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Transfer of tropane alkaloids (atropine and scopolamine) into the milk of subclinically exposed dairy cows

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ABSTRACT

Tropane alkaloids are toxic secondary metabolites found in agricultural weeds, like jimson weed (*Datura stramonium*) and henbane (*Hyoscyamus niger*). The intake of food and feed contaminated with tropane alkaloids led to several incidents of poisoning in humans and animals. Because data on the transfer of tropane alkaloids into the milk of exposed dairy cows were not available in scientific literature, the present study was carried out. Four cows received tropane alkaloids (atropine sulfate and scopolamine hydrobromide trihydrate) orally in three increasing dosage levels for five days each. The total doses of pharmacologically active alkaloids (sum of L-hyoscyamine and scopolamine) at the levels 1, 2, and 3 were 93, 186, and 279 μg/kg body weight/day, respectively. None of the applied dosage levels induced obvious clinical symptoms in the cows. The alkaloid content in composite milk of individual cows was measured for each milking time. Even though the maximum mean transfer rates (atropine: 0.037%, scopolamine: 0.007%) were very low, a transfer of tropane alkaloids into milk could already be proven at the lowest dosage level. At the highest dosage level, the biologically active substances were detected in milk at a mean level of 1.60 ± 0.07 μg/kg. The results obtained in this study indicate that under particular circumstances contaminated raw milk may contribute to the exposure of consumers to tropane alkaloids.

1. Introduction

Tropane alkaloids (TA) are toxic secondary metabolites occurring in several plant families. In Central Europe, jimson weed *(Datura stramonium*) belongs to the most relevant agricultural weeds containing these phytotoxins (Alletsee, Weller, & Altmann, 2006). *Datura stramonium* can be found in all Central European countries and even in the southern parts of Scandinavia (Global Biodiversity Information Facility, 2020). Therefore, infestations of agricultural areas with this plant are a frequent source of TA in food and feed (Aboling et al., 2019; Disel, Yılmaz, Kekec, & Karanlık, 2016; Fretz et al., 2007).

As a member of the tardily sprouting solanaceous plant family, jimson weed is especially difficult to detect and control in summer crops like millet, maize, and buckwheat (Söchting & Pfundheller, 2018). Products containing these cereals are particularly prone to contamination with TA leading to the intoxication of consumers (Perharič et al., 2005) and repeated public product recalls. This is in line with 42 TA-associated notifications reported in the European Rapid Alert System for Food and Feed (RASFF) since 2012 (status February 2021).

More than 200 TA are known, but atropine (a racemic mixture of D- and L-hyoscyamine) and scopolamine are the best investigated compounds of this group. The pharmacological effects of atropine in mammals are mainly caused by L-hyoscyamine (EMEA, 1998). Based on their parasympatholytic mode of action, the original substances as well as chemical modifications of TA have been used for medical treatment since a long time (Brown & Taylor, 2006). However, their non-specific binding characteristic to muscarinic receptors can induce several side effects beyond the desired therapeutic benefit (Bhattarcharjee et al., 2013). Clinical symptoms include pupil dilatation, dryness of the mouth mucosa, indigestions, respiratory depression, as well as central nervous system-mediated effects like restlessness and seizures (Lüllmann & Mohr, 2001).

Regarding this existing hazard, the European Food Safety Authority (EFSA) published a risk assessment on the human acute exposure to TA

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(EFSA, 2013). As the onset of pharmacological effects of these substances is observed shortly after oral intake, an acute reference dose (ARfD) for the sum of L-hyoscyamine and scopolamine was established at a level of 0.016 μg/kg body weight. This group ARfD is based on the No Observed Adverse Effect Level (NOAEL) in a human volunteer study (Perharič et al., 2013) and is defined as the quantity of a substance that can be ingested, usually during one meal or one day, without appreciable health risk to the consumer (FAO/WHO, 2002). Based on the analytical results of 7391 food samples of plant origin using the upper bound approach, it was estimated that the dietary exposure of infants to TA could be up to three times the group ARfD. Bread and other grain milling products have been found to be the main contributors to the co-exposure of atropine and scopolamine (EFSA, 2018).

Until now, only plant-based foods are considered for the risk assessment, while the contribution of food from animal origin to the exposure of consumers to TA currently remains unknown. Even though the potential transfer of atropine to human breast milk has been assumed (EMEA, 1998), there are no studies available on the transfer of these compounds from feed to bovine milk and products thereof. Nevertheless, several cases of jimson weed intoxication in cattle have been published. Bofill, Bofill, Such, Piqué, & Guitart (2007) described acute intoxications in two Spanish dairy cattle farms caused by *Datura*-contaminated maize silage in one case and crushed maize in the other case. In 2018, approximately 120 fattening bulls at one German farm were poisoned by *Datura*-contaminated maize silage (Aboling et al., 2019). Furthermore, Nelson et al. (1982) conducted a 14-day feeding study with 11 yearling heifers receiving three different jimson weed seed dosages. All studies described the intoxicated cattle with special regard to animal health aspects; therefore, toxin residues in meat and milk were not considered. However, the cases of jimson weed poisoning in bovines led to the question whether TA may have a negative impact on the safety of animal products from intoxicated cattle.

Livestock with obvious signs of acute intoxication would certainly be excluded from food production (EFSA, 2008). In contrast, a transfer of TA from feed to the milk of subclinically exposed animals would be of special concern regarding food safety aspects. Therefore, the objective of this study was to examine the transfer of TA from feed into bovine milk.

2. Material and methods

2.1. Experimental animals

The experiment was conducted at the agricultural research station of the Max Rubner-Institut in Schädtbek from March 25 to April 15, 2019. Eight lactating, healthy, non-gravid dairy cows, German Holstein black and white breed, were allocated to the experimental and control groups according to the performance parameters shown in Table 1.

During the trial, experimental and control cows were kept together in a separate section of the outdoor climate free stall barn with deep straw bedding cubicles. All animals had free access to a mixed ration of grass and maize silage and received additional concentrate feed from a computer-controlled feeding station according to the individual milk yield.

The cows were milked in a tandem milking parlor (GEA Farm Technologies, Bönen, Germany) twice a day at the regular milking times (7 a.m. and 5 p.m., milking intervals: 10 and 14 h). During the experiment and the following 28-day withdrawal period, the milk of the experimental group was disposed of after sampling.

Table 1
Animal data.

Number	Body weight (kg)	BCS[1]	Number of lactations	Days in milk (DIM)[2]	Milk yield[3] (kg/day)
Experimental cows					
1	690	3.50	3	144	37.9
2	790	3.25	2	189	29.6
3	700	3.50	2	167	25.1
4	670	3.00	1	251	16.5
Control cows					
5	650	2.00[4]	3	165	41.3
6	730	3.25	2	127	32.8
7	640	3.00	2	166	25.7
8	670	3.00	1	318	22.4

[1] BCS = Body Condition Score (according to Edmonson et al., 1989, details see Supplementary file A);
[2] At the beginning of the trial;
[3] Average during the trial;
[4] BCS of 2.0 indicates undercondition of cow 5 due to a high milk yield during lactation and has no effect on the transfer studies, because the cow belongs to the control group

2.2. Official authorizations

The animal experiment was approved by the Ministry of Energy, Agriculture, the Environment, Nature, and Digitalization of Schleswig-Holstein, Germany (reference number V244-59456/2018; 111–11/18). The Federal Office of Consumer Protection and Food safety (BVL) determined a 28-day withdrawal period for milk and meat of the experimental group after the end of the supplementation (reference number 305.36004.0.252501). This was confirmed by the department responsible for the monitoring of veterinary drugs at the Federal State Laboratory of Schleswig-Holstein, Germany (reference number GeB3-725.4.3.02.02).

2.3. Supplementation

To ensure an exact and safe dosage for the experimental animals according to the individual body weight, chemically defined atropine sulfate (CAS 5908-99-6) as a racemic mixture (optical rotation −0.06°) and scopolamine hydrobromide (CAS 6533-68-2) were used as pure substances in pharmacopoeia quality from Fagron GmbH (Barsbüttel, Germany) instead of natural plants. This allowed the precise calculation of the TA transfer rates into milk.

In order to analyze the dose-dependent transfer of the TA, the four cows received atropine sulfate and scopolamine hydrobromide in three increasing dosage levels. The compounds were administered at each dosage level for five days. Dosage level 1 followed the suggestion of Löscher (2006) for the oral long-term therapy of heart diseases in cattle with atropine sulfate. In contrast to atropine sulfate, scopolamine hydrobromide is not used therapeutically, and there were no data available on an adequate dosage of this substance in cattle. Therefore, the dosage of pure scopolamine was generally set to half the dose of atropine sulfate. At levels 2 and 3, these dosages were doubled and tripled, respectively (Table 2). When comparing the dosages of the pure substances, these corresponded to an atropine: scopolamine ratio of 1: 0.6.

The sum of L-hyoscyamine and scopolamine applied at level 3 (279 μg/kg b.w.) corresponded to the NOAEL for cattle (300 μg/kg b.w.) as suggested by EFSA (2008). The applied salts atropine sulfate and scopolamine hydrobromide were assumed to dissociate quickly after intake; hence, their pharmacological action should be identical to the natural plant toxins. The substances were administered orally using capsules (Science Services GmbH, Munich, Germany) individually prepared for each cow and dosage level by adding 10% solutions of atropine-sulfate and scopolamine hydrobromide in purified water to 12 g of ground dairy concentrate feed as adsorbent. The capsules were firmly sealed using citric acid (1% solution). According to the recommendations for long-term oral therapy (Löscher, 2006), the daily dose was divided into three equal parts and applied every 8 h (05:00 a.m., 1:00 p.m. and 9:00 p.m.).

Table 2
Dosage of atropine and scopolamine.

Level	Dosage (μg/kg body weight/day)				
	Atropine sulfate[1]	Scopolamine hydrobromide trihydrate[2]	Atropine	Scopolamine	Sum of pharmacologically active alkaloids L-hyoscyamine + scopolamine[5]
1	100	72	86	50	93
2	200	144	171	100	186
3	300	217	257	150	279

[1]Atropine sulfate 676.82 g/mol;
2Scopolamine hydrobromide trihydrate 438.3 g/mol;
[3]Atropine = racemate of D-/L-hyoscyamine 289.38 g/mol;
[4]Scopolamine 303.35 g/mol;
[5]As the atropine sulfate used in this study complied with the guidelines of the European Pharmacopoeia, the pharmacological active isomer l-hyoscyamine corresponded to 50% of the atropine amount.

2.4. Clinical examination

All animals were examined daily by a veterinarian to detect early signs of TA intoxication. According to the published side effects of TA intake (Aboling et al., 2019; Lüllmann & Mohr, 2001), the pupillary light reflex, muzzle humidity, heart rate, respiratory rate, rumen activity, and behavior were recorded (Supplementary file A). The daily milk yield and concentrate feed intake were also monitored.

2.5. Sampling

The sampling was started two days before and ended five days after the supplementation. In the experimental group, milk samples of each cow were taken daily at both milking times from the composite milk as a mixture of all four quarters after completion of milking. In the control group, the milk was sampled prior to supplementation, at each dosage level, and during the washout period at four consecutive milking times in each case.

Each sample was divided into two 100 mL aliquots for TA analysis. One of these was frozen at −20 °C as a retain sample and the other was stored for up to 60 h at 4 °C until further processing for laboratory analysis. First, these milk samples were heated up to 20 °C in a water bath and homogenized by shaking. Then, the samples were skimmed to improve physical sample stability during frozen storage. An aliquot of 50 mL was taken for centrifugation at 2200 g for 40 min at 4 °C. Skimmed milk and sediment were separated by decantation. The skimmed milk was frozen and stored at −20 °C until analysis. Prior to laboratory analysis, the frozen milk samples were left in the refrigerator overnight (4 °C), then thawed at room-temperature and afterwards homogenized by shaking.

2.6. TA analysis in milk

Chemicals and reagents used were of analytical grade. Methanol (CAS 67-56-1) was purchased from Honeywell/Riedel-de Haën (Seelze, Germany). Atropine standard solution (102.5 μg/mL; CAS 51-55-8) was supplied by Biopure (Tulln, Austria). Scopolamine hydrobromide trihydrate, >98% (CAS 6533-68-2) and formic acid (98%; CAS 64-18-6) were obtained from Sigma Aldrich (Taufkirchen, Germany). Ultrapure water (18.2 MΩcm-1) supplied by the Milli-Q® Advantage A10® System (Merck, Darmstadt, Germany) was used throughout analysis.

For sample centrifugation, a Jouan KR4i (Thermo Fisher Scientific, Waltham, USA) was used. The micro centrifugation was performed by operating a Himac CT15RE (Hitachi Koki Co., Ltd., Tokyo, Japan). All analytical experiments were carried out on a Shimadzu LC 20 system (consisting of an online degasser, 2 binary pumps, an autosampler with a variable injection system and a column thermostat; Shimadzu, Duisburg, Germany) coupled to a mass spectrometer 4000 QTrap (AB SCIEX, Darmstadt, Germany) with electrospray ionization. Separation was performed on a Luna 5 μm Phenyl-Hexyl-column ID 2 mm × 150 mm (Phenomenex, Aschaffenburg, Germany).

The method of Tsiplakou et al. (2014) served as the point of departure for the method extension to determine atropine and scopolamine in milk. A portion of 2 mL homogenized milk was weighed into a centrifuge tube. A volume of 4 mL extraction solvent (methanol/formic acid, 99/1 v/v) was added and the mixture was vortexed for 1 min. After centrifugation for 5 min at 4200 g and 4 °C, a volume of 2 mL was transferred to a micro reaction tube and frozen at −20 °C. After at least 12 h, the sample was once more centrifuged for 10 min at 18,000 g and −10 °C. The resulting supernatant served as sample ready for injection into the chromatographic system.

The separation was carried out on the reversed phase column using a mixture of water/formic acid (999/1 v/v; solvent A) and methanol (solvent B) as mobile phases at a flow rate of 0.5 mL/min. Gradient elution started with a mobile phase mixture of 10% B and held for 2 min, reached 80% B in 10 min, held for 2 min and went back to the initial conditions within 1 min. After 2 min equilibration time, the system started automatically with the next sample. A volume of 10 μL was injected while cooling the samples at 15 °C in the autosampler. The oven temperature was set to 40 °C.

For mass spectrometry, the ESI interface was used in positive ionization mode at 600 °C with the following general settings for the determination of atropine and scopolamine: curtain gas 20 psi, nebulizer gas 50 psi, auxiliary gas 55 psi, ionization voltage 3500 V, collision gas medium, multi reaction monitoring (MRM), and dwell time 150 msec. For each analyte, two mass transitions were recorded applying individually optimized MS/MS conditions. For atropine, the MRM transitions at the retention time of 6.2 min were 290.0 m/z (precursor ion) to 124.1 m/z (product ion, quantifier) and 93.1 m/z (product ion, qualifier). Scopolamine was recorded at a retention time of 4.9 min with the precursor ion 304.2 m/z and the product ions of 138.2 m/z (quantifier) and 156.2 m/z (qualifier). Different concentrated atropine and scopolamine standard solutions in solvent (methanol/water/formic acid 60/40/0.4 v/v/v) were injected for system calibration and quantification of atropine and scopolamine using an external standard calibration in solvent. The calibration range was 0.1–5.0 ng/mL for both substances.

In order to validate the determination method, different concentrations of atropine and scopolamine were used. The repeatability was examined by analyzing the peak areas of two concentrations levels 0.1 ng/mL and 0.25 ng/mL in milk samples (0.3% fat content) spiked before extraction in tenfold repetitions and calculating the relative standard deviations. The intermediate repeatability was calculated from repeated measurements (n = 10) with standard solutions of 0.5 ng/mL and 5 ng/mL atropine and scopolamine on three different days. Recovery experiments were performed with spiked milk samples (0.3 and 3.5% fat content) containing 0.1 ng/mL and 0.25 ng/mL atropine and scopolamine. These spiked milk samples were treated as described above and injected and analyzed in triplicate, whereby each sample was injected 3 times from the same vial. The matrix effect (signal suppression/enhancement SSE) was quantified by multiplying the quotient between the area of the spiked matrix standard and the signal area of the standard in solvent by 100, as described by Steiner et al. (2020). The limit of detection was based on a signal to noise S/N ratio of 3 and the quantification limit on a S/N ratio of 10.

2.7. Statistical analysis

The calculation was carried out using the statistic package SAS (Version 9.4) with the GLM (general linear model) procedure, for which additive constants were included to an overall mean by considering at the same time a regression relationship to a covariate. In this procedure, the Tukey-Test for multiple comparison of means was used. All values

below the LOD (0.025 μg/kg) or the LOQ (0.075 μg/kg) were set as half the corresponding value, i.e. 0.0125 or 0.0375 μg/kg, respectively. Depending on the model, the following factors were included as additive constants: "cow", "milking time", "dosage level", "sort of alkaloid", "day within dosage level", and as covariate the "milk yield". A probability of $p<0.05$ was used to establish the statistical significance.

3. Results

3.1. Supplementation

In three of the four experimental animals, the supplementation was carried out without any difficulties until the end of the trial. Cow 4 was accidentally overdosed on day 3 at dosage level 3 and thereafter developed signs of a TA intoxication, so that further supplementation was stopped. The sampling of this cow was carried out as planned, while the clinical observation was intensified until complete recovery. As the aim of this study was to analyze the transfer of TA into milk of subclinically exposed dairy cows, the values of cow 4 were only included in the statistical analysis until the day before the overdosing (level 3, day 2). The findings concerning the intoxication are described in a separate section (3.6).

3.2. Clinical symptoms

In the four control animals, none of the clinical parameters were conspicuous during the whole trial. Table 3 specifies the altered parameters in the experimental group and the day of onset of the symptoms.

The symptoms were only detected through careful clinical examination and did not influence the behavior of the animals, the milk yield, or the intake of concentrates. Therefore, the supplementation of the three regularly dosed cows was continued until the end of dosage level 3.

3.3. Analytical method to detect atropine and scopolamine in milk

The method published by Tsiplakou et al., in 2014 regarding the determination of mycotoxins in feed and milk samples was used and extended to determine atropine and scopolamine in milk samples. To the best of our knowledge, the analysis of atropine and scopolamine in milk samples has been described for the first time in this study. A small volume of milk was vigorously shaken with acidified methanol, centrifuged, and frozen overnight. After further centrifugation at minus 10 °C additional co-extractives as well as the major part of solidified fat were removed. Usually, the amount of the remaining residue from the -10 °C cooled centrifugation was low, because the milk samples used were skimmed prior to analysis. Further experiments with milk samples containing a higher fat percentage let us conclude that the described freezing out procedure is also applicable to milk samples containing higher fat contents. An aliquot of the twice centrifuged extraction solvent served as the sample ready to inject into the chromatographic system without any more filtration steps. Performance characteristics and statistical parameters of the applied method are shown in Table 4. The recovery was evaluated and reached values from 96% to 99% at different concentration levels. For both alkaloids, the LOD and the LOQ were 0.025 and 0.075 ng/mL, respectively. Matrix effects were expressed by SSE and showed very little influence, i.e. 96 % for atropine and 98% for scopolamine. Very good performance characteristics were achieved for this easy to perform, quick, and cost-effective determination method with a high sensitivity and a linear range between 0.1 and 5.0 ng/mL.

Table 3
Clinical symptoms in cows of the experimental group. For detailed information see Supplementary file A.

Clinical symptoms				
		Level	Day	Reduced rumen activity
2	5	Cow 2, 4		
3	1	Cow 2, 4		
3	2	Cow 1, 2, 4		
3	3[1]	Cow 1, 2, 3		Cow 1, 2, 3
3	4[1]	Cow 1, 2	Cow 3	Cow 1, 2, 3
3	5[1]	Cow 1, 2	Cow 3	Cow 1, 2, 3

[1] Cow 4 excluded

3.4. Detection of atropine and scopolamine in skimmed milk

As there was no evidence of atropine or scopolamine in milk during the anamnesis period or in milk samples of the control cows during the trial, it was assumed that a contamination of the regular animal feed with these alkaloids did not occur. In contrast, atropine and scopolamine were detected in the skimmed milk of all experimental cows during the supplementation. The resulting concentrations in skimmed milk are illustrated for each alkaloid and each cow separately in Fig. 1. The atropine concentrations exceeded the LOQ in all milk samples of all experimental animals following the first supplementation (Fig. 1). In contrast, only the scopolamine concentration in the milk of cow 3 exceeded the LOQ at dosage level 1 (Fig. 1), whereas the concentrations were below the LOQ in the milk of the other cows. At dosage level 2, the scopolamine concentration exceeded the LOQ in the milk samples of all experimental cows. The concentration of scopolamine was always lower than that of atropine. The time courses in Fig. 1 illustrate how the concentrations vary between the morning and the evening milking. Especially the scopolamine concentrations tended to be higher in the morning. However, for both alkaloids this effect was not statistically significant. Regarding the individual scopolamine concentrations, there were significantly lower contents in the milk of cow 1 and 2 ($p<0.05$). Interestingly, these cows had the highest average milk yield during the trial (37.9 and 29.6 kg/d).

The concentrations of atropine and scopolamine increased with each level (Fig. 1, Table 5). In addition, the atropine concentration tended to increase during the five supplementation days at dosage levels 2 and 3. This effect was less pronounced in the case of scopolamine. During the subsequent washout period, the concentrations quickly decreased and were below the LOD for atropine and scopolamine after five and two milking times, respectively.

To simulate the concentration of both alkaloids in composite milk, the mean atropine and scopolamine contents were calculated per cow as weighted mean based on the milk yield and concentrations at both milking times. The mean concentrations in the daily production of the experimental group were calculated as Least Square Mean (LSM) (Table 5). The results of cow 4 were excluded from the calculation beginning with the day of overdosing (level 3, day 3).

Additionally, the respective mean values for days 3–5 were calculated as LSM to improve comparability between the three dosage levels. It became apparent that the concentrations of both alkaloids in milk significantly increased as the dosage level increased ($p<0.05$). Although atropine and scopolamine were administered at a ratio of 1:0.6, the highest atropine mean value (2.55 ± 0.06 μg/kg) was almost eight times higher than the highest scopolamine mean value (0.32 ± 0.01 μg/kg) (Table 5).

The highest concentrations of both alkaloids were observed on the last day of the supplementation (level 3, day 5). However, the highest daily values were not detected in the milk of the same animal: the maximum atropine concentration (3.00 μg/kg) was measured in cow 2 and the maximum scopolamine concentration (0.51 μg/kg) in cow 3 (Fig. 1).

Table 4
Performance characteristics and statistical parameters of the method: linear range, coefficient of regression (R), limit of detection (LOD), limit of quantification (LOQ), repeatability, intermediate precision, recovery and matrix effect in skimmed milk expressed as signal suppression / enhancement (SSE). Skimmed milk with 0.3 % fat content was used for repeatability. For recovery experiments, skimmed milk (0.3 % fat)[1,2] and milk samples (3.5 % fat content)[5,6] were spiked.

Analyte	Linear range [ng/mL]	R	LOD [ng/mL]	LOQ [ng/mL]	Repeatability [%] n = 10	Intermediate precision [%] n = 10	Recovery [%] n = 3 / 2	Matrix effect SSE [%]
Atropine	0.1 – 5.0	0.9998	0.025	0.075	3.0 [1] 0.9 [2]	3.4 [3] 1.9 [4]	97 [1] / 95 [5] 99 [2] / 99 [6]	96
Scopol-amine	0.1 – 5.0	0.9999	0.025	0.075	2.7 [1] 1.3 [2]	1.5 [3] 1.0 [4]	96 [1] / 97 [5] 97 [2] / 95 [6]	98

Concentration in spiked skimmed milk: [1] 0.1 ng/mL; [2] 2.5 ng/mL
Concentration of standard in solvent: [3] 0.5 ng/mL; [4] 5.0 ng/mL
Concentration in spiked milk with 3.5 % fat content: [5] 0.1 ng/mL [6] 2.5 ng/mL

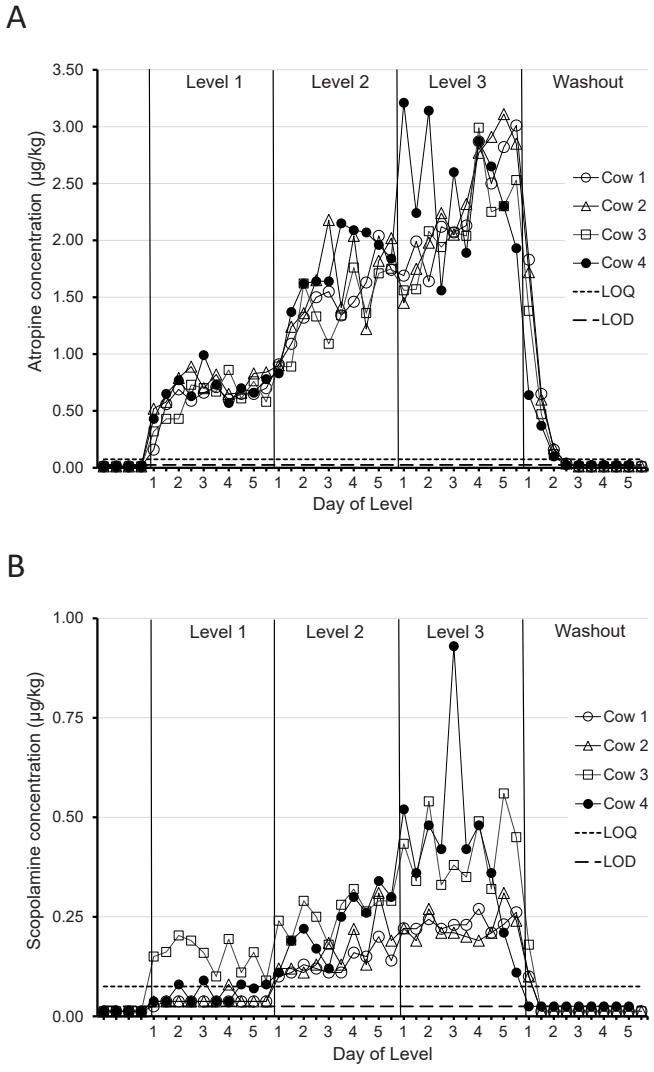

Fig. 1. Concentration of atropine (A) and scopolamine (B) expressed in µg/kg in skimmed milk of the individual cows. Major ticks correspond with morning milkings, minor ticks indicate the evening milkings. Cow 4 was overdosed on day 3 of level 3 and the supplementation was stopped on the following day (level 3, day 4).

3.5. Transfer rates

The transfer rates of atropine and scopolamine into milk were calculated as the ratio of the total excretion via milk to the alkaloid intake for days 3–5 at each dosage level (Table 6). Due to the fact that some scopolamine measurements were below the LOQ in level 1, the mean values for the scopolamine-transfer rates could not be calculated at this dosage level.

With values well below 0.1%, the transfer rates of both compounds into milk were generally very low. Basically, the transfer rate of atropine was approximately five times higher than that of scopolamine. The transfer rate of atropine at dosage level 1 was significantly lower than those at dosage levels 2 and 3 ($p<0.05$). This effect could not be observed with scopolamine. The factors “cow” and “milk yield” had no significant effect on the transfer rates of atropine and scopolamine.

3.6. Overdosing of cow 4

On day 3 at dosage level 3, cow 4 was accidentally overdosed. During the morning application at 5:00 a.m., it regurgitated the capsule. As this capsule appeared to be slightly crushed but still intact, the reserve capsule was administered and properly swallowed by cow 4. Presumably, the cow took up part of the atropine and scopolamine from the crushed capsule, as it developed signs of a TA intoxication shortly afterwards.

3.6.1. Clinical symptoms

On the first two days of dosage level 3, the cow showed no signs of a TA intoxication, but a moderate mydriasis. Three hours after the morning application on day 3 of dosage level 3, the cow had a moderate mydriasis and showed a reduced rumen activity. The milk yield of the morning milking was reduced by 2.7 kg (approx. 25%) compared to the two previous days. During the clinical examination at 12:00 p.m., the cow was slightly sleepy and showed an obviously slowed down intake of roughage. Due to these symptoms, the supplementation at 1:00 p.m. was skipped. In the following hours of the same day, rumen activity and behavior normalized, whereby only mydriasis and the reduction of milk yield in the evening milking (5.1 kg instead of 6.9 kg) were still observed. Therefore, the supplementation was resumed at 9:00 p.m. Three hours after the morning application on the following day (level 3, day 4), the cow showed distinct signs of a TA intoxication. The most remarkable symptoms were the drowsiness of the animal and its inability to carry out targeted food and water intake. In addition, the rumen activity was again significantly reduced. Therefore, further supplementation was stopped. After treatment with a rumen stimulant and an energy drink, the clinical symptoms improved within one day. Three days after the end of the supplementation (washout period, day 3), the behavior and rumen activity of the cow had normalized and the milk yield increased. Only the moderate mydriasis remained for two more days. On the last day of the washout period, the cow had completely recovered.

3.6.2. Concentration of atropine and scopolamine in skimmed milk during the intoxication

The clinical symptoms of cow 4 combined with the atropine and scopolamine concentrations in skimmed milk are shown in Table 7. As

Table 5
Mean concentrations of atropine and scopolamine in the daily milk of the experimental group; measured in skimmed milk, calculated as Least Square Mean (LSM) with standard deviation (s).

Level	Day	Atropine [µg/kg]				Scopolamine [µg/kg]			
		Per day		Day 3 to 5		Per day		Day 3 to 5	
		LSM	S	LSM	s	LSM	s	LSM	s
1	1	0.44	0.10			0.07[2]	0.02		
	2	0.69	0.10			0.09[2]	0.02		
	3	0.75	0.10			0.07[2]	0.02		
	4	0.66	0.10	0.69[a]	0.05	0.08[2]	0.02	0.08[a 2]	0.01
	5	0.72	0.10			0.07[2]	0.02		
2	1	1.00	0.10			0.14	0.02		
	2	1.50	0.10			0.18	0.02		
	3	1.59	0.10			0.17	0.02		
	4	1.73	0.10	1.70[b]	0.05	0.23	0.02	0.23[b]	0.01
	5	1.86	0.10			0.27	0.02		
3	1	1.95	0.10			0.32	0.02		
	2	2.12	0.10			0.35	0.02		
	3[1]	2.17	0.11			0.27	0.03		
	4[1]	2.80	0.12	2.55[c]	0.06	0.30	0.03	0.32[c]	0.01
	5[1]	2.83	0.11			0.35	0.03		
Wash-out period	1[1]	1.25	0.11			0.08[2]	0.03		
	2[1]	0.16[2]	0.11			<LOD			
	3[1]	<LOD				<LOD			
	4[1]	<LOD				<LOD			
	5[1]	<LOD				<LOD			

Different letters within a column show significant differences between the levels ($p < 0.05$).
LOD: limit of detection.
[1] Data of cow 4 excluded after overdosing;
[2] calculated by including values below the limit of quantification.

Table 6
Mean atropine and scopolamine transfer rates calculated as Least Square Mean (LSM) for each dosage level on days 3 to 5.

Level	Cows	Transfer rates in %			
		Atropine		Scopolamine	
	(n)	LSM	s	LSM	s
1	4	0.030[a]	<0.001	n.c.	n.c.
2	4	0.036[b]	0.001	0.007[a]	<0.001
3		0.037[b]	0.001	0.007[a]	<0.001

n.c. not calculable; different letters within column show significant differences ($p < 0.05$).

the supplementation was stopped on day 4 at dosage level 3, the washout period of cow 4 started two days earlier compared to the regularly dosed cows.

The increase of the scopolamine concentration in skimmed milk went parallel to the manifestation of clinical symptoms. On the presumed day of overdosing, the scopolamine content in milk (0.74 µg/kg) had the highest measured value of the whole trial. In contrast, the atropine concentration was already high in the morning milk of days 1 and 2 at dosage level 3 (Fig. 1) and remained below the previous values on the day of overdosing. After cessation of supplementation, the concentration of atropine and scopolamine in the milk fell below the corresponding LOD values after seven and four milking times, respectively. Thus, the washout period of each alkaloid in the case of cow 4 was two milking times longer than that of the regularly dosed animals.

4. Discussion

Currently, only plant-based foods are regarded as a potential source of TA in feed and food (EFSA, 2018). Based on the potential health risk to the consumer, the European Commission recommended a monitoring of TA in several foodstuffs of plant origin in the Member States (EU Commission Recommendation, 2015/976). Maximum levels for

Table 7
Clinical symptoms and concentration of atropine and scopolamine in skimmed milk of cow 4 at the dosage level 3 and during the washout period. For detailed information see Supplementary file A.

Level	Day	Pupil diameter	Rumen activity	Milk yield[3] (kg/day)	Concentration of TA in skimmed milk (µg/kg)	
					Atropine	Scopolamine
3	1	moderate mydriasis	normal	17.8	2.85	0.46
	2	moderate mydriasis	normal	18.1	2.54	0.46
	3[1]	moderate mydriasis	moderately reduced	13.6	2.34	0.74
	4[2]	moderate mydriasis	moderately reduced	11.0	2.79	0.48
Washout period[4]	1	moderate mydriasis	moderately reduced	11.2	2.15	0.21
	2	moderate mydriasis	moderately reduced	12.9	0.50	<LOD
	3	moderate mydriasis	normal	16.1	<LOQ	<LOD
	4	moderate mydriasis	normal	16.2	<LOD	<LOD
	5	moderate mydriasis	normal	19.5	<LOD	<LOD
	6	normal	normal	21.9	<LOD	<LOD

[1] Day 3, level 3: day of suspected overdosing;
[2] Day 4, level 3: stop of supplementation;
[3] Whole milk;
[4] Washout period began two days earlier if compared to the regularly dosed animals.

atropine and scopolamine (each 1.0 μg/kg food) were established for processed cereal-based foods and baby foods for infants and young children containing millet, sorghum, buckwheat, or their derived products by Regulation (EU) No. EU Commission Regulation, 2016/239.

Presently, the contribution of other food groups to the TA exposure of consumers remains unclear. Therefore, the primary aim of this study was to analyze the transfer of TA from feed into the milk of TA-exposed dairy cows. Based on the results obtained, the potential health risk to the consumer due to an exposure to TA via cow milk is discussed.

4.1. Animal experiment

4.1.1. Chemical substances

The use of pure substances instead of natural plant material containing TA allowed the exact dosing per animal based on the individual body weight. This seemed necessary due to the narrow therapeutic index of TA and the limited knowledge available in literature regarding their potential adverse effects in cows. In *Datura stramonium* plants the scopolamine concentration is generally lower than the atropine concentration. The actual ratio of the alkaloids varies depending on the age of the plant, the plant section, and the plant origin (Friedman & Levin, 1989; Miraldi et al., 2001). The applied atropine/scopolamine ratio of 1:0.6 was an attempt to match the natural conditions. As shown in Fig. 1, the quick transfer into milk only 2 h after the first application demonstrates that the applied atropine sulfate and scopolamine hydrobromide trihydrate salts were rapidly released from the bolus and subsequently became bioavailable within a very short period of time. Under natural conditions, the bioavailability of TA from plants and seeds might be reduced compared to that of the pure substances, because the natural alkaloids need to be released from plant components. Nevertheless, the possibility of a harmful TA uptake from contaminated feed is underlined by poisoning incidents in cattle previously described in literature (Aboling et al., 2019; Bofill et al., 2007).

Since this trial was intended to simulate the effects caused by the intake of TA-containing plants, both alkaloids were applied simultaneously. Consequently, it is not possible to assign the clinical signs to one single substance. The pharmacological effects of atropine mainly derive from the active stereoisomer L-hyoscyamine; thus, only 50% of the daily atropine dosage can be assumed to be effective (EFSA, 2013).

4.1.2. Supplementation

As it was planned to expose the cows to subclinical dosages of TA, none of the three dosage levels (Table 2) exceeded the NOAEL in cattle of 300 μg/kg body weight (EFSA, 2013). As expected, the milk yield, feed intake, and behavior were not altered in the regularly dosed cows. The moderate alterations of the pupil diameter (at dosage level 2), muzzle humidity, and rumen activity (at dosage level 3) were only detected by a careful clinical examination. Under practical conditions, these cows would most likely remain unnoticed by the livestock owner and their milk would be placed on the market. The results of this study support the current NOAEL in cattle, as the dosage at level 3 (279 μg/kg b.w.) was very close to the NOAEL value established by EFSA (2013).

In contrast to the regularly dosed cows, cow 4 showed distinct signs of a TA-intoxication shortly after the suspected overdosing on day 13. These results are in accordance with the findings of Aboling et al. (2019), who described distinct clinical symptoms in fattening bulls at a calculated dosage of 468 μg/kg body weight. Nevertheless, just as in the present study, the TA dosage causing clinical symptoms could only be roughly estimated and the exact intake level remains uncertain. Thus, the toxic dose cannot be derived from our data.

4.2. Transfer rates and concentration of atropine and scopolamine in skimmed milk

Even though the regularly dosed cows showed no signs of intoxication, atropine and scopolamine were detected in the skimmed milk of all animals from the very first day at the lowest dosage level 1. However, there were significant differences between the two alkaloids, which are reflected in the level of the measured concentrations in milk and the respective transfer rates.

4.2.1. TA concentration in milk

Basically, the atropine concentrations were significantly higher than the scopolamine concentrations in milk throughout the whole trial. The highest calculated atropine group mean value (2.83 μg/kg skimmed milk) was about eight times higher than the highest scopolamine group mean value (0.35 μg/kg skimmed milk). Even though scopolamine was supplemented at 60% of the atropine dosage, the scopolamine concentration in milk was only 10–15% of the atropine concentration (Table 5). Thus, the substance-specific transfer rate determines how high the concentration of the respective alkaloid measurable in milk will be.

4.2.2. Transfer rates

The transfer rates of both alkaloids were very low; the mean atropine transfer rate at dosage level 3 (0.037%) was about five times higher than the mean scopolamine transfer rate (0.007%) (Table 6). The higher atropine transfer rate can possibly be explained by the substance-specific pharmacokinetics. In humans, the bioavailability of atropine is 50% and its elimination half-life is 3.9–4.3 h after oral application (Böhm, 2016; Sauer, 2001). Regarding scopolamine, the bioavailability in humans is only 30% and the elimination half-life 2.9 h. These differences are probably caused by a substantial presystemic metabolism of scopolamine in the gastrointestinal tract and liver of humans (Renner et al., 2005), thereby leading to a significantly reduced bioavailability of this substance after oral intake.

The very low transfer rates of TA are in line with studies on the transfer of pyrrolizidine alkaloids from feed to milk that were less than 0.1% for most compounds (Hoogenboom et al., 2011; Mulder et al., 2020). Differences attributed to specific metabolic pathways are also observed for individual alkaloids of this group of plant toxins.

There are currently no data available on the metabolism of TA after an oral intake in ruminants; only the half-life time of atropine sulfate after intramuscular injection in sheep is known (1.6 h; EMEA, 1998). Nevertheless, the differences detected between both alkaloids in this study could also be explained by a better oral bioavailability of atropine compared to scopolamine in cattle. This assumption needs to be investigated in further trials.

4.2.3. Dosage level

As expected, the concentration of both alkaloids in skimmed milk increased significantly with increasing dosages from level to level. Accordingly, the transfer rate of atropine also increased significantly from level 1 to level 2, while there was no significant increase between level 2 and 3 (Table 5). The obtained data did not show a clear-cut dose-dependency of the atropine transfer rate. No statement can be made in this regard for scopolamine, as it was not possible to calculate a mean scopolamine transfer rate at dosage level 1.

The design of the study included increasing dosages in the same cows without any washout period between dosage levels. However, considering the short plasma half-life time of atropine sulfate after intramuscular injection in sheep (1.6 h; EMEA, 1998) and the rapidly decreasing TA concentrations in milk after the end of the supplementation (Fig. 1, Table 5), TA-residues from the previous level should not contribute to the transfer rates in the following level.

4.2.4. Duration of application

The data indicate a certain influence of the duration of application on the concentrations of the alkaloids in milk. However, this effect was not detected at all dosage levels. Considering dosage level 1, the concentrations of both atropine and scopolamine did not increase significantly between days 2 and 5 (Fig. 1). The dosage at this level seemed to correspond to the steady state, in which case drug intake, receptor

binding, metabolism, and excretion are balanced. This assumption seems likely, as the daily dosage at level 1 was based on the recommendations for a long-term therapy with atropine sulfate (Löscher, 2006).

When the therapeutic dosage range was exceeded at levels 2 and 3, the concentration of both alkaloids showed a tendency to increase during the five supplementation days (Fig. 1, Table 5). Hence, the steady state was apparently not yet achieved at these dosage levels. As the supplementation of each dosage level only lasted five days, higher concentrations in milk with a prolonged application cannot be excluded.

However, it is uncertain whether the experimental animals would also develop clinical symptoms during a longer supplementation at dosage levels 2 or 3. In previous studies, a prolonged exposure of cattle to contaminated feed led to more pronounced clinical symptoms, especially reduced rumen activity (Aboling et al., 2019; Nelson et al., 1982). Due to the subsequently reduced feed intake combined with the short half-life times of TA, the intoxication is described as self-limiting. Therefore, the interruption of supplementation after development of clinical symptoms in cow 4 followed by a quick recovery of the cow seems to comply with the natural course of an intoxication under field conditions.

4.2.5. Milking times

The observed differences between the concentrations of atropine and scopolamine in the morning and the evening milking may be caused by the different time intervals between supplementation and milking time. In the morning, this interval was only 2 h, while it was almost twice as long in the evening. Consequently, the time for absorption, distribution, and metabolism of the alkaloids differed between both milking times, which could explain the different concentrations measured. An effect of the interval between supplementation and milking time on the concentration of contaminants in milk was also demonstrated for pyrrolizidine alkaloids (Hoogenboom et al., 2011). In addition, the different milking intervals of 10 and 14 h led to a slightly lower milk yield with a higher fat content in the evening, which could have also influenced the measurable alkaloid concentrations. Under practical conditions, these differences would not be relevant due to the continuous intake of potentially contaminated feed and the mixture of morning and evening milk in the bulk tank.

4.2.6. Individual differences

Due to the small number of experimental animals used, there were individual differences which need to be considered. Interestingly, cows 3 and 4 with the lowest milk yield showed significantly higher scopolamine concentrations than cows 1 and 2 ($p<0.05$). Based on the existing data, it can only be hypothesized that higher milk yields generally lead to a dilution of the scopolamine eliminated via milk.

Regarding cow 4, there was a remarkably high scopolamine content in the skimmed milk (0.74 mg/kg skimmed milk) on the day of the overdosing (level 3, day 3), which cannot only be explained by a concentration process due to the reduction of milk yield on that day. It is known that gastrointestinal microbes in ruminants can detoxify several plant compounds (Smith, 1992). Despite this detoxification capacity, toxic compounds can also inhibit the microbial growth (de Oliveira et al., 2010). Based on the reduced rumen activity on the day of the overdosing, alkaloid-induced changes of the ruminal microbiome may be responsible for the altered metabolism and absorption of scopolamine. This could have led to elevated concentrations in milk. On the other hand, the cow exhibited a more rapid increase of atropine concentrations in milk compared to the other animals beginning with the third dosage level (level 3, days 1 and 2; Fig. 1, Table 5), which might indicate a higher sensitivity of this individual animal to TA under the regular dosing. Interindividual differences regarding the enzymatic degradation of atropine have been previously described in several rabbit breeds (Tucker & Beattie, 1983; van Zutphen, 1972). So far, based on the current data, no definitive statement can be made about the reasons for the inter-individual differences observed in the experimental cows.

4.3. Potential effects on consumer health

In general, the pharmacological effects of TA are observed shortly after intake. The substances neither accumulate nor exhibit any chronic toxicity. Therefore, EFSA derived an "acute reference dose" (ARfD) of 0.016 μg/kg b.w. for the sum of L-hyoscyamine and scopolamine to evaluate the potential health risk from TA in food (EFSA, 2013).

When assessing the possible health impact of the mean alkaloid concentrations in milk, it is necessary to convert the measured atropine contents into L-hyoscyamine. Since it is currently not known, in which proportion D- and L-hyoscyamine appear in milk, the conversion method used by EFSA (2013) was applied, in which 50% of the atropine content is assumed to be L-hyoscyamine. Based on this calculation, the maximum mean value for the sum of L-hyoscyamine and scopolamine was 1.60 ± 0.07 μg/kg skimmed milk on days 3–5 at dosage level 3 (Table 5).

When assessing the health risks stemming from the consumption of TA-contaminated milk, it must be considered that the milk of most farms is processed to a large extent in dairies obtaining their raw milk from different sources, thereby leading to a dilution of possible contaminants. Therefore, the occurrence of dairy products contaminated with TA in retail seems rather unlikely. On the other hand, although the consumption of raw or certified raw milk by children is strongly discouraged, the increasing tendency of dairy farms to directly market their raw milk has to be considered.

For children, who can drink considerable amounts of milk during the day, even raw milk contaminated with low amounts of TA could significantly contribute to their total daily TA exposure. However, up to now, there is no information available regarding the actual levels of TA in raw milk samples in Germany. Based on the maximum mean concentrations of L-hyoscyamine and scopolamine calculated in milk in our experimental set-up, a child weighing 20 kg would need to drink 200 g of this contaminated milk to reach the ARfD of 0.016 μg/kg b.w. derived by EFSA (2013). This calculation should be considered as an initial approach for a possible exposure assessment, as no chiral separation of the hyoscyamine enantiomers was carried out. The maximum concentration of L-hyoscyamine used for this calculation was estimated on the basis of the EFSA Scientific Opinion on tropane alkaloids in food and feed (EFSA, 2013), and the actual concentration can possibly differ. However, the difference in fat content between raw full fat milk and skimmed milk as well as the concomitant slightly different TA contents in the two types of milk can be neglected. At the present time, based on the fact that the actual levels of TA in raw milk in Germany remain unknown, a comprehensive risk assessment based on realistic TA exposure levels cannot be performed. Moreover, the stability of TA in raw milk over a longer period of time, the influence of the usual processing steps on TA levels in milk, as well as the frequency and level of occurrence of TA in feed need to be determined in order to assess the practical relevance of the results obtained in the present study.

In the above-mentioned context, it is necessary to consider a possible heat denaturation of TA by pasteurization or cooking. Several authors described different degrees of TA heat denaturation in various foods such as bread, buckwheat porridge, pasta, and tea (Friedman & Levin, 1989; Perharič et al., 2013; Marín-Sáez et al., 2019a, 2019b) depending on heating time and temperature. However, pasteurization and UHT-treatment of milk had no effect on other plant toxins such as pyrrolizidine alkaloids (de Nijs et al., 2017). Although such data are currently not available for TA, these observations could be an indication for a certain heat stability of TA in milk.

4.4. Relevance of the results of the present study in relationship to Datura maximum contents in feeding stuffs

According to the EU Directive, 2002/32/EC on undesirable substances in animal feed, products intended for animal feed may contain a

maximum of 1000 mg *Datura* weed seeds per kg relative to a feeding stuff with a moisture content of 12%. It should be noted, that the TA content in *Datura* seeds can vary between 182 and 3400 mg/kg dry matter (Caligiani et al., 2011; Friedman & Levin, 1989; Miraldi et al., 2001). Assuming a high daily intake of 12 kg of concentrate feed contaminated with 1000 mg of *Datura* seeds per kg, the TA intake of a dairy cow would therefore be between 2.18 and 40.8 mg per day which would be lower than the dosages used in this study. Nevertheless, the cases of poisoning of dairy cows and bulls by contaminated maize silage (Aboling et al., 2019; Bofill et al., 2007) show that not only the seeds, but also the plant itself has to be considered as a source of contamination. In the poisoning case described by Aboling et al. (2019), the maize silage contained 10.47 mg of TA (L-hyoscyamine plus scopolamine) per kg fresh mass. In order to achieve the dosages applied in our study at level 3, a cow would have to consume approximately 18 kg of such contaminated maize silage. Depending on the design of the ration, this could be a realistic scenario.

Until now, the EU has only set maximum limits for the contamination of animal feed with *Datura* seeds. This approach disregards the fact that alkaloid levels in jimson weed leaves and flowers can be as high or even higher than in the seeds (Miraldi et al., 2001). Our own analyses on individual parts of plants (leaves, stems, and seeds, among others) confirm the above-mentioned observation (C. Schwake-Anduschus, unpublished data). With this in mind, farmers should be encouraged to pay attention to the occurrence of *Datura* plants on their cropland and apply weed control methods measures at an early stage. Taking this into account, an extension of the present statutory limits to the entire plant should be considered. This aspect could become particularly important if the global climate change leads to a further spreading of thorn apple in regions where it did not grow up to now.

5. Conclusions

In the present study at the experimental station Schädtbek of the MRI, the transfer of TA from feed to milk was demonstrated in cattle for the first time. The highest possible subclinical dosage was determined to be 279 μg/kg b.w.

With values well below 0.1%, the transfer rates of atropine and scopolamine were very low. Nevertheless, one has to consider that TA can lead to pharmacological effects in humans when consumed in the microgram range. On the one hand, milk of most farms is processed to a large extent in dairies obtaining their raw milk from different stocks. Since the mixing of various raw milks leads to a dilution of possible contaminants, the occurrence of dairy products contaminated with TA in retail can most probably be disregarded. On the other hand, the situation might be different when raw milk is sold by direct marketing at the farm level.

Based on the fact that only four cows were exposed to TA, the data described in the present study should be viewed as a first step towards a final risk assessment. Further studies on feed and food are necessary for a comprehensive risk assessment of TA present in milk and products thereof. To analyze the actual content of biologically active L-hyoscyamine in milk and milk products, the quantification methods should include the chiral separation of the enantiomers. In subsequent studies, it would be of particular interest to determine whether the prolonged intake of TA may lead to higher alkaloid concentrations in milk of cows without clinical symptoms and how the ruminal microbiome may influence the absorption and metabolism of the orally ingested TA. Analysis of additional matrices such as blood, urine and faeces may help to explain individual differences regarding TA metabolism and excretion. In addition, the behavior of TA during heating and further processing of milk to dairy products has to be determined. Moreover, the analysis of TA content in milk and feed samples from dairy farms could help to estimate current TA exposure levels.

CRediT authorship contribution statement

J. Lamp: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization. **K. Knappstein:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization. **H.-G. Walte:** Formal analysis, Writing – review & editing. **T. Krause:** Conceptualization, Methodology, Writing – review & editing. **P. Steinberg:** Writing – review & editing. **C. Schwake-Anduschus:** Conceptualization, Methodology, Investigation, Writing – review & editing.

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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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.foodcont.2021.108056.

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