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In vitro biodegradation of hepatotoxic indospicine in *Indigofera spicata* and its degradation derivatives by camel foregut and cattle rumen fluids

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Abstract

The known accumulation of the hepatotoxin indospicine in tissues of camels and cattle grazing *Indigofera* pasture plants is unusual in that free amino acids would normally be expected to be degraded during the fermentation processes in these foregut fermenters. In this study, *in vitro* experiments were carried out to examine the degradability of indospicine of *Indigofera spicata* by camel and cattle foregut microbiota. In the first experiment, a 48 h *in vitro* incubation was carried out using foregut fluid samples that were collected from 15 feral camels and also a fistulated cow. Degradability of indospicine ranged between 97 − 99% with the higher value of 99% for camels. A pooled sample of foregut fluids from three camels that were on a roughage diet was used in a second experiment to examine the time-dependent degradation of indospicine present in the plant materials. Results indicated that camels' foregut fluids have the ability to biodegrade approximately 99% of the indospicine in *I. spicata* within 48 h of incubation and produced 2-aminopimelamic acid and 2-aminopimelic acid. The time-dependent degradation analysis showed rapid indospicine degradation (65 nmol/h) during the first 8−18 h of incubation followed by a slower degradation rate (12 nmol/h) between 18 h and 48 h. Indospicine degradation products were also degraded towards the end of the experiment. The results of these *in vitro* degradation studies suggest that dietary indospicine may undergo extensive degradation in the foregut of the camel resulting in trace levels after 48 hours. The retention time for plant material in the camel foregut varies depending on feed quality, and the results of this study together with the observed accumulation of indospicine in camel tissues suggests that although indospicine can be degraded by foregut fermentation, this degradation is not complete before the passage of the digesta into the intestine.

KEYWORDS: *In vitro degradation; indospicine; camel; 2-aminopimelamic acid; 2-aminopimelic acid; Indigofera spicata*

INTRODUCTION

Indigofera plants are high in protein and generally considered to be edible by livestock and highly digestible.[1] However, the usage of *Indigofera* plants as animal fodder has been constrained by the presence of the natural hepatotoxin, indospicine (**1**), in some *Indigofera* species.[2,3] Ingestion of indospicine (**1**) has caused a severe liver disease to simple stomached animals, including dogs,[4] mice[5] and rabbit.[6]

The dense and diverse microbial population of the rumen can decompose complex plant materials and has the ability to destroy and modify toxins present in ingested plants.[7] Amino acids are normally metabolized by deamination, followed by incorporation of the liberated ammonium (NH_4) into microbial protein in the compartmental stomach of ruminants by rumen microorganisms.[8,9] It is not unreasonable to expect that indospicine (**1**) (Figure 1), an amidino analogue of the amino acid arginine (**2**), would be similarly metabolized. However, several animal studies have revealed that it is not just simple stomached animals that are susceptible to indospicine (**1**) toxicosis, since ruminants like sheep,[10,11] goats[12] and cattle[10] also develop signs of toxicity after prolong dietary ingestion of toxin indospicine. Indeed, a considerable body of research is available on indospicine (**1**) hepatotoxicosis in animals and there is no distinct difference between ruminants and non-ruminants in their susceptibility to the toxin.[5,6,10-16]

The camel is unique in being a non-ruminant herbivore animal with compartmental stomach and extensive foregut fermentation processes, and some researchers refer camels as "pseudo-ruminants".[17,18] Although camel fermentation processes are similar to that in ruminant animals, camels have different feeding behaviors and harbor different microflora in their compartmental stomach, with little similarity to cattle.[19] Such differences may be of particular relevance to the detoxification of secondary compounds found in plants that the camel feeds on.

There are more than 700 species of *Indigofera* plants found around the world, mostly in subtropical and tropical regions,[20] with most of these plants having unknown levels of indospicine (**1**). Consequently, indospicine (**1**) may be overlooked or neglected as a possible source of reported plant poisonings to livestock in subtropical and tropical regions. Reports on liver disease and deaths of dogs fed indospicine-contaminated horse[4] and camel[21] meats have raised concerns about herbivores feeding on *Indigofera* plants.[21] The accumulation of indospicine as the free amino acid in tissues of cattle and camels fed *Indigofera* plant material has been demonstrated in feeding trials,[22,23] leading to speculation on the capacity of indospicine to be degraded in camel and bovine gastric systems.

To date, no work has been reported on the ability of camel foregut microflora to degrade indospicine (**1**), and if it does, to what extent indospicine (**1**) is degraded. Considering the complexity and cost of *in vivo* studies on a camel, an *in vitro* fermentation process approach was employed to generate an initial set of data and to develop a better understanding of the fate of ingested indospicine (**1**) in the camel foregut. The current paper presents results from series of *in vitro* experiments carried out to investigate the degradability of indospicine (**1**) present in *I. spicata* by camel foregut fluid and cattle rumen fluid.

MATERIALS AND METHODS

Source of *I. spicata*. Mature flowering *I. spicata* plant material was collected from a site in Brisbane on 19 December 2013, air dried and hammer milled (1 mm screen size). A separate pressed plant sample was submitted for botanical identification to the Queensland Herbarium which confirmed the species identity (BRI AQ797866).

Source of Inocula. For total degradability of indospicine (**1**), Experiment 1, foregut digesta fluid was opportunistically obtained from camels that had been slaughtered at abattoir as part of normal commercial practices. A group of 15 feral camels of different ages and sexes,

harvested from the Atula Station area (Northern Territory, Australia) were transported to the Meramist Pty Ltd abattoir in Caboolture, Queensland where they were kept in a paddock that had native trees and native pasture for two weeks before slaughter. In addition to the native trees and pasture the camels were offered a grassy lucerne (*Medicago sativa*) hay. The camels were then deprived of feed, but had access to fresh water, during the 24 h period immediately prior to being slaughtered (on 7th November 2013). The foregut digesta were collected immediately post-mortem from these 15 mature camels. Each of these 15 samples was filtered separately through four layers of cheesecloth. A sterile control foregut digesta was obtained by autoclaving at 121 °C at 15 psi for 15 min.

Cattle rumen digesta were collected from a 3-year-old fistulated steer that had been fed on a 50:50 mixture of tropical grass pasture and Lucerne hay (Animal Ethics Approval: DAF SAFS/259/12/DAFF/DAIRY AUST). The digesta were filtered through four layers of cheesecloth into a 1.8 L Thermos flask previously filled with warm water to maintain the temperature of the foregut fluid. A sterile control rumen digesta was obtained by autoclaving at 121 °C at 15 psi for 15 min.

For the time-dependent degradation rate, Experiment 2, three feral camels were similarly sourced from Atula Station area before being slaughtered on 29th July 2014 at Caboolture. A pooled sample of camel foregut digesta were prepared immediately after post mortem collection from three mature camels and filtered through four layers of cheesecloth.

Preparation of the Basal Medium 10 and the *Indigofera spicata* Substrate. The homogenized milled sample of *I. spicat*a was weighed into a series of Hungate tubes. A pre-reduced basal medium 10 (BM10) broth was prepared according to Caldwell and Bryant[24] with no carbohydrate source except *I. spicata*. The BM10 media (5 mL) was dispensed into the Hungate tubes containing the *I. spicata* substrate (0.3 g per tube) under CO_2, which were then

sealed with rubber stoppers and aluminium caps, and sterilized at 121 °C at 15 psi for 15 min before being stored at room temperature until used.

For the *in vitro* incubation studies, foregut fluids (5 mL), as from both camels and cattle were added to the pre-prepared sterilized medium and incubated in a shaking incubator at 39 °C for 48 h. All the foregut fluid samples were injected into the tubes immediately after filtering, within 20 min of collection. These samples were then transported immediately to Health and Food Science Precinct laboratory, Brisbane, Australia for incubation. The temperature of the tubes was maintained at 39 °C with warm water. Needles (21G) were used to release the gas after 24 h and 48 h of fermentation to simulate gas belched from the stomachs of cattle and camel. Next, the tubes were frozen at −20 °C after 48 h (48 h *in vitro* foregut digestibility study, Experiment 1), and 6, 12, 18, 24, 36 and 48 h (the time-dependent degradation of indospicine (**1**) study, Experiment 2) of incubation to stop the reaction. Another three sets of tubes were maintained as controls, one set contained autoclaved camel foregut fluid with *I. spicata*, a second set contained autoclaved cattle foregut fluid with *I. spicata*, and a third set contained freshly filtered camel foregut fluid with *I. spicata*, and was immediately submerged in ice before being stored at −20 °C for a 0 h control. The initial values of indospicine (**1**) concentration after sterilization were subtracted from each measured value to give the actual total value of degraded indospicine (**1**) value.

Indospicine and Degradation Products UPLC−MS/MS Analysis. Foregut fluid, *I. spicata* and BM10 media (pre- and post-incubation) were prepared and analyzed for indospicine (**1**) and indospicine (**1**) degradation products (2-aminopimelamic acid (**3**) and 2-aminopimelic acid (**4**)) according to the UPLC−MS/MS method developed by us.[25,26] Indospicine (**1**) (99% purity) and 2-aminopimelamic acid (**3**) (86% purity) were provided by Prof. James De Voss and Dr. Robert Lang.[27] DL-α-aminopimelic acid (**4**) was purchased from Sigma-Aldrich (Sigma-Aldrich, Castle Hill, Australia). Amicon Ultra 0.5 mL, 3K centrifugal

filter units (Merck Millipore, Kilsyth, Australia) were pre-rinsed and centrifuged (10 000 rpm, 20 min) with reverse osmosis water (2 x 300 μL) to remove trace glycerine, then inverted and spun for 1 min at 1,000 rpm. A slight modification of sample preparation was done in this present work, in that an aliquot of the ion-pairing agent; heptaflurobutyric acid (HFBA, 10% (v/v), 50 μL) was added into the Hungate tubes containing *I. spicata* substrate, BM10 media and foregut fluid prior to homogenization using a Polytron T25 Basic homogenizer (Labtek, Brandale, Australia) for 15 s, so that the final concentrations of the samples contained 0.1% of HFBA (similar to the published method). The homogenized samples were chilled (4 ºC) for 20 min and then centrifuged at 4500 rpm for 20 min at 18 ºC. Dilutions of 1:10 or 1:100 were also used before ultracentrifugation and UPLC−MS/MS analysis with 0.1% HFBA, depending on the level of indospicine (**1**), 2-aminopimelamic acid (**3**) and 2-aminopimelic acid (**4**) present. Aliquots of the diluted extracts (1.0 mL), spiked with 100 μL of the internal standard D_3-L-indospicine (**3**) (1 mg/L in 0.1% HFBA) were vortexed for 10 s. A portion (450 μL) was transferred into pre-rinsed Amicon Ultra 0.5 mL, 3K centrifugal filters, which were centrifuged (10 000 rpm, 20 min), and the filtrates then transferred to a limited volume insert for UPLC−MS/MS analysis.

Statistical Analysis. Data were analyzed using Genstat Software. A one-way ANOVA was performed and an α-value of 0.05 was used to assess the significance amongst the means. The standard error of means (SEM) was also reported together with the mean values.

RESULTS

Analysis of indospicine in media preparations. Application of the previous sample preparation method used in meat studies[25] had an apparent matrix suppression effect in the current analysis, with a much higher indospicine (**1**) levels extracted from plant material in BM 10 preparations. Consequently, an additional 50−fold dilution was employed for the

UPLC−MS/MS indospicine (**1**) analysis to minimize matrix suppression effect of media and plant materials. The addition of the isotopically labelled D_3-L-indospicine as an internal standard further counters this matrix effect.[27]

Degradation of indospicine by autoclaving. Foregut fluids from camels and cattle used for the experimental control were analyzed prior to autoclaving, and no indospicine (**1**) was detected. No matrix interference was observed for the elution time window of indospicine (**1**) quantitation. Autoclaving processing at 121 °C at 15 psi for 15 min has degraded nearly 47% of indospicine (**1**) in the plant material reducing the tubes content from 397 μg to 211 μg indospicine (**1**) (Table 1). Substitution of reverse osmosis (RO) deionized water for the BM 10 resulted in a similar effect, with no significant degradation differences being observed between water and the BM 10 solutions.

***In vitro* degradability of indospicine.** The results of the degradation test for the 15 camels and the fistulated steer (Table 2) show that all camel inocula produced extensive degradation of indospicine (**1**) during the 48 h incubation period. Degradation rates for the camel inocula averaged 99% with the indospicine (**1**) residue quantitated at 0.08 − 0.84 μg DM. Individual variation was small, and differences were not significant ($P > 0.05$). Interestingly, degradation of indospicine (**1**) by cattle rumen fluid was 97%, slightly lower than that of camel. Incubation for 48 h with sterilized cattle and camel foregut fluid led to no degradation in the experimental control samples (Table 2). Hence even though the pre-experimental sterilisation by autoclaving unexpectedly reduced the indospicine concentration, this effect did not accelerate the metabolism kinetics and indospicine levels after 48 hrs with sterilised foregut fluid were not statistically different from time zero control.

Time-dependent degradation of indospicine. The time-dependent degradation of indospicine (**1**) with camel foregut fluid (Figure 2) showed an increasing degradation to a maximum level of 99.3% (8.9 ± 1.3 nmol) at 48 h of incubation. Results of this experiment are

similar to those observed for the 48 h *in vitro* incubation (Table 2). Approximately 70% of indospicine (**1**) was degraded by 18 h of incubation. A rapid degradation rate (65 nmol/h) of indospicine (**1**) was recorded between 8 h and 18 h, while a much lower degradation rate (12 nmol/h) occurred between 18 h and 48 h. Plotting in [indospicine (**1**)] against [time] provided an estimated first-order degradation rate constant of being 0.0989 h^{-1}.

Indospicine degradation products. Camel foregut fluid degraded indospicine (**1**) and produced 2-aminopimelamic acid (**3**) and 2-aminopimelic acid (**4**) in the *in vitro* time-dependent incubation experiment. The concentrations of the major indospicine (**1**) degradation product, 2-aminopimelamic acid (**3**), increased from 1483 ± 168 nmol at 0 time of incubation to 2097 ± 131 nmol at 12 h of incubation (Figure 3) and declined gradually after 12 h of incubation reaching a final concentration of 309 ± 62 nmol at 48 h of incubation. The concentrations of 2-aminopimelic acid (**4**) remained relatively lower (86 – 183 nmol) than indospicine (**1**) and its amide throughout the incubation period. Even though the concentrations of 2-aminopimelic acid (**4**) were low, increment of 2-aminopimelic acid (**4**) concentration continued up to 18 h of incubation before started to decline until the end of the incubation time.

DISCUSSION

This is the first report that examines the *in vitro* degradation of indospicine (**1**) using camel foregut fluid and cattle rumen fluid. Indospicine (**1**) from *I. spicata* which is known to contain relatively higher indospicine (**1**) concentration amongst *Indigofera* spp.,[2] was degraded by both camel foregut and cattle rumen fluid. Plant material containing the naturally occurring form of indospicine (**1**) (L-2-amino-6-amidinohexanoic acid) was used in our experiments, rather than pure indospicine (**1**), since the *in vitro* activity of an aqueous solution of indospicine (**1**) may not necessarily be similar to indospicine (**1**) contained in the plant material. Like other amino acids, indospicine (**1**) can be synthesized in either 2 enantiomers (D and L), and only the

L is naturally occurring.[5] It was therefore important that the L form was used in this study (rather than either racemic or D-indospicine (**1**)).

A relatively small depletion (19%) of indospicine (**1**) has been reported when indospicine (**1**) contaminated horse meat is subjected to 120 °C at 26 psi for 90 min.[28] The greater loss of ≥42% for indospicine (**1**) that we found in this study suggests that indospicine (**1**) contained in the matrix of *I. spicata* is thermally unstable (at least in the presence of the aqueous media and water). The higher concentrations of the amide derivative 2-aminopimelamic at 0 time of incubation (Figure 3) indicates partial hydrolysis of indospicine under conditions of the autoclave sterilization process.

Amidines such as indospicine (**1**) are known to be hydrolyzed firstly to the amide and then subsequently to the corresponding acid (Figure 4), with the first step usually being faster than the second. Typically amidine hydrolysis occurs under milder conditions than the corresponding nitriles, amides or esters, and is more rapid under alkaline than acidic conditions, with unsubstituted amidines (such as indospicine (**1**)) being considered much more reactive than *N*-substituted amidines.[29] Previous research has reported the hydrolysis of pure indospicine (**1**) to 2-aminopimelamic acid (**3**) in water at 120 ºC for 20 h or with 0.1M Na_2CO_3 at 50 ºC for 15 h.[5] The amide was further hydrolyzed to 2-aminopimelic acid (**4**) by a treatment with 1N HCl at 120 ºC for 2 h. Alternatively, acid hydrolysis of indospicine (**1**) using 6N HCl at 120 ºC for 20 h afforded 2-aminopimelic acid (**4**) directly.[5]

In this study, it was considered that feral (non-domesticated) camels roaming unrestricted areas of central Australia could have a huge variation in their foregut fluid microflora profiles, while cattle kept in the same feeding ground with the same feed and living conditions would have only slight differences.[30] Hence, digesta from 15 camels were used. However, the observed extensive indospicine (**1**) degradation by the digesta from all 15 camels infers the pre-dominant microflora may be similar between individual camels within a mob,

particularly so as the 15 animals grazed the same small paddock for the 2 weeks prior to slaughter. More interestingly, similar indospicine (**1**) degradation was observed in the digesta from the one fistulated steer, suggesting the ability to degrade indospicine (**1**) is similar across both animal species.

Even though non-proteinogenic amino acids (such as indospicine (**1**)) can be toxic to monogastric animals, it is believed that ruminants, with the help of ruminal microbial metabolism of amino acids, have the potential to degrade such toxic amino acids ingested from plants.[31] Indospicine (**1**) is an analogue of arginine (**2**) and although no mammalian enzymes seem capable of degrading it,[7] it is not unreasonable to expect indospicine (**1**) would be metabolized in the rumen in a similar fashion to other amino acids. Free amino acids are known to be absorbed rapidly by rumen microbes and used as such or deaminated. This indospicine (**1**) might be metabolized via deamination to form ammonia as the main nitrogen source for cellulolytic bacteria similar to other amino acids present in the rumen.[8,9,31] Alternatively, indospicine (**1**) might be taken up by the non-structural carbohydrate bacteria as their nitrogen sources, when the concentration present is similar to that of other amino acids.[31] The camel foregut is only weakly acidic,[32] and it is very unlikely indospicine will be degraded under mild acid conditions as indospicine has been demonstrated to be stable under more acidic aqueous conditions *in vitro*.[26]

The non-time dependent 48 h *in vitro* degradation showed that indospicine (**1**) in *Indigofera* plant material can be successfully degraded by both bovine rumen and camel foregut microflora, with residual indospicine (**1**) concentration in our camel foregut fluid after 48 h being lower than that in cattle foregut fluid. The observed *in vitro* degradation of indospicine (**1**) by camel foregut and bovine rumen fluid is consistent with other research, which demonstrated that ruminant animals are relatively less susceptible to certain feedborne toxins than are simple stomached animals, due to ruminal degradation of toxins present in the feed.[33]

Although, results of the present *in vitro* incubation experiments have shown an extensive degradation of indospicine in both cattle rumen and camel foregut fluids, evidence from the field suggests less efficient metabolism of indospicine occurs *in vivo*. This results in considerable amounts of the indospicine (**1**) from ingested *Indigofera* plant material is being absorbed and deposited in camel meat tissue as the free amino acid,[21,22,25,34] and that the exposure of cattle to *Indigofera* has resulted in mild indospicine (**1**)-induced liver damage,[10] and that indospicine similarly accumulates in tissues of cattle fed *Indigofera*.[23] This had initially led us to anticipate that cattle rumen and camel foregut microflora may not have the capacity to degrade indospicine (**1**); however, this first non-time dependent *in vitro* degradation study has demonstrated the contrary. Therefore, the means by which indospicine (**1**) is able to reach muscle and other tissue still requires further clarification.

The second *in vitro* experiment, a time-dependent *in vitro* degradation of camel foregut fluid has demonstrated that total indospicine (**1**) degradation may only occur after 48 h of *in vitro* incubation (Figure 2), with a rational inference being that cattle rumen microflora may do the same. A number of researchers have reported the mean retention time of ingesta in the rumen of ruminant animals eating a range of diets is around 25 h.[35,36] The findings of the present time study indicate that a considerable amount of indospicine (**1**) may escape degradation and enters the intestine, since approximately 20% indospicine (**1**) would remain non-degraded at 25 h in our degradation study (Figure 2). This residual indospicine (**1**) could then be absorbed in the small intestine into the circulatory system and accumulate in the cattle and camel tissues. Even though different plant materials have differential passage times in the foregut,[36,37] the camel is known to retain low quality forage longer, up to 49 h, than it does good quality forage.[38] *Indigofera* plants are highly nutritious[39] and such plant material may have a relatively shorter passage time, which would result in a considerable amount of indospicine (**1**) passing

into the intestine without being degraded. Water soluble indospicine may also have a shorter retention time moving into the intestine, and be readily absorbed and distributed to tissues.

This study was concerned with the indospicine (**1**) level in the supernatant fluid after *in vitro* incubation, by separating the hydrolysate from microbial cells. It is hypothesized that this toxic arginine (**2**) amino acid analogue, indospicine (**1**), may be readily taken up by ruminal microorganisms and metabolized. Even though indospicine (**1**) is an analogue of arginine (**2**), the absence of the internal guanidino nitrogen would necessitate different metabolic pathways to that of arginine (**2**): arginine (**2**) → citrulline → ornithine → proline → 5-aminovaleric acid → volatile fatty acids + ammonia.[40] The result of our current *in vitro* camel foregut fluid metabolism study of indospicine (**1**) is the first to show indospicine (**1**) degradation produced 2-aminopimelamic acid (**3**) and 2-aminopimelic acid (**4**). What is interesting in this study is that the indospicine (**1**) metabolites were also undergone further hydrolysis during the incubation. The initial step in indospicine (**1**) metabolism would no doubt be the formation of 2-aminopimelamic acid (**3**) (equivalent to citrulline formation), but without the nitrogen adjacent to the amide, no ornithine equivalent will be produced. This amide (2-aminopimelamic acid (**3**)) is, in fact, a homolog of the amino acids asparagine and glutamine and should therefore be similarly metabolized. Glutamine is, for example, is metabolized through loss of ammonia to form glutamate/pyro-glutamate (5-oxoproline) and then itself is directly deaminated to butyrate and acetate with further production of ammonia and carbon dioxide.[41] Initial steps in rumen metabolism would thus be expected to similar to the depicted hydrolysis of indospicine (**1**) (Figure 4) with concurrent production of ammonia as the main nitrogen source, which will then be utilized by foregut bacteria for the synthesis of microbial protein.

However, these indospicine (**1**) biodegradation findings raise intriguing questions regarding the other metabolism pathways of indospicine (**1**); a presumption where a toxic

amino acid (indospicine (**1**)) is degraded to non-toxic metabolites (2-aminopimelamic acid (**3**) and 2-aminopimelic acid (**4**)) as hypothesized above is not always true. The breakdown of mimosine (toxic amino acid) from *Leucaena* to 3,4-dihydroxy pyridone (3,4-DHP) and 2,3-dihydroxy pyridone (2,3-DHP) is a good example of the degradation of a toxic amino acid to a toxic derivative.[42,43] Cattle grazing on *Leucaena* in Australia experienced severe toxic effects because they lack of the microorganism that can further degrade the potent goitrogen-DHP, produced from the degradation of mimosine.[42] However, ruminants feeding on *Leucaena* in Hawaii and Indonesia do not demonstrate severe toxicosis due to the presence of such rumen microorganisms and the artificial transfer of these bacteria to cattle in Australia has provided protection against mimosine toxicity. This shows that the ability of the rumen to degrade ingested amino acid toxins varies within the same animal species and is dependent on the microflora present in the rumen.

Hence, the degradation of the hepatotoxin-indospicine (**1**) observed in this study must be interpreted with caution because indospicine (**1**) may be metabolized into another toxic metabolite, and accumulation of such deleterious amino acids in the tissues of animals is not uncommon.[31] Even though indospicine (**1**) is not incorporated into mammalian protein, incorporation of this amino acid into protein synthesis cannot be ruled out, since it is another typical toxic mechanism and no evidence shows this is not possible.[44] In *in vitro* studies have shown that indospicine (**1**) has for instance been incorporated into a bacterial protein by *Escherichia coli*.[45] Therefore, there is a possibility that indospicine (**1**) and its metabolites (**1**) may be taken up by non-structural carbohydrate bacteria, and incorporated in the production of microbial protein without being metabolized. These microbial proteins may be later digested in the intestine of the ruminant animals or camels, leading to release a free indospicine (**1**) or its metabolites. Such a pathway may be another potential route for indospicine (**1**) and its metabolites to escape foregut degradation and lead to absorption and accumulation in animal

tissue. Despite promising results of hepatotoxic indospicine (**1**) could be degraded by camel foregut fluid, little literature is available on the toxicological aspects of 2-aminopimelamic acid (**3**) and 2-aminopimelic acid (**4**), hence, questions remain on the toxicity of the degradation products of indospicine (**1**) and warranted further investigation.

In conclusion, a 15 min 120 °C sterilization processing degrades nearly half of indospicine (**1**) contained in the matrix of the *I. spicata*; while a non-time dependent 48 h *in vitro* degradation study using foregut fluid has shown the ability of camel and cattle foregut microflora to degrade dietary indospicine (**1**) to trace levels after 48 h. While a time-dependent *in vitro* study demonstrated a cumulative degradation during the 48 h period with a formation of its degradation products, 2-aminopimelamic acid (**3**) and 2-aminopimelic acid (**4**). A bypass mechanism must be present, due to a relatively short retention time with good quality roughages and higher outflow rate of fluids and its soluble constituents, will facilitate indospicine (**1**) bypassing foregut degradation. Indospicine (**1**) residue may eventually uptake by the intestine and accumulates in the meat tissue. It is recommended that further research be undertaken to characterize indospicine (**1**) degradation products (2-aminopimelamic acid (**3**) and 2-aminopimelic acid (**4**)) so as to better understand the metabolic pathways and toxicity for indospicine (**1**) metabolism in camel. The isolation of microbes with enhanced indospicine (**1**) metabolism ability may lend themselves to the production of an inoculum to protect animals against the effects of the toxin indospicine (**1**) in a similar manner to the commercially produced inoculum of the rumen bacterium *Synergistes jonesii* which enables the utilization of Leucaena without adverse toxic effects.[46]

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REFERENCES

(1) Hassen, A.; Rethman, N. F. G.; van Niekerk, W. A.; Tjelele, T. J. Influence of season/year and species on chemical composition and *in vitro* digestibility of five *Indigofera* accessions. *Anim. Feed Sci. Technol.* **2007,** *136*, 312-322.

(2) Aylward, J. H.; Court, R. D.; Haydock, K. P.; Strickland, R. W.; Hegarty, M. P. *Indigofera* species with agronomic potential in the tropics. Rat toxicity studies. *Aust. J. Agric. Res.* **1987,** *38*, 177-186.

(3) Tan, E. T. T.; Materne, C. M.; Silcock, R. G.; D'Arcy, B. R.; Al Jassim, R.; Fletcher, M. T. Seasonal and species variation of the hepatotoxin indospicine in Australian *Indigofera* legumes as measured by UPLC−MS/MS. *J. Agric. Food Chem.* **2016,** *64*, 6613-6621.

(4) Hegarty, M. P.; Kelly, W. R.; McEwan, D.; Williams, O. J.; Cameron, R. Hepatotoxicity to dogs of horse meat contaminated with indospicine. *Aust. Vet. J.* **1988,** *65*, 337-340.

(5) Hegarty, M. P.; Pound, A. W. Indospicine, a hepatotoxic amino acid from *Indigofera spicata*: isolation, structure, and biological studies. *Aust. J. Biol. Sci.* **1970,** *23*, 831-842.

(6) Hutton, E. M.; Windrum, G. M.; Kratzing, C. C. Studies on the toxicity of *Indigofera endecaphylla*: I. Toxicity for rabbits. *J. Nutr.* **1958,** *64*, 321-337.

(7) Hegarty, M. P. Toxic amino acids in foods of animals and man. *Proc. Nutr. Soc. Aust.* **1986,** *11*, 73-81.

(8) Looper, C.; Stallcup, O.; Reed, F. Deamination of amino acids *in vivo* by rumen microorganisms. *J. Anim. Sci.* **1959,** *18*, 954-958.

(9) Moran, J., *Tropical Dairy Farming: Feeding Management for Small Holder Dairy Farmers in the Humid Tropics*. Landlinks Press: Collingwood, Victoria, Australia, 2005; p 291.

(10) Nordfeldt, S.; Henke, L. A.; Morita, K.; Matsumoto, H.; Takahash, M.; Younge, O. R.; Willers, E. H.; Cross, R. F. Feeding tests with *Indigofera endecaphylla* Jacq. (creeping indigo) and some observations on its poisonous effects on domestic animals. *Hawaii Agric.Exp. Stn., Tech. Bull* **1952,** *15*, 5-23.

(11) Maskasame, C. Toxicity and nutritional value of some promising pasture legumes in rats and sheep. MVSc Thesis, University of Queensland, Brisbane Australia, 1984.

(12) Suliman, H. B.; Wasfi, I. A.; Tartour, G.; Adam, S. E. I. The effects of *Indigofera hochstetteri* on goats. *Rev. Elev. Med. Vet. Pays Trop.* **1983,** *36*, 393-402.

(13) Christie, G. S.; Wilson, M.; Hegarty, M. P. Effects on the liver in the rat of ingestion of *Indigofera spicata*, a legume containing an inhibitor of arginine metabolism. *J. Pathol.* **1975,** *117*, 195-205.

(14) Kelly, W. R.; Young, M. P.; Hegarty, M. P.; Simpson, G. D., The Hepatotoxicity of Indospicine in Dogs. In *Poisonous Plants*, James, L. F.; Keeler, R. F.; Bailey, E. M.; Cheeke, P. R.; Hegarty, M. P., Eds. Iowa State University Press: Ames, Iowa, 1992; pp 126-130.

(15) Rosenberg, M. M.; Zoebisch, O. C. A chick test for toxicity in forage legumes. *Agron. J.* **1952,** *44*, 315-318.

(16) Rose, A. L.; Banks, A. W.; McConnell, J. D. Birdsville disease in the Northern Territory. *Aust. Vet. J.* **1951,** *27*, 189-196.

(17) Mukasa-Mugerwa, E., *The Camel (Camelus dromedarius): A Bibliographical Review*. International Livestock Centre for Africa: Addis Ababa, Ethiopia, 1981; Vol. 5, p 147.

(18) Ellard, K.; Seidel, P., *Development of a Sustainable Camel Industry*. Rural Industries Research and Development Corporation: Canberra, Australia, 2000; p 70.

(19) Samsudin, A. A.; Wright, A. D. G.; Jassim, R. A. Cellulolytic bacteria in the foregut of the dromedary camel (*Camelus dromedarius*). *Appl. Environ. Microbiol.* **2012,** *78*, 8836-8839.

(20) Williams, M. C. Nitro compounds in *Indigofera* species. *Agron. J.* **1981,** *73*, 434-436.

(21) FitzGerald, L. M.; Fletcher, M. T.; Paul, A. E. H.; Mansfield, C. S.; O'Hara, A. J. Hepatotoxicosis in dogs consuming a diet of camel meat contaminated with indospicine. *Aust. Vet. J.* **2011,** *89*, 95-100.

(22) Tan, E. T. T.; Al Jassim, R.; Cawdell-Smith, A. J.; Ossedryver, S. M.; D'Arcy, B. R.; Fletcher, M. T. Accumulation, persistence and effects of indospicine residues in camels fed *Indigofera* plant. *J. Agric. Food Chem.* **2016,** *64*, 6622-6629.

(23) Fletcher, M. T.; Reichmann, K. G.; Ossedryver, S. M.; McKenzie, R. A.; Carter, P. D.; Blaney, B. J. Accumulation and depletion of indospicine in calves (*Bos taurus*) fed creeping indigo (*Indigofera spicata*). *Anim. Prod. Sci.* **2016**, submitted.

(24) Caldwell, D. R.; Bryant, M. P. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. *Appl. Microbiol.* **1966,** *14*, 794-801.

(25) Tan, E. T. T.; Fletcher, M. T.; Yong, K. W. L.; D'Arcy, B. R.; Al Jassim, R. Determination of hepatotoxic indospicine in Australian camel meat by ultra-performance liquid chromatography–tandem mass spectrometry. *J. Agric. Food Chem.* **2014,** *62*, 1974-1979.

(26) Tan, E. T. T.; Yong, K. W. L.; Wong, S. H.; D'Arcy, B. R.; Al Jassim, R.; De Voss, J. J.; Fletcher, M. T. Thermo-alkaline treatment as a practical degradation strategy to reduce indospicine contamination in camel meat. *J. Agric. Food Chem.* **2016,** *64*, 8447-8453.

(27) Lang, C. S.; Wong, S. H.; Chow, S.; Challinor, V. L.; Yong, K. W. L.; Fletcher, M. T.; Arthur, D. M.; Ng, J. C.; De Voss, J. J. Synthesis of L-indospicine, [5,5,6-$^{2}H_3$]- L-indospicine and L-norindospicine. *Org. Biomol. Chem.* **2016,** *14*, 6826-6832.

(28) Simpson, G. D.; Hegarty, M. P., *Determination of Indospicine in Biological Material (issue 57 of Tropical Agronomy Technical Memorandum)*. CSIRO Division of Tropical Crops and Pastures: Brisbane Australia, 1987; p 11.

(29) de Wolfe, R. H., Kinetics and Mechanisms of Reactions of Amidines. In *Amidines and Imidates*, Patai, S., Ed. John Wiley & Sons Ltd.: Bristol, Great Britain, 1975; Vol. 1, pp 349-384.

(30) Manubolu, M.; Madawala, S. R.; Dutta, P. C.; Malmlöf, K. *In vitro* biodegradation of cyanotoxins in the rumen fluid of cattle. *BMC Vet. Res.* **2014,** *10*, 110.

(31) D'Mello, J. P. F., *Amino Acids in Animal Nutrition*. CABI: Oxon, United Kingdom, 2003; p 516.

(32) Kadim, I. T., *Camel Meat and Meat Products*. CABI: Oxon United Kingdom, 2012; p 258.

(33) Kurmanov, I. A., Fusariotoxicosis in Cattle and Sheep. In *An Encyclopaedic Handbook Vol. 3. Mycotoxic Fungi, Mycotoxins and Mycotoxicoses*, Wyllie, T. D.; Morehouse, L. G., Eds. Mercel Dekker: New York, 1977; pp 85-110.

(34) Tan, E. T. T.; Al Jassim, R.; D'Arcy, B. R.; Fletcher, M. T. Level of natural hepatotoxin (indospicine) contamination in Australian camel meat. *Food Addit. Contam.: Part A* **2016,** *33*, 1587-1595.

(35) Hummel, J.; Südekum, K. H.; Streich, W. J.; Clauss, M. Forage fermentation patterns and their implications for herbivore ingesta retention times. *Funct. Ecol.* **2006,** *20*, 989-1002.

(36) Rutagwenda, T.; Lechner-Doll, M.; Kaske, M.; Engelhardt, W.; Schultka, W.; Schwartz, H. Adaptation strategies of camels on a thornbush savannah pasture: comparison with other domestic animals. *Options méditerranéennes, série Séminaires* **1989**, 69-73.

(37) Van Soest, P.; Jeraci, J.; Foose, T.; Wrick, K.; Ehle, F. In *Comparative fermentation of fibre in man and other animals*, Fibre in human and animal nutrition. Proceedings of Dietary Fibre in Human and Animal Nutrition Symposium, Palmerston North, New Zealand, 1983; The Royal Society of New Zealand: Palmerston North, New Zealand, 1983; pp 75-80.

(38) Heller, R.; Lechner, M.; Weyreter, H.; Engelhardt, W. V. Forestomach fluid volume and retention of fluid and particles in the sastrointestinal tract of the camel (*Camelus dromedarius*). *J. Vet. Med. A.* **1986,** *33*, 396-399.

(39) Skerman, P. J., *Tropical Forage Legumes*. Food and Agriculture Organization of the United Nations: Rome, 1977.

(40) Sultana, H.; Hussain-Yusuf, H.; Takahashi, T.; Morita, T.; Onodera, R. A quantitative study on arginine catabolism by mixed ruminal bacteria, protozoa and their mixture in vitro. *J. Anim. Sci.* **2003,** *74*, 11-16.

(41) Russell, J. B.; Chen, G. Effects of monensin and pH on the production and utilization of pyro-glutamate, a novel product of ruminal glutamine deamination. *J. Anim. Sci.* **1989,** *67*, 2370-2376.

(42) Jones, R.; Lowry, J. Australian goats detoxify the goitrogen 3-hydroxy-4 (1H) pyridone (DHP) after rumen infusion from an Indonesian goat. *Experientia* **1984,** *40*, 1435-1436.

(43) Ghosh, M. K.; Samiran, B. Mimosine toxicity− a problem of Leucaena feeding in ruminants. *Asian J. Anim. Vet. Adv.* **2007,** *2*, 63-73.

(44) Mander, L.; Liu, H. W., *Comprehensive Natural Products ii: Chemistry and Biology*. 1st ed.; Elsevier Ltd.: Kidlington, United Kingdom, 2010; Vol. 1, p 977.

(45) Leisinger, T.; Haas, D.; Hegarty, M. P. Indospicine as an arginine antagonist in *Escherichia coli* and *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta-Nucleic Acids Protein Synth.* **1972,** *262*, 214-219.

(46) Graham, S.; Dalzell, S.; Ngu, N. T.; Davis, C.; Greenway, D.; McSweeney, C.; Shelton, H. Efficacy, persistence and presence of *Synergistes jonesii* in cattle grazing leucaena in Queensland: on-farm observations pre-and post-inoculation. *Anim. Prod. Sci.* **2013,** *53*, 1065-1074.

FIGURE CAPTIONS

Figure 1. Structure of indospicine (**1**); an amidino analogue of arginine (**2**), 2-aminopimelamic acid (**3**) and 2-aminopimelic acid (**4**)

Figure 2. Time-dependent degradation of indospicine by foregut microflora of camel for 48 h at 39 °C.

Figure 3. Hydrolysis of indospicine (**1**) and the formation of its degradation products (2-aminopimelamic acid (**3**) and 2-aminopimelic acid (**4**)).

Figure 4. Hydrolysis of indospicine **(1)** to corresponding amide (**3**) and acid (**4**).[4,26]

TABLE CAPTIONS

Table 1. Indospicine concentration for various controls of *in vitro* incubation study.

Table 2. Indospicine *in vitro* degradation at 39 °C for 48 h in camel and cattle foregut fluids.

FIGURE GRAPHICS

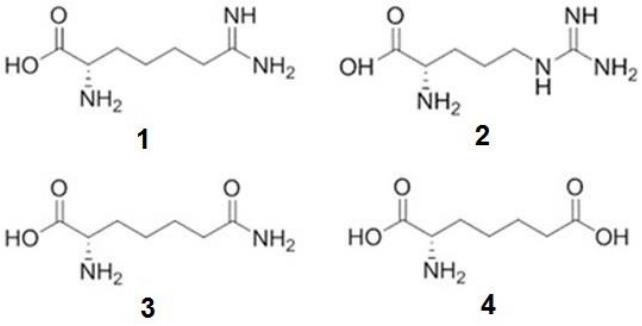

Figure 1

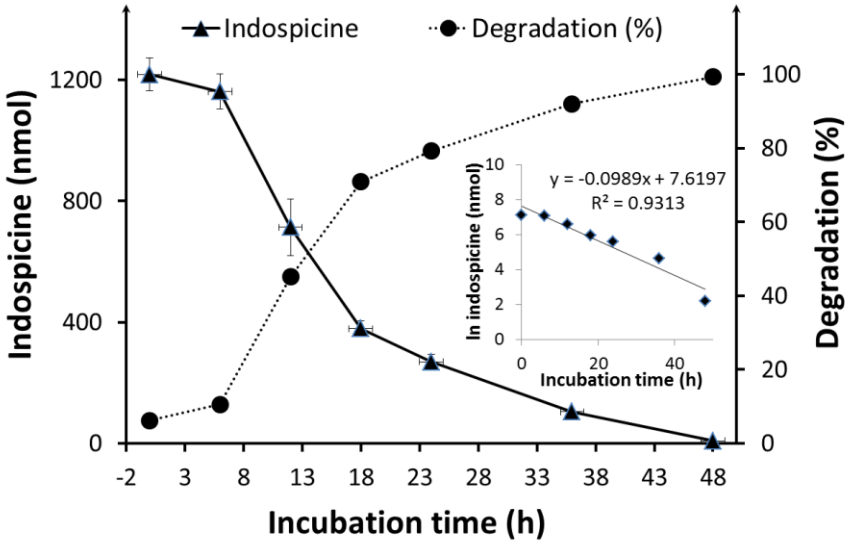
Indospicine
Degradation (%)
Indospicine (nmol)
Degradation (%)
Incubation time (h)
1200
800
400
0
100
80
60
40
20
0
-2
3
8
13
18
23
28
33
38
43
48
ln indospicine (nmol)
y = -0.0989x + 7.6197
R² = 0.9313
Incubation time (h)
10
8
6
4
2
0
0
20
40

Figure 2

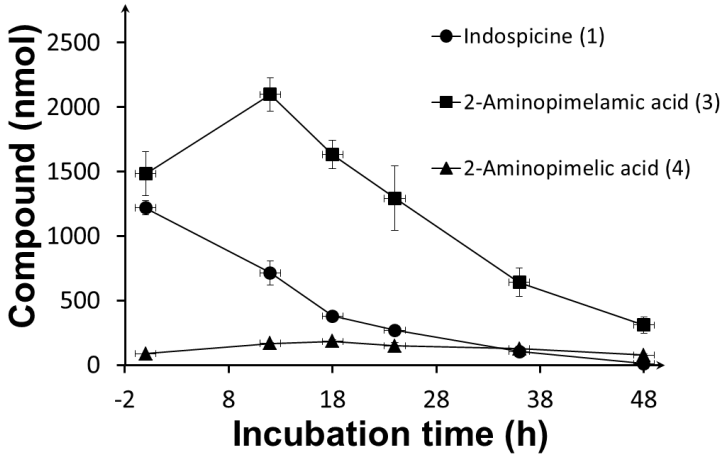
Indospicine (1)
2-Aminopimelamic acid (3)
2-Aminopimelic acid (4)
Compound (nmol)
2500
2000
1500
1000
500
0
-2
8
18
28
38
48
Incubation time (h)

Figure 3

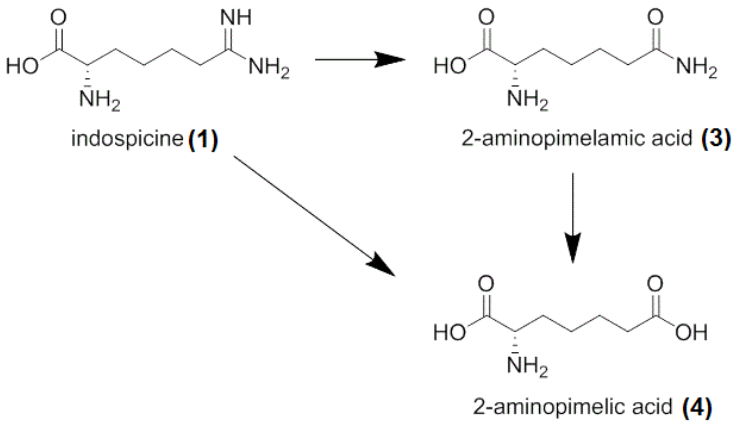
indospicine (1)
2-aminopimelamic acid (3)
2-aminopimelic acid (4)

Figure 4

TABLE GRAPHICS

Table 1

	replicate (n)	residue of indospicine (μg ± SD)	SEM
BM 10 and camel foregut fluid	2	N.D.	-
BM 10 and cattle rumen fluid	2	N.D.	-
BM 10 and *I. spicata* (before autoclaving)	4	397.4 ± 15.9^{a}	8.0
BM 10 and *I. spicata* (after autoclaving)[c]	4	210.8 ± 19.7^{b}	9.8
RO deionized water and *I. spicata* (after autoclaving)	4	232.4 ± 13.5^{b}	6.7

[a,b] Value with disparate letters differ significantly ($p < 0.05$). [c] Time zero control (T_0). BM 10: basal medium 10, RO: reverse osmosis

Table 2

foregut/ rumen fluid	replicate (n)	after 48 h incubation			indospicine degradability (%)
		residue of indospicine (μg ± SD)	SEM	remaining residue of indospicine (%)	
Camel 1	2	0.39 ± 0.00	0.00	0.18	99.82
Camel 2	2	0.84 ± 0.54	0.38	0.39	99.61
Camel 3	2	0.40 ± 0.03	0.02	0.18	99.82
Camel 4	2	0.12 ± 0.03	0.02	0.06	99.94
Camel 5	2	0.50 ± 0.16	0.11	0.23	99.77
Camel 6	2	0.24 ± 0.03	0.02	0.11	99.89
Camel 7	2	0.11 ± 0.02	0.01	0.05	99.95
Camel 8	2	0.14 ± 0.06	0.04	0.06	99.94
Camel 9	2	0.46 ± 0.08	0.06	0.21	99.79
Camel 10	2	0.08 ± 0.01	0.01	0.04	99.96
Camel 11	2	0.13 ± 0.04	0.03	0.06	99.94
Camel 12	2	0.10 ± 0.01	0.01	0.04	99.96
Camel 13	2	0.08 ± 0.02	0.01	0.04	99.96
Camel 14	2	0.20 ± 0.00	0.00	0.09	99.91
Camel 15	2	0.42 ± 0.19	0.14	0.19	99.81
Cattle	6	5.65 ± 0.26	0.11	2.74	97.26
Camel sterilized	3	216.47 ± 4.30[a]	2.48	100%	−2.69
Cattle sterilized	2	206.50 ± 7.04[a]	4.98	100%	2.04

[a]Not statistically different from time zero control (T_0 = 210.8 ± 19.7 μg, n = 4)

TABLE OF CONTENT