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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*

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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 C═C 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 1,2-unsaturated 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

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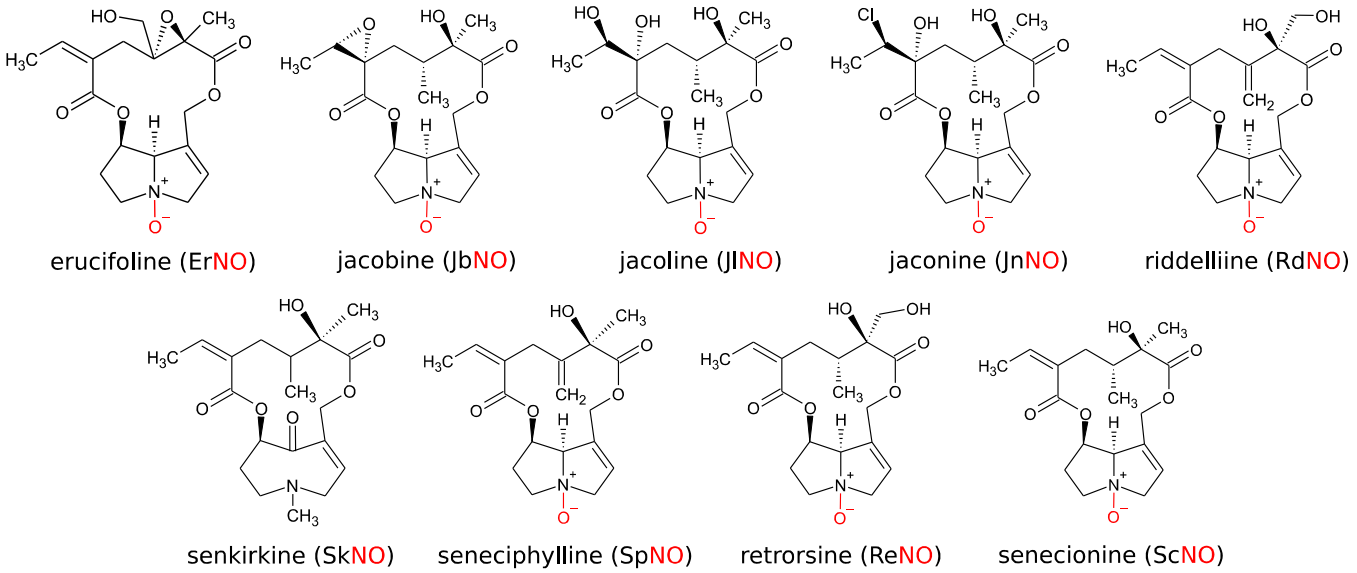

Figure 1. Structures of the major pyrrolizidine alkaloids produced by Senecio plants. Each compound occurs both as free base form and corresponding N-oxide (marked in red).

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.

MATERIALS 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

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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

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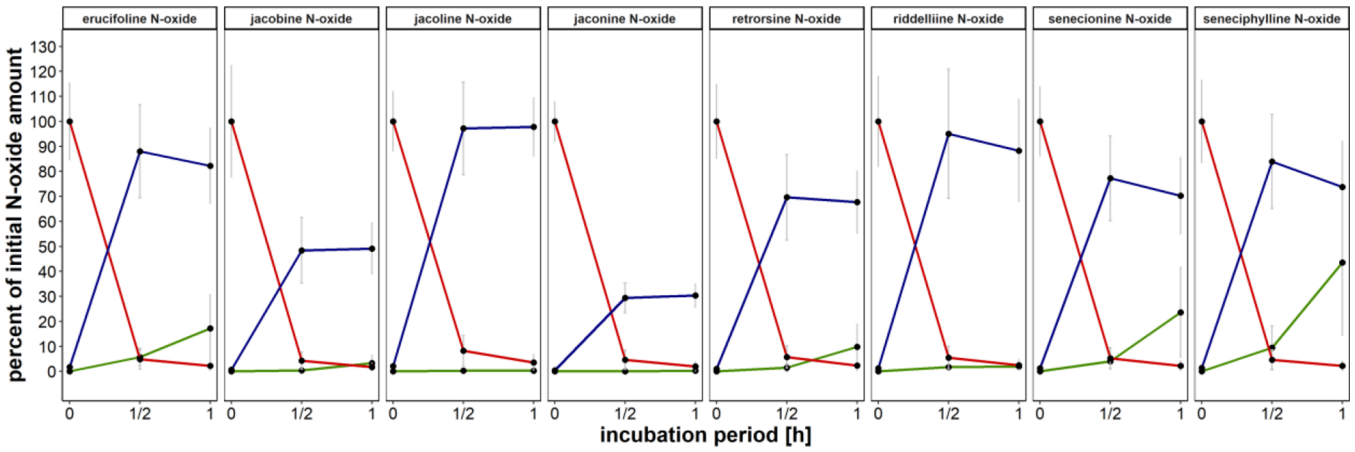

Figure 2. Incubation of a mixture of Senecio PANO standards (structures shown in Figure 1) with rumen inoculum from fistulated cows (n = 6). The concentration plots during the first hour of incubation show that the PANO concentration decreases (red lines), while at the same time the concentration of 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.

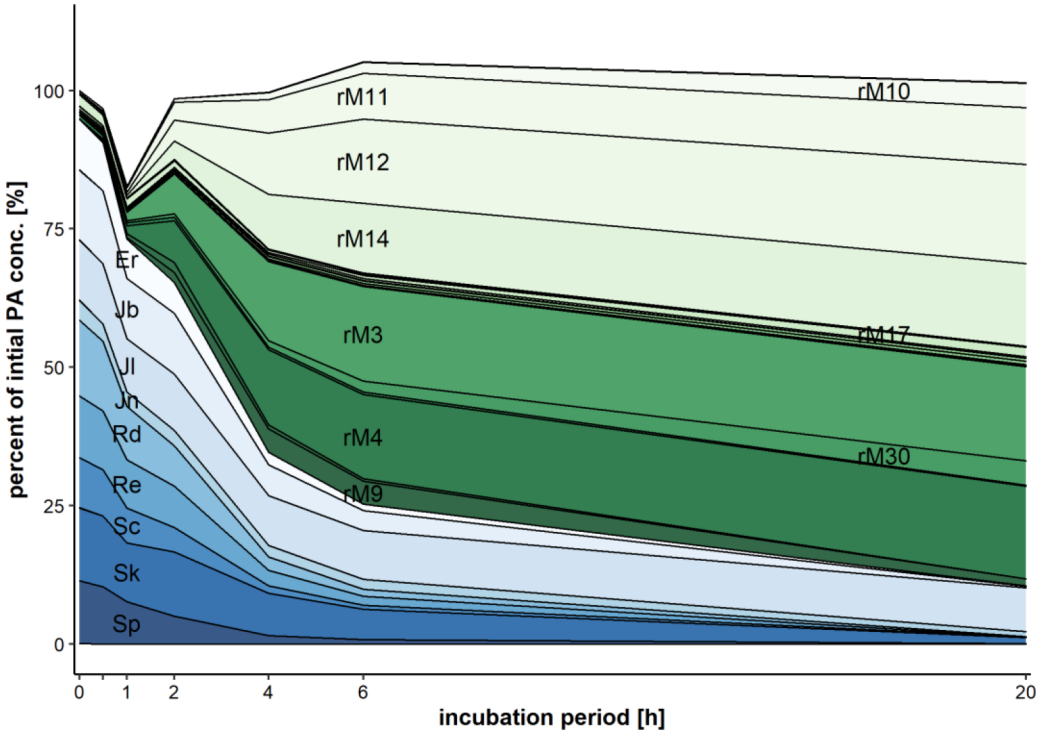

Figure 3. Incubation of a mixture of Senecio PA standards (structures shown in Figure 1) with inocula from rumen-fistulated cows. Samples were taken at t = 0, 0.5, 1, 2, 4, 6 and 20 h. Shown are the concentrations of the PAs and their ruminant metabolites (rM). Analytes in blue represent 1,2-unsaturated 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.

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

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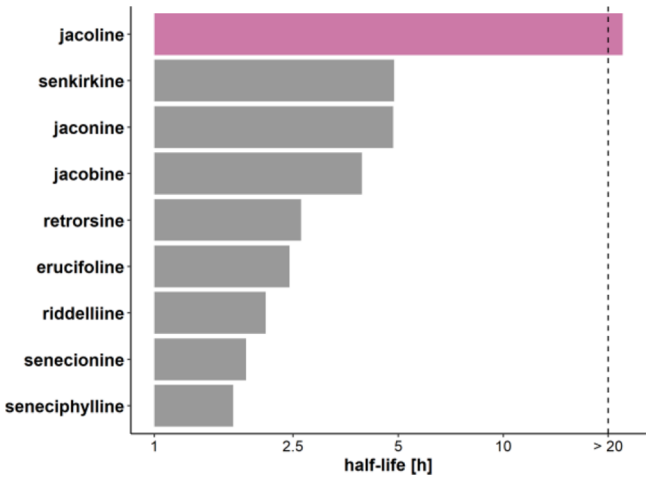

Figure 4. Half-lifes [h] of PAs determined by incubation with rumen inoculum from fistulated cows. Shown are the mean values resulting from incubations of Senecio PA and PANO mixtures performed in three biological replicates each, where each replicate was performed as a technical duplicate (in total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

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).

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 half-lives 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). 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).

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 1,2-saturated necine base structures formed during ruminal

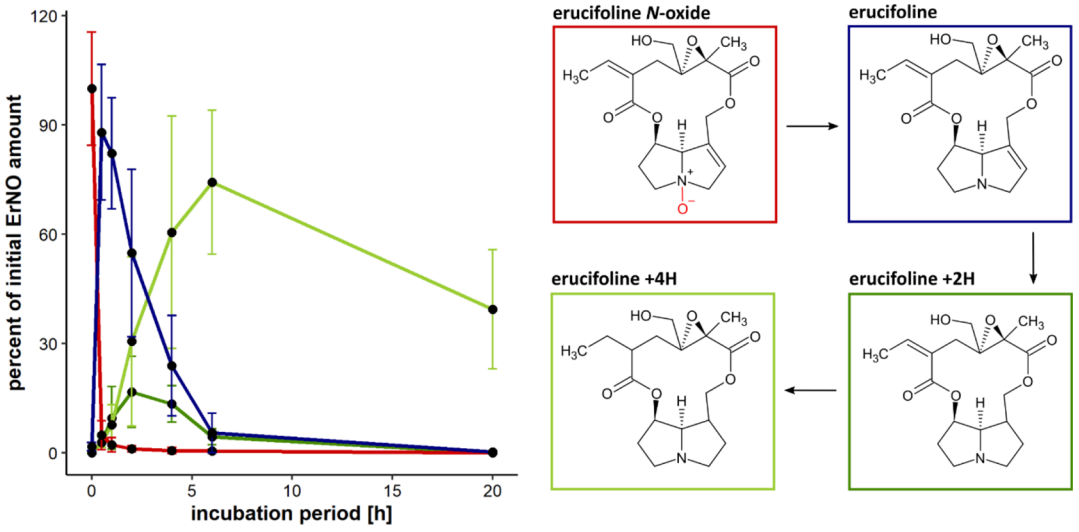

Figure 5. Overview of structural changes of pyrrolizidine alkaloids during rumen metabolism using erucifoline *N*-oxide (ErNO) as an example. All *N*-oxides present in Senecio plants are transformed into the corresponding PA, followed by the reduction of double bonds present in the necine base and necic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

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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.

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).

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

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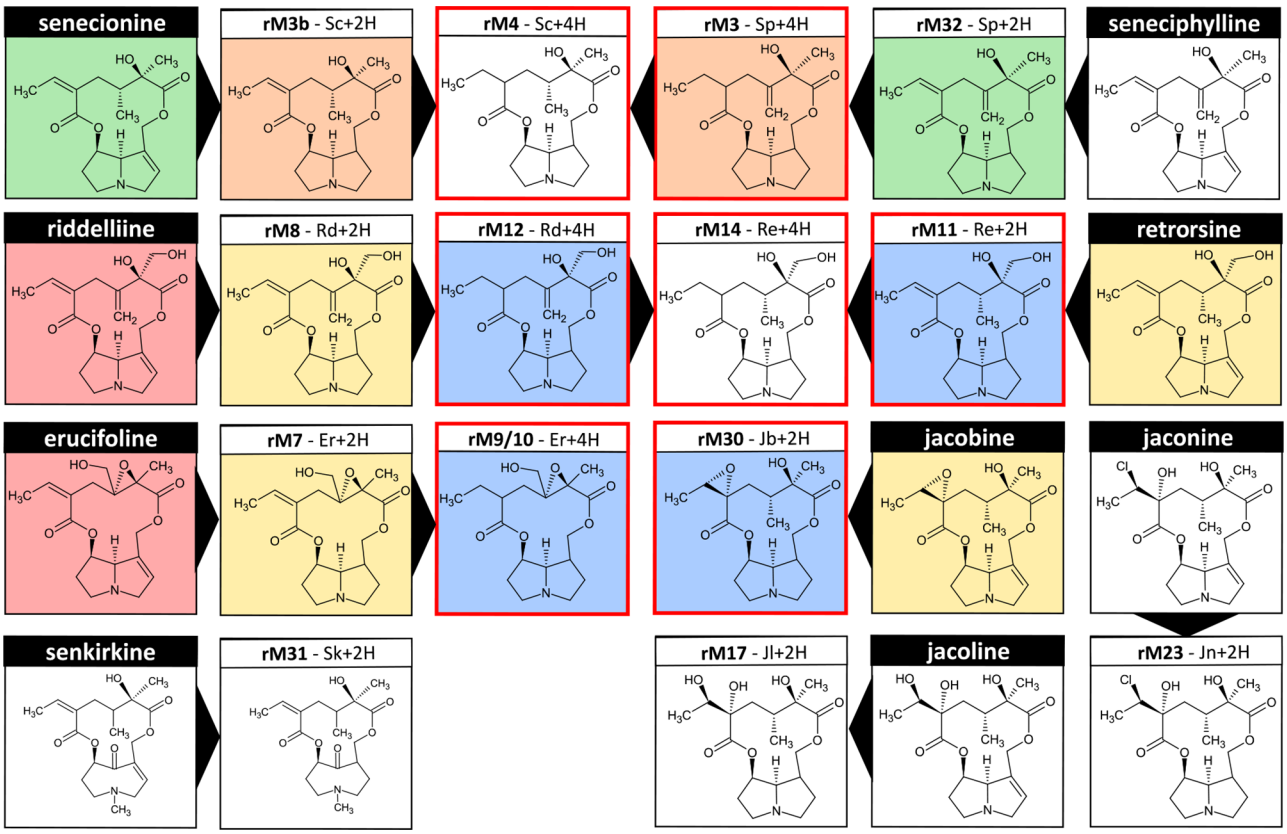

Figure 6. Structure proposals for identified main metabolites of rumen metabolism from Senecio PA (black headline). Metabolites with the same sum formula (molecular mass) are highlighted with the same color. Some metabolites can result from various PAs, such as rM3 or rM4, which can be formed by both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

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]

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 1,2-saturated 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.

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

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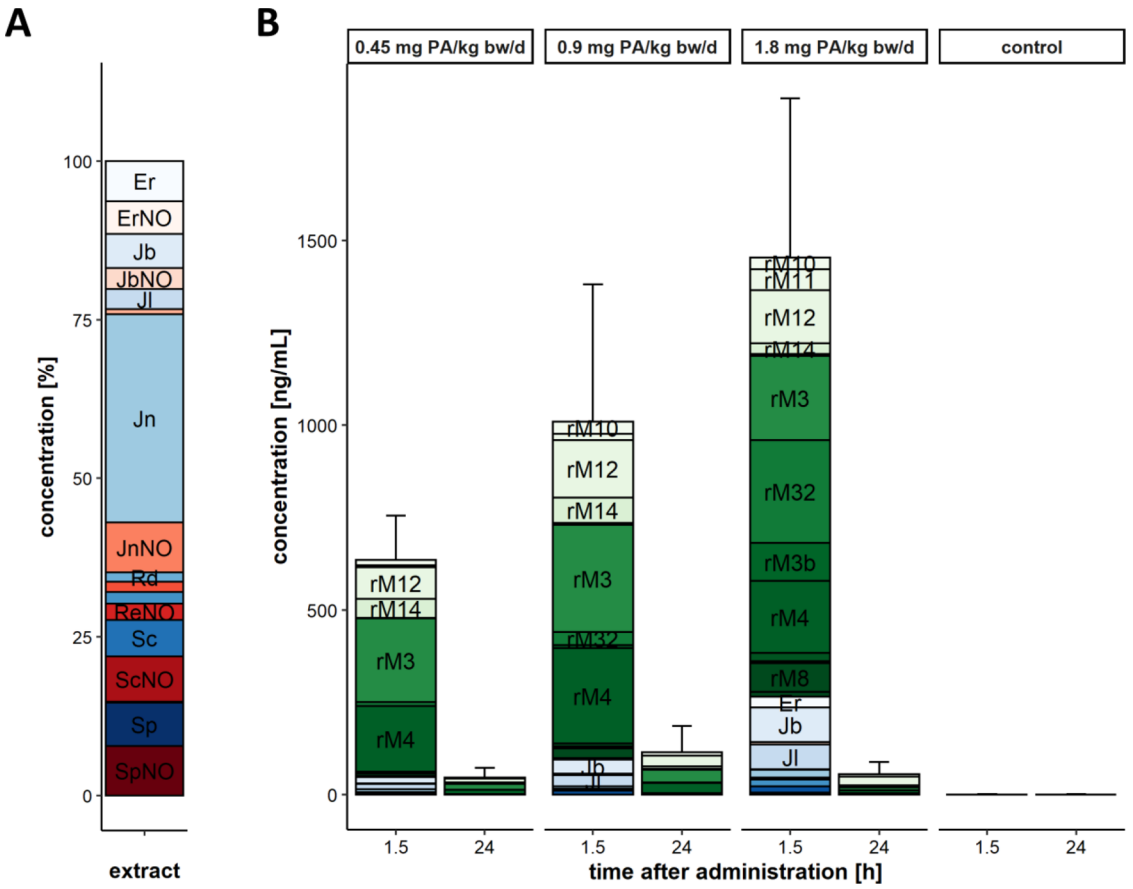

Figure 7. (A) PA (blue) and PANO (red) profile of a Jacobaea vulgaris extract administered to dairy cows via gavage in a 28-day feeding study and (B) Determined concentrations of 1,2-unsaturated PAs (blue) and PANOs (red) and their rumen metabolites (green) in ruminal liquids of tested cows. Three different 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).

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 1,2-unsaturated 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

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of PA in the rumen and consequently different times available for rumen metabolism and disappearance of PA.

■ ASSOCIATED CONTENT

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 half-lives of the pyrrolizidine alkaloids, composition of the *Jacobaea vulgaris* extract, and concentrations of 1,2-unsaturated PAs/PANOs and their rumen metabolites in ruminal liquids of the in vivo experiment (PDF)

■ AUTHOR INFORMATION

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, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, 38116 Braunschweig, Germany*

Sven Dänicke − *Institute of Animal Nutrition, Friedrich-Loeffler-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

The authors declare no competing financial interest.

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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

■ REFERENCES

(1) Landesamt für Landwirtschaft Umwelt und ländliche Räume des Landes Schleswig-Holstein Umgang mit dem Jakobs-Kreuzkraut. Meiden − Dulden − Bekämpfen. https://www.schleswig-holstein.de/DE/Fachinhalte/L/lebensmittel/Downloads/broschuere.pdf (accessed December 11, 2021).

(2) Gottschalk, C.; Kaltner, F.; Zimmermann, M.; Korten, R.; Morris, O.; Schwaiger, K.; Gareis, M. Spread of Jacobaea vulgaris and Occurrence of Pyrrolizidine Alkaloids in Regionally Produced Honeys from Northern Germany: Inter- and Intra-Site Variations and Risk Assessment for Special Consumer Groups. *Toxins* **2020**, *12*, 441.

(3) Jung, S.; Lauter, J.; Hartung, N. M.; These, A.; Hamscher, G.; Wissemann, V. Genetic and chemical diversity of the toxic herb Jacobaea vulgaris Gaertn. (syn. Senecio jacobaea L.) in Northern Germany. *Phytochemistry* **2020**, *172*, No. 112235.

(4) Möhler, H.; Diekötter, T.; Donath, T. W. Regulation of Jacobaea vulgaris by varied cutting and restoration measures. *bioRxiv* **2021**, DOI: 10.1101/2021.02.22.432250.

(5) Maskell, L. C.; Henrys, P.; Pescott, O. L.; Smart, S. M. Long-term trends in the distribution, abundance and impact of native "injurious" weeds. *Appl. Veg. Sci.* **2020**, *23*, 635−647.

(6) EFSA. Scientific Opinion on Pyrrolizidine alkaloids in food and feed. *EFSA J.* **2011**, *9*, 1−134.

(7) Mädge, I.; Gehling, M.; Schöne, C.; Winterhalter, P.; These, A. Pyrrolizidine alkaloid profiling of four Boraginaceae species from Northern Germany and implications for the analytical scope proposed for monitoring of maximum levels. *Food Addit. Contam., Part A* **2020**, *37*, 1339−1358.

(8) Mattocks, A. R. *Chemistry and toxicology of pyrrolizidine alkaloids*; Academic Press Inc., 1986.

(9) Molyneux, R. J.; Johnson, A. E.; Roitman, J. N.; Benson, M. E. Chemistry of toxic range plants. Determination of pyrrolizidine alkaloid content and composition in Senecio species by nuclear magnetic resonance spectroscopy. *J. Agric. Food Chem.* **1979**, *27*, 494−499.

(10) Mei, N.; Guo, L.; Fu, P. P.; Fuscoe, J. C.; Luan, Y.; Chen, T. Metabolism, genotoxicity, and carcinogenicity of comfrey. *J. Toxicol. Environ. Health, Part B* **2010**, *13*, 509−526.

(11) Ruan, J.; Yang, M.; Fu, P.; Ye, Y.; Lin, G. Metabolic activation of pyrrolizidine alkaloids: insights into the structural and enzymatic basis. *Chem. Res. Toxicol.* **2014**, *27*, 1030−1039.

(12) Mattocks, A. R. Toxicity of pyrrolizidine alkaloids. *Nature* **1968**, *217*, 723−728.

(13) Mattocks, A. R.; White, I. N. H. The conversion of pyrrolizidine alkaloids to N-oxides and to dihydropyrrolizine derivatives by rat-liver microsomes in vitro. *Chem.-Biol. Interact.* **1971**, *3*, 383−396.

(14) Miranda, C. L.; Cheeke, P. R.; Buhler, D. R. Effect of pyrrolizidine alkaloids from tansy ragwort (Senecio jacobaea) on hepatic drug-metabolizing enzymes in male rats. *Biochem. Pharmacol.* **1980**, *29*, 2645−2649.

(15) White, I. N. H.; Mattocks, A. R.; Butler, W. H. The conversion of the pyrrolizidine alkaloid retrorsine to pyrrolic derivatives in vivo and in vitro and its acute toxicity to various animal species. *Chem.-Biol. Interact.* **1973**, *6*, 207−218.

(16) Schrenk, D.; Gao, L.; Lin, G.; Mahony, C.; Mulder, P. P. J.; Peijnenburg, A.; Pfuhler, S.; Rietjens, I. M. C. M.; Rutz, L.; Steinhoff, B.; These, A. Pyrrolizidine alkaloids in food and phytomedicine: Occurrence, exposure, toxicity, mechanisms, and risk assessment - A review. *Food Chem. Toxicol.* **2020**, *136*, No. 111107.

(17) Petzinger, E. Pyrrolizidine alkaloids and seneciosis in farm animals. Part 2: clinical signs, species-specific sensitivity, food residues, feed contamination, limit values. *Tierarztl Prax Ausg G Grosstiere Nutztiere* **2011**, *39*, 363−372.

(18) Cheeke, P. R. Comparative Toxicity and Metabolism of Pyrrolizidine Alkaloids in Ruminants and Nonruminant Herbivores. *Can. J. Anim. Sci.* **1984**, *64*, 201−202.

(19) Cheeke, P. R. Toxicity and Metabolism of Pyrrolizidine Alkaloids. *J. Anim. Sci.* **1988**, *66*, 2343−2350.

(20) Aguiar, R.; Wink, M. Do naive ruminants degrade alkaloids in the rumen? *J. Chem. Ecol.* **2005**, *31*, 761−787.
(21) Loh, Z. H.; Ouwerkerk, D.; Klieve, A. V.; Hungerford, N. L.; Fletcher, M. T. Toxin Degradation by Rumen Microorganisms. A Review. *Toxins* **2020**, *12*, 664.
(22) Duringer, J. M.; Buhler, D. R.; Craig, A. M. Comparison of hepatic in vitro metabolism of the pyrrolizidine alkaloid senecionine in sheep and cattle. *Am. J. Vet. Res.* **2004**, *65*, 1563−1572.
(23) Huan, J. Y.; Miranda, C. L.; Buhler, D. R.; Cheeke, P. R. Species differences in the hepatic microsomal enzyme metabolism of the pyrrolizidine alkaloids. *Toxicol. Lett.* **1998**, *99*, 127−137.
(24) Wachenheim, D. E.; Blythe, L. L.; Craig, A. M. Characterization of rumen bacterial pyrrolizidine alkaloid biotransformation in ruminants of various species. *Vet. Hum. Toxicol.* **1992**, *34*, 513−517.
(25) Mulder, P. P. J.; Klijnstra, M. D.; Goselink, R. M. A.; van Vuuren, A. M.; Cone, J. W.; Stoopen, G.; Hoogenboom, R. L. A. P. Transfer of pyrrolizidine alkaloids from ragwort, common groundsel and viper's bugloss to milk from dairy cows. *Food Addit. Contam., Part A* **2020**, *37*, 1906−1921.
(26) Dickinson, J. O.; Cooke, M. P.; King, R. R.; Mohamed, P. A. Milk transfer of pyrrolizidine alkoloids in cattle. *J. Am. Vet. Med. Assoc.* **1976**, *169*, 1192−1196.
(27) Hoogenboom, L. A. P.; Mulder, P. P. J.; Zeilmaker, M. J.; van den Top, H. J.; Remmelink, G. J.; Brandon, E. F. A.; Klijnstra, M.; Meijer, G. A. L.; Schothorst, R.; Van Egmond, H. P. Carry-over of pyrrolizidine alkaloids from feed to milk in dairy cows. *Food Addit. Contam., Part A* **2011**, *28*, 359−372.
(28) Mould, F. L.; Morgan, R.; Kliem, K. E.; Krystallidou, E. A review and simplification of the in vitro incubation medium. *Anim. Feed Sci. Technol.* **2005**, *123*, 155−172.
(29) Mauricio, R. M.; Mould, F. L.; Dhanoa, M. S.; Owen, E.; Channa, K. S.; Theodorou, M. K. A semi-automated in vitro gas production technique for ruminant feedstuff evaluation. *Anim. Feed Sci. Technol.* **1999**, *79*, 321−330.
(30) FISA Studies on the transfer of pyrrolizidine alkaloids into livestock (PA-SAFE-FEED). https://www.fisaonline.de/en/find-projects/details/?tx_fisaresearch_projects%5Baction%5D=projectDetails&tx_fisaresearch_projects%5Bcontroller%5D=Projects&tx_fisaresearch_projects%5Bp_id%5D=13972&cHash=2314e223c70ca20108cf327f2195938a#more (accessed April 7, 2022).
(31) Ruan, J.; Liao, C.; Ye, Y.; Lin, G. Lack of Metabolic Activation and Predominant Formation of an Excreted Metabolite of Nontoxic Platynecine-Type Pyrrolizidine Alkaloids. *Chem. Res. Toxicol.* **2014**, *27*, 7−16.
(32) Hungerford, N. L.; Carter, S. J.; Anuj, S. R.; Tan, B. L. L.; Hnatko, D.; Martin, C. L.; Sharma, E.; Yin, M.; Nguyen, T. T. P.; Melksham, K. J.; Fletcher, M. T. Analysis of Pyrrolizidine Alkaloids in Queensland Honey: Using Low Temperature Chromatography to Resolve Stereoisomers and Identify Botanical Sources by UHPLC-MS/MS. *Toxins* **2019**, *11*, 726.
(33) Geburek, I.; Preiss-Weigert, A.; Lahrssen-Wiederholt, M.; Schrenk, D.; These, A. In vitro metabolism of pyrrolizidine alkaloids − Metabolic degradation and GSH conjugate formation of different structure types. *Food Chem. Toxicol.* **2020**, *135*, No. 110868.
(34) Geburek, I.; Schrenk, D.; These, A. In vitro biotransformation of pyrrolizidine alkaloids in different species: part II—identification and quantitative assessment of the metabolite profile of six structurally different pyrrolizidine alkaloids. *Arch. Toxicol.* **2020**, *94*, 3759−3774.
(35) Kolrep, F.; Numata, J.; Kneuer, C.; Preiss-Weigert, A.; Lahrssen-Wiederholt, M.; Schrenk, D.; These, A. In vitro biotransformation of pyrrolizidine alkaloids in different species. Part I: Microsomal degradation. *Arch. Toxicol.* **2018**, *92*, 1089−1097.
(36) Udén, P. The effect of intake and hay: Concentrate ratio upon digestibility and digesta passage. *Anim. Feed Sci. Technol.* **1984**, *11*, 167−179.
(37) Mambrini, M.; Peyraud, J. L. Retention time of feed particles and liquids in the stomachs and intestines of dairy cows. Direct measurement and calculations based on faecal collection. *Reprod., Nutr., Dev.* **1997**, *37*, 427−442.

https://doi.org/10.1021/acs.jafc.2c01332
J. Agric. Food Chem. 2022, 70, 10111−10120