights of the cows tested and the time points of sampling at 2.5 and 1.5 h after gavage administration were comparable. Nevertheless, in the present study, the summed concentration of 1,2unsaturated PAs was 100 ng/mL, and that was 3000 ng/mL in Mulder et al. The reason for this significant difference remains unexplained but might include differences in the rumen digestion and transit times for the administered form of PA (liquid extract vs plant material) with consequences in the degree and velocity of PA liberation from the different matrices.[36,37] Moreover, different lactational states accompanied by a different feeding regimen and level of DM intake might have been associated with varying mean retention times of PA in the rumen and consequently different times available for rumen metabolism and disappearance of PA.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.2c01332.

Chromatograms of erucifoline and selected metabolites, LOESS regression curves used for determining the halflives of the pyrrolizidine alkaloids, composition of the *Jacobaea vulgaris* extract, and concentrations of 1,2unsaturated PAs/PANOs and their rumen metabolites in ruminal liquids of the *in vivo* experiment (PDF)

Corresponding Author

Anja These − *Department Safety in the Food Chain, German Federal Institute for Risk Assessment, 10589 Berlin, Germany;* orcid.org/0000-0001-7306-2729; Phone: Tel: +493018412-28516; Email: anja.these@bfr.bund.de

Authors

Julian Taenzer − *Department Safety in the Food Chain, German Federal Institute for Risk Assessment, 10589 Berlin, Germany*

Matthias Gehling − *Department Safety in the Food Chain, German Federal Institute for Risk Assessment, 10589 Berlin, Germany*

Fenja Klevenhusen − *Department Safety in the Food Chain, German Federal Institute for Risk Assessment, 10589 Berlin, Germany;* orcid.org/0000-0001-6015-5459

Janine Saltzmann − *Institute of Animal Nutrition, FriedrichLoeffler-Institut, Federal Research Institute for Animal Health, 38116 Braunschweig, Germany*

Sven Dänicke − *Institute of Animal Nutrition, FriedrichLoeffler-Institut, Federal Research Institute for Animal Health, 38116 Braunschweig, Germany*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.2c01332

Notes

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Abbreviations

ANOVA, analysis of variance; CYP, cytochrome P450; LC, liquid chromatography; LOQ, limit of quantification; LOESS, locally weighted scatterplot smoothing; MeOH, methanol; MS/MS, tandem mass spectrometry; PA, free base form of pyrrolizidine alkaloids; PANO, *N*-oxide form of pyrrolizidine alkaloids; rM, ruminal metabolite; UHPLC, ultrahigh-performance liquid chromatography; vDIA, variable data-independent

Acquisition

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6.2. 2. Publication

Selective and low transfer of pyrrolizidine alkaloids from *Jacobaea vulgaris* Gaertn. into muscle and liver of dairy cattle, goat and sheep

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Julian Taenzer, Anja These, Karin Knappstein, Julika Lamp, Sven Dänicke, Janine Saltzmann, Christoph Gottschalk, Illya Fedotenko, Wolfgang Jira
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Selective and low transfer of pyrrolizidine alkaloids from *Jacobaea vulgaris* Gaertn. into muscle and liver of dairy cattle, goat and sheep

Julian Taenzer[a], Anja These[a], Karin Knappstein[b], Julika Lamp[b], Sven Dänicke[c], Janine Saltzmann[c], Christoph Gottschalk[a], Illya Fedotenko[d], Wolfgang Jira[d,*]

[a] Department Safety in the Food Chain, German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Straße 8–10, 10589 Berlin, Germany

[b] Department of Safety and Quality of Milk and Fish Products, Max Rubner-Institut (MRI), Hermann-Weigmann-Straße 1, 24103 Kiel, Germany

[c] Institute of Animal Nutrition, Friedrich-Loeffler-Institut, Bundesallee 37, 38116 Braunschweig, Germany

[d] Department of Safety and Quality of Meat, Max Rubner-Institut (MRI), E.-C.-Baumann-Straße 20, 95326 Kulmbach, Germany

* Corresponding author: wolfgang.jira@mri.bund.de

Highlights

- Low transfer of pyrrolizidine alkaloids in muscle and liver of dairy animals.
- Only the Senecio PA jaconine, jacoline and jacobine were detected.
- Contents of PA in muscle and liver were dose dependent.
- Resistance to ruminal metabolization is one explanation for selective transfer.
- Elimination of the major amount of PA from muscle and liver expected within 48 h.

Abstract

1,2-unsaturated pyrrolizidine alkaloids and their *N*-oxides (PA/PANO) are plant toxins that are produced by several hundreds of plant species. Due to the pronounced liver toxicity and carcinogenicity of certain PAs, their presence in food and feed has raised concern. However, since PA-producing weeds cannot generally be prevented on grassland and Senecio species often occur in high densities, the transfer of PAs into muscle and liver of dairy cattle, goat and sheep was determined. In 28-day feeding studies extracts of tansy ragwort (Jacobaea vulgaris Gaertn.) were administered orally in three dose levels per animal species. Of the administered Senecio PAs only jacobine, jaconine and jacoline could be detected in relevant quantities by LC-MS/MS in the muscle and liver tissues of the three animal species. The calculated transfer parameters show a low transfer of total PAs into muscle (transfer factors: 0.0004–0.0009) and liver (transfer factors: 0.0008–0.0030), which is lower than reported for other typical contaminants and residues in the literature. This selective transfer can be explained by the rumen passage, during which the other Senecio PAs are largely and rapidly transformed to fully saturated metabolites. These ruminal metabolites were not detected in muscle and liver. No evidence of bioaccumulation of PAs in muscle and liver was observed.

Keywords
Pyrrolizidine alkaloids; Feeding trial; Transfer parameters; *Senecio jacobaea* L.; Rumen metabolism

Introduction

Agricultural land may be infested with weeds that have the potential to harm humans and animals after ingestion, such as plants which can produce 1,2-unsaturated pyrrolizidine alkaloids and their *N*-oxides (PA/PANO). PA/PANO are protoxins that are bioactivated during liver metabolism by oxidation to electrophilic dehydropyrrolizidine alkaloids, which can rapidly react with functional groups of proteins and DNA, leading to veno-occlusive disease, liver necrosis, DNA mutations and possible tumor formation (Allgaier & Franz, 2015; COT, 2008; Wiedenfeld & Edgar, 2011).

In most cases plant-based foods and feed are contaminated by co-harvesting PA-producing plants from the Boraginaceae and Asteraceae families. The European Commission has recently implemented maximum levels in certain plant-based foods (European Union, 2023a). Since PAs occur in a variety of plants and many individual PA structures exist, the analytical scope of the methods for controlling these maximum levels needed to be defined. A spectrum of 35 PA/PANO was selected, which is composed of the marker PAs of naturally occurring PA-producing plants and thus enables official controls to detect various contamination profiles and to quantitatively determine the total PA content in foodstuffs (European Union, 2023a; Mädge et al., 2020). The present study deals with tansy ragwort (*Jacobaea vulgaris* Gaertn., syn. *Senecio jacobaea* L.), as this PA-producing plant is a typical problem of grassland used as pasture or for hay or silage production, especially in nature-preserved areas e.g. in northern Germany (Gottschalk et al., 2020). These plants belong to the genus *Senecio* of the family Asteraceae, which are known to produce the macrocyclic PAs shown in Fig. 1. Of these PAs produced by *Senecio* plants, only senkirkine, senecionine, retrorsine and seneciphylline (and the corresponding *N*-oxides) are included in the spectrum of the 35 PAs (European Union, 2023a). The growth of ragwort on pastures is of concern to farmers and authorities since these plants not only may affect animal health, but also pose a risk to consumers if PAs are transferred into food of animal origin. Investigations on the presence of PA/PANO in meat are rare. In a survey on the occurrence of PA/PANO in food across Europe, 273 meat and meat products were investigated, with no PA/PANO-levels above the limit of detection (LOD) being determined for all samples (Mulder et al., 2015). Studies on the occurrence of PAs in feedstuffs reported average PA contents between 0.010 mg/kg dry matter (DM) (Gottschalk et al., 2015) and 0.026 mg/kg DM (Bolechová et al., 2015). In a Dutch survey, 147 forage samples were analyzed, which showed similar average contents (Mulder, 2009). The highest concentrations were found in contaminated dried alfalfa samples and 10% of those even contained PA levels between 3 and 5 mg/kg (Mulder, 2009). An EFSA survey (European Food Safety Authority, 2011) found maximum PA levels of 23 mg/kg in forages and roughage (N = 252), but a mean content of only 0.29 mg/kg.

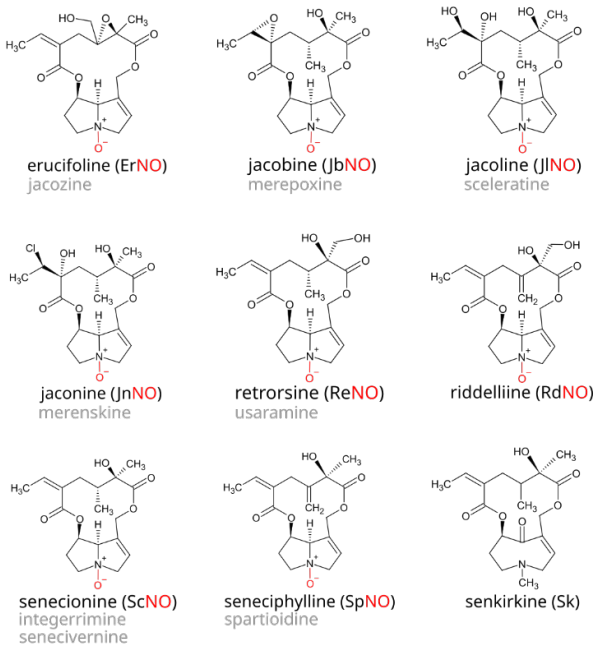

***Figure 1**: Structures of the major pyrrolizidine alkaloids produced by Senecio species. In plants, each compound is present both as a free base and as the corresponding N-oxide (red). In addition, constitutional isomers are formed, which are shown in grey. The compositions of the orally administered extracts of Jacobaea vulgaris Gaertn. and the individual dosages are given in Table 2.*

Studies on the stability of PA/PANO during feed preservation show that PANO are degraded during ensiling, reducing PA/PANO contamination by 80–90% (Gottschalk et al., 2015; Klevenhusen et al., 2022), but remain stable in dried feedstuffs such as hay and grass pellets. Overall, these data on PA-levels in feed indicate that highly contaminated feed may occur, but is rare in the area of intensive agriculture. This can be attributed to good field management in combination with the use of herbicides and fertilizers, which promote crop growth and suppress weed. Since the growth of PA-producing weeds cannot generally be prevented in nature, and a targeted reduction in the use of pesticides combined with current increasing drought may actually lead to increased growth of these plants, regulators need reliable data on the PA-transfer into food of animal origin.

Several studies on the transfer into milk were conducted with dairy cows that received different doses of PA-producing plants (Dickinson et al., 1976; Hoogenboom et al., 2011; Mulder et al., 2020). These studies showed that the transfer of PAs was relatively low (0.05–0.2%) and that jacoline was the major PA of *Senecio* that was transferred into bovine milk. In a study investigating the transfer of PAs to chicken eggs and meat, laying hens were fed with various PA-producing plants (Mulder et al., 2016). In hens fed with *Jacobaea vulgaris* Gaertn., the overall transfer rate to eggs was estimated to be 0.09%. At

the end of the supplementation, the concentrations in the meat were half as high as in the eggs. The levels in the liver were about 6 times higher than in the meat. PAs were not detectable in meat and liver of animals from the group that was fed uncontaminated feed 14 days after supplementation (Mulder et al., 2016).

In the present study, feeding trials were conducted with dairy cattle, goat and sheep as these are the main farm animals whose dietary source is grass – either fresh or preserved – and therefore exposure to PAs is possible. The aim was to determine transfer rates and to evaluate the risk for consumers. In 28-day-feeding studies, each animal species was divided in three PA dose groups which received different amounts of PA-extracts prepared of tansy ragwort (Fig. 1; Supplementary Table S1) by oral administration and an additional control group (N = 4, each). PA contents in muscle and liver were determined by LC-MS/MS.

Materials and methods

Official authorizations. All three trials were performed in agreement with the German Animal Welfare Act and accepted by the official agencies of the respective states where the studies were carried out. Cattle: Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Germany (protocol number 33.19-42502-04-19/3191). Sheep and goats: Ministry of Energy, Agriculture, the Environment, Nature and Digitalization of Schleswig-Holstein, Germany (protocol number 244–30394/2020 (63–5/19)).

Study design. Feeding studies with dairy cattle, goat and sheep were conducted within the framework of the joint research project "PA-SAFE-FEED" (Information System for Agriculture and Food Research, 2024). The study with cows was conducted by the Friedrich-Loeffler-Institut (Knoop et al., 2023) and the studies with sheep and goats were conducted at the agricultural research station of the Max Rubner-Institut (MRI) in Schaedtbek, Germany.

The studies were conducted as short-term 28-day exposure scenarios. To ensure a standardized PA exposure, the experimental animals received an extract of tansy ragwort (*Jacobaea vulgaris* Gaertn.). For this purpose, tansy ragwort was harvested and dried in 2019. The company Phytoplan (Heidelberg, Germany) generated a concentrated extract with PA/PANO from these plants using methanol, which was subsequently removed. The PA/PANO compositions of the extracts are shown in Table 1 and further details on supplementation are given in Table 2. Besides the PA/PANO content, the extract of tansy ragwort was rich in carbohydrates, which could possibly lead to ruminal dysbiosis associated with a ruminal acidosis (Sharp et al., 1982). To avoid erroneous inter-group „sugar effects", all animals had to receive the same amount of carbohydrates. Therefore, the highest dose group was supplemented with pure PA-extract. The reduced sugar intake of the two lower dosed groups was compensated by mixing their PA-extract with sugar cane molasses (Hansa Melasse Handelsgesellschaft, Bremen, Germany; HaGe, Prasdorf, Germany) and the control group received pure molasses according to the amount of

carbohydrates consumed by the three dose groups (Knoop et al., 2023). Before this described feeding study a dosage finding study with four goats and four sheep was conducted.

Table 1*: Percentage PA/PANO composition of extracts of Jacobaea vulgaris Gaertn. used for oral administration.*

Compound [%]	Cattle	Goat	Sheep
Erucifoline	6.4	7.4	4.1
Erucifoline *N*-oxide	4.9	4.1	11.9
Jacobine	5.7	6.5	6.6
Jacobine *N*-oxide	2.9	1.3	13.1
Jacoline	3.3	4.5	1.9
Jacoline *N*-oxide	0.9	1.0	1.6
Jaconine	33.9	30.2	15.4
Jaconine *N*-oxide	7.5	9.3	8.8
Retrorsine	1.8	2.0	1.1
Retrorsine *N*-oxide	2.3	2.1	2.9
Riddelliine	1.3	1.3	0.7
Riddelliine *N*-oxide	1.5	1.3	1.7
Senecionine	5.6	6.9	3.2
Senecionine *N*-oxide	6.9	6.0	10.8
Seneciphylline	7.4	9.8	4.3
Seneciphylline *N*-oxide	7.5	6.3	11.7
Senkirkine	0.2	0.1	0.2
PA [%]	65.6	68.7	37.4
PANO [%]	34.4	31.3	62.6
Total PA content [mg/g]	4.2	3.8	5.4

For further details on the preparation and characterisation of the crude extract, see Knoop et al. 2023.

***Table 2**: Details of the feeding study of the three animal species tested.*

Species	PA-dose (mg/kg bw/d)	Administrations per day (n)	Number of animals	Animals with premature termination of PA supplementation (n)	Period of dosage (d) *
Dairy cattle	0.00*	1	4	0	29
	0.47	1	4	0	29
	0.95	1	4	0	29
	1.91	1	4	0	29
Goat	0.00*	2	4	0	35
	1.33	2	4	0	29
	2.65	2	4	0	28
	5.30	2	4	1[a]	28
Sheep	0.00*	2	4	0	35
	1.50	2	4	0	29
	3.00	2	4	1[b]	28
	6.00	2	4	2[c]	28

a,b,c Premature termination on day: a) 21, b) 17, c) 10 and 22

* Control group receiving a molasses administration. This corresponds to a PA dose of < 0.0001 mg PA/kg bw/d

Dairy cattle: A number of 20 pluriparous, non-pregnant, clinically inconspicuous lactating German Holstein cows (170 ± 30 days in lactation; mean ± standard deviation (SD)) were randomly assigned to 5 treatment groups (N = 4 per group) with two control groups and the three PA-dose-groups. The molasses-control-group received similar amounts of molasses as compared to the total extract amount as a PA-dose-group. To the water-control-group a similar volume of tap water was administered to test the additional hypothesis that the sugar present in the PA extract would not exert an additional effect on the investigated endpoints. However, since clinical–chemical parameters did not indicate an additional effect of sugars present in the PA extract (Knoop et al., 2023), only the molasses control group was used for further evaluation (Table 1). Once daily, the following PA doses were administered to the three dose groups via an ororuminal tube after morning milking: 0.47, 0.95, and 1.91 mg total PA/kg body weight (bw) per day. Further, all cattle received the same total mixed ration, which consisted of maize silage (30%), grass silage (30%) and concentrate feed (40%) on a DM basis provided ad libitum (Knoop et al., 2023). Two hours after the last PA administration the dairy cattle were stunned by bolt shot and killed through bleeding to death.

Goat and sheep: Sixteen primiparous, non-pregnant, clinically inconspicuous lactating animals of each species (goats: 60 ± 16 days in lactation; sheep: 51 ± 8 days in lactation; mean ± SD) were used for this study. The goats were crossbreds (German Improved Fawn, German Improved White, Thuringian goat) and the sheep East Friesians. The animals were randomly assigned to 4 treatment groups (N = 4 per group) with one control group and three PA dose groups. The control group (group 1) received molasses corresponding to the amount of sugar consumed by the highest dose group 4. Furthermore, molasses was added to the daily PA doses of group 2 and 3 to compensate their lower sugar intake compared to group 4. To improve the acceptance of the supplementation, the daily dosage (sheep: 1.50 mg, 3.00 mg, 6.00 mg PA/kg bw; goat: 1.32 mg, 2.63 mg, 5.26 mg PA/kg bw) was divided into two equal doses and administered to the animals via mouth syringe at 8 a.m. and 6 p.m. (Table 1). All animals were adapted to the molasses by supplying increasing amounts one to two weeks before start of the experiment. The animals were kept in an outdoor climate barn with deep straw bedding boxes. The goats' boxes were additionally equipped with a wooden climbing platform. All animals had free access to clover grass silage and salt. Additionally, they received 500 g rolled wheat (goats) or oat (sheep) and 10 g mineral food per day supplied during milking. Milking was performed twice a day at 7 a.m. and 5 p.m. (milking intervals: 10 and 14 h) using a bucket milking machine (Minimelker, Schlauerbauer Melktechnik, Liebenscheid, Germany). Small ruminants were euthanized 14 h after the last administration under general anesthesia by injection of pentobarbital. One goat of the highest dosage group (a), one sheep of the medium dosage group (b) and two sheep of the highest dosage group (c) were prematurely excluded from supplementation on days 21 (a), 17 (b), and 10/22 (c), but were further sampled (Table 2).

Sampling. Liver and muscle samples of all animals were taken at the end of the feeding study. One muscle type (tenderloin) was sampled in dairy cattle and three muscle types (back, shoulder and haunch) were sampled in sheep and goats for comparison of the PA/PANO contents. Additionally, rumen fluid was sampled during the experiment to study the metabolism of PA/PANO in the rumen as contribution to explain the results in muscle and liver. Samples from dairy cattle were taken directly before and 1.5 h after oral administration of the test substances on days 7, 14 and 28 of the trial using a gavage (*Taenzer et al., 2022*). Rumen fluid samples from the goats and sheep were taken on days 7, 14 and 21 with a time interval of 1.0 h after administration. Control samples were taken for all three species before the start of the study. All samples were frozen and stored at −20 °C until PA analysis.

Chemicals. The PA/PANO standards erucifoline (Er), erucifoline *N*-oxide (ErN), jacobine (Jb), jacobine *N*-oxide (JbN), retrorsine (Re), retrorsine *N*-oxide (ReN), riddelliine (Rd), riddelliine-*N*-oxide (RdN), senecionine (Sc), senecionine *N*-oxide (ScN), seneciphylline (Sp), seneciphylline *N*-oxide (SpN) and senkirkine (Sk) were obtained from PhytoLab (Vestenbergsgreuth, Germany) and jacoline (Jl), jacoline *N*-oxide (JlN), jaconine (Jn) and merenskine *N*-oxide (MkN) from Cfm Oskar Tropitzsch (Marktredwitz, Germany). Stock solutions (c = 1 mg/mL) of each PA/PANO were prepared with methanol. Aliquots of

the stock solutions were diluted in methanol to prepare a mixed solution containing all substances (c = 5 µg/mL), which was successively diluted with methanol to obtain c = 1.0, 0.25, 0.1, 0.025 and 0.005 µg/mL. The stock solutions and the mixed solutions were stored at −20 °C in the dark. Methanol (LC/MS hypergrade, Merck), formic acid for LC/MS from VWR International (Darmstadt, Germany), water (LC/MS Optigrade, Promochem), acetonitrile (LC/MS Optigrade, Promochem) and ammonium formate (eluent additive for LC/MS, VWR) were purchased from the corresponding chemical suppliers.

Analysis of PA/PANO in muscle and liver. For analysis of liver or muscle the slightly thawed samples were cut into small pieces (about 0.5 cm) with a scalpel, subsequently frozen at −20 °C or −80 °C and then freeze-dried. The weight loss of each sample after lyophilisation was recorded to enable the calculation of PA/PANO-concentration related to fresh tissue. The resulting material (about 3–4 g) was ground in a Tube Mill control with disposable grinding chambers from IKA-Werke (IKA, Staufen, Germany) to obtain a fine powder. An amount of 10 mg of the freeze-dried material was mixed with 1 mL of the extraction buffer (1% of formic acid in methanol), shaken at room temperature for 15 min and centrifuged for 10 min at 12,000 g. An amount of 500 µL of the supernatant was carefully dried in a nitrogen stream at 50 °C and 100 µL of water containing 0.1% formic acid and 5 mM ammonium formate (solvent A) was added, shaken on a Vortex, centrifuged for 15 min at 12,400 g, filtered through a 0.2 µm centrifugal filter (modified nylon, 0.2 µm) from VWR International (Darmstadt, Germany) and transferred into a 1-mL tapered glass vial and stored at −20 °C until further analysis.

For the preparation of the matrix-matched standard solutions, uncontaminated samples of the respective matrix and the corresponding control group were used and essentially the same steps were followed as described above, except that 1 mL of the extraction buffer (1% of formic acid in methanol) was replaced with 980 µL of the same extraction buffer along with 20 µL of the corresponding PA/PANO standard mixture (0.005, 0.025, 0.1, 0.25 and 1.0 µg/mL) to obtain calibration standards of 0.01, 0.05, 0.2, 0.5 and 2 µg/g of the corresponding freeze-dried tissue.

Chromatographic separation was performed with a Dionex UltiMate 3000 RS HPLC from Thermo Scientific (Waltham, USA). The column temperature was 30 °C and the injection volume was 2 µL. The analytical column used was a Kinetex EVO C18 100 Å (150 × 3 mm, particle size: 5 µm) from Phenomenex (Aschaffenburg, Germany). The mobile phase consisted of solvent A: 0.1% formic acid and 5 mM ammonium formate in water; and solvent B: 0.1% formic acid, 5 mM ammonium formate and 95% acetonitrile in water. The LC run (total time: 23 min) started with a gradient 2%–9.5% B for 10 min, followed by another gradient to 39% B in 5 min and an isocratic step at 100% B for 1.5 min. An isocratic step at 2% B continued until the end of the run. The flow rate was 400 µL/min.

The detection of PA/PANO (N = 17; Fig. 1) was carried out on an AB Sciex QTrap 5500 (Darmstadt, Germany) in the positive ESI mode using scheduled multiple reaction monitoring (MRM). The following source parameters were used: temperature 550 °C, ion spray voltage +4000 V, curtain gas flow 30 psi, ion source gas 50 psi. The Q1 and Q3 quadrupoles were set to unit resolution. To enable the

unique identification of each analyte, two transitions (quantifier and qualifier) for each compound were measured. Details of the scheduled MRM method are shown in Supplementary Table S2. Data acquisition and processing were carried out with Analyst 1.7.1 (Sciex, Darmstadt, Germany) and the Sciex OS-MQ software (version 1.7.0.36606; Sciex, Darmstadt, Germany).
Quantitation of the PA/PANO was achieved with a 5-point matrix-matched standard calibration as described above. A weighted calibration was used, and it was checked whether the accuracy of the back-calculated concentration of the respective calibration level using the calibration curve was in the range of 80–120%. Each sample was analyzed in duplicate and the mean value of the calculated PA-concentrations was used. The quantitation of JnN was performed with the co-eluting isomeric standard MkN. The LOD and LOQ of the analyzed PA/PANO for muscle and liver are shown in Supplementary Table S3 and the results of the method validation experiments in Supplementary Table S4. Similar to the rumen fluid muscle and liver samples were analyzed for the metabolites identified in the rumen. PA contents below the LOQ were also reported, since this approach is acceptable for monitoring purposes and exposure assessments according to EU Regulation 2023/2783 (European Union, 2023b).

Analysis of rumen fluid samples. Analysis of rumen fluids has been described previously (Taenzer et al., 2022). Briefly, reactivation of metabolic activity after thawing was prevented by adding 75 µL of methanol to 425 µL of the sample. After filtration through a centrifugal filter (modified Nylon 0.2 µm, VWR, Radnor, Pennsylvania) at 23,500 g for 10 min (Eppendorf 5424 R centrifuge with an FA-45-24-11 rotor, Hamburg, Germany) samples were analyzed directly. Additionally to the method described by Taenzer et al. (2022), rumen fluids from sheep and goats were analyzed for other ruminal metabolites than described in that study. These metabolites had similar characteristics (unsaturated necine base) as the ones formerly identified. Quantitative analysis was made for the most abundant metabolites in the respective species. A more detailed overview of the metabolites can be found in Supplementary Table S5.

Calculation of transfer rates, transfer factors, biotransfer factors and margin of exposure. For the calculation of the transfer parameters (transfer rate (TR; Equation (1)), transfer factor (TF; Equation (2)) and biotransfer factor (BTF; Equation (3))) the equations from Krause et al. (2022) were used. The weights of the different tissues were estimated on the basis of the relative organ weights (percent of body weight) as published for cattle (Lin et al., 2020) and sheep and goat (Li et al., 2021): cattle (muscle: 36.1%), sheep (muscle: 24.78%; liver: 1.27%), goat (muscle: 38.58%; liver: 1.89%). The weights for the livers of the cattle were available from the weighing after slaughtering. In the following, these formulas are shown with the parameters used in this study to calculate the transfer parameters. Transfer parameters were calculated for each animal individually and were subsequently averaged independently of the PA exposed groups. Transfer parameters were calculated for the sum of all PA/PANOs and

additionally for selected PAs individually. For these three, the *N*-oxide and the corresponding free base were examined together.

(1) $$TR[\%] = \frac{cont_{PA/PANO}\left[\frac{mg}{kg}\right] \cdot weight_{tissue}[kg] \cdot \frac{1}{t}\left[\frac{1}{d}\right]}{dose_{PA/PANO}\left[\frac{mg}{d}\right]} \cdot 100\ [\%]$$

(2) $$TF = \frac{cont_{PA/PANO}\left[\frac{mg}{kg}\right]}{dose_{PA/PANO}\left[\frac{mg}{d}\right]\ /\ feed\ \left[\frac{kg}{d}\right]}$$

(3) $$BTF\left[\frac{d}{kg}\right] = \frac{cont_{PA/PANO}\left[\frac{mg}{kg}\right]}{dose_{PA/PANO}\left[\frac{mg}{d}\right]}$$

With.

$cont_{PA/PANO}$: content of PA/PANO in the examined tissue

$dose_{PA/PANO}$: dose of PA/PANO that was administered to the animals

$weight_{tissue}$: weight of the examined tissue

feed: feed intake by experimental animals as dry matter

The Margin of Exposure (MOE) was calculated as proposed by the EFSA (European Food Safety Authority, 2005). Human intake was estimated with consumption data from the EFSA Comprehensive European Food Consumption Database, filtering “Germany” as survey country and the exposure hierarchy (L4) for: “bovine fresh meat” or “sheep fresh meat”, and measured PA/PANO levels in our study. As reference point the benchmark dose level ($BMDL_{10}$) for PAs proposed by the EFSA was used (Hardy et al., 2017).

(4) $$MOE = \frac{BMDL_{10}\left[\frac{\mu g/kg\ bw}{d}\right]}{consumption\left[\frac{g/kg\ bw}{d}\right] * cont_{PA/PANO}\left[\frac{\mu g}{g}\right]}$$

With.

$cont_{PA/PANO}$: content of PA/PANO in the examined tissue

$consumption$: estimated amount of muscle or liver consumed by consumer group

Results and discussion

Determination of PA concentrations in tissue of muscle and liver. At the end of the feeding study, all animals were killed, with cattle slaughtered 2 h after the last gavage and goats and sheep euthanized

14 h after the last administration. This means that in addition to the different killing times after the last administration, different killing methods were used, which led to higher blood contents in the tissues of the euthanized animals. The PA-concentrations were determined in muscle and liver samples of all species (Supplementary Tables S6 and S7) and mean values per dosage group are presented in Fig. 2. Only jacobine, jacoline and jaconine were detected in muscle and liver of sheep and goats. In cattle, which were slaughtered at a shorter interval after the last PA-supplementation, in muscle additionally small amounts of senkirkine and in liver low contents of erucifoline, retrorsine, seneciphylline and senkirkine were detected. The analysis of muscle parts of back, haunch and shoulder of goats and sheep also showed only marginal differences in the PA-profiles and -contents within the same animal (Supplementary Table S8).

The reasons for the selective transfer of certain PAs into muscle and liver are still unclear. It is not known so far, whether this selectivity is determined more by the stereochemistry, or rather by the physicochemical properties or even simply by the number of atoms that can be attacked. The selectivity can also possibly be explained by the metabolic activities in the rumen (Section 3.2), since the analysis of the respective rumen liquid samples showed that jacobine, jacoline and jaconine represent a major part of the detectable PAs while the other administered PA/PANOs were mostly metabolized (Fig. 4, Table 1). Considering the high influence of the rumen metabolism on PAs, it has to be discussed if a scenario using contaminated feed instead of a PA-extract would result in a longer rumen passage time of the PA/PANOs and therefore in a more effective ruminal degradation of PAs.

Neither jacobine, jacoline nor jaconine are among the 35 PAs listed in the Regulation (EU) 2023/915 for the control of maximum levels in herbal products and would therefore have remained undetected during market monitoring. This can be explained by the fact that the analytical scope specified in the regulation reflects the marker PAs of weeds that are known to contaminate plant foods. Since the PA-profile is altered within the animals, other markers are needed for a potential control of PAs in animal derived food. (Dickinson et al., 1976; Hoogenboom et al., 2011; Mulder et al., 2016, 2020).

PA-profiles in muscle and liver samples of sheep and goats were very similar, but concentrations found in liver samples were generally two times higher than in muscle (Fig. 2). In the livers of dairy cows, concentrations were on average approximately five times higher than in muscle. In addition, differences in the ratios of jacobine, jacoline and jaconine were found in dairy cattle compared to sheep and goat (Fig. 2) with higher concentrations of jaconine in dairy cows. We suspect that these differences are caused by deviations in the timing of killing (killing by slaughter for cattle 2 h after last administration and killing by euthansia for sheep and goats 14 h after last administration) rather than differences between species. This assumption is supported by the results of a dose-finding study conducted for goat and sheep prior to the present study and by the results of the four animals in the present study whose PA supplementation had to be terminated prematurely (Table 2). In both instances, the time between the last PA-administration and the death of the animals was different than in the presented study (Fig. 3). With increasing time difference between administration and killing, the total PA concentration

in liver and muscle decreases rapidly, and the data shown in Fig. 3 indicate that PAs do not accumulate in the tissues of the animals and are no longer detectable roughly after 48 h. These results are supported by results from other studies (Fletcher et al., 2011; Mulder et al., 2016). Consequently, the PA concentrations in the muscle of cattle determined in this study represent a worst-case scenario, as these cattle were already slaughtered 2 h after the last administration, whereas in agricultural marketing there is probably a longer time span between PA exposure and arrival at the slaughterhouse. It is questionable if these results from dairy animals can be directly be transferred to fattening animals since the excretion via milk can alter the transfer into meat.

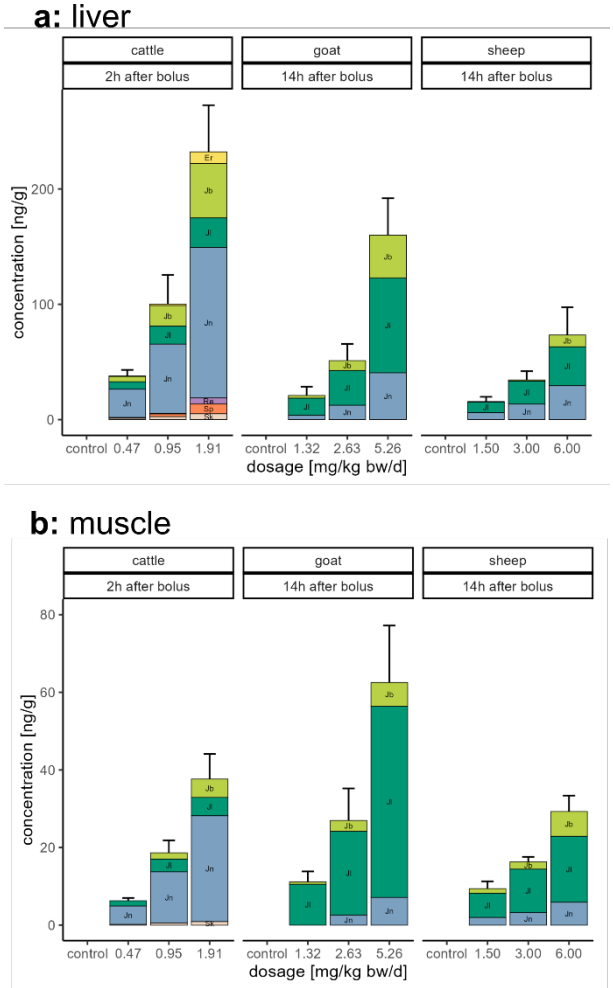

***Figure 2**: Detected PAs and their mean contents [ng/g wet weight] in tissue of liver (top) and muscle (bottom) of dairy cattle (left), goat (middle) and sheep (right) within the three dosage groups and in the respective control group. Error bars are calculated as standard deviations of the sum of jaconine (Jn), jacoline (Jl) and jacobine (Jb), erucifoline (Er), retrorsine (Re), seneciphylline (Sp) and senkirkine (Sk) in all animals per dosage group (compositions of the administered extracts are shown in Table 1; number of animals per group are given in Table 2).*

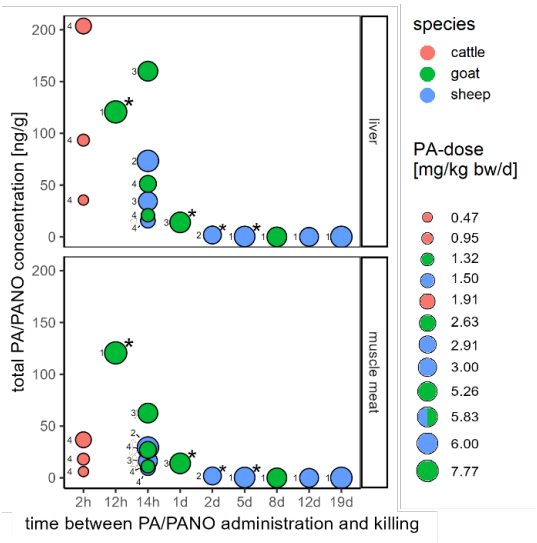

***Figure 3**: Determined total PA contents [ng/g] in liver (top) and muscle (bottom) in dairy cattle (red dots), goats (green dots) and sheep (blue dots) depending on the time difference between last administration and killing. In addition, the data from the dose-finding study for sheep and goats are shown and marked with "*". The superscript number indicates the number of animals from which the data originate.*

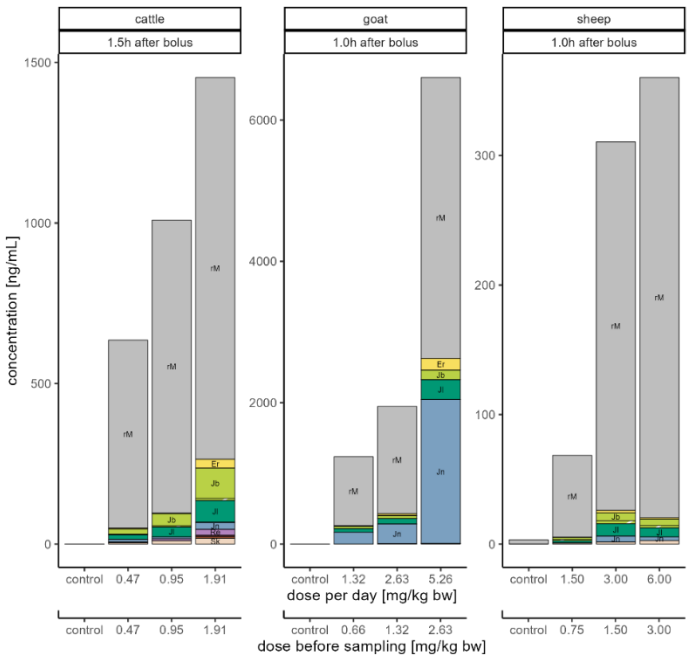

***Figure 4**: Concentrations of PAs and their ruminal metabolites (rM) in rumen liquids of dairy cattle (left), goat (middle) and sheep (right) within the three dosage groups and in the respective control group. Shown are the mean values of the sampling days during the feeding trial (day 7, 14, 21). Samples were collected 1.5 h after administration in dairy cattle and 1.0 h in sheep and goats. The daily PA-dose was administered as a single shot for cattle, but was divided into two portions of half the quantity each for goat and sheep (Table 2). With: erucifoline (Er), jacobine (Jb), jacoline (Jl), jaconine (Jn), retrorsine (Re) and senkirkine (Sk).*

In a study from Northern Australia the quality of muscle of weaning calves fed with *Senecio brigalowensis* (previously classified as *S. lautus*) plants corresponding to PA doses of 2.5 mg PA/kg BW/day was investigated (Fletcher et al., 2011). The transfer of PAs into liver and muscle was low and limited to otonecine-type PAs, such as senkirkine (Fig. 1). Additionally, a significant lower PA content in muscle and liver was determined in animals killed after the second half of the supplementation phase compared to the first half, probably because of the maturing of ruminal digestion in calves. The authors concluded that the risk to health of persons consuming meat and liver from stock exposed to PA-containing plants is negligible (Fletcher et al., 2011). In a study with laying hens, diets containing various *Senecio* species were fed for 28 days (referring to approximately 0.8 mg PA/kg BW/day). In animals of the *Jacobaea vulgaris* Gaertn. group, that was slaughtered 14 days after the last treatment, the breast muscle and liver were free of PA (last day of treatment: 6.5 ± 1.9 µg/kg; 14 days after treatment: 0.0 ± 0.0 µg/kg). In contrast, high levels of PA were still detectable in animals fed with *Senecio inaequidens*, which produces high amounts of otonecine type PAs (last day of treatment: 67.1 ± 26.2 µg/kg; 14 days after treatment: 3.3 ± 1.1 µg/kg) (Mulder et al., 2016). This demonstrates that the selective transfer of PAs common for *Senecio* plants into animal tissue is mainly focused on

jacoline and otonecine-type PAs, but is not restricted to ruminants. In addition, this study shows that there is no bioaccumulation in liver or muscle.

Determination of PA concentrations in rumen liquids. Rumen metabolism might significantly influence the absorption of PA/PANO and thus their content in subsequent tissues. The rumen metabolism of cattle has already been investigated in another study as part of the PA-SAFE-FEED project and can be summarized as follows: the biotransformation starts with a rapid conversion of the *N*-oxide form into the corresponding free bases, which are converted to rumen metabolites that are hydrogenated in both the necine base and the necic acid moiety (Taenzer et al., 2022). A reduction of *N*-oxides to parent PA was already observed by Mattocks (1971) when incubating retrorsine-*N*-oxide with rat gut homogenates. In our investigations, the pattern and concentration of PAs and their rumen metabolites were compared in cattle, goats and sheep. The concentrations of PAs and their rumen metabolites were determined for all animal species in the ruminal fluid (Supplementary Table S9). During the study, dairy cattle were sampled three times (on days 7, 14, and 28) 1.5 h after administration, whereas goats and sheep were sampled on days 7, 14, and 21, with a time difference of 1.0 h between administration and sampling. Because PA profiles and concentrations were similar between sampling days and between animal replicates, mean values are shown for the different dosage groups (Fig. 4). As the daily dose for goats and sheep was divided into two doses per day, the doses administered prior to sampling were similar between species (e.g., the highest dose groups received 1.91, 2.63, and 3.00 mg/kg bw/d for dairy cattle, goat and sheep, respectively). The determined concentrations of PA/PANO and their rumen metabolites are not easily comparable due to anatomical and physiological differences such as relative rumen volume, saliva production or rumen passage rates of ingesta. Nevertheless, the lowest PA concentrations in the rumen as well as the lowest concentrations of PA rumen metabolites were determined for sheep (approx. 20% of dairy cattle), suggesting that their PA conversion rate and passage rate is the highest among these three ruminants. Data of the present study confirm the previously reported differences in kinetics which were explained by differences in the activity and density of PA-degrading microorganisms in the rumen (Duringer et al., 2005; Wachenheim et al., 1992). Surprisingly, the highest PA concentrations were found in the ruminal samples of goats (approx. 160% of dairy cattle). This is in contrast to the study of Wachenheim et al. (1992), who found the highest transformation rate in sheep, followed by goats in a similar range. In cattle, the concentrations were an order of magnitude lower. However, these differences might originate from the differences in the design of both studies. In the study of Wachenheim et al. (1992), for example, the dairy cattle, goat and sheep breed are not specified, so they could be different from those used in this study, which may have a different microbiome or different retention times in the rumen. Additionally, the way of PA/PANO-administration: plant material vs. extract may influence the results.

With regard to the PA-pattern detected in rumen fluids after 1–1.5 h, a high similarity between species can be observed. In terms of the PA profile administered, mainly jaconine, jacoline, and jacobine were

detected with traces of erucifoline, retrorsine and senkirkine, while all *N*-oxide forms were undetectable, probably due to ruminal degradation (Fig. 1, Fig. 2). These observations are in agreement with the results obtained in the systematic investigation of rumen metabolism of *Senecio* PAs (Taenzer et al., 2022). In that study, *in vitro* batch culture incubations with rumen inoculum from cattle were used to determine half-lives of the individual PAs. Results showed that seneciphylline and senecionine degraded most rapidly, while riddelliine, erucifoline, and retrorsine were in the middle range. Slower degradation rates and thus higher resistance to rumen metabolization were observed for jacobine, jaconine, senkirkine, and especially jacoline, which was almost resistant to degradation. This *in vitro* degradation was observed during the incubation of bovine ruminal fluid, but was also tested with sheep rumen with similar results (data not shown).

Therefore, we conclude that the sieving effect of the ruminal metabolism explains that only certain PAs will be transported to the liver where they undergo further metabolism. Thus, the PA transfer into tissue of ruminants is mainly reflected by the xenobiotic activity of rumen and liver and the PA pattern detectable in muscle is also detectable in milk (Knoop et al., 2024). No metabolites identified in the rumen fluids could be detected in the liver and muscle tissue of these animals.

Calculation of transfer factors, biotransfer factors and transfer rates. For regulatory purposes and risk assessment, transfer parameters of a contaminant can be used to estimate the expected concentration of this compound in the respective animal tissue at a given concentration in the feed. The data of the present feeding study were used to calculate the transfer factors (TF, the ratio of the concentration of a compound in an animal product to the concentration of the compound in animal feed), biotransfer factors (BTF, ratio of steady-state compound concentration between animal tissues and feed) and transfer rates (TR, daily fraction of a compound that is excreted, for instance via milk, divided by the daily intake with the diet when the steady state has been reached) (Krause, 2022). These transfer parameters were calculated for the total PA and the individual PAs in the muscle and liver tissue of dairy cattle, goat and sheep and are listed in Table 3. For PAs that were administered but not detectable in the tissues, their LODs (Supplementary Table S2) were used to estimate the maximum value of the respective transfer parameter.

***Table 3**: Transfer parameters such as the transfer rate (TR), transfer factor (TF) and the biotransfer factor (BTF) were determined for muscle and liver of dairy cattle, goat and sheep. In addition to the total PA individual values were calculated for single PA compounds. For those PAs that were administered but not detectable in muscle or liver, the LODs of the method were used to estimate the maximum transfer values.*

		Muscle			Liver		
	PA	TR [%]	TF	BTF [d/kg]	TR [%]	TF	BTF [d/kg]
Cattle[a]	Total PA	0.62	0.00049	0.00003	0.16	0.00291	0.00016
	Sk	6.90	0.00539	0.00030	1.87	0.03312	0.00179
	Jl	2.52	0.00195	0.00011	0.55	0.00953	0.00052
	Jn	1.11	0.00088	0.00005	0.24	0.00422	0.00023
	Re	<0.87	<0.00075	<0.00004	0.04	0.00075	0.00004
	Jb	0.59	0.00045	0.00002	0.33	0.00586	0.00032
	Er	<0.32	<0.00027	<0.00001	0.03	0.00065	0.00003
	Sc	<0.29	<0.00025	<0.00001	<0.01	<0.00024	<0.00001
	Sp	<0.24	<0.00021	<0.00001	0.03	0.00051	0.00003
Goat[b]	Total PA	0.35	0.00091	0.00018	0.04	0.00192	0.00038
	Jl	6.06	0.01575	0.00317	0.44	0.02337	0.00467
	Sk	<1.34	<0.00348	<0.00060	<0.10	<0.00581	<0.00101
	Re	<1.06	<0.00275	<0.00048	<0.05	<0.00275	<0.00048
	Jb	0.54	0.00140	0.00028	0.11	0.00602	0.00120
	Er	<0.38	<0.00098	<0.00017	<0.02	<0.00098	<0.00017
	Sc	<0.34	<0.00089	<0.00015	<0.02	<0.00089	<0.00015
	Sp	<0.27	<0.00071	<0.00012	<0.01	<0.00071	<0.00012
	Jn	0.05	0.00014	0.00003	0.02	0.00092	0.00018
Sheep[b]	Total PA	0.11	0.00043	0.00007	0.01	0.00084	0.00013
	Jl	1.99	0.00797	0.00121	0.17	0.01312	0.00200
	Re	<0.63	<0.00251	<0.00039	<0.03	<0.00251	<0.00039
	Sk	<0.45	<0.00181	<0.00028	<0.03	<0.00301	<0.00046
	Sc	<0.18	<0.00072	<0.00011	<0.01	<0.00072	<0.00011
	Er	<0.17	<0.00063	<0.00010	<0.01	<0.00063	<0.00010
	Sp	<0.16	<0.00062	<0.00010	<0.01	<0.00062	<0.00010
	Jn	0.10	0.00038	0.00006	0.02	0.00135	0.00021
	Jb	0.08	0.00030	0.00005	0.00	0.00024	0.00004

Animals were killed at a) 2h and b) 14h after the last administration of PA.

In general, the highest transfer rate in all tissues and for all animal species was determined for jacoline (Table 3). Senkirkine was also detectable in muscle and liver of cattle, which were slaughtered 2 h after the last PA-administration. As the proportion of senkirkine in the PA-extract was very low, the calculated transfer parameters are high. This result is in line with the transfer study with laying hens in which the transfer was calculated for eggs, muscle and liver as being the ratio between the level in the respective food product and the feed. The authors pointed out that PAs with high transfer, such as jacoline and otonecine-type PAs, e.g. senkirkine, are relatively stable to metabolic degradation and are therefore detectable in animal tissue (Mulder 2016).

Differences for the transfer of the three PAs could be observed between the three species (Table 3). Jacobine and jaconine in particular show a higher transfer in cattle than in goats and sheep, which can be explained by the longer time span between the last PA dose and killing of the goats and sheep (14 h) compared to cattle (2 h) as well as the higher metabolic stability of jacoline compared to jacobine and jaconine.

In order to classify the transfer of PAs into muscle determined in this study in comparison to other substance groups, literature data was used. As for muscle usually transfer factors are calculated, this parameter was used for classification and compared with the arithmetic mean of published transfer factors for residues and contaminants (Leeman et al., 2007). As can be seen from Fig. 5, the transfer of PAs into muscle and liver is very low and is in the range of pesticides or mycotoxins or is even one order of magnitude lower.

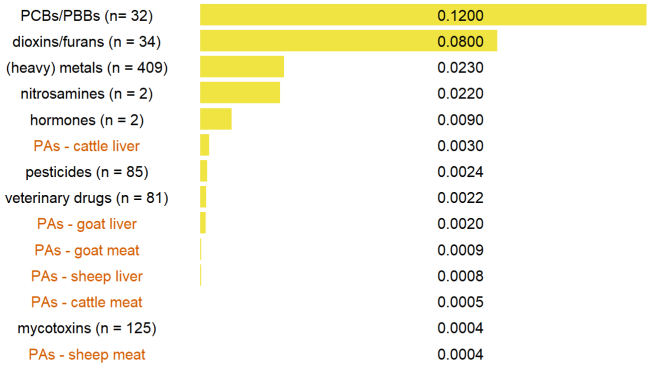

***Figure 5**: Transfer factors determined for total PAs in muscle and liver of dairy cattle and sheep (given in red) compared to median transfer factors for selected contaminants and residues published by Leeman et al., 2007 (given in black).*

Estimation of acceptable PA burden in grassland infested with PA plants. The detection of PAs in animal tissue (as exemplified by muscle and liver) upon transfer of PAs from contaminated feed raised the question which PA burden in grassland infested with PA plants could be acceptable. For a rough estimate the administered PA doses were converted into the PA level that would be required in the feed corresponding to the administered dose. The average body weight of dairy cattle in this study was 650 kg, i.e., in the lowest dosage group (0.47 mg PA/kg bw/d) on average 293 mg PA absolute were daily administered per animal and 1242 mg PA (1.91 mg PA/kg bw/d) in the highest dosage group. With an average daily total mixed ration (TMR) of 19 kg DM for dairy cattle, the PA doses administered correspond to PA concentrations in the feed of between 16 and 65 mg/kg (Table 4). Since, according to current knowledge, contamination of concentrate feed with PAs is rare, it was also calculated for a PA exposure of the animals mainly via the roughage. Assuming that the average roughage portion of a 650 kg cattle is 13 kg DM (Knoop et al., 2023), the lowest administered PA dose corresponds to a PA concentration of 24 mg/kg DM and the highest to 96 mg PA/kg DM (Table 4). The same calculations

were made for sheep, based on an average body weight of 66 kg. With an average dry matter intake of 2.2 kg and an average roughage portion of 1.7 kg, the PA doses administered correspond to the PA concentrations in the feed shown in Table 4.

***Table 4**: Margin of Exposure (MOE) calculated as the ratio of the $BMDL_{10}$ of 237 µg PA/kg body weight (bw) per day and the mean dietary exposure estimated from the mean total PA concentration in muscle (sum of all individual PAs, lower bound approach) per dose group and the mean chronic consumption of bovine and sheep muscle (consumers only). In addition, the total PA concentration determined in the muscle of dairy cattle and sheep per dose group is given and the PA doses administered were converted into the corresponding PA concentrations in the feed (PA exposure of the animals is exclusively via roughage and on a total mixed ration (TMR) basis).*

	Bovine meat			Sheep meat		
Study parameters						
Dose group	1	2	3	1	2	3
Total mean PA conc. in muscle	0.006	0.018	0.037	0.009	0.016	0.029
Adm. PA-dose [mg/kg bw/d]	0.47	0.95	1.91	1.5	3	6.00
Corresp. PA conc. TMR [mg/kg]	16	33	65	46	93	185
Corresp. PA conc. roughage	24	48	96	60	120	240
Meat consumption [g/kg bw/d]						
Infants	0.585			-		
Children	0.66			0.534		
Adults	0.791			0.863		
Elderly	0.668			0.814		
Margin of Exposure (MOE)						
Infants	66,000	22,000	11,000	-	-	-
Children	59,000	20,000	10,000	47,000	27,000	15,000
Adults	49,000	17,000	8,000	29,000	17,000	9,000
Elderly	58,000	20,000	10,000	31,000	18,000	10,000

In a second step, we calculated the ratio of an $BMDL_{10}$ of 237 µg/kg bw per day as derived by EFSA (2017) as the appropriate toxicological reference point and the estimated PA exposure based on PA levels measured in muscle and liver and mean consumption data available from the EFSA Comprehensive European Food Consumption Database for "consumers only" (European Food Safety Authority, 2023). Based on the mean consumption data available from the EFSA Comprehensive European Food Consumption Database (European Food Safety Authority, 2023), the proportion of consumers who consume beef and sheep meat is between 40% and 1%. The proportion of liver consumers is much lower, generally less than 1%. For goat meat and liver no consumption data was available, so that the exposure assessment was only carried out for bovine and sheep meat. The mean total PA levels determined in bovine and sheep muscle for the respective PA dose groups were used as lower bound approach, where PA results below LOD account as zero. As shown in Table 4, only for the highest dose group MOE values around 10,000 were obtained. Thus, concluding from these data, a PA burden in grassland infested

with PA plants up to levels even in the highest dose range used in this study would not result in ratios below 10,000. If risk management measures are to be taken to decrease the exposure of consumers further, the reduction of PA-producing plants in the feed and/or the feeding of animals with PA-free feed for a period of at least 48 h before slaughter could be an option.

In standard risk assessment for substances that are both genotoxic and carcinogenic, the quotient of an appropriate toxicological reference point and the human exposure to this substance is referred to as an MOE. For risk assessment of the occurrence of 1,2-unsaturated PA in food, EFSA selected the benchmark dose lower confidence limit 10% ($BMDL_{10}$) of 237 µg/kg bw per day, derived for the incidence of liver haemangiosarcoma in female rats exposed to riddelliine as reference point for the chronic risk assessment of PAs with the assumption of equal potency for all other 1,2-unsaturated PAs (EFSA 2017). Following the concept, an MOE of 10,000 or higher is interpreted as to be of low concern from a public health point of view and might reasonably be considered a low priority for risk management actions (European Food Safety Authority, 2005).

Conclusions

In the present 28-day feeding studies with dairy cattle, goats and sheep, the transfer of Senecio PAs into muscle and liver tissue was investigated. The data showed a low and selective transfer of jacobine, jacoline, and jaconine. In cattle, additionally low amounts of senkirkine were detected in muscle and low contents of erucifoline, retrorsine, seneciphylline and senkirkine in liver. This selectivity most likely was due to the effective metabolism of certain PAs during rumen passage and was observed for all three species of ruminants by analysing rumen fluids originating from this trial. Of the *Senecio* PAs tested, jacoline had the highest transfer rate, and was detectable for the longest time in muscle and liver after administration. High transfer rates of certain PAs into the tissue of ruminants can be explained by their limited biotransformation in the rumen and liver. Since bioactivation during hepatic metabolism is a prerequisite for toxicity, the question arises whether these PAs should be considered of less concern since their bioactivation potential appears to be low. An assessment was made whether the PA concentrations in muscle determined in the present feeding study may pose a risk to the consumer. The MOE values were calculated based on the total PA concentrations in the muscle of dairy cattle and sheep, which were determined 2 h and 14 h after the last administration, respectively, in combination with the mean consumption data and the $BMDL_{10}$ of 237 µg/kg bw per day published by EFSA (for consumers only). In addition, different scenarios were considered in order to convert the administered PA doses into PA concentrations in the feed of dairy cattle and sheep and to make the data useable for risk assessment. Concluding from these data, for dairy cattle a PA content of 65 mg/kg in the daily TMR or of 96 mg/kg in roughage and for sheep 185 mg/kg in the daily ration or 240 mg/kg in roughage should not result in MOE values below 10,000 even in the case the animals are slaughtered shortly after exposure. Based on the obtained data, there is no hint for PA accumulation in muscle or liver, and the major amount of the PA load is expected to be eliminated within 48 h and is thus no longer detectable in dairy animal tissues.

With regard to the health risks for humans, feeding PA-free feed before slaughter could therefore be a measure if the animals have been exposed to high levels of PA.

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CRediT authorship contribution statement

Julian Taenzer: Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Anja These:** Writing – original draft, Validation, Investigation. **Karin Knappstein:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Julika Lamp:** Writing – review & editing, Resources, Methodology, Investigation. **Sven Dänicke:** Writing – review & editing, Project administration, Investigation, Funding acquisition, Conceptualization. **Janine Saltzmann:** Writing – review & editing, Project administration, Investigation, Funding acquisition. **Christoph Gottschalk:** Writing – review & editing, Funding acquisition, Conceptualization. **Illya Fedotenko:** Writing – review & editing, Investigation. **Wolfgang Jira:** Writing – review & editing, Validation, Project administration, Funding acquisition, Data curation.

Declaration of competing interest

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

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Data availability

Data will be made available on request.

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6.3. Other journal publications with the participation of Julian Tänzer

Effects of ensiling conditions on pyrrolizidine alkaloid degradation in silages mixed with two different Senecio spp.
Klevenhusen, F., These, A., Taenzer, J., Weiß, K., & Pieper, R. (2022).
Archives of Animal Nutrition, 76(2), 93–111.
https://doi.org/10.1080/1745039X.2022.2084321

Short-term exposure of dairy cows to pyrrolizidine alkaloids from tansy ragwort (Jacobaea vulgaris Gaertn.): effects on health and performance.
Knoop, K., Knappstein, K., Kaltner, F., Gabler, A. M., Taenzer, J., These, A., Kersten S., Meer U., Frahm J., Kluess J., Hüther L., Gottschalk C., Knudsen K., Saltzmann J. & Dänicke, S. (2023).
Archives of Animal Nutrition, 77(5), 363–384.
https://doi.org/10.1080/1745039X.2023.2261806

6.4. Conference contributions

Flash-poster presentation
Comparative ruminal metabolism of pyrrolizidine alkaloids in cheep and cattle
J. Tänzer, J. Saltzmann, K. Knoop, S. Dänicke, K. Knappstein, J. Lamp, C. Gottschalk, A. These
77. Tagung der Gesellschaft für Ernährungsphysiologie, 2023

Selektiver und geringer Transfer von Pyrrolizidinalkaloiden von *Jacobaea vulgaris* Gaertn. in das Fleisch und die Leber von Milchtieren (Kuh, Schaf, Ziege)
J. Tänzer, A. These, K. Knappstein, J. Lamp, S. Dänicke, J. Saltzmann, C. Gottschalk, I. Fedotenko, W. Jira
52. Deutsche Lebensmittelchemietage, 2024

Presentations
Untersuchungen zur gastrointestinalen Permeabilität von Pyrrolizidinalkaloiden in Rindern
J. Tänzer
„Was wurde aus Alma“ – Abschluss-Symposium zum Organ-Sharing-Project 2020, 2022

Poster

Can the different toxicity of pyrrolizidine alkaloids in cows and pigs be explained by their intestinal permeability?

J. Tänzer, A. These, S. Geiger, J.R. Aschenbach, S. Hessel-Pras, A.M. Enge, J. Buchmüller, I. Röhe, R. Pieper

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