Effect of *in-vitro* ruminal fermentation and HCL/pepsin digestion on loline

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Abstract

Loline, an alkaloid produced by Neotyphodium endophytes in pastoral grass species with potential antimicrobial properties, has several known derivatives; N-formyl loline (NFL), N-acetyl loline (NAL), N-acetyl norloline (NANL), N-methyl loline (NML), and loline base, though the availability and form during rumen fermentation and digestion is unclear. *Festuca pratensis* seeds either with (LOL) or without (NIL) lolines (150-200 µg/mL) were incubated in either sterile or viable rumen fluid or in either HCL/pepsin (pH 2) or water (pH 7). At 72 hours LOL in sterile rumen fluid had 23%, 26%, and 45% more NFL (P=0.05), NANL (P=0.04), and loline base (P=0.01) respectively, with 27% more total loline present (P=0.06), and 65% more NML (P=0.09) compared with LOL in viable rumen fluid. Loline tended to alter the fermentation pattern as NIL produced 11% more ammonia (P=0.07), and 5% less propionate (P=0.06) than LOL. In HCL/pepsin there were 38%, 49%, and 39% more total lolines (P=0.05), NANL (P=0.02), and NFL (P=0.04) respectively, compared with water. Rumen and abomasal digestion appear to have a small effect on loline's form and concentration with only slight alteration to the rumen fermentation pattern. Presence of lolines had little evidence of an antimicrobial effect.

Keywords: loline; alkaloid; ruminal digestion; abomasal digestion

Introduction

Some secondary plant metabolites have pharmaceutical activities that may benefit animals experiencing a pathogenic challenge. Lolines are an alkaloid produced by *Neotyphodium* fungi in grass species and are suggested to possess antimicrobial, insecticidal, and anthelmintic properties (Schardl et al. 2007; Bacetty et al. 2009; Muponda 2014) while considered non-toxic to mammals (Bush et al. 1993; Gooneratne et al. 2012). There are several naturally occurring loline derivatives: N-formyl loline (NFL), N-acetyl loline (NAL), N-acetyl norloline (NANL), and N-methyl loline (NML). Metabolism of loline in the rumen could influence rumen metabolism (Westendorf et al. 1993) although no information is available as to the fate of their forms during digestion, and if any active loline forms have the potential to survive digestion and reach the intestine to affect microflora, and provide a pharmacological benefit. The objectives of this study were to use *in-vitro* methods to determine the fate of lolines during digestion.

Materials and methods

Meadow fescue seed (Barrier U2, Cropmark Seeds Ltd, Rolleston, NZ) with (LOL) or without (NIL) loline alkaloids were ground through a 1-mm sieve (ZM200, Retsch) at 18,000 RPM. Loline-containing seed contained 16.7 mg/g of total loline and the NIL seed contained 0 mg/g loline, with similar nutrient composition (table 1).

Experiment 1: Loline in rumen fluid

Degradation of loline (NFL, NAL, NANL, and NML) in rumen fluid was measured using NIL or LOL seed in a 2x2 factorial, replicated twice, with four treatments of either: NIL seeds in sterile rumen fluid (NS), seeds with loline in sterile rumen fluid (LS), NIL seeds in viable rumen fluid (NV), and seeds with loline in viable rumen fluid (LV).

Incubations were conducted an anaerobic DAISY incubator containing four fermentation jars each containing 2000 mL fluid (DAISY II-200/220, ANKOM Technology Co. Ltd. NY, USA). A buffer solution was prepared according to the operating instructions of ANKOM (Marten & Barnes 1980) and consisted of two pre-warmed (39˚C) solutions; buffer A (KH_2PO_4, $MgSO_4$•7 H_2O, NaCl, $CaCl_2$•2 H_2O, and urea) and buffer B (Na_2CO_3 and Na_2S•9 H_2O) that were mixed as a 5:1 ratio (buffer A: B) with a final pH of 6.8. Each jar contained 40 grams of ground seed, 1600 mL of buffer solution, 400 mL rumen fluid, and were purged with CO_2. The 40 grams of seed was calculated to give an approximate amount of 150-200 µg/mL of loline in LOL treatments to ensure detection by gas chromatography (GC). Jars were maintained at 39˚C and were continuously rotated in the incubator throughout a 72-hour fermentation period.

Rumen fluid was collected from a non-lactating cannulated dairy cow grazing perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) pasture (Lincoln University Animal Ethics Committee #2018-05). Approximately 1000 mL was filtered through cheesecloth into a warmed thermos flask 16 hours prior to, and immediately before incubations. Rumen fluid collected 16 hours prior was sterilized by autoclaving at 121˚C for 20 minutes and was then sealed and stored in the refrigerator (approximately 4˚C). The sterilized rumen fluid was reheated (39˚C) using a water bath prior to the start of incubations.

Fermentation jars were sampled at 0, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, 24, 48, 72 hours. At each time, 5 mL of fluid was removed through the septa on the lid to prevent air from entering the jars. Two mL was sampled for loline analysis and 1.5 mL for each of volatile fatty acids (VFA, propionate, acetate, butyrate, iso-valeric, iso-butyrate) and

Table 1 Nutrient analysis of meadow fescue seed and loline compounds with and without loline.

	Seed type[1]		
	LOL	NIL	P-value
Seed analysis (% DM)			
ADF	15.5	16.0	–
NDF	48.2	45.9	–
DM	92.4	90.7	–
OM	96.8	95.2	–
N	2.8	2.9	–
Apparent DMD[2] (%)			
Sterilized	51.3 ± 3.3	52.8 ± 0.29	0.73
Viable	68.0 ± 0.67	69.1 ± 0.41	0.31
Loline[3] (mg/g)			
NFL	11.7	0	–
NAL	2.8	0	–
NANL	1.6	0	–
NML	0.5	0	–
Total loline	16.7	0	–

[1]Meadow fescue seed with (LOL) or without (NIL) loline
[2]Dry matter digestibility (DMD) of seed (LOL, NIL) incubated for 72 hours in sterilized by autoclaving or microbial viable rumen fluid
[3]N-formyl loline (NFL), N-acetyl loline (NAL), N-acetyl norloline (NANL), Loline base (Loline), N-methyl loline (NML)

ammonia analysis. Ammonia samples were immediately acidified with 10 µL of HCL and all samples were stored frozen (-20˚C) until analysis.

Volatile fatty acid analysis was detected using a GC (model GC-2010, Shimadzu, Kyoto, Japan). 100 µl of centrifuged rumen sample had 20 µl of 2-Methylvaleric acid internal standard and 40 µl metaphosphoric acid added, vortexed, then diluted with 50/50 acetone/water after which the sample was vortexed, centrifuged and filtered using a 0.22 µm filter prior to GC injection as according to the procedures of Ottenstein and Bartley (1971), and Blanch and Drew (1985). Briefly, 1 µL was injected using an autosampler (AOC-20i) at a split ratio of 1:3 at the injection port (240ºC). The VFAs were separated on a bore capillary column (SGE BP21 30 m x 530 µm x 1.0 µm) with a flow of 5.23 mL/min of He using 2-methylvaleric acid as an internal standard. Initial oven temperature was 105ºC held for 4 min and then increased by 15ºC/min to 230ºC, where it was held for 5 min. The flame ionization detector was maintained at 240ºC.

Ammonia concentration in fluid samples was determined by an enzymatic UV method using a Randox NH_3 kit and the Randox Rx Daytona analyzer (United Kingdom). Samples were prepared as described by Neeley and Phillipson (1988).

Fluid samples for loline analysis were lyophilized and then extracted using 2 mL of methanol containing 60 µg/mL 4-phenomorpholine (Sigma Aldrich) as an internal standard. Samples were placed on an orbital shaker at 200 RPM for 1 hour and then centrifuged at 11,200 x g for 5 minutes, after which time 1 mL of supernatant was transferred to a GC vial and analyzed within 24 hours. Samples were analyzed using a GC (model GC-2010, Shimadzu) equipped with a flame ionized detector and were introduced via 1 µL split-less injection. Lolines were separated on a ZB-5 capillary column (30 m x 0.25 mm x 0.25 µm, Phenomonex). Hydrogen was used as a carrier gas at a flow of 6 mL/min, H_2 and airflows at the detector were 40 and 400 mL/min respectively. Initial oven temperature was 40˚C and was increased to 320˚C at a rate of 20˚C a min and was held for 5 min. Retentions for the loline derivatives were NML, 4-phenomorpholine (6.7 min), NAL (8.3 min), NFL (8.4 min), and NAL (8.6 min). Limit of detection was ~30 µg/g or 1 µg/mL.

At the end of fermentation, non-degraded seed were filtered through a Dacron bag, rinsed with cold water and dried in a 60˚C oven for 48 hours to enable calculation of apparent dry matter digestibility (DMD).

Experiment 2: Degradation of loline in HCL/pepsin fluid

Degradation of loline in HCL/pepsin fluid to simulate abomasal conditions was measured using NIL or LOL seed. Experiment was 2x3 factorial replicated twice with three treatments: control (W) seed incubated in water pH of 7.0, and seeds incubated in HCL/pepsin at a pH of 2.0 (HPL), and seeds incubated in HCL/pepsin at a pH of 3.0 (HPH).

Seeds were replicated using 125 mL conical flasks in an incubator (Minitron, INFORS HT, Switzerland). Each flask contained 1.5 grams of ground seed, with 75 mL of either deionized water or HCL/pepsin solution. The 1.5 grams of seed was calculated to give an approximate amount of 150-200 µg/mL of loline to ensure GC detection. The HCL/pepsin solution was prepared according to the method of Gargallo et al. (2006), to give a simulated abomasal fluid that consisted of 0.1 *N* HCL and 1 g/L of pepsin. Solution was pH adjusted and warmed to 39˚C prior to incubation. Flasks were maintained at 39˚C and shaken continuously throughout a two-hour incubation period.

Flasks were assigned a sample collection time point of either 0, 0.5, 1, or 2 hours. Flasks containing HCL/pepsin were neutralized using NaOH at the end of their incubation period to stop digestion and 2 mL was sampled from each flask and frozen (-20˚C) lyophilized and stored at -20˚C until loline analysis as described previously.

Statistical analysis

All data were analyzed and compared using repeated-measures REML in Genstat (18th edition). In experiment 1 loline metabolites were only compared between sterile and viable rumen fluid incubated with LOL seed. Ammonia, VFA and apparent DMD were compared using rumen fluid viability and seed type (LOL or NIL) and time as factors. In experiment 2, loline metabolites were compared using repeated-measures analyses among all three treatments. Treatment, time, and treatment x time were considered to be fixed effects with loline-within-treatment as a random effect. Significance was declared at $P<0.05$ and trends at $0.05<P<0.10$.

Results

Experiment 1: Loline in rumen fluid

Concentrations of loline forms (NFL, NANL, NML, NAL, loline base, total lolines) during incubation in sterile or viable rumen fluid are given in Fig. 1. Loline compounds increased over time (P=0.01) for all treatments with no evidence of their degradation over 72 hours. An interaction between rumen fluid type and time for NFL (P=0.05) and NANL (P=0.04) reflected increased concentrations in LS compared with LV. At 72 hours, LS had 23%, 26%, and 45% more NFL (P=0.05), NANL (P=0.04), and loline base (P=0.01) respectively, with 27% more total loline present (P=0.06), and 65% more NML (P=0.09) compared with LV. No differences were observed for NAL concentrations.

Figure 1 Degradation of A) N-formyl loline (NFL), B) N-acetyl loline (NAL), C) N-acetyl norloline (NANL), D) Loline base (Loline), E) N-methyl loline (NML), F) Total lolines incubated in sterile or viable rumen fluid. Treatments are meadow fescue seed with loline in sterile rumen fluid (LS ●); meadow fescue seed with loline in viable rumen fluid (LV ○).

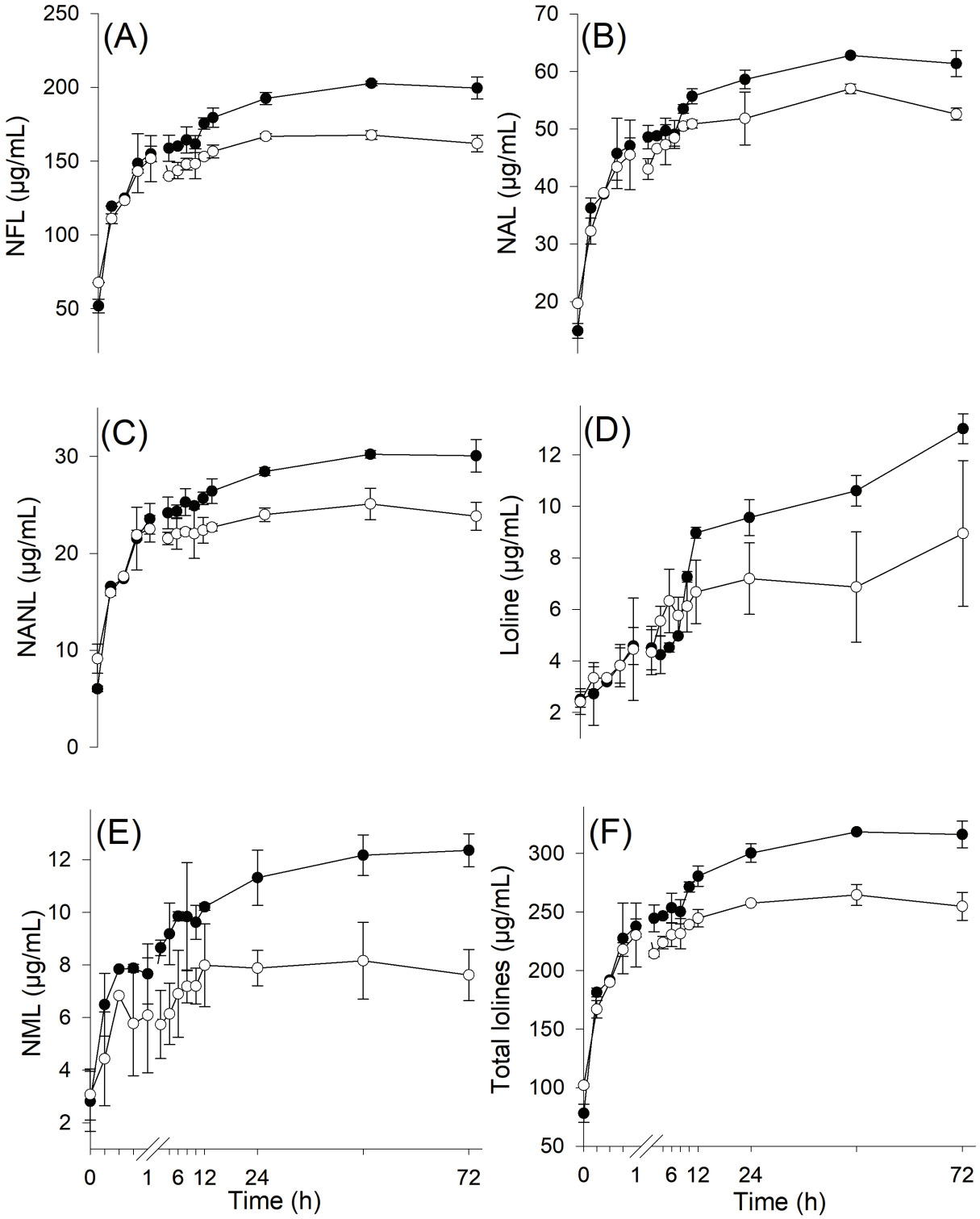
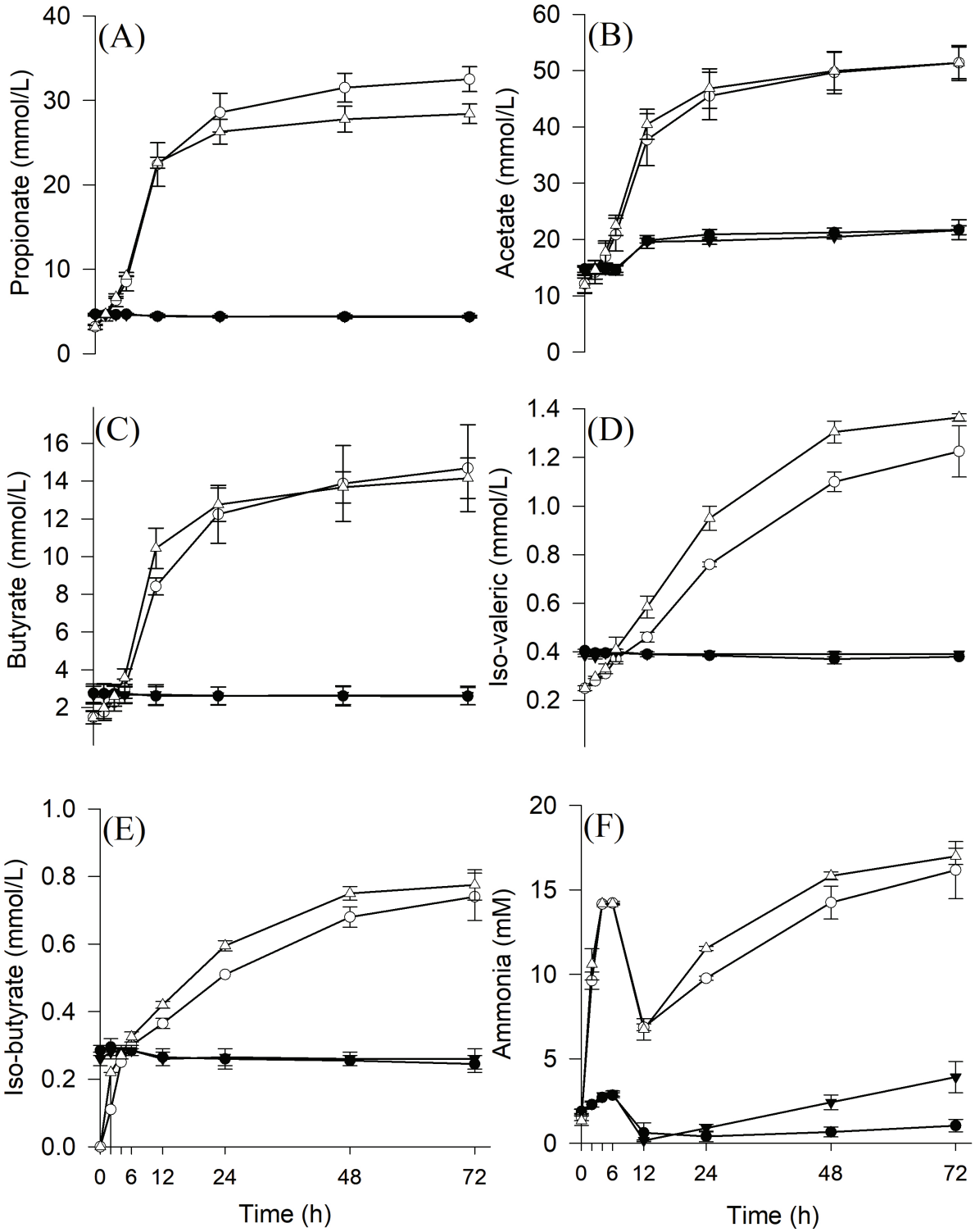
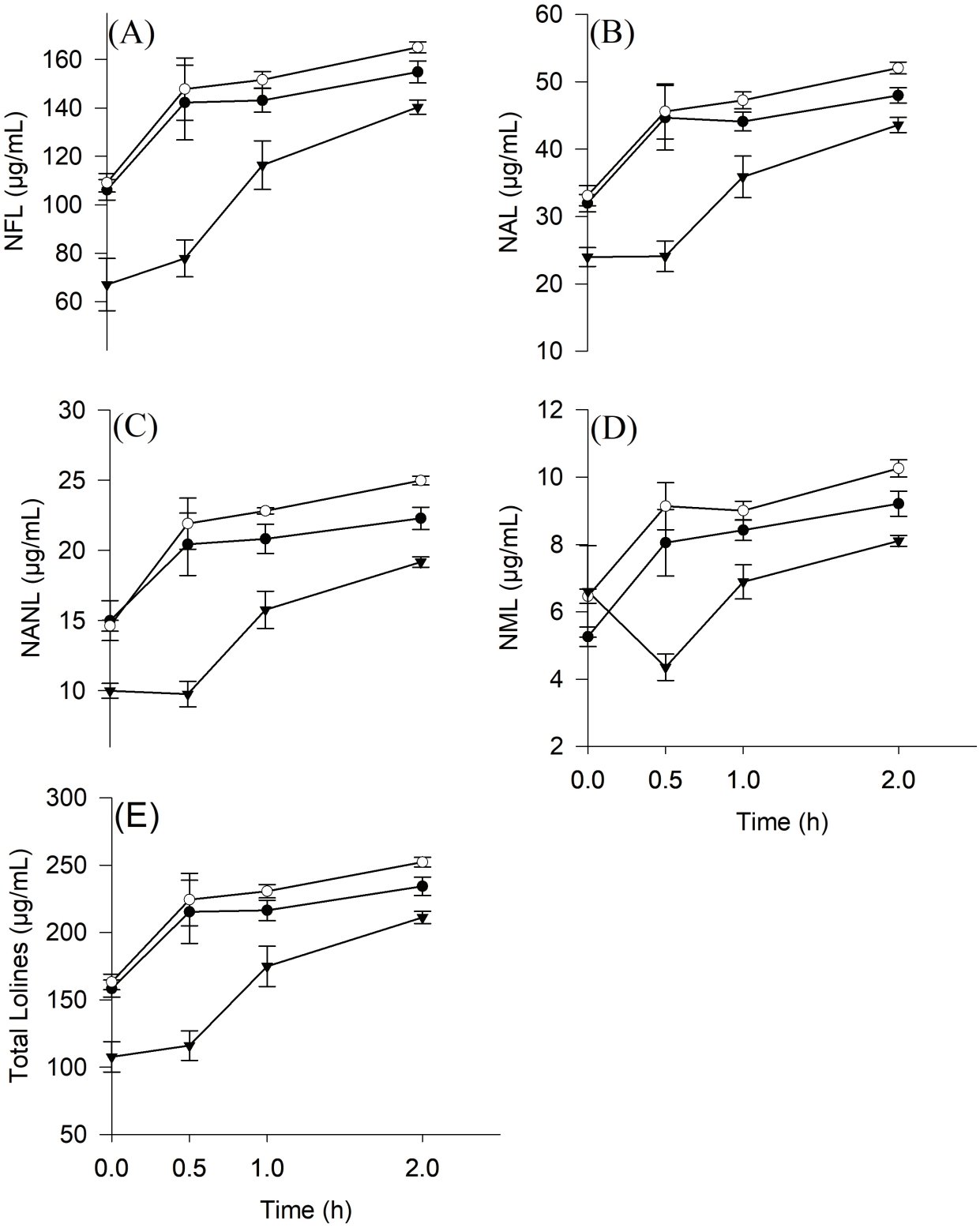


Figure 2 Volatile fatty acid and ammonia concentration in sterile or viable rumen fluid. Treatments are meadow fescue seed with loline in sterile rumen fluid (LS ●); meadow fescue see with loline in viable rumen fluid (LV ○); meadow fescue seed without loline in sterile rumen fluid (NS ▲); meadow fescue see without loline in viable rumen fluid (NV △).



Volatile fatty acid (propionate, acetate, butyrate, iso-valeric, iso-butyrate) concentrations during incubation are given in Fig. 2. All VFAs increased over time (P=0.01) in all treatments. No treatment (NIL or LOL) x fluid (viable or sterile rumen fluid) x time interaction occurred for acetate (P=0.71), butyrate (P=0.55), isobutyrate (P=0.65), isovaleric (P=0.37), valerate (P=0.54), and acetate:propionate (P=0.48) ratios. However, NIL tended to produce 11% more NH_3 than did LOL (P=0.07) and LOL produced 5% more propionate than did NIL (P=0.06). Seeds incubated in viable rumen fluid produced more (P=0.01) VFA and ammonia compared with sterile rumen fluid. Among NIL and LOL and rumen fluid treatments, no difference was observed in acetate (P=0.95), butyrate

Figure 3 Degradation of A) N-formyl loline (NFL), B) N-acetyl loline (NAL), C) N-acetyl norloline (NANL), D) N-methyl loline (NML), E) total lolines incubated in HCL/pepsin at varying pHs or water. Treatments are meadow fescue seed with loline incubated in HCL and pepsin at pH 3 (HPH ●); meadow fescue seed with loline incubated in HCL and pepsin at pH 2 (HPL ○); meadow fescue seed with loline incubated in water (W ▲).



(P=0.96), isobutyrate (P=0.18), isovaleric (P=0.13), valerate (P=0.60), and propionate (P=0.26) concentrations.

Apparent DMD was 51 ± 3.3%, 68 ± 0.67%, 53 ± 0.29%, and 69 ± 0.41% for LS, LV, NS, and NV respectively. Overall, apparent DMD was lower in sterile rumen fluid (P=0.01) but not affected by loline (P=0.49).

Experiment 2: Loline in HCL/pepsin fluid

Changes in the concentrations of loline forms during incubation in water and HCL/pepsin fluid are given in Fig. 3. There was a treatment x time interaction (P=0.04) reflecting less NML being produced at time 0.5 h for W compared with HPH, and HPL treatments. Loline metabolites increased over time (P=0.01) for all treatments.

The HCL treatments yielded 38%, 49%, and 39% more total lolines (P=0.05), NANL (P=0.02), and NFL (P=0.04) respectively, and tended (P=0.12) to yield 33% more NAL, and 26% more NML compared with W. No differences in loline concentration were observed between HPH and HPL.

Discussion

Loline concentration increased in both simulated abomasum and rumen fluid appearing to survive digestion, however, apparent differences in recovery between sterilized and viable rumen fluid suggest some degradation appears to occur. Differences in loline concentrations after 12 hours between sterilized and viable rumen fluid suggest that rumen microbes degraded loline, with 95% of lolines recovered in sterilized rumen fluid compared with only 79% in viable rumen fluid. Loline is water soluble and similar recovery concentrations were expected unless microbes had a role in their degradation. It is known that microbes play a substantial part in detoxifying compounds such as alkaloids (Weimer 1998), and microbial adaption to loline has been suggested (Gooneratne et al. 2012), and would explain the similar loline concentrations recorded during the first 12 hours, and differences thereafter. Although the seeds themselves were not sterilized, the differences between viable and sterilized rumen fluid indicate this may have occurred in the rumen viable microbes rather than introduced microbes with the seed. Furthermore, loline-catabolizing microbes exist and are found on the surface of plant leaves with ability to live exclusively off loline (NFL) as its main carbon and nitrogen source (Roberts & Lindow 2014). Loline derivative NFL and some NAL is metabolized to the simple loline base in rumen fluid (Westendorf et al. 1993; TePaske & Powell 1993; Gooneratne et al. 2012) and loline catabolizing microbes consume little loline base (Roberts & Lindow 2014), explaining why loline base didn't plateau after 48 hours. Given that no loline base was detected in the seed prior to the incubation, but was found in rumen liquor, suggests some conversion occurred. Loline base was not detected in the abomasal fluid and no apparent loline digestion occurred. More loline, 76% and 70% recovered in HPL and HPH treatments respectively was found compared with water (63% recovery). Presumably reflecting pH-dependent solubility of loline resulting in release differences from seeds. This indicates that administration of rumen by-passed loline would survive digestion and could potentially have pharmaceutical effects on gut microbial populations.

Evidence of an antimicrobial effect was limited; loline only slightly altered the rumen fermentation pattern. There was a tendency for more ammonia produced in NIL than LOL, and LOL produced more propionate than NIL. However, apparent DMD was similar between treatments. Loline antimicrobial effects have been suggested with insecticidal (Schardl et al. 2007; Bush et al. 1993) and nematocidal (Bacetty et al. 2009; Muponda 2014) properties. However, many studies (Westendorf et al. 1993; Bush et al. 1993; Muponda 2014) documenting loline effects either failed to report concentrations or include other alkaloids, hence confounding results. Given an approximate loline concentration of 330 µg/mL in rumen fluid, an influence on rumen microbes was anticipated, although potentially the concentration was too low for any pharmaceutical effect, as loline effects appear dependent on the organisms and route of administration. Root loline concentrations > 450 µg/g in meadow fescue have been shown to act as a feed deterrent to grass grub and negatively affect larval body mass (Patchett et al. 2011). Further, in sheep, loline appears in the urine within 15 minutes of oral dosing (Gooneratne et al. 2012) suggesting rapid metabolism and potentially limiting pharmaceutical effect *in vivo*.

Rumen and abomasal digestion appear to have a small effect on the form and concentration of loline and only slight alteration to the rumen-fermentation pattern. Presence of lolines showed little evidence of an antimicrobial effect on rumen microbes. Therefore, with loline's ability to survive digestion without altering digestive physiology, pharmaceutical effects on gut-dwelling organisms are possible, however, require future studies

Acknowledgements

The authors gratefully acknowledge Cropmark Seeds Ltd, Rolleston, NZ and Brian Patchett and Tim Gillanders for supplying meadow fescue seed and analysis of samples.

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