Metabolism of loline in ruminants and their potential effects on microflora, and gastrointestinal nematodes

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Kelly Ann Froehlich

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Loline is an alkaloid produced by *Epichloë* endophytes in pastoral grass species with potential antimicrobial properties. Loline has several known derivatives; N-formyl loline (NFL), N-acetyl loline (NAL), N-acetyl norloline (NANL), N-methyl loline (NML), and loline base. Although considered non-toxic to mammals there is little information available about its metabolism or potential antimicrobial effects. Therefore, through a series of experiments this study investigated concentration and forms of loline metabolized in rumen fermentation and digestion, its absorption through gastrointestinal epithelia, and distribution in the body (tissues, blood, etc), and lastly determine if any anti-microbial effects exist and their impact on rumen microflora and gastrointestinal nematodes.

Chapter 3 used *in-vitro* methods to determine the fate of lolines during rumen fermentation and digestion through examining forms and concentrations, and its effects on rumen microbes through the production of volatile fatty acids and ammonia. *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 appeared 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 on rumen microbes.

Chapter 4 was an extension of chapter 3 to explore lolines effect on rumen fermentation using an *in-vitro* gas production technique in two experiments. Experiment 1 treatments were *Festuca pratensis* seeds either with (LS) or without (NS) loline (150-200 µg/mL) as sole substrate and experiment 2 used *Lolium perenne* as a substrate with treatments being ryegrass only (RGS) or ryegrass with (RGL) or without (RGN) a loline seed extract incubated in buffered ruminal fluid. Cumulative gas production was measured over 24-hours and apparent dry matter digestibility and pH were collected at the end. In experiment 1, NS had a 9% greater gas yield than LS after 24-hours (*P*=0.0001) with no difference in apparent dry matter digestibility (P=0.11). In experiment 2 predicted potential gas production was not different (P=0.67) for ryegrass treatments using an Ørskov nonlinear model. However, the fractional rate of gas production was greater (P=0.0001), in RGN (0.112 ± 0.0002) than RGL (0.109 ± 0.0002), and RGL was greater than RGS (0.093 ± 0.0002). Apparent dry matter digestibility was greater in RGS than RGL (P=0.05) and RGN (P=0.02). Loline had little antimicrobial effect on rumen microbes as shown by potential gas production but the addition of the water-soluble fraction of seed extracts to rumen fluid increased the soluble pool readily available to microbes, changing the fermentation kinetics. Therefore, loline could be explored as a potential pharmaceutical on gut-dwelling organisms without negatively affecting rumen fermentation or function.

Loline metabolism in ruminants is believed to be rapid, surviving digestion and relying on hepatic metabolism prior to fast but low urinary excretion. It is unclear however where loline is absorbed and information on its distribution throughout tissues is not available. Chapter 5 investigated passive absorption of *Festuca pratensis* seed extract (1034 µg/mL) and caffeine (22.1 µg/mL) suspended in a solution in either rumen, abomasal, duodenal, ileum, large intestine, or colon tissues mounted in an Ussing chamber. Loline distribution was measured in gastrointestinal (small intestine, abomasum), organs (kidney, liver), and blood (plasma, red blood cells) of lambs deliberately bottle-fed loline.

Little passive absorption occurred in ruminal or abomasal tissues (<2%). Ileum tissues appeared to have the greatest absorption capacity (5%, P=0.04) at 2 hours incubation compared with abomasal tissues (0.11%). Of the loline forms, loline base and NFL were passively absorbed across all gastrointestinal tissues with NAL and NANL only crossing small intestine tissues. Recovery of loline *in-vivo* was low for NFL in blood plasma (46 µg/g DM), and greatest for loline base (296 µg/g DM), and loline base was the only metabolite found in liver (126 µg/g DM) and kidney (112 µg/g DM) of lambs. The low recovery of loline in organs and blood are in line with its reported fast metabolism however, the low absorption rates through gastrointestinal tissues were unexpected. Potentially indicating either the majority of loline is not passively absorbed or membrane integrity was affected as suggested by lack of caffeine absorption.

Chapter 6 investigated the validation of using loline as a natural anthelmintic for the gastrointestinal nematodes in sheep of *Trichostrongylus colubriformis, Teladorsagia circumcincta,* and *Haemonchus contortus* through a series of *in-vitro* and *in-vivo* studies. *In-vitro* experiments revealed that larval migration of *T. colubriformis* was not affected by *Festuca pratensis* seed extract (P=0.29) nor was there an extract x concentration interaction (P=0.52). However, *T. circumcincta* establishment was decreased (P<0.01) by 59% in excised tissues of lambs fed loline compared with non-treated lambs. This suggested loline could have potential anthelmintic properties on larvae when in close contact with tissues or blood. In a pilot study, the numbers of L4 mucosal browsers *T. colubriformis* and *T. circumcincta in-vivo* were reduced by 23, and 75% in lambs treated with a loline seed extract via abomasum 10 days post infection. However, dosing loline 23 days post larval infection when *T. colubriformis* and *T. circumcincta* were an adult had no effect. In a follow up pilot study, adult *H. contortus in-vivo* was reduced 34 and 36% in lambs fed loline 23 days post larval infection either orally or via abomasum compared with controls. Further investigation examined the effect of loline seed extract given orally *in-vivo* and compared non-treated lambs (CON) or lambs treated with *Festuca pratensis* seed extract without loline (NIL) in lambs infected with a mix of L4 *T. circumcincta*, *T. colubriformis*, and adult *H. contortus.* Lambs were dosed *H. contortus* on day 0, and L3 *T. circumcincta*, *T. colubriformis* on day 14 from dosing *H. contortus.* Loline was dosed orally starting on

day 13, and every other day until slaughter on day 28. Worm burdens of *T. circumcincta* (P=0.96), *T. colubriformis* (P=0.43), and *H. contortus* (P=0.15) showed no difference in lambs loline treated compared with CON or NIL. Furthermore, no treatment difference (P=0.39) was observed in faecal egg counts, weight gain (P=0.51), feed intake (P=0.18) between treatments. However, average growth efficiency (kg LWG/ kg DM intake) was 0.18 in CON treated lambs which was less (P=0.01) than LOL (0.24) or NIL (0.23) treated lambs. Overall, it was concluded there is limited evidence to support an *in-vivo* anti-parasitic effect of lolines despite *in-vitro* studies indicating potential benefits when parasites are in either a developmental stage in close contact with gastric mucous layers or adult stage consuming blood. The discrepancy between *in-vivo* and *in-vitro* results may be dependent on the length of time loline has in contact with larvae and potentially mode of ingestion or the forms of loline present.

In summary, these results demonstrate that rumen and abomasal digestion have little effect on loline form and concentration. There were slight alterations of ammonia, propionate, and gas production in the presence of loline in the rumen but overall showed limited antimicrobial effect on rumen microbes and, perhaps more importantly, microbial digestion did not affect the level of loline present. Surviving digestion with little effect on rumen fermentation meant loline could be explored as a potential pharmaceutical on gut-dwelling organisms without negative effects. Unexpectedly, only small amounts of loline were passively absorbed across gastrointestinal epithelia with the most absorption occurring in ileum tissues compared with abomasal tissues, indicating the majority of loline is not a passively absorbed. *In-vivo* only small amounts of NFL and loline base were found in kidney, liver, and blood plasma of lambs that may reflect its suggested fast metabolism, which is supported by, loline base being the metabolite found in the greatest concentration. Although, loline metabolites are found *in-vivo* there was limited evidence to support an anti-parasitic effect of loline although some benefit may arise when parasites are in close contact with the gastric mucous layers or at a blood feeding stage.

Keywords: loline, alkaloid, meadow fescue, ruminal digestion, abomasal digestion, gas production, Ussing chambers, gastrointestinal parasites

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Randel you took the burden of the most ranting, so sorry, but thank you. I needed someone to tell me to focus on finishing and stop thinking – it was pretty good advice.

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Publications and presentations during the course of this study

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Chapter 1

Introduction

1.1 Introduction

Some secondary plant metabolites act as a source of pharmaceuticals, which may benefit animals experiencing a pathogenic challenge. Many antimicrobials, such as antibiotics, have a limited lifespan before becoming unviable due to resistance. Resistance and viability has put antibiotics and antimicrobials under increasing scrutiny, restriction, and regulation. This is especially so for food-producing animals, creating a demand to push away from traditional medicine to increase food safety. However, relatively few viable antimicrobial agents exist, creating a need for natural alternatives, in which plant secondary metabolites, such as alkaloids, could potentially provide. Lolines are one such alkaloid, and are suggested to possess antimicrobial, and insecticidal (Schardl et al., 2007, Bacetty et al., 2009) effects while generally considered non-toxic to mammals (Bush et al., 1993, Gooneratne et al., 2012). Currently, limited information is available on the effects of lolines in ruminants or is confounded with presence of other alkaloids detrimental to animal health upon herbivory. Lolines are produced as a secondary metabolite by *Neotyphodium*, recently updated to *Epichloë*, fungal endophytes (Leuchtman et al., 2014) associated with many pastoral grass species important to livestock. Lolines antimicrobial properties are of interest, particularly the potential to be a natural anthelmintic against parasites, such as gastrointestinal nematodes. Seed extracts containing alkaloids have shown to affect egg hatching and larval motility of free-living nematodes (Muponda, 2014). Moreover, lolines specifically, have shown nematocidal properties to plant parasitic nematodes (Bacetty et al., 2009). Relatively little is known about loline metabolism, however, it is known that, following ingestion, lolines are rapidly absorbed, metabolized, and excreted by ruminants (Westendorf et al., 1993, Gooneratne et al., 2012). However, elucidation is needed to understand where and what forms loline are metabolized, and if any active loline forms have the potential to reach the intestine to affect gut microflora, and gastrointestinal nematodes.

1.2 Thesis objectives and hypotheses

Using *in-vitro* methods the objectives of this thesis was to determine the fate of lolines during ruminal and abomasal digestion and the loline forms passively absorbed in various gastrointestinal epithelia. Furthermore, to determine lolines distribution *in-vivo* and lolines antimicrobial effects to ruminal microbes and the gastrointestinal parasites *Trichostrongylus colubriformis*, *Teladorsagia circumcincta*, and *Haemonchus contortus* using *in-vitro* and *in-vivo* methods.

Previous research has suggested loline to have antimicrobial and insecticidal properties however, appears to be non-toxic to mammals with loline either being rapidly metabolized/excreted. Therefore, it was hypothesized that ruminal and abomasal digestion would have an effect on the forms and concentration of loline however, would not affect gastrointestinal microflora. Furthermore, loline being a small water-soluble molecule would be passively absorbed across gastrointestinal epithelia, and accumulation of loline would occur in various tissues. Specifically, absorption through the foregut and accumulation in the liver where it is presumably metabolized. Lastly, loline will have an anti-parasitic effects against *Trichostrongylus colubriformis*, *Teladorsagia circumcincta*, and *Haemonchus contortus* in loline-fed lambs.

Chapter 2

Literature Review

2.1 Introduction

Some secondary plant metabolites act as a source of pharmaceuticals, which may provide a natural alternative to the relatively few antimicrobials available to animals experiencing a pathogenic challenge. Loline is one secondary plant metabolite, which is produced by the fungal endophyte *Epichloë* and is generally considered non-toxic to mammals (Bush et al., 1993, Gooneratne et al., 2012). However, lolines have suggested antimicrobial, and specifically nematocidal properties to plant parasitic nematodes (Schardl et al., 2007, Bacetty et al., 2009) an effect, which may also benefit animals challenged with gastrointestinal nematodes. Although in order to be effective, information on the fate of lolines once ingested and the availability to the animal is required.

This literature review provides a background of how secondary plant metabolites are produced, its association with plants and specifically chemistry, occurrence, detection and biological activities of loline.

2.2 Endophytes

Endophytes are naturally occurring microorganisms that live in plant tissues as bacteria, fungi, actinomycetes, or viruses. It is widely accepted that every plant species on earth hosts at least one to several hundred endophyte species (Compant et al., 2016, Ahmed Wani et al., 2015, Hassan Aly et al., 2011). Based on their taxonomy, functional diversity, biology, and mode of transmission, endophytes can be classified into two broad categories, systemic/true or transient/non-systemic. Systemic endophytes co-evolved with the plant host to live mutualistically, and asymptomatically with vertical and sometimes horizontal modes of transmission. Whereas, non-systemic endophytes live a transient lifestyle that can be mutualistic but under certain environmental conditions some can change to parasitic and uses horizontal mode of transmission (Ahmed Wani et al., 2015).

The recruitment of endophytes in plants is often dependent on geographical region and environmental stresses, host genotype, and resource availability (Ahmed Wani et al., 2015). With higher latitude regions representing several endophyte classes but fewer species and tropical regions containing fewer classes but many species (Arnold and Lutzoni, 2007). In general, most endophytes are beneficial and can manipulate plants to help combat both abiotic and biotic stresses, such as reducing severity of pathogenic infections (Compant et al., 2016). Manipulation of plants by endophytes broadly occurs through two mechanisms: 1) through production of a wide array of chemical and functional metabolites, or 2) altering/inducing gene expression of plant defence and metabolic pathways (Ahmed Wani et al., 2015). In return, endophytes usually receive protection and nutrition (Tan and Zou, 2001).

2.2.1 Life cycle

Plant-endophyte interactions and successful establishment of a relationship to exploit niches in living plant tissues is generally poorly understood. Endophytes have below ground interactions including, rhizosphere (plant roots), laimosphere (below ground stems), and also above ground interactions (phyllosphere) encompassing caulsphere (stems), anthosphere (flowers), carposphere (fruits), spermosphere (seeds), and plant endosphere compartment (Compant et al., 2016). Transmission from generation to generation is either through vertical or horizontal means (Figure 2.1).

Vertical transmission occurs via seeds and/or vegetative propagules/tillers (Ahmed Wani et al., 2015). For example, in grasses, fungal endophyte mycelia (vegetative part of a fungus) are found concentrated in leaf sheaths around the basal meristem of tillers (Musgrave, 1994). As new tillers are produced the endophyte follows, growing into the leaf blade and sheath and into the inflorescences, seeds and seed embryo upon the plant reaching a reproductive age (Philipson & Christey, 1986, Sikkonen et al., 2004). By comparison, horizontal transmission is accomplished mainly through the production of contagious sexual spores with some evidence indicating transmission occurring through asexual spores (Sikkonen et al., 2004).

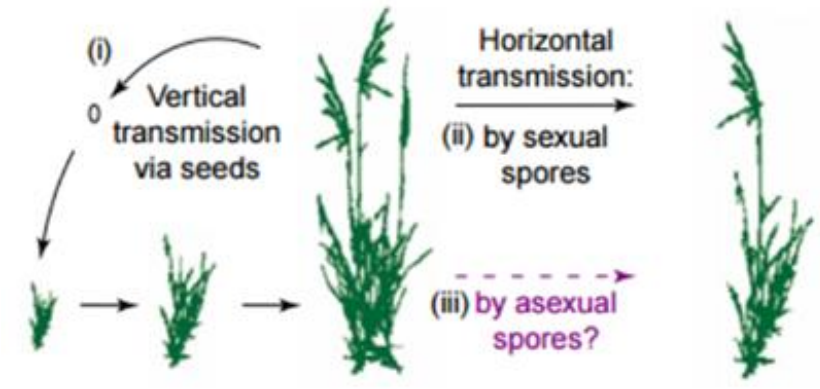


Figure 2.1 Vertical and horizontal transmission of grass endophytes
Source: Sikkonen et al., 2004

2.2.2 Functional metabolites

There are many types of metabolites that can be produced by endophytes and these have a wide variety of effects on the host plant. These metabolites include reactive oxygen species, phytohormones, volatile organic compounds, and alkaloids (Ahmed Wani et al., 2015). Metabolites such as alkaloids encompass a wide range of chemical groups that are often categorized as secondary metabolites as they are not directly involved in the growth and development of the host but offer other benefits. Alkaloids found in many grass-endophyte relationships have been subject to much research, specifically, amine and amide are common chemical group of alkaloids produced by fungal endophytes infecting cool season grasses (Tan and Zou, 2001). The secondary metabolites produced by these endophytes are known to promote a variety of beneficial plant enhancements from greater resistance to mammalian and insect herbivores, pathogens, nematodes, and increased drought tolerance and competiveness (Bush et al., 1997).

2.2.3 Grass-Fungal Endophytes

Fungal endophytes are categorized into two general groups, clavicipitaceous and non-clavicipitaceous which can be further broken down into four distinctive groups (Rodriguez et al., 2009). The clavicipitaceous family hosts the endophytes of *Epichloë* genera, some which were formally classified as *Neotyphodium* (Leuchtmann et al., 2014) and are common in several cool-season grasses in the Poaceae family (Schardl et al., 2007). These endophytes have been linked to a variety of important forages that has been known to cause livestock toxicosis and are transmitted

both asexually and sexually. Asexual species colonize the ovules, seeds, and flowering meristems, relies on vertical transmission for propagation and is asymptomatic (Bush et al., 1997, Kuldau & Bacon, 2008). Sexual endophytic species use horizontal transmission of contagious spores that mimics plant growth regulators that suppresses the host seeding production known as “choke disease”. Endophytes in the *Epichloë* genera are known to produce a variety of secondary alkaloid metabolites that can generally be classified into a few different groups; namely, peramine (pyrrolopyrazine), ergot (ergopeptines), lolitrems (indolediterpenes), and pyrrolizidine (Blankenship et al., 2001). These groups of alkaloids encompass a vast majority of different compounds that have a variety of effects on grazing mammals.

Ergot (Ergopeptines) alkaloids, are composed of an ergolene ring system and is represented by many different compounds. It is typically found in tall fescues and produced by the endophyte *Epichloë coenophiala* (Menna et al., 2012). One well known compound is ergovaline, known for its toxicity to grazing mammals causing a reduction in weight gain, reproduction, and milk production, elevated body temperature, and restricted blood flow especially to the extremities which can cause necrosis and sloughing of hoof, tip of ears, and tail (Sigel and Bush, 1996, Strickland et al., 1996, Thompson & Studemann, 1993). Ergovaline is thought to affect blood prolactin and melatonin levels and has also been associated with a high affinity for D_2 dopamine receptors causing altered neurological processes (Bush et al., 1997, and Larson et al., 1995). Reported clinical signs develop when diets contain 300-500 ppb in horses, 400-750 ppb in cattle, and 500-800 ppb in sheep of ergovaline (Aldrich-Markham et al., 2007).

Lolitrems (indolediterpenes) are also composed of many different compounds. Lolitrem B is a well-known lolitrem that is produced naturally by *Epichloë festucase* var. *lolii* in ryegrass (Menna et al., 2012). Lolitrem B is tremorgenic to mice, sheep, cattle, horses, donkeys, deer, alpaca, and goats (Gallagher et al., 1981, Menna et al., 2012) causing a phenomena denoted as ryegrass staggers. Similar to ergovaline, lolitrem affects animal performance and health with threshold values for clinical symptoms estimated at 1,800-2,000 ppb in sheep and cattle (Aldrich-Markham et al., 2007).

In dairy cows, it reduces milk yield (Bluett et al., 2005), causes weight loss, decreased live weight gains, causes heat stress, and increased scouring and consequently increased dags and risk for fly strike in sheep (Menna et al., 2012) resulting in poor animal performance, and an estimated annual loss of $200-800 million to U.S. cattle producers alone (Siegel et al., 1984).

Peramine (pyrrolopyrazine) along with loliterm is produced naturally by *Epichloë festucase* var. *lolii* in ryegrass with reported levels of 14-18 ppm (Menna et al., 2012). It is a water-soluble alkaloid with insecticidal effects, such as reduced feeding of argentine stem weevil. Despite having insecticidal effects it is suggested to have low mammalian toxicities to grazing livestock (Menna et al., 2012).

Pyrrolizidine alkaloids are a diverse group of compounds in which many have been associated as heptatoxins and carcinogens (Cheeke, 1988). Following consumption, livestock often experience liver damage and associated toxicity symptoms. It has also been suggested that pyrrolizidines affect vitamin and mineral metabolism (Cheeke, 1988). However, lolines are a unique pyrrolizidine compound that does not appear to detrimentally affect livestock health upon consumption but has strong insecticide activity (Schardl et al., 2007). Unfortunately, a majority of studies on loline in ruminants are confounded with other endophyte metabolites, such as egrovaline, that are known to cause toxic affects to animals (Bush et al., 1993).

2.2.4 Grass Types

Grasses in the family Poaceae have been established as popular forage and pasture species. Some common grasses that can be found in New Zealand and U.S. with its associated endophyte and alkaloids are listed in Table 2.1. Meadow Fescue is one forage species that is known to produce only lolines without other alkaloid metabolites that cause detrimental staggers in grazing livestock (Menna et al., 2012). Consumption of tall fescue and perennial ryegrass is often associated with “tall fescue toxicosis” and “ryegrass staggers” caused by the presence of ergopeptines, lolitrems, and peramines. Ultimately, resulting in poor animal welfare and performance. In the 1980’s and early ‘90s this translated to an estimated loss of $200-$800 million annually to U.S. cattle producers (Siegel et al., 1984, Hoveland et al., 1993), and more than $40 million to N.Z. producers (Prestidge et al.,

1991) for ryegrass staggers. With estimated losses of more than $1 billion for the entire U.S. livestock industry in 2011 (Strickland et al., 2011) for fescue toxicosis. Historically, attempts to establish endophyte-free grasses to improve welfare of grazing livestock came at the expense of growth and productivity of the plants. Both abiotic and biotic stress tolerances are lowered in endophyte free-grass resulting in a lowered grass production (Bush et al., 1997, Joost, 1995), making presence of endophytes imperative for high performing pastures (Easton, 1999). However, novel strains of endophytes have been commercially marketed including AR1 and AR37 that provide some of the benefits of having an endophyte present without majorly affecting livestock productivity or welfare (Fletcher & Sutherland, 2009, Popay & Thom, 2009).

Table 2.1 Natural grass and associated fungal endophyte species and alkaloids that they produce
Source: Cook and Lewis 2001, Menna et al. 2012, Leuchtmann et al. 2014

	Endophyte		**Alkaloids**			
Grasses	**Species**	**Former name**	**Ergopeptines**	**Lolitrems**	**Peramine**	**Lolines**
Perennial ryegrass (*Lolium perenne*)	*Epichloë festucase* var. *lolii*	*Neotyphodium lolii*	Yes	Yes	Yes	No
Tall Fescue (*Lolium arundinaceaum/ Festuca arundinacea*)	*Epichloë coenophiala*	*Neotyphodium coenophialum*	Yes	No	Yes	Yes
Meadow Fescue (*Lolium pretense/Festuca pratensis*)	*Epichloë uncinatum*	*Neotyphodium uncinatum*	No	No	No	Yes

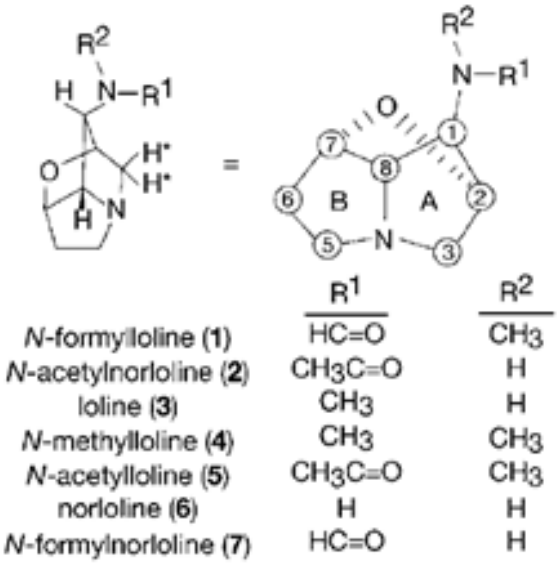
2.3 Loline Chemistry, Occurrence and Detection

Structurally, lolines are a unique class of saturated pyrrolizidines that do not appear to be detrimental to mammals, have broad insecticidal activity, and can accumulate up to 2% of the plant dry matter (Blankenship et al., 2005). An area of interest is the bioavailability and potential for anthelmintic properties in mammals, especially ruminants. However, a lot is still unknown, loline metabolism in ruminants and the effects on microflora, and gut health need to be better understood in order to determine potential for having anti-microbial properties. Lolines are found in the genera of *Lolium,* and *Festuca* (family Poaceae) grass species such as tall fescue and meadow grass and are associated with a variety of endophytes (Schardl et al., 2007). Loline alkaloids have also been found in *Argyreia mollis* (Convolvulaceae) and *Adenocarpus* (Fabaceae) plants but have not been associated with endophytes (Tofern et al., 1999, Schardl et al., 2007).

2.3.1 Chemical structure

Typically, pyrrolizidine alkaloids are unsaturated and possess a CH_2OR group at C1 (Cheeke, 1988). Loline and its derivatives are a saturated pyrrolizidine alkaloid that share a common exo-1-aminopyrrolizidine with an ether bridge between the 2nd and 7th carbon (Schardl et al., 2007). Unlike unsaturated pyrrolizidine alkaloids, lolines are not known to be heptatoxic and carcinogenic to animals and humans (Cheeke, 1988, Bush et al., 1993).

Naturally occurring loline and derivatives shown in Figure 2.2 are loline, N-formyl loline (NFL), N-acetyl loline (NAL), N-methyl loline (NML), norloline, N-acetyl norloline (NANL), and N-formyl norloline (NFNL) (Bush et al., 1993 and Justus et al., 1997).



	R^1	R^2
N-formylloline (**1**)	HC=O	CH_3
N-acetylnorloline (**2**)	CH_3C=O	H
loline (**3**)	CH_3	H
N-methylloline (**4**)	CH_3	CH_3
N-acetylloline (**5**)	CH_3C=O	CH_3
norloline (**6**)	H	H
N-formylnorloline (**7**)	HC=O	H

Figure 2.2 Naturally occurring loline and derivatives
Source: Blankenship et al. 2005

2.3.2 Biosynthesis

Loline biosynthesis has been investigated for many years (Bush et al., 1997, Blankenship et al., 2001). Blankenship et al. (2001) elucidated that growing *Epichloë uncinatum* on culture medium produced N-acetylnorloline and N-formylloline showing that loline production is a product of the fungal endophyte and not of plant origin. Illuminating where loline is produced has allowed the advancement of how it is produced. Genetic analysis has shown that loline is encoded by a single locus, designated as *LOL* (Schardl et al., 2012). Eleven genes have been identified with the *LOL* gene cluster that is strictly associated with the biosynthesis of loline (Pan et al., 2014). Feeding of *Epichloë uncinatum* cultures with radiolabeled and stable-isotype-labelled amino acids has further shown that L-homoserine and L-proline are precursors to the carbon and nitrogen loline backbone (Blankenship et al., 2005). Proposed loline biosynthetic pathway and associated genes are presented in Figure 2.3.

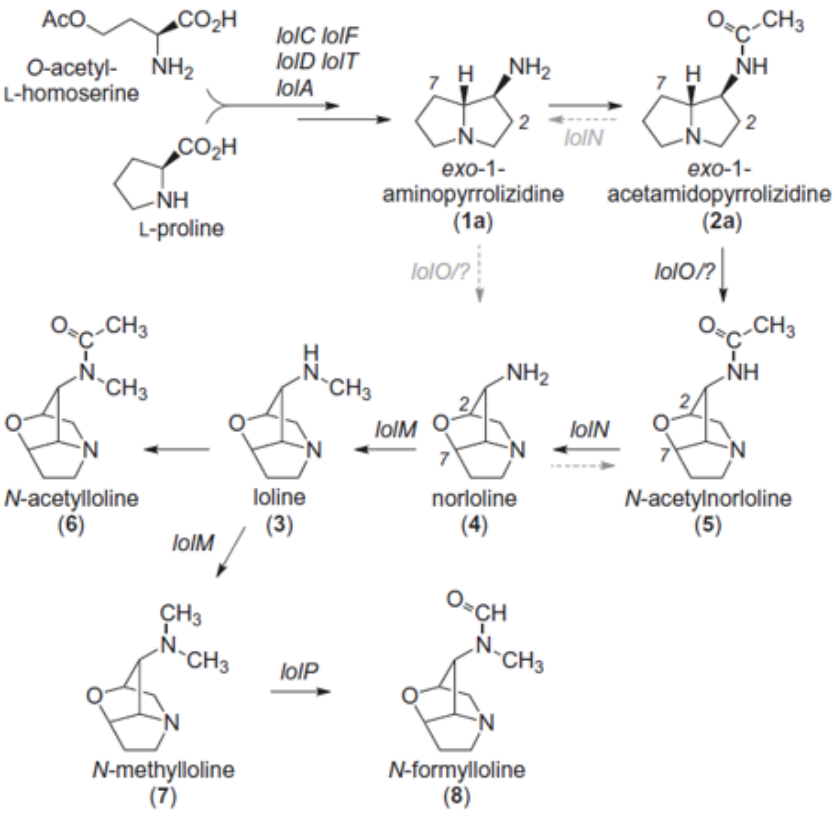


Figure 2.3 Proposed biosynthetic pathway and associated genes for loline
Source: Pan et al. 2014

2.3.3 Concentration of lolines

Different natural loline derivatives can co-exist in plants in varying amounts. In tall fescue and meadow fescue infected with their natural co-existing endophyte concentrations of lolines are as follows; N-formyl loline (NFL), N-acetyl loline (NAL), and N-acetyl norloline (NANL) (Table 2.2) (Ball & Tapper, 1999, Patchett et al., 2011a, De-Wen et al., 2006). N-formyl loline is the greatest, accounting for concentrations greater than 60% of the total alkaloids in meadow fescue stems. N-methyl loline has also been detected in varying lines of meadow fescue but only in trace (Justus et al., 1997) to low concentrations (5-235 µg/g) (Patchett et al., 2011a).

Table 2.2 Natural loline derivatives concentrations in meadow and tall fescue

Species	Endophyte		NFL	NAL	NANL
Tall fescue	*E. coenophiala* **	NI	1043	351	91
	E. coenophiala *	AI	987	557	
	E. coenophiala *	NI	898	211	
Meadow fescue	*E. uncinatum* **	NI	909	329	284
	E. uncinatum *	NI	4360	1254	

AI = Artificially infected, NI = Naturally infected
N-formyl loline (NFL), N-acetyl loline (NAL), N-acetyl norloline (NANL)
*Bush et al. 1993
**Ball & Tapper, 1999

Lolines also vary in different plant parts. In flowering tall fescue, NAL and NFL concentrations have been reported as follows rachis (578 µg/g) > stem (255 µg/g) > leaf sheath (181 µg/g) > leaf blade (74 µg/g) (Bush et al., 1993). Similarly, average concentrations of loline are greater in the stem (2800 µg/g) than in the leaf (823 µg/g) of meadow fescue. In roots up to 290 µg/g NFL has been reported in tall fescue (Bush et al., 1993) and in varying amounts in meadow fescue (Justus et al., 1997, Patchett et al., 2011b). Justus et al. (1997) found trace amount of lolines (NFL and NAL) in meadow fescue roots however, concentrations of up to 600 µg/g have been reported (Patchett et al., 2011b). It appears plant concentrations can vary with season (Table 2.3). It is thought that alkaloids, such as loline, can be translocated within the plant. Earlier studies found loline in low concentrations in the root and leaf blade of tall fescue but with no obvious presence of the endophyte indicating translocation from site of synthesis (Bush et al., 1993). Patchett et al. (2011b) reported increased

root concentrations in late autumn (600 μg/g), corresponding to a decrease in crown and shoot loline concentrations, suggesting a transportation from shoots to roots.

Concentration of alkaloid metabolites are influenced by environmental conditions as well as plant and endophyte species (Menna et al., 2012). Typically, alkaloid concentrations are relatively high in late spring/early summer with a slight decrease in midsummer, and the highest concentration occurring in autumn. With loline, Studemann et al. (1985) reported NAL and NFL had the greatest accumulation in late summer and the lowest concentration occurring in winter in tall fescue. In meadow fescue, peak loline concentrations occurs in late spring in both leaf (1240 μg/g) and stem (4080 μg/g) with a secondary peak in late summer, and then a decline in autumn (Patchett et al., 2011a), which corresponded with an increase loline concentration in the roots (Patchett et al., 2011b, Table 2.3). Similarly, Justus et al. (1997) described plant growth and loline accumulation in meadow fescue in two stages. Stage one, the reproductive stage, alkaloid (loline) formation occurs with the growth of new leaves in spring and increases until seed dispersal in summer. Following, stage two, a rapid vegetative growth is achieved in which there is an increase in loline alkaloids but quickly declines after leaf senescence in the autumn.

Table 2.3 Concentrations of lolines by plant part and season

	Total loline (μg/g)		
Season	**Stem***	**Leaf***	**Root****
Early spring	3180	670	
Late spring	4080	1240	71
Early summer	2600	610	165
Late summer	2690	1160	
Early autumn	2600	760	180
Late autumn	1670	500	600

Total loline = N-formyl loline + N-acetyl loline + N-acetyl norloline + N-methyl loline
*Patchett et al. 2011a
**Patchett et al. 2011b

Other environmental factors also seem to influence alkaloid accumulation. Tall fescue under water stress conditions increases loline (NAL & NFL) concentrations (Belesky et al., 1989). Under field conditions increases in NAL and NFL in tall fescue were observed in high nitrogen paddocks compared with low nitrogen paddocks (Beleskey et al., 1989) but was not replicated in a glasshouse (Bush et al., 1993). Kennedy and Bush (1983) observed over a 10 week period NFL and NAL

concentrations in tall fescue are greatest in 21/15°C day/night temperatures with decreased concentrations at higher or lower temperatures. Concentrations of NAL and NFL also appear to increase in the regrowth of cut forage (Kennedy and Bush, 1983). In meadow fescue infected with *Epichloë uncinatum* or *Neotyphodium siegelii*, an increased concentration of loline alkaloids were observed in younger plant tissue after insect damage or mock herbivory (clipping) (Gonthier et al., 2008, Zhang et al., 2009), an effect which may be attributed to amino acid availability (asparagine and glutamine), the precursors to loline production (Zhang et al., 2009).

2.3.4 Detection

Loline alkaloids have been detected by numerous methods. These include paper chromatography, thin-layer chromatography on silica gel or alumina plates, and high speed countercurrent chromatography (Schardl et al., 2007). Nuclear magnetic resonance and x-ray crystallography have been used to determine the relative configuration and structure of loline (Powell & Petroski, 1992). More typically, a quantitative analysis of lolines is needed which can be determined by capillary gas chromatography with a flame ionization detector (GC), and/or can be combined with mass spectroscopy (MS) (Porter, 1995, Schardl et al., 2007).

Analysis using GC-MS first involves the extraction of loline alkaloids from samples. Retention times increase with molecular mass. From shortest to longest retention time is norloline, loline, N-methylloline, N-formylnorloline, N-acetylnorloline, N-formylloline, and N-acetylloline (Yates et al., 1990). Typically, samples are extracted with organic solvents under basic conditions using dichloromethane:methanol:ammonia (75:25:0.5) (Schardl et al., 2007, Yates et al., 1990).Yates et al. (1990) described this procedure for analysis of loline in plant tissue but has been modified to handle other solid biological samples such as faeces (Gooneratne et al., 2012).

Internal and external standards are used in loline GC-MS analysis for calibration and identification of samples. Internal standard is added to the extraction solution, and quinoline or phenylmorpholine have both been used (Blankenship et al., 2001, Schardl et al., 2007, Yates et al., 1990). However, phenylmorpholine seems to be more commonly used, and has been specifically used for analysis of

meadow fescue (Gooneratne et al., 2012), and works well in the range for loline alkaloids (Yates et al., 1990).

External standards for development of a standard curve are derived from loline dihydrogen chloride crystallized from meadow fescue seed. A modified procedure from Petroski et al. (1989) and Blankenship et al. (2001) has been described by Patchett (2007). Melting point and nuclear magnetic resonance (NMR) can be used to confirm purity, authenticity, and chemical structure. Loline dihydrogen chloride has a published melting point of 243-248°C (Petroski et al., 1989).

2.4 Biological Activities of Loline

2.4.1 Loline effects on insects, parasites and environment

Lolines appear to be a general broad spectrum insecticide. Depending on the species of insect lolines apparently act as a metabolic toxin and as a feeding deterrent (Bush et al., 1997). N-formyl loline and NAL are the principle natural loline alkaloids produced in meadow and tall fescue (Bush et al., 1993), with NFL being the predominant alkaloid (Yates et al., 1990). Research on insect toxicity has focused on these natural lolines. Riedell et al. (1990, 1991) reported naturally occurring lolines with shorter acyl chains (1-2 carbons) and synthetic derivatives with longer acyl chains (10-14 carbons) to have the greatest insecticidal activity. Specifically, NFL has been reported as a contact and ingested broad spectrum insecticide to several insects from different orders in a dose dependent manner (Dahlman et al., 1997). In milkweed bugs, NFL appeared to be about ten times more toxic than other purified ergocryptine or ergonovine alkaloids (Yates et al., 1989). Loline and derivatives have had apparent effects on larvae development of rice leaf bug (Shiba & Sugawara 2008), argentine stem weevil (Patchett et al., 2008a, Popay et al., 2009), Japanese beetle (Patterson et al., 1991), horn fly (Dougherty el al., 1998), and grass grub (Patchett et al., 2008b). It is toxic to greenbug and bird-cherry-oat aphids (Siegel et al., 1990). It has also been reported that beef cattle grazing endophyte-infected tall fescue that produces loline had a lower population of horn and face flies (Brown et al., 1993). Lolines also have multi-trophic effects Fall armyworms fed tall fescue infected with *Epichloë coenophiala* and parasitized with one of two Euplectrus parasitoids had reduced pupal mass and

survival of the Euplectrus parasitoids. The same results occurred when fall armyworms were fed an artificial diet laced with purified NAL or NFL (Bultman et al., 1997).

It is possible the anti-parasitic properties may extend to gastrointestinal parasites of livestock. Alkaloids such as loline, are thought to effect antagonizing receptors in the central nervous system of the nematode causing death (Roy et al., 2010, Jain et al., 2013, Dubois et al., 2019). However, it is unknown if loline could influence multiple stages of a lifecycle in ruminants. Most gastrointestinal nematodes have a similar direct lifestyle using a ruminant's gut lumen to mate and produce eggs, that are deposited in the faeces. In favourable conditions eggs in the faecal pat develop into first stage larvae (L1) where they feed and further develop, moulting into L2 and then to L3 larvae. Larvae L2 and L3 have a retained sheath providing protection from the external environment as they migrate from faecal pat to pasture. Unlike L1 larvae, L2 and L3 sheath prevents larvae from feeding and thus any potential to consume toxic compounds. Specifically, seed extracts of loline containing grasses such as meadow and tall fescue has shown anthelmintic affects disrupting the egg hatching and larval motility (L1) of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* nematodes. High concentrations (1:1 ratio of water to seed extract) lysed eggs and affected L1 larvae motility. However, these results appeared independent of presence of endophyte or endophyte type and identification of the causal agent was not explored (Muponda, 2014). Beyond, L1 stage it is unknown if loline would have any observed anthelmintic affects. The third larval stage (L3) is the infective stage to grazing ruminants, and once ingested behaviour slightly differs depending on specie. For example, *Teladorsagia circumcincta* is closely associated with host tissue and metabolites, burrowing into the abomasal mucosal surfaces to develop into L4 larvae. As an adult, it returns to browse the mucosal surfaces. Other nematodes such as *Haemonchus contortus* or *Trichostrongylus colubriformis* are not as closely associated during the development into an adult. *Trichostrongylus colubriformis,* a mucosal browser, mainly develops and feeds on the mucosal surfaces of the small intestine. While *Haemonchus contortus*, after adult development will lacerate abomasal wall and feed on host blood and any potential blood metabolites (Jackson et al., 2004). As will be described in the next section, loline is found *in-vivo* with potential for effects on other life stages of parasites.

Below ground loline alkaloids, and specifically endophyte infected grasses, are thought to influence grass grubs, and nematodes (Patchett et al., 2011c, Bacetty et al., 2009). Root loline concentrations > 450 µg/g in meadow fescue acted as a feed deterrent to grass grub, negatively affecting larval body mass (Patchett et al., 2011c). Combined NAL & NFL is nematocidal to *Pratylenchus scribneri*, a plant parasitic nematode at concentrations 100 and 250 µg/mL (Bacetty et al., 2007). Nematode behaviour is significantly affected by alkaloids contained in the roots of *Epichloë coenophiala* infected tall fescue. Combined root extracts of tall fescue alkaloids at 50-400 µg/mL was a strong repellent, and only a weak repellent at 5-50 µg/mL to *Pratylenchus scribneri*. Specifically, loline mix at 10-200 µg/mL and NFL at high concentrations (50-200 µg/mL) was a strong repellent (Bacetty et al., 2009).

Endophyte-infected roots differ in their primary and secondary metabolism influencing emission of volatile organic compounds (VOCs) which many nematodes and below ground insects rely on to locate potential hosts (Rostas et al., 2015, Perry 1996). Alkaloids such as lolines are not volatile compounds but the associated endophytes may have just as an important influence on soil insect behaviour. Loline producing *Epichloë uncinatum* in a meadow fescue/perennial ryegrass hybrid has shown to decrease VOCs attracting fewer soil insects than non-infected grass (Rostas et al., 2015).

Presence of endophytes in grasses have been known to alter plant and soil communities negatively attributed to an allopathic effect from leached alkaloids. For instance, it is thought that the alkaloids can inhibit the growth of other plants (Rudgers & Orr, 2009). In tall fescue *Epichloë coenophiala* endophyte in clay loam soils negatively affects archaea, gram-positive bacteria, microbial utilization, and soil structure (Buyer et al., 2011, Jenkins et al., 2006), as well as mycorrhizal colonization of roots (Mack & Rudgers, 2008). It has been suggested that residual alkaloids are toxic to microbial/detritivore communities affecting decomposition rates in endophyte infected tall fescue litter and faeces of sheep grazing endophyte infected perennial ryegrass (Lemons et al., 2005, Cripps & Edwards, 2013). Although, attributing alkaloids to reduced decomposition rates has been questioned when Siegrist et al. (2010) found only minimal loline concentrations in litter bags containing endophyte infected tall fescue. An alternative explanation could be attributed to more

easily degradable fibre/smaller neutral detergent fibre (NDF) fraction, as non-endophyte infected grasses have equivalent or elevated NDF content when compared with endophyte infected grasses (Zabalgogeazcoa et al., 2006). Similarly, a greater NDF fraction was found in the faeces of sheep grazing non-infected perennial ryegrass (Cripps & Edwards, 2013). Although not significant, ADF levels were lower in the faeces of sheep grazing non-infected perennial ryegrass, making the proportion of hemicellulose greater and therefore, easier decomposed (Cripps & Edwards, 2013). Furthermore, *Epichloë* endophytes have inhibited the growth of several plant pathogenic fungi but the effect was not attributed to loline (66% pure NFL & NAL), peramine, or ergot alkaloids specifically and was suggested that other antimicrobial compounds may be involved (Siegel & Latch, 1991). Endophytes may have a broader ecological effect that extend beyond their bioactive alkaloids.

Despite loline alkaloids having bioactive effects on plant and soil communities there is always exceptions to the rule. Indication of niche bacterial microflora on the leaves of endophyte infected (*Epichloë*) tall fescue with the ability to catabolize lolines exist (Roberts & Lindow, 2014). It has been observed that these bacterial strains are exclusively found on *Epichloë* infected grasses and could use NFL as the sole nitrogen and carbon source however, catabolism of loline base did not occur (Roberts & Lindow, 2014). Although loline catabolism is an uncommon occurrence it is a niche that can be exploited by specific bacterial microflora that is highly selected for as a result of plants with loline producing endophytes.

2.4.2 Loline effects on mammals

Many studies of loline effects on mammals are confounded with the presences of other endophyte metabolites such as ergot alkaloids (Bush et al., 1993). It is generally accepted that lolines are non-toxic (Schardl et al., 2007 & Bush et al., 1993, Gooneratne et al., 2012). Both NFL and NAL were thought to be a contributing factor in equine fescue oedema however, no connections were made between presence/absence of symptoms and detection of loline metabolites (Rudolf 2018, 2019). Therefore, research is still limited, and a lot is unknown about the effects loline has on the metabolism and performance of ruminants, specifically rumen microflora. Biological activities in

alkaloids are affected by changes in pH which alters the chemical structure (Aniszewski, 2007). Moreover, lolines may be influenced by ruminal metabolism (Westendorf et al., 1993), although whether this is caused from ruminal pH or other factors is unknown.

Murine studies

Effects of loline in murine models has shown to be non-toxic with a few exceptions. Intravenous injections of 400 mg/kg of 'festucine' now known as loline base (Aasen & Culvenor, 1969) are lethal (Yates & Tookey, 1965). However, oral administration of 'festucine' at 1000 mg/kg of BW has been shown to not have any apparent effect (Yates & Tookey, 1965). Dannhardt & Steindl (1985), Casabuono & Pomilio (1997), and Finch et al. (2016) also found no apparent effects of oral administration in mice. Whereas, intraperitoneal administration, of pure loline dihydrogen chloride at 200 mg/kg BW and two loline mixes and derivatives ranging 31.3 – 125 mg/kg BW in mice were shown to be non-toxic and asymptotic (Dannhardt & Steindl, 1985, Casabuno & Pomilio, 1997). Finch et al. (2016) fed meadow seed containing an average of 62.4, 28.2, 326.6 mg/kg BW daily of NAL, NANL, and NFL, respectively for a total intake 415 mg/kg BW daily of lolines with no apparent effect on gross pathology, histology, haematology, blood chemistry, heart rate, blood pressure or motor coordination of mice. However, weight gain and feed consumption decreased initially but was equivalent to mice fed non-endophyte feed towards the end of the study (Finch et al., 2016). Jackson et al. (1996) concluded that loline alkaloids might have some depressive effect on feed intake in rats. This was investigated between nine different treatments looking at NFL, and NAL separately, and in with combination of each other and ergot alkaloids (Table 2.4). Similarly, depressed feed intake in rats was most noticeable at the start of the feeding period, and in this case, for NFL containing diets compared with the positive control (endophyte-free tall fescue) (Jackson et al., 1996). Although, total loline alkaloid in treatment diets varied from 18-28 mg/d and ergot alkaloids were never reported, this could reflect total consumed alkaloids. Interestingly, average daily gains did not necessarily follow feed intakes and were higher in some treatments (NFL+NAL & NFL+NAL+ergot) than expected (Table 2.4). This suggested a growth-stimulating factor associated with NFL loline alkaloid, although no explanation has been alluded. Epididymides, testes, hypothalamus, corpus striatum weights along

with, prolactin and alkaline phosphatase content was measured with no statistical differences between treatments (Jackson et al., 1996).

Table 2.4 Intake, weights, average daily gains, feed/gain of mice fed combinations of loline and ergot Source: Jackson et al. 1996

	Total loline intake	Trial intake, g/d	Beginning weight, g	Trial ADG, g/d	Feed/gain, g
NFL	27.8	14.2^{bc}	182.8	1.424^{bc}	10.3
NAL	18.0	14.9^{a}	175.9	1.189^{cd}	12.9
NFL+NAL	22.3	14.6^{abc}	174.3	1.769^{a}	8.4
NFL+ergot	23.8	14.0^{c}	174.3	1.344^{bc}	10.5
NAL+ergot	20.0	14.9^{a}	183.5	.964^{d}	1.2
NFL+NAL+ergot	21.6	14.8^{ab}	174.8	1.518^{ab}	10.7
Ergot	0	15.0^{a}	174.6	.983^{d}	16.6
+ Control	0	15.0^{a}	182.4	1.133^{cd}	14.0
- Control	25.5	12.4^{d}	179.3	.554^{e}	26.0
SE		.2	7.4	.102	2.8

Means in the same column with different superscripts differ (P < 0.5)
N-formyl loline (NFL), N-acetyl loline (NAL)
+ Control = endophyte-free tall fescue seed plus solvent
- Control = endophyte-infected tall fescue seed plus solvent

In-vitro endocrinology studies

Several *in-vitro* culture studies have examined the effects of lolines with mixed results. Loline dihydrochloride was found to have no affinity for α1 adrenergic, serotonin, muscarinic, nicotinic, or benzodiazepine biogenic amine receptors in calf brain (Dannhardt & Steindl, 1985). A dose dependent effect was observed with a high concentration (10^{-4} M) of loline base suppressing prolactin. The use of a D_2 dopamine receptor antagonist (domperidone and sulpiride) blocked the effect of loline preventing a prolactin lowering receptor effect in rats indicating that loline may be a D_2 dopamine agonist (Strickland et al., 1994). The inhibition of prolactin was an unexpected result as a previous study in rats found no effect of loline derivative alkaloids (NFL, NAL, and NML) at similar concentration on pituitary prolactin (Strickland et al., 1992). Furthermore, Larson et al. (1999) found NFL and NAL to have no binding to rat D_2 receptors or affect cyclic AMP system at nanomolar to millimolar concentrations.

A greater contractile response to α_2 adrenergic receptor of the lateral saphenous veins was observed in cattle grazing endophyte (*Epichloë coenophiala*) infected tall fescue and was suggested that effects

of α_2 adrenergic tissue receptor would have to be neutralized to prevent adverse effects (Oliver et al., 1998). In lolines specifically, Solomons et al. (1989) found partial inhibition of norepinephrine-elected vasoconstriction in cattle of a concentrated loline mix (NAL and NFL) of 3×10^{-5} M. Similarly, NAL elicits only minimal vasoconstriction in bovine lateral saphenous veins (Klotz et al., 2008) and no arterioconstriction in equine (Abney et al., 1993). However, discrepancies have been found in NAL vasoconstriction responses. Unlike Klotz et al. (2008) NAL caused vasoconstriction in bovine lateral saphenous veins and indicated antagonist activity against serotonergic and alpha-2 adrenergic receptor sites (Oliver, 1997). Adrenergic receptor α_2 is involved in metabolic reactions such as regulation of tissue metabolism and heat regulation that could result in poor animal performance and growth. These have been associated with endogenous catecholamines, norepinephrine and epinephrine, which can stimulate vascular smooth muscle growth that can be contributed to vascular complications (Strickland et al., 1996). Strickland et al. (1996) studied the effect of various alkaloids including N-acetyl loline on growth of active and quiescent bovine vascular smooth muscle *in-vitro*, and reported N-acetyl loline stimulated growth of vascular smooth muscle cells at higher concentrations (10^{-8}, 10^{-9}, 10^{-11} M) in quiescent cells but inhibited the growth of vascular smooth muscle cultures at 10^{-8}, 10^{-9} M (Strickland et al., 1996).

Toxicology/Metabolism

Long-term or residual effects of loline in ruminants are currently unknown and residual or accumulation in tissues have been suggested (Gooneratne et al., 2012, Klotz et al., 2008). Recently, Rudolph et al. (2019) detected concentrations ranging from ND (not detected) up to 19.8 pg/mg and 41.3 pg/mg for NFL and NAL, respectively, in horsehair. This suggests long-term or residual accumulation of loline. Loline metabolism in ruminants is balanced to be rapid, breaking down in the rumen and/or liver followed by a rapid but low urinary excretion (average of 3.9 – 6.1% recovery, Table 2.5). Gooneratne et al. (2012) suggested this could result in accumulation of potential toxic concentration of loline alkaloids in animal tissue. Klotz et al. (2008) echo this concern stating a potential disadvantage of the many *in-vitro* culture studies is that diets of animals prior to collection

of tissues is unknown. Potentially this variation could contribute to discrepancies in research if residual effects occur in the tissues of ruminants.

Cytotoxic research with semisynthetic loline derivatives, reported N-acyl derivatives of loline with 12-18 carbon chain length to be cytotoxic to three human solid tumour cell lines and in a brine-shrimp assay. Shorter chain lengths of less than 12 carbons of acyl loline derivatives had weak to insignificant bioactivities (Petroski et al., 1994). This is however in contrast to *in-vitro* studies looking at metabolic activation of naturally occurring pyrrolizidine alkaloids and found that loline type of alkaloids most likely pose no toxic harmful effects due to their metabolism into metabolites with no ability to interact with cellular macromolecules (Ruan et al., 2014a). The discrepancies could be the result of chemical differences. Currently there is no standard procedure for creating synthetic lolines and because of their nature has been difficult for biochemists to recreate (Cakmak et al., 2011).

Toxic potency of natural pyrrolizidine alkaloids are dependent on numerous factors mainly dependent on enzymatic factors and chemical structure. Naturally, occurring pyrrolizidines occur as esters consisting as a necine base and one or two necine acids. These pyrrolidines are non-toxic per say but require 'bioactivation', upon ingestion are metabolized into pyrrolic esters and dependent on its structure and properties posing toxicity issues (Ruan et al., 2014b, Stegelmeier, 1999). Bioactivation occurs primarily in the liver through a series of oxidative-reductive reactions and conjugation (Mattocks, 1986, Dominguez-Bello, 1996, Stegelmeier, 1999). It seems as if the liver detoxification is similar among all chemical types of pyrrolizidines (PA), but the end results are different depending on the chemical structure. As such, pyrrolizidine can be classified into three chemical groups: retronecine, otonecine, and platynecine. Retronecine and otenecine are referred to as unsaturated pyrrolizidine and have 1, 2 double bond in the necine base and are attributed as hepatotoxins. Detoxification of these two chemical groups of PAs are well established and upon reaching the liver are 'activated' by hepatic cytochrome P450 enzymes (Figure 2.4). This oxidation pathway results in a highly reactive unstable pyrrolic esters (dehydropyrrolizidine) that wants to grab electrons by binding and forming thioethers to compounds making itself stable (Mattocks and Juke,

1990). This is accomplished in one of two ways; either through binding of glutathione to form glutathione conjugates or forming pyrrole-protein adducts (Mattocks, 1986, Stegelmeier, 1999, Ruan et al., 2014a, b).

Formation of glutathione conjugates is considered the PA detoxification pathway as the combination results in a water-soluble compound that is excreted with no effects. Formation of pyrrole-protein adducts however causes acute toxicity to mainly the liver but also other organs and lungs. Furthermore, these adducts can form with DNA leading to genotoxicity (Ruan et al., 2014b). Measuring pyrrolic esters *in-vivo* is difficult because of their instability therefore metabolism of PAs can only be determined indirectly by glutathione conjugates or pyrrole-protein adducts (Ruan et al., 2014a). Silver nitrate in conjugation with an alcohol (ethanol/methanol) is used to break the stable thioether bond without binding to the pyrrole and resulting in a stable, measurable pyrrolic ester (Mattocks and Juke, 1990).

Platynecines on the other hand are a saturated PA with only a single bond in the necine base (Stegelmeier, 1999). Their structural difference was thought to contribute to their non-toxicity resulting in no hepatic metabolic activation unlike their unsaturated counterparts (Mattocks, 1986). However, this is proven not to be the case and that saturated PA metabolism is dependent on the same oxidative hepatic cytochrome P450 enzymes. The main difference is that the resulting pyrrolic esters formed (dehydropyrrolizidine) cannot undergo the same reactions as unsaturated PAs because of the lack of a double bond in the necine base, resulting in a stable, water-soluble dehydroPA carboxylic acid. Unlike unsaturated dehydropyrrolizidine metabolites, dehydroPA carboxylic acid is unreactive with no need to form glutathione conjugates or protein adducts and is readily excreted (Ruan et al., 2014a).

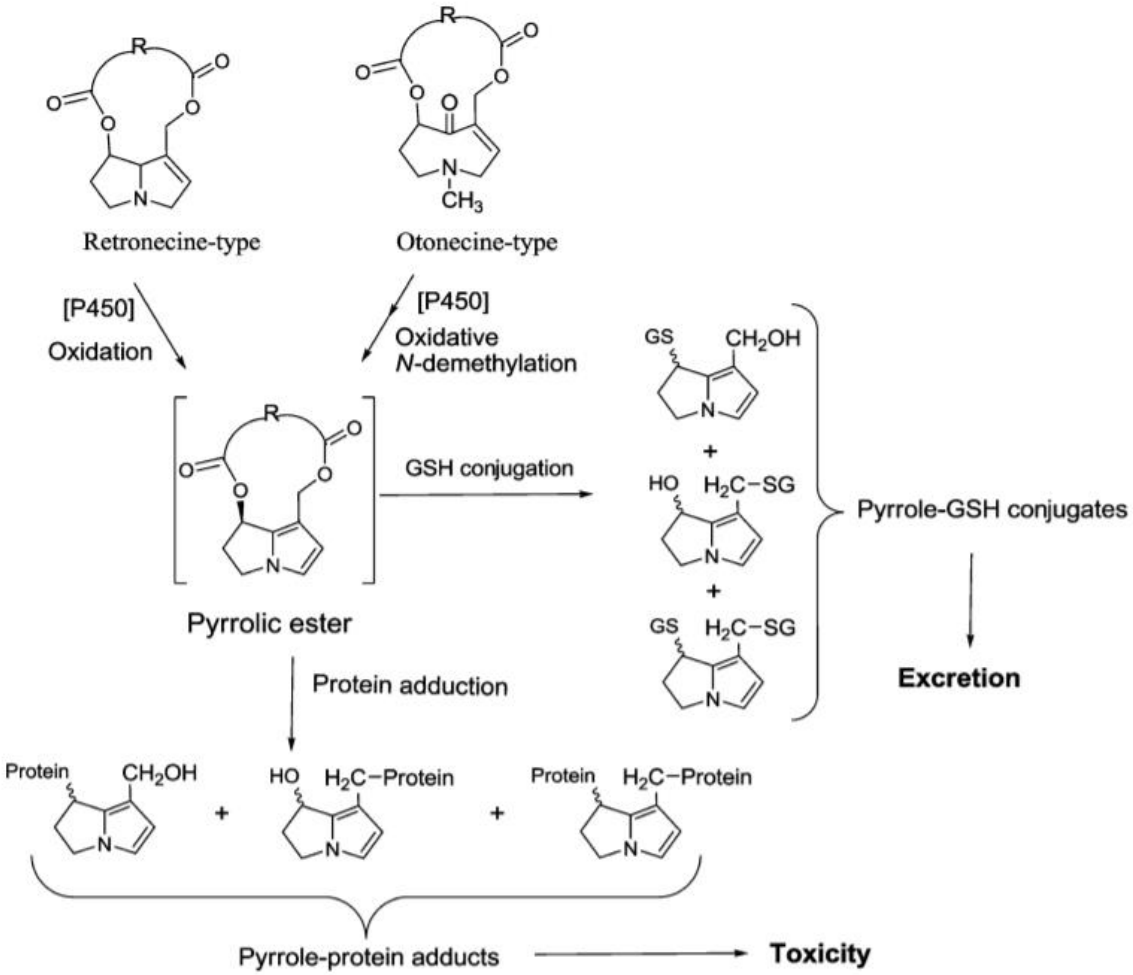


Figure 2.4 Metabolism of pyrrolizidine alkaloids
Sources: Ruan et al. 2014b

Large Animal Studies

In ruminants, loline alkaloids appear to have a rapid uptake, metabolism, and excretion with no symptoms of toxicity (Gooneratne et al., 2012, Westendorf et al., 1993). Feeding sheep either 954 mg (EI945) or 2346 mg (EI2346) of NFL and NAL daily, results in over 90% of NFL and NAL unaccounted for prior to the abomasum, with an average 5% recovery suggesting some alkaloids are metabolized in the small intestine (Table 2.5). Interestingly, although not fed, loline base had the highest recovery rate in the abomasum followed by NAL, and NFL (Westendorf et al., 1993). Similarly, in cattle grazing endophyte infected tall fescue and sheep orally dosed with meadow fescue seeds showed conversions of loline metabolites to loline base (TePaske et al., 1993, Gooneratne et al., 2012). *In-vitro* experiments have revealed that NFL incubated in rumen fluid is metabolized and converted to loline base (Westendorf et al., 1993), while low concentrations of loline has been found

in the urine of cattle (TePaske et al., 1993), sheep (Westendorf et al., 1993, Gooneratne et al., 2012), and horses (Takeda et al., 1991). In a cow exhibiting fescue toxicosis, total loline concentration of 83 µg/mL was detected in urine. Predominant loline alkaloids found were loline base (70 µg/mL), NFL (8.6 µg/mL), followed by NAL (4.4 µg/mL). Similarly, Gooneratne et al. (2012), reported over half (52.9%) of the loline excreted in urine was loline base followed by NFL (20.5%) although NML had the greatest recovery in terms of percent intake (16.6 & 15.7%) (Table 2.5). Excretion of lolines in urine occurs rapidly, within 15 minutes of dosing sheep and had a peak at 2 hours with a minor peak occurring after 12 hours indicating a temporary sequestration and/or continued slow metabolism (Gooneratne et al., 2012). Excretion of lolines in sheep faeces have not been detected (Westendorf et al., 1993) or only in low amounts (3.8% & 0.5%, Table 2.5) which was attributed to an unknown interfering substance during analysis (Gooneratne et al., 2012). Differences of loline metabolites recovered in faeces could be the result of a more complete breakdown of metabolites not detected in analysis or a faster metabolism due to the adaptation of rumen microbe populations and/or activation of liver biotransformation enzymes when sheep are chronically exposed (Gooneratne et al., 2012). This would explain why no loline was detected in EI945, EI2346, and only low amounts in the chronic treatment outlined in Table 2.5 as sheep in these treatments were fed over a period of time. An alternative explanation could be the sequestration of loline found in the hair of horses grazing endophyte-infected grasses. Predominant loline metabolites found were NFL and NAL ranging from ND to 19.8 pg/mg and 41.3 pg/mg, respectively, in horsehair (Rudolf et al., 2019). Attempts to find loline alkaloids in a cow's blood plasma was unsuccessful (TePake et al., 1993), but has been found in the blood serum of horses. Rudolf et al. (2018) found an average of 12 ng/mL of NAL (range of 0.05 ng/mL to over 140 ng/mL) and 8.2 ng/mL of NFL (range of 0.11 ng/mL to 222 ng/mL) in horses grazing endophyte-infected grasses. Although detected in serum it is also thought that metabolites of pyrrolizidine alkaloids are bound to red blood cells by binding to thiol groups in hemoglobin (Seawright et al., 1991). This, however, would contrast with the observations of Ruan et al. (2014a), that unsaturated pyrrolizidine metabolites are easily excreted with no apparent need to

bind with protein adducts. This research was however not specifically conducted with lolines warranting a need to further understanding of loline metabolism.

Table 2.5 Recovery of loline fed to sheep

		EI945*	EI2346*	Acute**	Chronic**
Diet (mg/sheep)					
	NFL	11,056	28,240	1,040	7,056
	NAL	4,064	9,296	402	2,548
	NANL			196	1,235
	NML			32	216
	Total	**15,120**	**37,536**	**1,670**	**11,047**
		% recovery from intake			
Abomasum					
	NFL	3.6	3.2		
	NAL	10.4	7.5		
	Total	**5.5**	**4.3**		
Urine					
	NFL			3.5	1.2
	NAL			2.1	0.9
	NANL			11.5	4.3
	NML			16.6	15.7
	Total			**6.1**	**3.9**
Faeces					
	NFL			2.3	0.2
	NAL			9.8	1.6
	Total	**0**	**0**	**3.8**	**0.5**

*Westendorf et al., 1993
**Gooneratne et al., 2012
N-formyl loline (NFL), N-acetyl loline (NAL), N-acetyl norloline (NANL), Loline base (Loline), N-methyl loline (NML)

Summary

Overall, endophytes provide many potential benefits in terms of health, growth, and development of plants. However, further research is needed to determine the effect of novel endophytes on grazing animals including a greater understanding of the metabolism. Particularly the fate of lolines during digestion and the likelihood of active forms reaching the intestine to have an effect on microflora, gastrointestinal parasites. Lolines have potential to provide many of the benefits of traditional alkaloids while being non-toxic to ruminants. However, symptomless consumption of these alkaloids during short-term investigations using ruminants may not reflect underlying concerns regarding metabolism and excretion of loline in end products which could have effects on animal performance, and gut health.

Chapter 3

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

This chapter was published in New Zealand Journal of Animal Science and Production volume 79 titled “Effect of in-vitro ruminal fermentation and HCL/pepsin digestion on loline” in 2019.

3.1 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 objective of this study was to use *in-vitro* methods to determine the fate of lolines during digestion.

3.2 Materials and methods

Meadow fescue seed (*Festuca pratensis*, 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 3.1).

Table 3.1 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)

3.2.1 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 in an anaerobic DAISY incubator containing four fermentation jars each containing 2000 mL fluid with the following buffers and rumen 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 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 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 (240°C). The VFA's

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 105°C held for 4 min and then increased by 15°C/min to 230°C, where it was held for 5 min. The flame ionization detector was maintained at 240°C.

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, H2 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).

3.2.2 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.

3.2.3 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 $0.05<P<0.10$.

3.3 Results

3.3.1 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 Figure 3.1. 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.

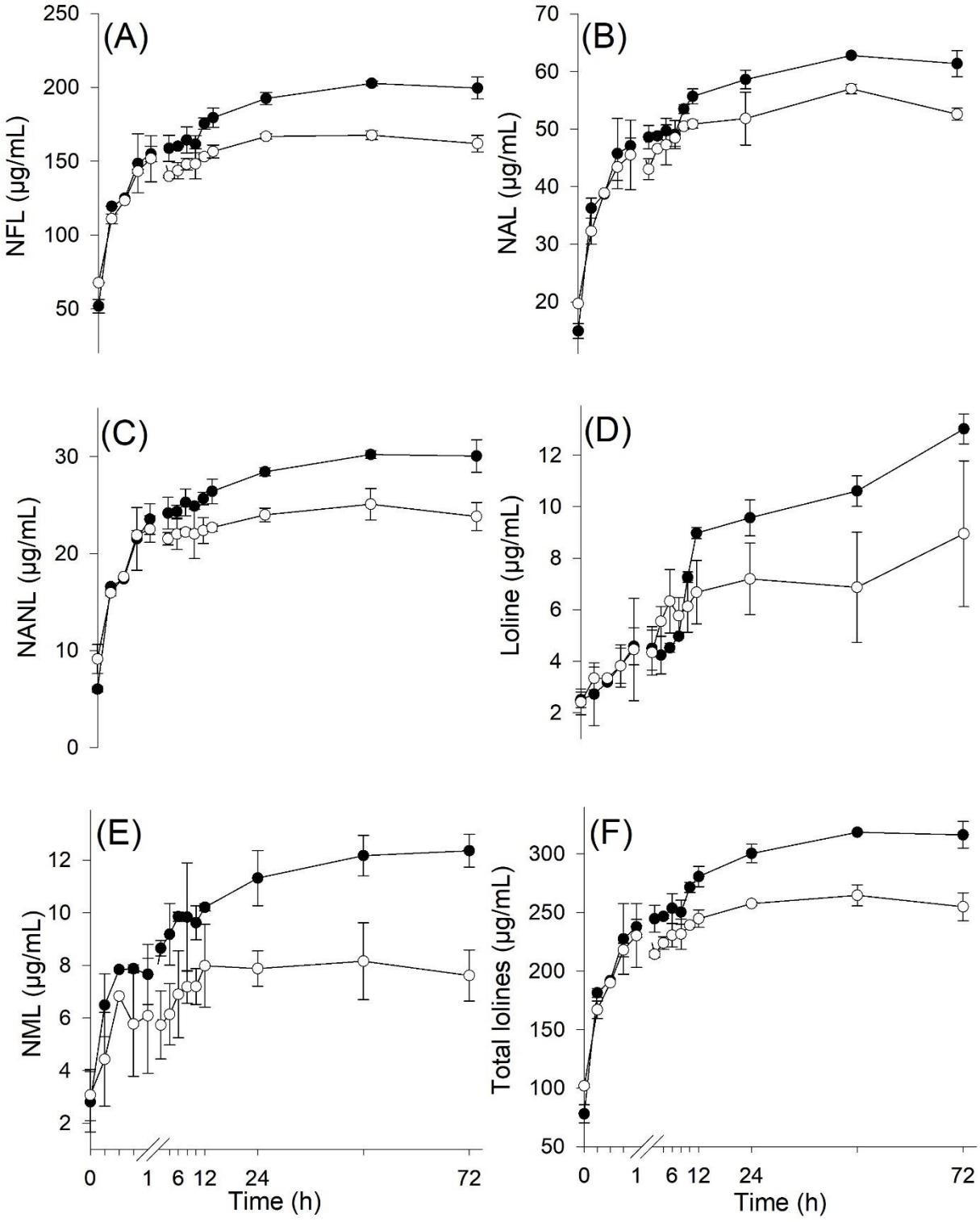


Figure 3.1 Degradation of A) N-formyl loline (NFL), B) N-acetyl loline (NAL), C) N-acetyl norloline (NANL), D) Loline base (Loline), E) N-methyl loline (NML), F) Total lolines incubated in sterile or viable rumen fluid. Treatments are meadow fescue seed with loline in sterile rumen fluid (LS); meadow fescue seed with loline in viable rumen fluid (LV).

●LS ○LV

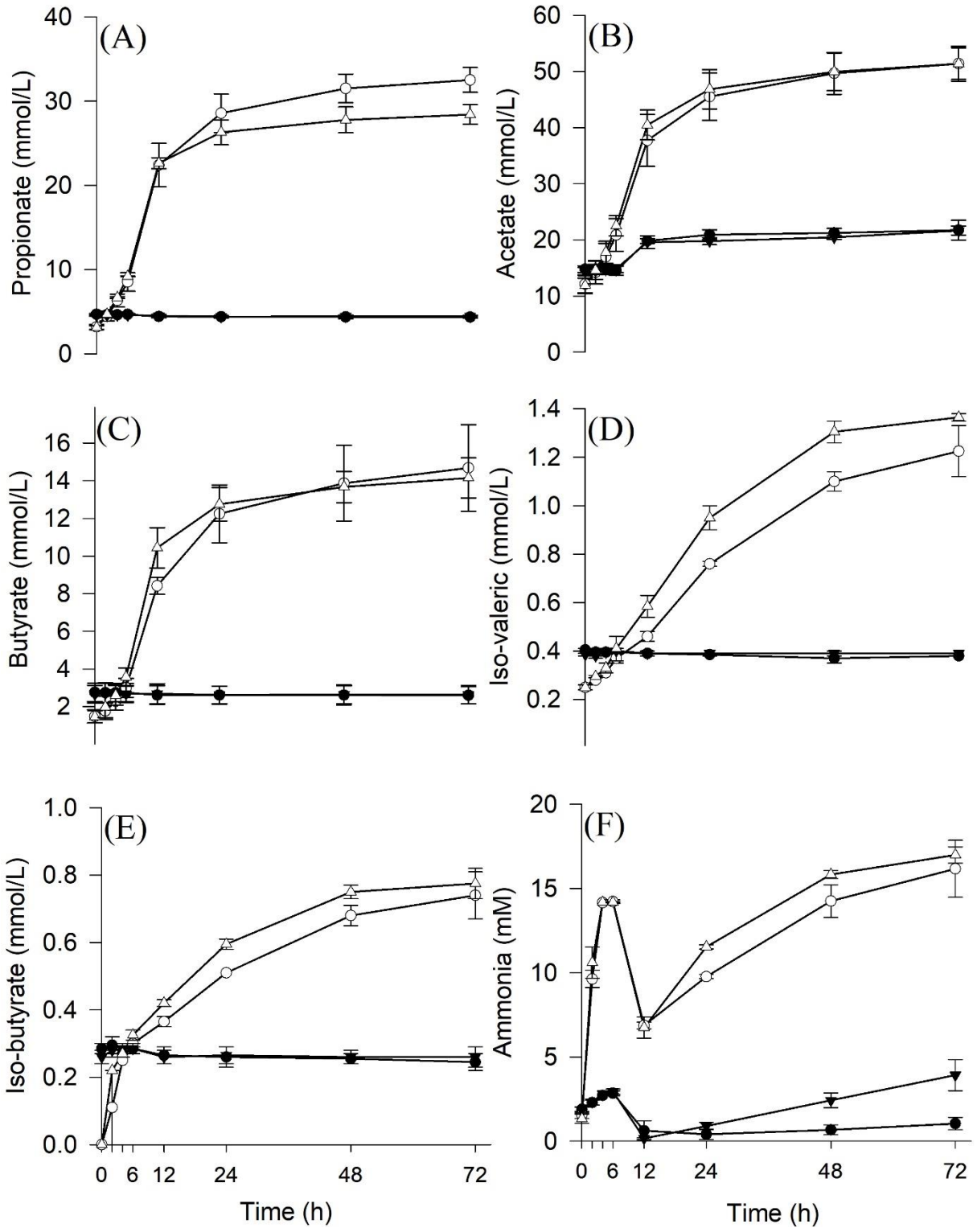


Figure 3.2 Volatile fatty acid, A) Propionate, B) Acetate, C) Butyrate, D) Iso-valeric, E) Iso-butyrate, and F) ammonia concentration in sterile or viable rumen fluid. Treatments are meadow fescue seed with loline in sterile rumen fluid (LS); meadow fescue see with loline in viable rumen fluid (LV); meadow fescue seed without loline in sterile rumen fluid (NS); meadow fescue see without loline in viable rumen fluid (NV).

●LS ○LV ▲NS ΔNV

Volatile fatty acid (propionate, acetate, butyrate, iso-valeric, iso-butyrate) concentrations during incubation are given in Figure 3.2. All VFA's 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 (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).

3.3.2 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 Figure 3.3. 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.

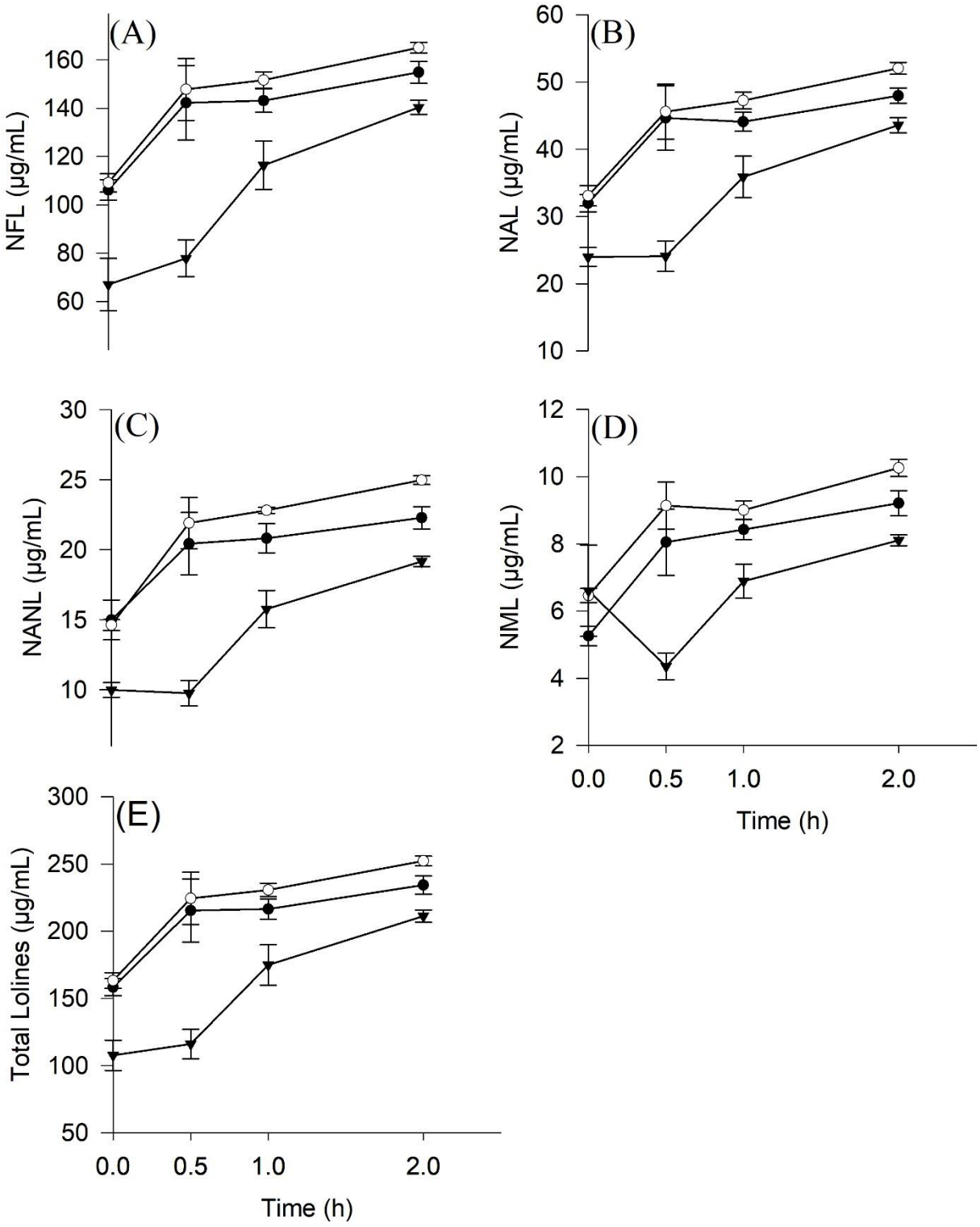


Figure 3.3 Degradation of A) N-formyl loline (NFL), B) N-acetyl loline (NAL), C) N-acetyl norloline (NANL), D) N-methyl loline (NML), E) total lolines incubated in HCL/pepsin at varying pHs or water. Treatments are meadow fescue seed with loline incubated in HCL and pepsin at pH 3 (HPH); meadow fescue seed with loline incubated in HCL and pepsin at pH 2 (HPL); meadow fescue seed with loline incubated in water (W).

●HPH ○HPL ▲W

3.4 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 exist. 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 does 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% recovery in HPL and HPH treatments respectively was found compared with water (63% recovery). Presumably reflecting pH dependent solubility of loline resulting in release differences but not forms. 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 affect, 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). In sheep, loline appears in the urine within 15 minutes of orally 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.

Chapter 4

Loline containing seeds or loline seed extract effects on *in-vitro* gas production and apparent dry matter digestibility

4.1 Introduction

Plants naturally can contain a wide array of secondary metabolites (Demain, 1999) which can cause a variety of detrimental effects in mammals (Bush et al., 1997, Cheeke, 1988). However, some of these secondary plant metabolites can also contain pharmaceutical activities that could benefit animals long-term even if it has short-term consequences (Athanasiadou and Kyriazakis, 2004). One secondary metabolite of interest is lolines. Lolines are an alkaloid produced by *Epichloë* fungi in grass species and has several naturally occurring derivatives: N-formyl loline (NFL), N-acetyl loline (NAL), N-acetyl norloline (NANL), and N-methyl loline (NML). Although loline is considered non-toxic to mammals (Bush et al. 1993; Gooneratne et al. 2012), alkaloids have been suggested to be toxic to microbial communities (Lemons et al., 2005, Cripps & Edwards, 2013), and limited research has examined lolines influence on rumen microbes. It has been suggested that loline is ruminally degraded and could influence rumen metabolism (Westendorf et al., 1993, Gooneratne et al., 2012). The results of Chapter 3 showed not only did loline survive but also did so without altering apparent feed digestibility (chapter 3, Froehlich et al., 2019). Although, there was a slight tendency for loline to produce less ammonia and propionate than the control, long term this would not have a detrimental effect to the rumen fermentation pattern (chapter 3, Froehlich et al., 2019). However, further research could tease out any negative effects on rumen fermentation by observing gas production. Loline has suggested antimicrobial, insecticidal, and anthelmintic properties (Schardl et al. 2007; Bacetty et al. 2009; Muponda 2014) and interest in its anthelmintic properties to gastrointestinal nematodes warrants an understanding of any potential detrimental effects. Any negative ruminal affect could outweigh any pharmacological effect on gut-dwelling organisms. Currently, limited information exists on the effect of loline on fermentation products but no information exists on lolines potential effects on *in-vitro* gas production. Rumen fermentation of truly degraded substances

yields three products 1) short chained fatty acids, 2) fermentative gases, and 3) microbial mass production (Blümmel et al., 1997), all three should be considered to determine lolines effects on ruminal microbes and therefore potential long-term effects. Therefore, the objectives of this study were to use an *in-vitro* method to determine loline effects on rumen fermentation by measuring subsequent gas production and was hypothesized addition of loline would have no negative impact on ruminal fermentation.

4.2 Materials and Methods

Meadow fescue seed (Barrier U2, Cropmark Seeds Ltd, Rolleston, NZ) with or without loline alkaloids were ground through a 1-mm sieve (ZM200, Retsch) at 18,000 RPM. Loline-containing seed contained 16.7 mg/g DM of total loline and the NIL seed contained 0 mg/g DM of loline, with similar nutrient composition (Table 4.1).

4.2.1 Experiment 1: Gas produced in ground seeds with or without loline

Loline (NFL, NAL, NANL, and NML) effects on rumen fermentation was measured as gas produced with two treatments of either: seeds without loline (NS) or seeds with loline (LS) as a substrate. The experiment was conducted over two runs on separate days. Within each replicate, samples were measured in triplicate (three fermentation jars per run) and fermentation jar was considered the replicate.

Incubations were conducted using an anaerobic gas module (Gas Production System, ANKOM Technology Co. Ltd., NY, USA). Prior to the start of the experiment approximately 1000 mL of 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 #2018-24), and filtered through cheesecloth into a warmed (39°C) flask. Buffer solution was also prepared according to the operating instructions of ANKOM (Marten & Barnes 1980) and consisted of two 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 fermentation jar contained either 2 grams of ground NS or LS, 80 mL of buffer solution, 20 mL rumen fluid, and were purged with CO_2. The 2 grams of seed was calculated to provide approximately 330 µg/mL of loline in LS treatment to maintain similar concentrations measured in a previous experiment examining degradation of loline in rumen fluid (Froehlich et al., 2019, chapter 3). Fermentation jars were maintained at 39˚C and were continuously shaken at 60-RPM in an incubator (Minitron, INFORS HT, Switzerland). Gas pressure in psi and temperature were

automatically collected every 2 minutes and relayed to a computer throughout the 24-hour fermentation period.

At the termination of the experiment, bottles were opened and pH was measured using a pH probe (Thermo Scientific Orion 2-Star, USA), and non-degraded substrate were filtered through Dacron bags, rinsed with cold water and dried in a 60˚C for 48 hours to enable calculation of apparent dry matter digestibility (DMD).

4.2.2 Experiment 2: Gas production produced from ryegrass and seed extract with or without loline

Loline extract effects on rumen fermentation was measured as gas produced with three treatments of either: ryegrass as control (RG), ryegrass with non-loline containing seed extract (RGN), or ryegrass with loline containing seed extract (RGL). The experiment was conducted over two runs on separate days for 24 hours. Within each replicate, samples were measured in triplicate (three fermentation jars per run) and fermentation jar was considered the replicate.

Ground loline containing seed was prepared as described earlier. Loline seed extract was made by soaking ground seed in deionized water (DI, 10 mL water to 1 g seed) and shaken in an incubator (Minitron, INFORS HT, Switzerland) at room temperature for one hour. After an hour soak the seed slurry was centrifuged at 48,400 x g, the liquid decanted, and resultant liquid passed through a 0.45µm cellulose nitrate filter. Resultant liquid was lyophilized, stored frozen (-20˚C), and analysed for loline as described below (Table 4.1).

Experiment 2 was conducted as described in experiment 1 except instead of seeds, 1 gram of ryegrass was used as a substrate with either without or with loline seed extract added. Extract amount was calculated to provide approximately 330 µg/mL of loline in the loline treatment and an equivalent amount of extract was used for the non-loline containing seed. These concentrations were used to maintain consistency between experiments.

4.3 Data Processing and Statistical Analysis

All data were compared and analysed in R (Version 3.6.0). Gas production data was first converted from psi to mL gas produced using the ideal gas law and Avogadro's number. Apparent DMD and pH were compared using each experiments respective treatments as factors and differences were separated using least significant difference test. In experiment 1, gas produced was analysed by a linear mixed effect regression to compare NS and LS treatment. With an interaction, term specified for the coefficient of fermentation time and with fermentation jar nested in treatment run as a random effect. Significant differences of the linear coefficient by treatments were explored and tested using the 'emtrends' function of the 'emmeans' package (Lenth, 2018). In experiment 2, gas production data was fit to the nonlinear equation $P = b(1 - e^{-ct})$ (Ørskov and McDonald, 1979) to determine gas rate and extent. Where P is the gas production at time t, b is the theoretical asymptote of the curve (potential gas production, mL per g DM), and c is the fractional rate of gas production (%/hour). Treatment interactions were specified for the b and c parameters and were considered to be the fixed effects, and fermentation jar nested in treatment run as a random effect. Tukey's honestly significant difference test was used to separate least square treatment means for the b and c model parameters using the 'emmeans' package (Lenth, 2018). Statistical difference was set at $P \leq 0.05$.

4.4 Results

Chemical composition of meadow fescue seeds used in experiment 1 and seed extracts for experiment 2 are reported in Table 4.1. Nutritive composition of meadow fescue seeds were similar between LS and NS.

Table 4.1. Nutrient analysis of meadow fescue seed and loline compounds with and without loline

	Seed type[1]	
	Loline containing seed	Non loline containing seed
Seed analysis (g/kg DM)		
Acid Detergent Fibre	155	160
Neutral Detergent Fibre	482	459
Dry matter	924	907
Organic matter	968	952
Nitrogen	28	29
Fat	12.4	11.1
Loline[1] (mg/g)		
NFL	11.7	0
NAL	2.8	0
NANL	1.6	0
NML	0.5	0
Total loline	16.7	0
Loline extract[2] (µg/mL)		
NFL	984	0
NAL	320	0
NANL	144	0
NML	53	0
L	16	0
Total loline	1518	0

[1] N-formyl loline (NFL), N-acetyl loline (NAL), N-acetyl norloline (NANL), Loline base (L), N-methyl loline (NML) in meadow fescue seed

[2] N-formyl loline (NFL), N-acetyl loline (NAL), N-acetyl norloline (NANL), Loline base (L), N-methyl loline (NML) extract made from soaking meadow fescue seeds in water and freeze-drying liquid

4.4.1 Experiment 1: Gas produced in ground seeds with or without loline

Gas produced for LS and NS incubated in rumen fluid are presented in Figure 4.1. A treatment x time interaction occurred (P=0.0001) with LS producing less gas over time compared with NS. The NS treatment produced 3.3 ± 0.007 mL gas per hour and tended to be greater than LS treatment of 3.0 ± 0.007 mL gas per hour (P=0.063). At the end of the fermentation (24 hour) NS had a 9% greater gas yield than LS.

At the end of the fermentation, neither pH (P=0.2) or apparent DMD (P=0.11, Table 4.2) were different. pH was 5.41 ± 0.05, and 5.5 ± 0.05 for LS and NS respectively, and the apparent DMD values being 46.2 ± 1.5% and 50 ± 1.5%, for LS, and NS, respectively.

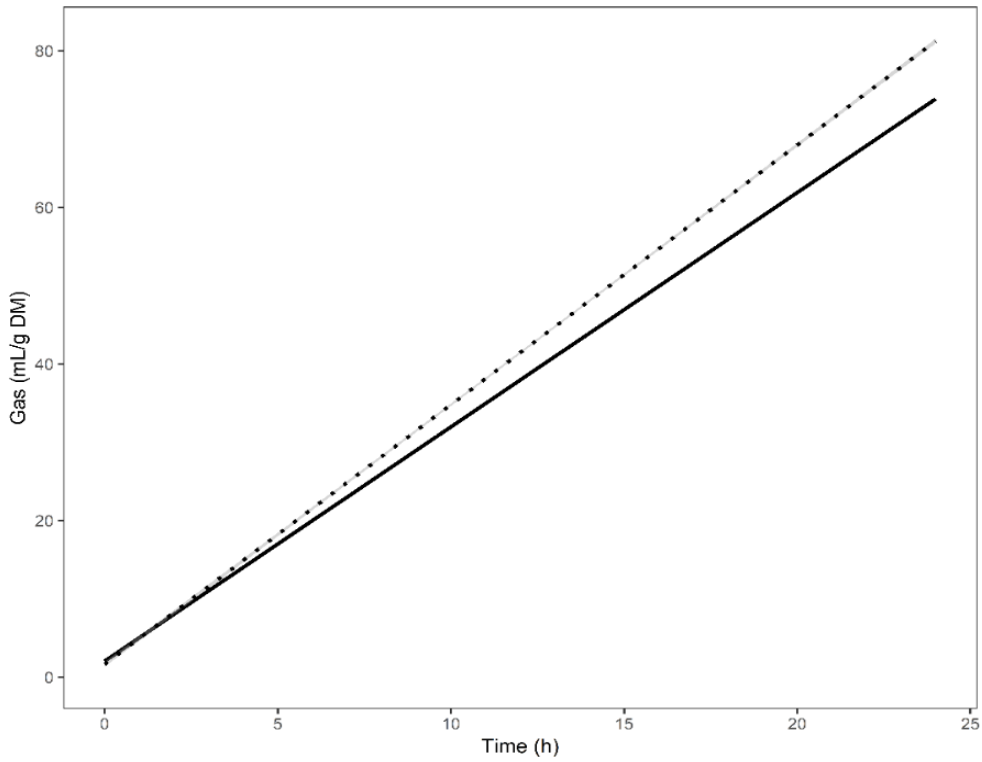


Figure 4.1 Gas production of meadow fescue seed either with (LS) or without loline (NS). Non loline containing seed extract ....., or Loline containing seed extract ____

Table 4.2 Gas production constants, pH and apparent DMD of ryegrass and meadow fescue seed with and without loline compounds

	Experiment 1[1]		Experiment 1[2]		
	LS	NS	RGS	RGL	RGN
Apparent DMD[3]	46.2 ± 1.5%	50 ± 1.5%	73.2 ± 6.5%[a]	63.3 ± 6.1%[b]	60.7 ± 6.1%[b]
pH	5.41 ± 0.05	5.5 ± 0.05	6.15 ± 0.03	6.17 ± 0.03	6.17 ± 0.03
Gas production constants[4]					
b	--	--	106.0 ± 3.5	102.2 ± 2.8	101.8 ± 2.8
c	--	--	0.093 ± 0.0002[a]	0.109 ± 0.0002[b]	0.112 ± 0.0002[c]

[1]Meadow fescue seed with loline (LS) or without loline (NS)
[2]Ryegrass substrate as control (RGS) or added loline (RGL) or without loline seed extract (RGN)
[3]Apparent dry matter digestibility (DMD)
[4]Gas production constants from the nonlinear equation P = b(1 - e –ct), b is the asymptote of the curve (potential gas production), and c is the fractional rate of gas production (b), the amount of insoluble material degraded per unit of time
a,b,c Means within rows with unlike superscripts differ, P < 0.05.

4.4.2 Experiment 2: Gas production produced from ryegrass and seed extract with or without loline

Gas produced for RGS, RGL, and RGN incubated in ruminal fluid are presented in Figure 4.2. The asymptote of the curve or potential gas production reached if time was infinite (parameter b, mL g-1 DM, Table 4.2) was 106.0 ± 3.5 for RGS and was not different from RGL, viz 102.2 ± 2.8 (P=0.62), or RGN viz 101.8 ± 2.8 (P=0.67). Likewise, RGL and RGN were not different (P=0.99). The fractional rate of gas production or the amount (%) of insoluble material degraded per unit of time (parameter c, %/h-1) was however different between treatments (P=0.0001) with, RGN having the greatest fractional rate of 0.112 ± 0.0002, followed by RGL at 0.109 ± 0.0002, and 0.093 ± 0.0002 for RGS.

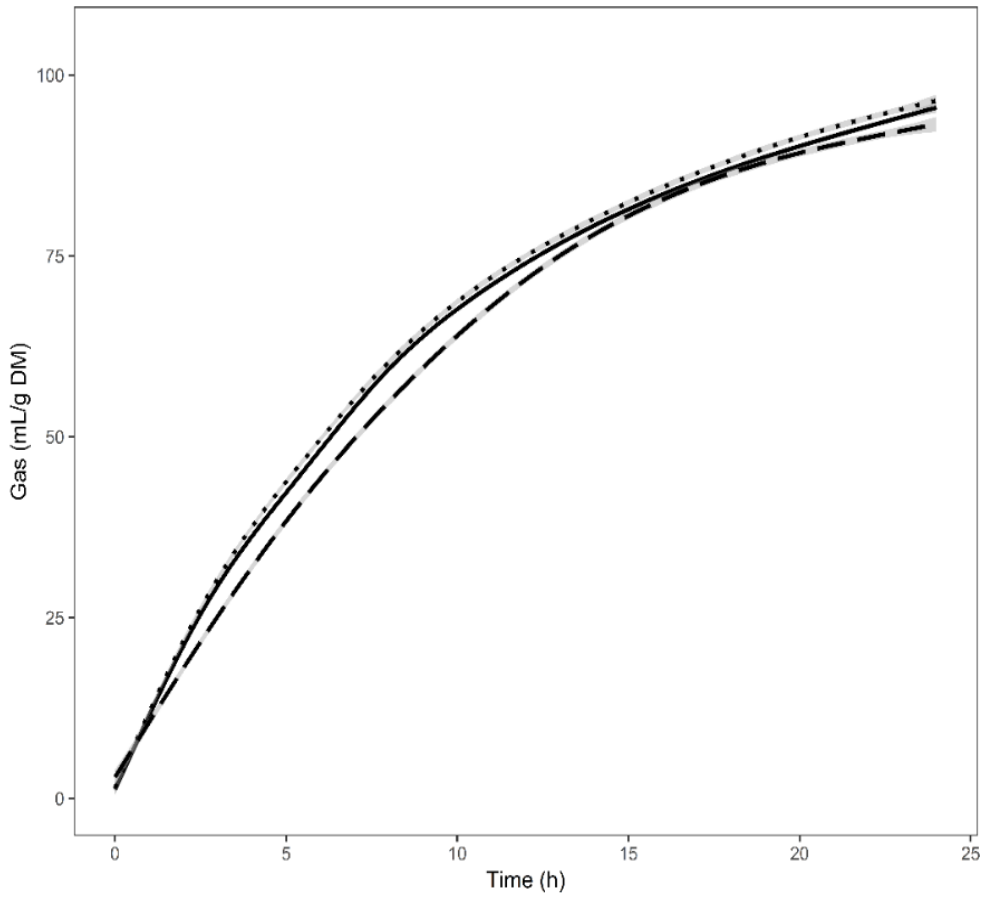


Figure 4.2 Gas production from ryegrass, or ryegrass with added meadow fescue seed extract either with or without loline. Ryegrass _ _ _, ryegrass with Loline containing seed extract ___, ryegrass without loline containing seed extract ....

At the end of the fermentation, pH was 6.15 ± 0.03 for RGS and was not different from RGL pH of 6.17 ± 0.03 (P=0.21), or RGN pH of 6.17 ± 0.03 (P=0.36), Likewise, RGL and RGN were not different (P=0.68).

Apparent DMD was 73.2 ± 6.5%, 63.3 ± 6.1%, and 60.7 ± 6.1% for RGS, RGL, and RGN respectively. Overall, apparent DMD was greater in RGS than RGL (P=0.05) and RGN (P=0.02), while RGL and RGN were not different (P=0.51).

4.5 Discussion

Evidence of an antimicrobial effect was limited; however, there was some indication the presence and degradation of loline could change the microbial ecology of the rumen. More gas was produced in NS compared with LS however, meadow fescue non-loline containing or loline containing seed extracts added to a ryegrass substrate had no difference in potential gas production. Furthermore, apparent DMD was not different between LS and NS nor between ryegrass with added non-loline containing or loline containing seed extracts. Given calculated loline concentrations between seeds and extracts were similar and apparent DMD was not affected and only slight difference in gas production between NS and LS, but not extracts, suggest little antimicrobial effect due to loline. Moreover, in a previous study LS and NS incubated at similar concentrations to those used here yielded similar VFA profiles (Froehlich et al., 2019, chapter 3). Volatile fatty acid production and gas production is highly correlated (Getachew et al., 2002, Blümmel & Orskov, 1993) therefore, it may be expected that differences would occur if there was an antimicrobial effect.

Slight differences in LS and NS gas production, and decreased fractional rate for loline containing extract added to a ryegrass substrate perhaps suggests the presence of loline, and degradation of loline could have changed the microbial ecology of the flasks. Loline catabolizing microbes exist (Roberts and Lindow, 2014) and slight ruminal microbial degradation of loline has been suggested (Froehlich et al., 2019, chapter 3). Truly degraded substances yield three products 1) short chained fatty acids, 2) fermentative gases, and 3) microbial mass production (Blümmel et al., 1997). A limitation of *in-vitro* gas systems is that it cannot measure microbial mass production directly, which when measured could indicate a change in the microbial ecology. Such changes have been observed when lupanine and sparteine alkaloids were added to a hay substrate at 5-mM, whereby microbial protein synthesis efficiency increased by suppressing the growth of microbes with a high maintenance energy requirement while decreasing both gas production and true digestibility (Makkar et al., 2005). An earlier *in-vitro* experiment observed a tendency for increased ammonia in NS than LS incubated in the same type/concentration of medium buffer and rumen fluid (Froehlich et al., 2019, chapter 3). If loline had increased microbial protein synthesis efficiency a decrease in

ammonia production could be a result of a higher incorporation of ammonia to microbial protein and subsequent decrease in gas production as observed in LS compared with NS although the changes are relatively small with only a 9% difference.

Addition of an extract changes the kinetics of gas production demonstrated by the fractional rate of gas produced. Potential gas production was not different; however, the rate or amount of insoluble material degraded per unit of time was different with RGN having the greatest rate followed by RGL, and RGS. This presumably reflects the crude extract of RGL and RGN would consist of a variety of easily fermentable carbohydrates. Water extracts of forages release many different materials such as minerals, proteins, organic acids and various simple sugars that are structurally simple and thus more easily fermentable by rumen microbes (Stefanon et al., 1996, Doane et al., 1997). Every feedstuff has a 'soluble pool' of readily available nutrients and addition of meadow fescue seed extracts would add to this pool changing the fermentation kinetics. Addition of extract to ryegrass substrate affected apparent DMD, and could be explained by the soluble pool of the extract. It is known that adding large amounts of starch or sugars decreases fibre digestion (Merten and Loften, 1980), with microbes preferentially selecting the easily fermentable substrates (Aguiar and Wink, 2005). Furthermore, the same ryegrass substrate was used for all treatments and the fermentation environment was similar (temperature, pH). The observation that pH remained above 6 and temperature around 37˚C reflects that microbial activity and therefore change in fermentation kinetics was unlikely to have been inhibited by pH or temperature (Stewart et al., 1977).

In summary, the water insoluble fraction of the seeds containing loline affected microbe's behaviour and thus gas production. However, adding seed extracts to a ryegrass substrate showed no difference in amount of gas produced but did change fermentation kinetics. Loline appears to have little effect on rumen microbes, but the addition of the water-soluble fraction of seeds increased the soluble pool of readily available substrates altering the fermentation kinetics. Therefore, loline can be used as a potential pharmaceutical on gut-dwelling organisms without negatively affecting rumen fermentation.

Chapter 5

Passive absorption across gastrointestinal tissues *in-vitro* and *in-vivo* distribution of loline in lambs

5.1 Introduction

Some secondary plant metabolites have pharmaceutical activities, which may benefit animals experiencing a pathogenic challenge. Lolines are an alkaloid produced by *Epichloë* 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 generally 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), N-methyl loline (NML), and loline base. A majority of lolines appear to survive ruminal and abomasal digestion *in-vitro* without altering digestive physiology (chapter 3, Froehlich et al. 2019). Meadow fescue seed extracts tended to produce less ammonia and propionate than control (chapter 3), and only slightly altered gas production and fermentation kinetics (chapter 4). This was not suggested to affect rumen fermentation long term, meaning loline could be fed to ruminants without negatively affecting their performance. Furthermore, loline appears to remain intact during digestion (chapter 3), therefore fed orally could have a pharmaceutical effect on gut-dwelling organisms. However, it is unknown where loline forms are absorbed from the gastrointestinal tract. Loline metabolism in ruminants appears to be rapid, and relies on hepatic metabolism prior to excretion (Ruan et al., 2014). Low amounts of loline (NFL, NAL, NANL, NML, and loline base) has been found in urine within 15 minutes of sheep orally dosed (Gooneratne et al., 2012), and NFL and NAL in blood serum (Rudolph et al., 2018), and hair (Rudolph et al., 2019) of horses grazing endophyte containing grasses. Current detection of loline in the blood of ruminants has been unsuccessful (TePake et al., 1993) and although can be expected considering its presence in urine. The rapid excretion of loline metabolites suggests potential rumen absorption and therefore it is unknown if orally consumed if any loline forms have the potential to reach the intestine to affect microflora and provide a pharmacological

benefit. The objective of this study was to use an *in-vitro* method to determine where and what loline forms might be passively absorbed in the digestive process coupled with observing where loline is distributed *in-vivo* to give an indication of its potential metabolism.

5.2 Materials and Methods

Meadow fescue seed (Barrier U2, Cropmark Seeds Ltd, Rolleston, NZ) with 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 (Table 5.1). A seed extract was prepared as described in chapter 4. Concentration of loline extract is displayed in Table 5.1.

Table 5.1. Meadow fescue seed analysis with and without loline

Item[1]	NFL	NAL	NANL	NML	Loline	CAF	Total
Seed extract, µg/g	984	320	144	53	16	---	1518
Solution, µg/ml	715	166.6	85.1	42.1	25.2	22.1	1034.1

[1]Analysis conducted by Cropmark Seeds
N-formyl loline (NFL), N-acetyl loline (NAL), N-acetyl norloline (NANL), Loline base (Loline), N-methyl loline (NML), Caffeine (CAF)

5.2.1 Experiment 1: Passive absorption of loline among varying tissues *in-vitro*

Apparent passive absorption of loline (NFL, NAL, NANL, and NML) across digestive tissues was measured *in-vitro* with Ussing chambers. Loline seed extract was placed on the mucosal side of tissues (rumen, abomasal, duodenal, ileum, large intestine, colon) also known as the donor side of the chambers and apparent passive absorption was measured on the serosal or receptor side. The experiment was replicated twice and repeated thrice per replication using tissue from three different sheep each time. The first replication consisted of three, 12 week old, suckling lambs fed ad libitum lucerne pellets and the second replication were 10 month old lambs grazing perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) pasture.

This experiment was carried out similar to Matthews and Webb (1995) looking at only passive absorption of loline. Tissues were freshly obtained from lambs harvested at Lincoln University. Tissues were rinsed and stored (up to 15 hours) in krebs ringer phosphate (KRP; NaCl, KCl, $CaCl_2$ adjusted to pH of 7.3-7.4, Umbreit et al., 1964) solution to transport to the lab. Musculature from the serosal sides of the tissues were removed and an approximate 2x2 cm tissue was mounted between two L-shape glass chambers of an ussing chamber (Figure 5.1). The chambers have an outside water

insulator that was circulated continuously at 37˚C keeping the chamber/tissues warm. Fifteen mL of KRP solution was added to both sides of the chamber. The KRP on the donor side contained an approximate 1034 µg/ml loline and 22 µg/mL of caffeine as an internal marker (Table 5.1). Caffeine, like loline, is an alkaloid of similar molecular weight and because of its hydrophilic nature is passively absorbed making it an ideal marker choice for this study (Nicolazzo et al., 2003). Air was pumped into the chamber serving as a gas lift constantly circulating solution on either side of the chamber.

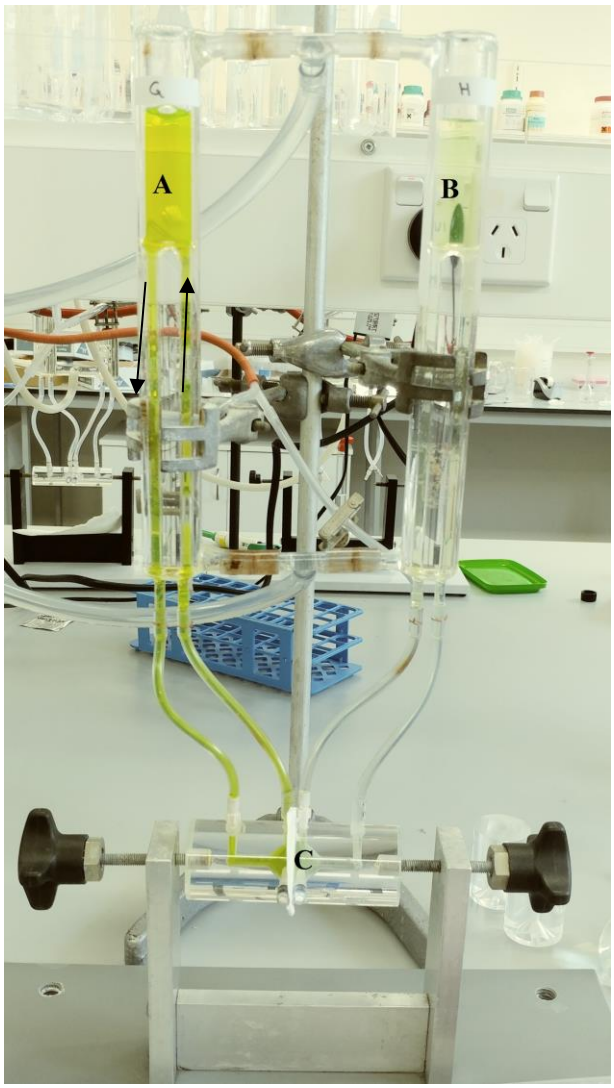


Figure 5.1. Ussing Chamber
Solution in donor side (A) has loline and caffeine, and flux across epithelia tissues is measured in receptor side (B). Tissues are mounted between the two chambers at the bottom (C) held together by the clamps. Chambers are kept warm by an outside water insulator that is circulated continuously with warm water hooked up to a water bath (not pictured). Air is bubbled into chambers to keep solution circulating (arrows).

Fluid in the chambers was sampled four times over a 2-hour period (0, 0.5, 1, 2 hours). A 200 µL sample was taken from both the donor and receptor side of the chamber and stored frozen (-20°C) for loline and caffeine analysis. 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 analysed within 24 hours. Samples were analysed 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, H2 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. Caffeine was analysed on GC using the loline procedure and concentrations were calculated using loline standards and therefore, can only be a qualification that it was detected.

5.2.2 Experiment 2: Loline distribution *in-vivo*

Distribution of loline was measured in gastrointestinal (small intestine, abomasum), organs (kidney, liver), and blood (plasma, red blood cells) of two, 12 week old, lambs. Loline seed extract was dosed twice 60 hours apart in 200 mL of milk at a rate of 52.5 mg/kg BW (Gooneratne et al., 2012). Lambs were trained to drink milk from a bottle with a teat to stimulate the oesophageal groove reflex delivering milk/loline directly to the abomasum. Approximately, 12 hours after the last loline dose lambs were slaughtered by stunning with a captive bolt followed by exsanguination (Lincoln University Animal Ethics Committee #2018-34) . Average lamb live weight pre-harvest was 22.75 ± 0.5 kg. Small intestine tissues were obtained 1 metre distal to pylorus, abomasum from proximal to omasum, liver from the left lateral section, and the kidney a cross section was taken. Samples were rinsed with phosphate-buffered saline (PBS) and frozen (-20°C) until freeze-drying.

Blood samples were separated into plasma and red blood cells by centrifugation at 1200 x g for 10 minutes to obtain plasma, and then washed with PBS three times to obtain red blood cells. Samples were pooled together and stored frozen (-20°C) until loline analysis, after which, samples were lyophilized and analysed using the procedure previously described in experiment 1.

5.2.3 Statistical Analysis

All data were analysed and compared using ANOVA in Genstat (18th edition). Treatment means were separated using Tukey's multiple comparison. Proportion of loline metabolites in the donor versus the receptor side of the chamber was calculated and proportion of each individual loline metabolite was compared between different tissue types at each time point (0.5, 1, 2 h). Tissue and time were considered fixed effects with loline within tissue as random effect. Any tissues with zero passive absorption were removed from analysis. Significance was declared at $P<0.05$ and trends at $0.05<P<0.10$.

5.3 Results

5.3.1 Experiment 1: Passive absorption of loline among varying tissues *in-vitro*

Proportion of loline forms (NFL, NAL, loline base, and total lolines) and caffeine as percent of donor are given in the following Figure 5.2 – 5.7. N-methyl loline was not included, as passive absorption was not detected in NML in any of the tissues at any of the time points.

N-formyl loline was absorbed across all tissues (rumen, abomasal, duodenal, ileum, large intestine, and colon). Absorption of NFL was similar at hour 0.5 (P=0.29) and 2 (P=0.11) among tissues with greater (P=0.05) 1 hour absorption in ileum tissues (3.5%) compared with abomasal tissues (0.24%).

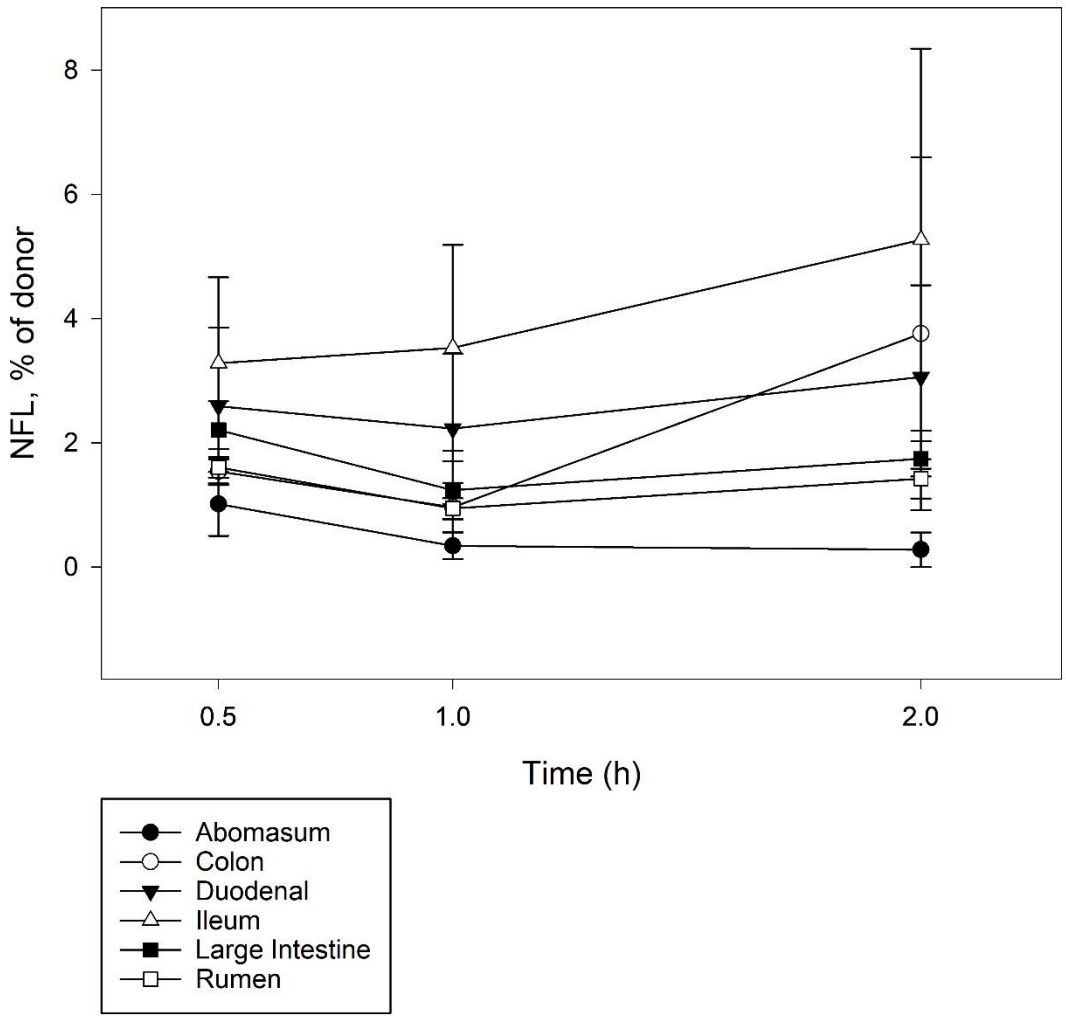


Figure 5.2. Passive absorption of N-formyl loline (NFL) in rumen, abomasal, duodenal, ileum, large intestine, or colon tissues.

No NAL crossed the abomasum, rumen or colon at 0.5, 1, or 2 hours. Passive absorption of NAL was similar at 1 (P=0.45), and 2 (P=0.36) hours and only occurred in ileum and duodenal tissues. At 0.5 hours NAL was measured in large intestine, duodenal, and ileum tissues and was similar (P=0.53).

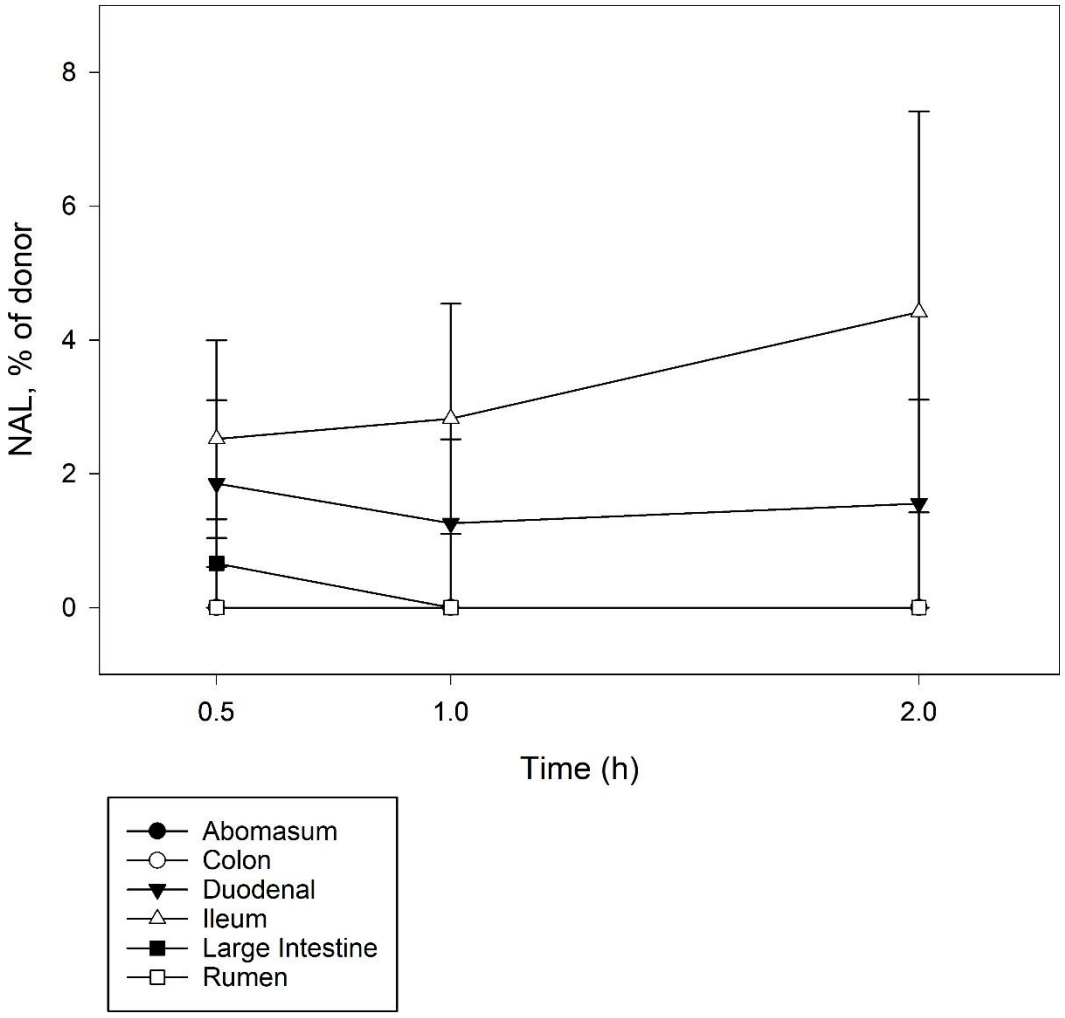


Figure 5.3. Passive absorption of N-acetyl loline (NAL) in rumen, abomasal, duodenal, ileum, large intestine, or colon tissues.

An average 1.64% of NANL was passively absorbed across duodenal tissue at 0.5 hours however; none was detected in other tissues at 0.5 hours therefore no pairwise comparisons could be made. No difference was observed at 1 (P=0.29) and 2 (P=0.97) hour between ileum and duodenal tissues in which NANL was measured.

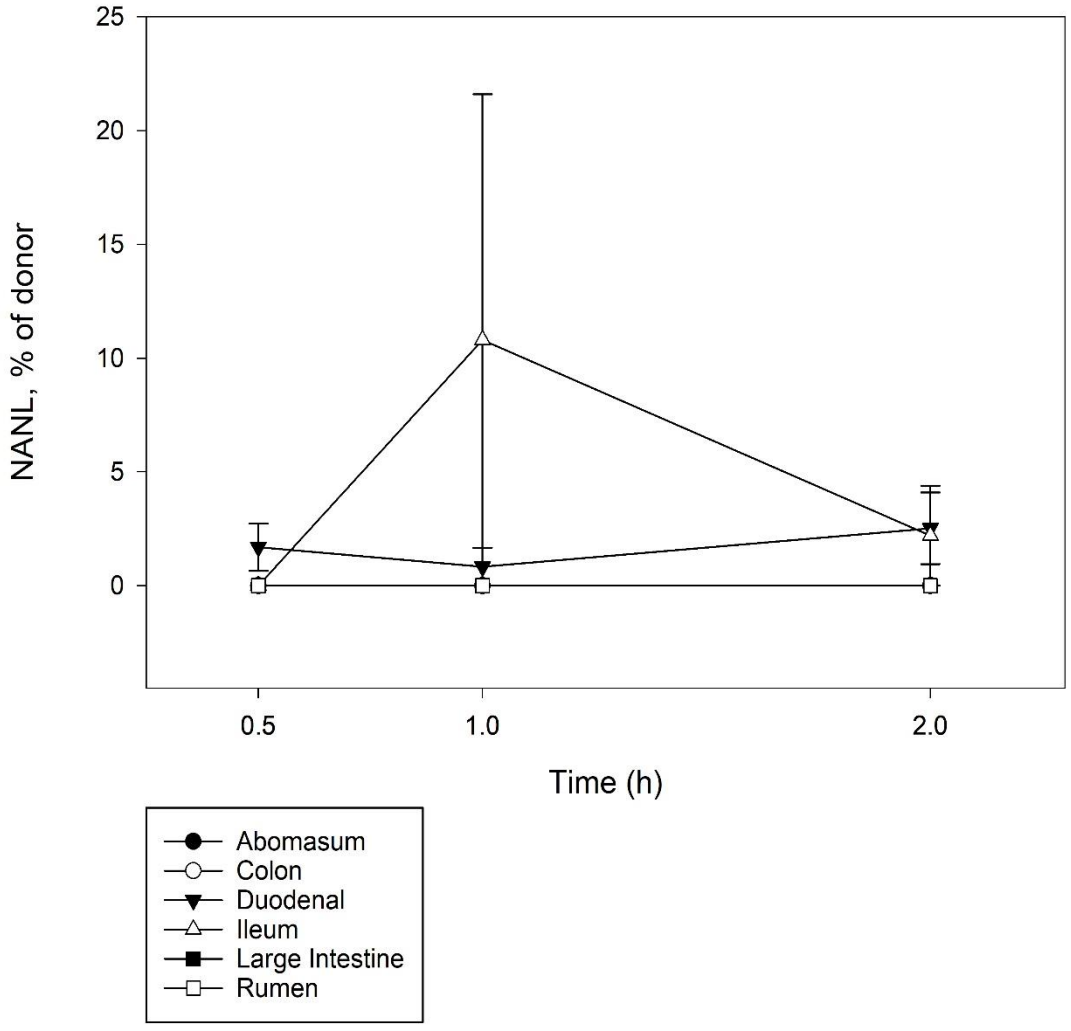


Figure 5.4. Passive absorption of N-acetyl norloline (NANL) in rumen, abomasal, duodenal, ileum, large intestine, or colon tissues.

Loline base was absorbed across all tissues (rumen, abomasal, duodenal, ileum, large intestine, and colon). No differences was observed in lolines base passive absorption at 0.5 (P=0.43), 1 (P=0.37), and 2 (P=0.35) hours.

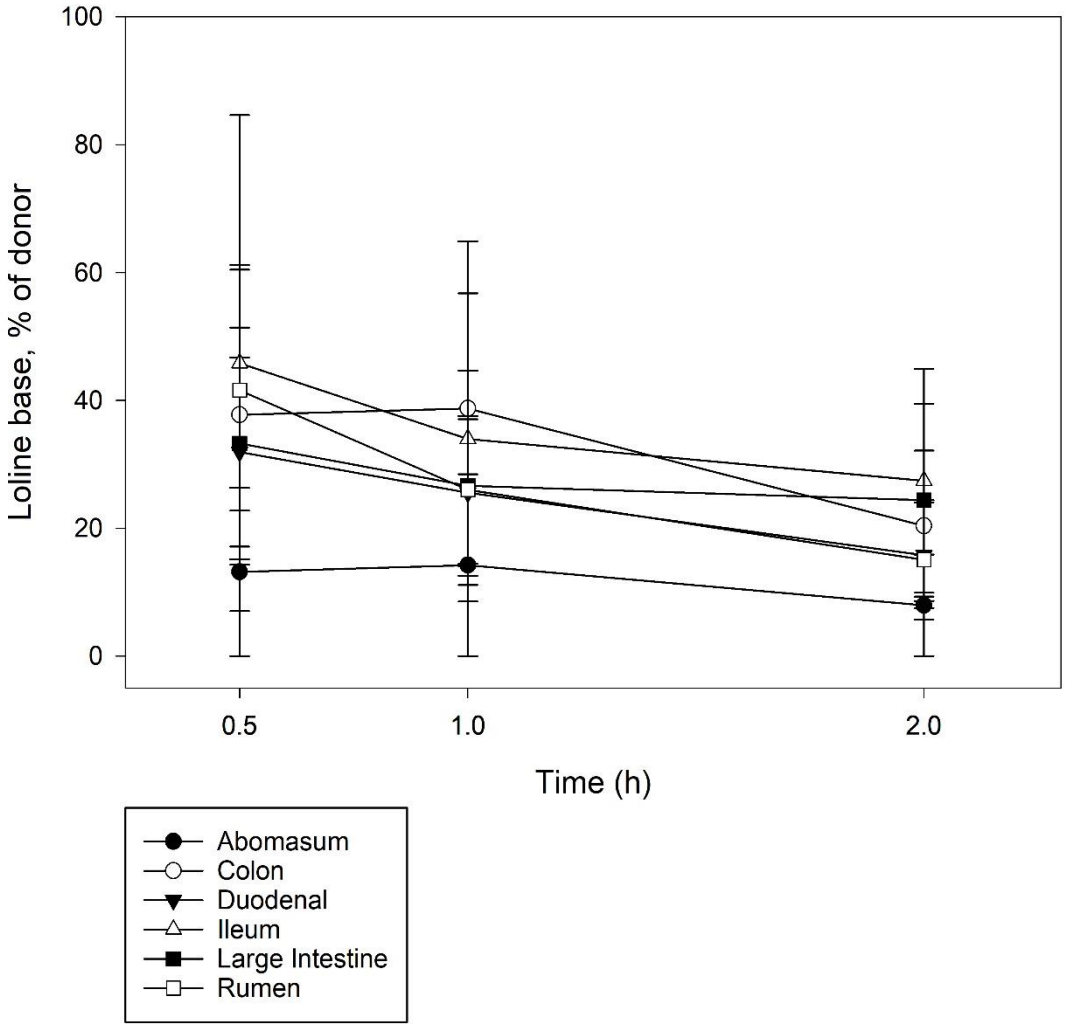


Figure 5.5. Passive absorption of Loline base (Loline) in rumen, abomasal, duodenal, ileum, large intestine, or colon tissues.

Total caffeine recovery on average was 207% greater in all chambers during the experiment than what was measured in the stock solution. No difference was observed at 0.5 (P=41), and 1 (P=0.14) hour. However, ileum was different from all tissues at 2 (P=0.02) hour.

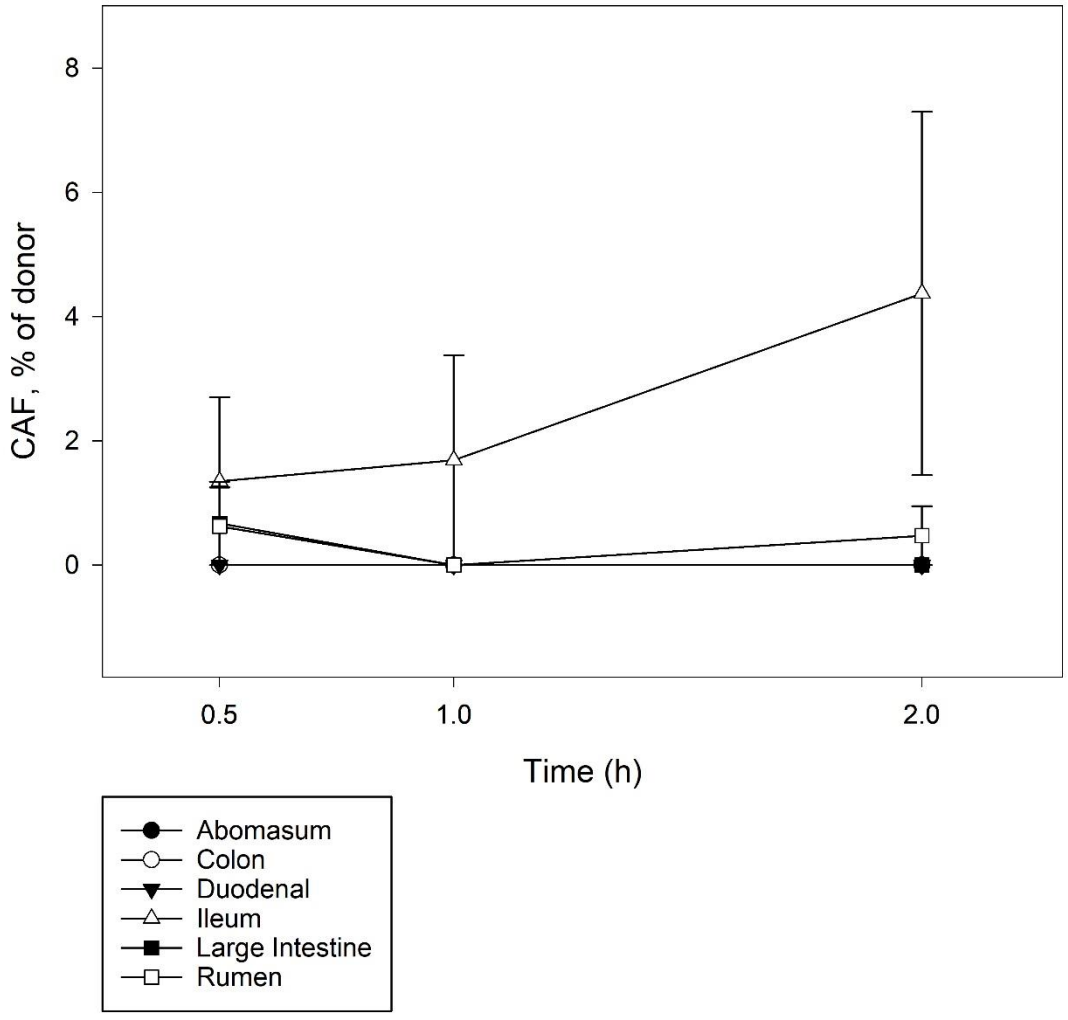


Figure 5.6. Passive absorption of Caffeine in rumen, abomasal, duodenal, ileum, large intestine, or colon tissues.

Overall, some loline was passively absorbed in all tissues (rumen, abomasal, duodenal, ileum, large intestine, and colon). Total loline absorption was similar in all tissues at 0.5 (P=0.15), and 1 (P=0.14) hour, with more (P=0.04) total lolines being absorbed in ileum tissues (5.1%) compared with abomasal tissues (0.11%) at 2 hours. Recovery of total loline (calculated as percent from stock solution) in receptor and donor sides of the chamber combined varied between tissues and was 94%, 85%, 96%, 87%, 96%, and 94% for abomasum, colon, duodenal, ileum, large intestine, and rumen, respectively.

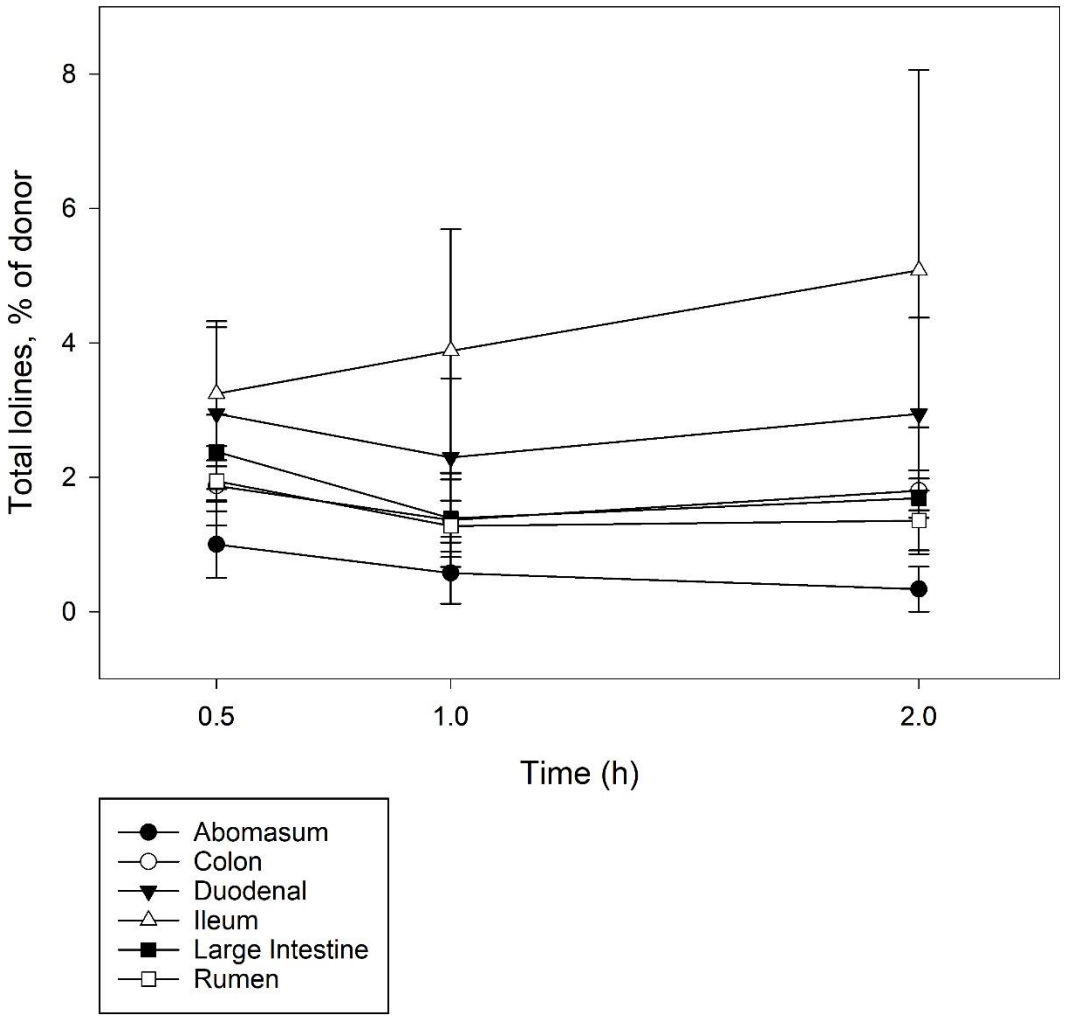


Figure 5.7. Passive absorption of Total lolines in rumen, abomasal, duodenal, ileum, large intestine, or colon tissues.

5.3.2 Experiment 2: Loline distribution *in-vivo*

Lambs were an average weight of 22.8 kg and dosed with 1,194.4 mg of loline to give the desired 52.5 mg/kg LW. No loline of any form was found in the abomasal or small intestine tissues (Table 5.2). Recovery of loline *in-vivo* was low for NFL in blood plasma (46 µg/g DM), and greatest for loline base (296 µg/g DM), and loline base was the only metabolite found in liver (126 µg/g DM) and kidney (112 µg/g DM) of lambs.

Table 5.2. Loline distribution in various gastrointestinal, organs, and blood samples

	Loline µg/g DM					
Sample	NFL	NAL	NANL	NML	Loline	Total Lolines
Gastrointestinal						
Abomasum	0	0	0	0	0	0
Small Intestine	0	0	0	0	0	0
Organs						
Liver	0	0	0	0	126	126
Kidney	0	0	0	0	112	112
Blood						
Plasma	46	0	0	0	296	342
Red blood cells	0	0	0	0	0	0

N-formyl loline (NFL), N-acetyl loline (NAL), N-acetyl norloline (NANL), Loline base (Loline), N-methyl loline (NML)

5.4 Discussion

Five percent of loline was passively absorbed in ileum tissues with surprisingly lower absorption occurring in the foregut. Limited metabolism of loline in rumen or HCL/pepsin fluid (Froehlich et al., 2019), its fast appearance (< 15 minutes) in sheep urine (Gooneratne et al., 2012), and low recovery (5%) in abomasal contents (Westendorf et al., 1993) suggests rapid absorption in foregut. However, this was not supported by these results with less than 2% of loline passively absorbed in ruminal or abomasal tissues. This either suggests the majority of loline is not passively absorbed or the foregut is only a minor absorption site.

It is plausible for a drug/nutrient to have more than one absorption pathway (Peck et al., 2014). Ruminal absorption of alkaloids, such as ergot (ergolines, and ergopeptines), are primarily an active process (Hill et al., 2001). Moreover, despite loline's fast appearance in sheep urine (Gooneratne et al., 2012) only 6% of an acute dose was recovered in 72-hours. Despite lolines quick appearance, such a small amount is indicative of little absorption in the foregut. For the ergot alkaloid metabolite ergovaline the small intestine plays an important role in absorption with the epithelial cells lining the intestine capable of transporting the alkaloid intact (Klotz & Nicol, 2016). This particular metabolite crosses the rumen epithelium at a slow rate, and less than 1% of the daily dose administered was able to cross in 3-hours (Foote et al., 2014). This contrasts Hill et al. (2004), who suggested the rumen to be a major absorption site for ergolines, and ergopeptines. Although it is worth noting that absorption appears to be structurally dependent, and depends on many factors including dissolution characteristics, digesta contents, molecular size and pH. Compounds in a similar chemical class does not guaranteed similar processes (Caspary et al., 1992, Peck et al., 2014). Hessel et al. (2014) demonstrated this using caco-2 cells representative of the human small intestine epithelium barrier. Senecionine and senkirkine alkaloids seem to use a passive transcellular process whereas heliotrine and echimidine are an active process even though they are all pyrrolizidine alkaloids their structural differences affects their bioavailability. Perhaps explaining some of the differences in loline forms between tissues. Loline base and NFL were passively absorbed in all tissues, and NAL in ileum and duodenal tissues in small amounts.

No NML and little NANL was absorbed across the various epithelial tissues. This is slightly surprising given all five of the major loline metabolites (NFL, NAL, NANL, NML, and loline base) are excreted in sheep urine within fifteen minutes (Gooneratne et al., 2012). However, for the majority of the metabolites less than 5% were passively absorbed across tissues meaning that some may be below detection levels. Loline base had the highest passive absorption rate ranging from 20-40%, following a similar trend found *in-vivo*. Despite not dosing detectable amounts of loline base, it is the greatest quantity found in the urine of sheep (Gooneratne et al., 2012), and was the only metabolite found in lambs kidney, and liver 15 hours after dosing. The absence of its presence in blood plasma indicates the tissues maybe a site of metabolism. A greater recovery of loline base perhaps reflects loline derivatives metabolizing to the simple loline base (Westendorf et al., 1993, TePaske & Powell, 1993, Gooneratne et al., 2012).

The positive control caffeine was limitedly detected on the receptor side of the Ussing chamber. Caffeine, like loline, is an alkaloid of similar molecular weight and because of its hydrophilic nature permeates transcellular by passive diffusion route (Nicolazzo et al., 2003, Smetanovà et al., 2009). It has a fast gastrointestinal absorption rate with serum and plasma caffeine concentrations reaching maximum levels at 0.5 hours of oral dosing, and a bioavailability of nearly 100% (Muraoka et al., 1998). Therefore, no detection in the receptor solution was unexpected. Potentially this indicates decreased membrane integrity affecting study validity, although this does not explore why some loline was passively absorbed.

Measuring flux of drugs and nutrients across a biological membrane is complicated and has its limitations. This study was only to qualify the potential of loline being passively absorbed and the site of absorption. Ussing chambers or parabiotic chambers are a useful technique to study absorption of various drugs, and nutrients across various tissues and have been used in numerous animal studies (Boudry, 2005, Clarke, 2009) including ergot alkaloid studies (Hill et al., 2001, Ayers et al., 2009). However, one major limitation is maintaining membrane integrity of tissues (Li, 2013), with tissue viability lasting up to 3 hours after slaughter (Clarke, 2009). Although, considered an important

limitation of active transport studies, loss of integrity has the potential to affect passive absorption studies as both paracellular and transcellular passive routes are dependent on membrane integrity (Li, 2013). Histology of pig gastrointestinal tissues two hours after slaughter showed desquamated cells on the luminal surfaces and edema however; tight junctions (paracellular route) were intact (Boudry, 2005). This may affect results by either increasing or potentially decreasing amounts that would occur naturally *in-vivo*. Given the harvest and storage of tissues in KRP solution for up to 15 hours this may have potentially limited passive absorption of caffeine and some loline. However, might have given some insight. Limited detection of caffeine suggests membrane integrity could have affected its transcellular passive routes. If that route is closed, and the tissues are not alive, therefore not active, potentially indicates loline is paracellular absorbed. The membrane was not 'leaky' as measurement of caffeine as well as all of the loline metabolite in the receptor side would have been detected, which was not the case.

In the organs (kidney, liver) and blood (plasma) of lambs 15 hours after dosing the main metabolites found were loline base and some NFL in blood. This correlates well with literature describing lolines excretion/metabolism patterns. Loline appearance in urine means it has a presence in blood. In horse serum an average concentration of 8.2 ng/mL and 12 ng/mL of NFL and NAL, respectively, has been identified (Rudolph et al., 2018) and to date attempts of quantifying loline in ruminant blood has been unsuccessful (TePake et al., 1993). The amounts of loline base and some NFL in blood plasma of lambs in this study compares well with the loline excretion pattern described by Gooneratne et al. (2012). All five loline metabolites were excreted but peaked at 2 hours post dosing, this was followed by a slower excretion of mainly loline base, and some NFL indicative of its slower hepatic metabolism (Gooneratne et al., 2012). It is known that saturated pyrrolizidines such as loline are metabolised in the liver by the oxidative hepatic cytochrome P450 enzymes to a water-soluble dehydropyrrolizidine carboxylic acid (Ruan et al., 2014). As expected, some loline was present in the kidney and liver, in 112 and 126 µg/g DM respectively. It could be suggested lolines first pass (metabolism) through the liver is high meaning loline bioavailability is low. This could be based on the low amounts of loline found in blood plasma and organs of lambs 15 hours after dosing, and low amounts in horse serum

(Rudolph et al., 2018) and recovered in urine and faeces of sheep (Gooneratne et al., 2012). To date loline metabolism in ruminants appears to be rapid, and readily excreted (Gooneratne et al., 2012, Ruan et al., 2014). However, both NFL and NAL have been found in horsehair of concentrations of up to 41.3 pg/mg (Rudolf et al., 2019). This perhaps suggests sequestration of loline *in-vivo* and warrants further investigation.

In conclusion, little passive absorption occurred in ruminal or abomasal tissues (<2%), with the ileum tissues appearing to have greatest absorption capacity (5%). Of the loline forms, loline base and NFL were passively absorbed across all gastrointestinal tissues with NAL and NANL only crossing small intestine tissues. Recovery of loline in tissues was low but in line with similar patterns to *in-vivo* studies characterizing excretion patterns of loline, with loline base being the main metabolite found in the liver, kidney, and blood plasma of lambs. The small loline amounts found in organs and blood are in line with its fast metabolism however, the low absorption rates through gastrointestinal tissues were unexpected. These results indicate either the majority of loline is not passively absorbed or membrane integrity was affected as suggested by lack of caffeine absorption.

Chapter 6

Loline effects on gastrointestinal nematodes

6.1 Introduction

Alleviation of the reliance on anthelmintic to control gastrointestinal parasites in livestock may be combated using the pharmaceutical activities of some plants (Githiori et al., 2006, Waller, 2006, Williams et al., 2014, Worku et al., 2017). Plants can contain a wide array of secondary metabolites arranged in a variety of chemical classes (Demain, 1999, Santos et al., 2019) that may provide an alternative, cost-effective treatment to gastrointestinal parasites limiting both drug use, and development of anthelmintic resistance (Githiori et al., 2006, Williams et al., 2015). Any plant part can contain secondary metabolites produced from naturally occurring symbiotic microorganisms (bacteria, fungi, actinomycetes, or virus) called endophytes (Compant et al., 2016). As a result, plants may be a novel source of bioactive compounds although research is needed to identify effective compounds to integrate into parasite control programs (Santos et al., 2019), without long-term detrimental effects on livestock health or wellbeing (Athanasiadou & Kyriazakis, 2004). One suggested bioactive compound is loline, an alkaloid produced by *Epichloë* fungi in fescue grass species and is 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). Chapters 3 and 4 showed loline to have a minimal effect on rumen microbes. Furthermore, loline appears to remain intact during digestion (chapter 3), and little loline is passively absorbed from the gastrointestinal tract of lambs (chapter 5). Therefore, loline has the availability to have a pharmaceutical effect on parasites in the gut without negatively affecting livestock performance. However, further research is needed to determine if loline has a biological effect against parasites. There are several naturally occurring loline derivatives: N-formyl loline (NFL), N-acetyl loline (NAL), N-acetyl norloline (NANL), and N-methyl loline (NML). Specifically NFL, the predominant alkaloid in loline containing plants (Yates et al., 1990), is a contact and ingested broad-spectrum insecticide (Dahlman et al., 1997). However, focused research has been on a mix of loline

derivatives. Larvae development of a variety of insects (Siegel et al., 1990, Patterson et al., 1991, Dougherty el al., 1998, Shiba & Sugawara 2008, Patchett et al., 2008a, Popay et al., 2009, Patchett et al., 2008b), as well as egg hatching and larval motility of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta nematodes* (Muponda, 2014) have been affected by mixed loline extracts. Specifically, a combined NAL & NFL is nematocidal to the plant parasitic nematode *Pratylenchus scribneri* at 100 and 250 µg/mL concentration (Bacetty et al., 2007). Therefore, the objective of this study was to investigate and validate using loline as a natural anthelmintic for the gastrointestinal nematodes in sheep of *Trichostrongylus colubriformis, Teladorsagia circumcincta,* and *Haemonchus contortus* through a series of *in-vitro* and *in-vivo* studies.

6.2 Materials and Methods

6.2.1 *In-vitro* experiment 1: Loline effects on larval migration

Antimicrobial effects of *T. colubriformis* L3 larvae migration was determined with eight treatments of either: NIL or LOL seed extract at serial dilutions.

T. colubriformis larvae were exsheathed using 160 µl of a hypochlorite solution in a 15 mL tube. Larvae were washed three times, twice with water, and one final washing with a 0.85% NaCl to remove excess hypochlorite. For each wash, tubes were filled to 10 mL and were centrifuged at 1300 x g and the supernatant decanted. Centrifugation occurred in short 10-15 second pulses to prevent larvae clumping. After the final NaCl washing, larvae were re-suspended using a 1 ml syringe by drawing and then forcefully expelling larvae back into the tube and adjusted so that 100 µl contained 200-250 larvae. A serial dilution of loline extract was made (16,000, 8,000, 4,000, 2,000 ppm) and larvae were incubated in each concentration at 37˚C for 2 hours. The same amount of NIL extract was used and diluted as the loline extract to serve as control. At the end of the two hour incubation 1 ml of the different loline/NIL extract concentrations were pipetted into a 24 test well plate in duplicate down an inner tube that contain a nylon mesh filter of 25 µm fitted into the plate wells. Larvae were again incubated at 37˚C for 2 hours after which filters were carefully removed from the plate wells. Remaining larvae inside of the nylon mesh filters were washed into a new well as the 'retained' larvae. Both retained and larvae that migrated through the mesh filter were stained with iodine and counted using an inverted microscope.

The experiment was replicated twice on separate days and each time samples were duplicated. Percent larvae migration was calculated by number of larvae migrated divided by the number of total larvae (migrated plus number larvae retained) x 100. All data were analysed and compared using ANOVA in Genstat (18th edition). Loline effects on larvae migration were compared between extract (LOL or NIL) and concentration (16,000, 8,000, 4,000, 2,000 ppm) were considered the factors.

6.2.2 *In-vitro* experiment 2: Establishment of *T. circumcincta* in abomasal tissues

Establishment of exsheathed *T. circumcincta* L3 larvae in abomasal tissues was determined using an *in-vitro* direct challenge method as described by Jackson et al. (2004). Experiment was replicated 3 times, and the treatments were either abomasal tissues of a lamb fed a loline lace milk (LOL) or loline naïve lamb tissue (CON) incubated with *T. circumcincta*.

Abomasal tissues were sourced from two, 12 week old suckling lambs. One lamb was milk fed loline at 52.5 mg/kg 14 hours prior, the other served as a loline naïve lamb. Abomasal tissues were removed immediately after slaughter, emptied of contents and washed with physiological saline solution removing the majority of digesta. Three, 2x2 cm sections of abomasal tissue from the fundic folds were removed from each sheep and placed into a well of a 6 well plate with the mucosal surface facing up. Warm Hank's medium was added to the wells surrounding but not submerging abomsal tissues. A barrel of a 10 ml syringe with needle end removed was placed into the centre of the tissue providing an isolation cylinder to contain the *T. circumcincta* larvae. The syringe barrel was held in place by the lid of the 6 well plate secured with rubber bands. Exsheathed *T. circumcincta* L3 larvae in 0.5 ml of saline was placed into the syringe barrel chamber onto the mucosal surface of the abomsal tissues. The larvae were exsheathed as described previously and adjusted so that a 1 ml contained 4,000 larvae. Once *T. circumcincta* larvae was placed onto the abomsal mucosal surface plates were transferred to a container with a lid which was gassed with pure oxygen for one minute. The container was sealed to maintain the high oxygen concentration and placed into a dark incubator maintained at 38˚C for 3 hours. From time of slaughter to placement in incubator was no longer than 20 minutes.

Following the 3 hour incubation, the syringe chamber and abomasal tissues were rinsed with physiological saline into 50 ml centrifuge tubes removing any larvae not associated with the abomsal tissue and were vigorously washed by immersing 30 times in 25 ml of saline. Tissues were placed in a separate 50 mL centrifuge tube and digested with 50 ml of 1% pepsin, 1% HCL solution at 38˚C for 12 hours. All samples were adjusted to 50 ml. A 4% sample was taken and counted under a microscope,

and estimated tissue associated populations were calculated by adding together worm counts and dividing digest counts by total multiplied by 100.

All data were analyzed in Genstat (18th edition) using a two sample t-test with tissues from LOL or CON fed lambs as factors and percentage establishment of larvae in abomasal tissue as the response variable.

6.2.3 *In-vitro* experiment 3: Establishment of *H. contortus* in abomasal tissues

Establishment of *H. contortus* in abomasal tissues was determined using the same method as described in *in-vitro* experiment 2 (Section 6.2.2). The treatments were either abomasal tissues of a lamb fed a loline lace milk or loline naïve lamb tissue incubated with *H. contortus*. Abomasal tissues were sourced from three, 14-15 week old suckling lambs. Two lambs were milk fed loline at 52.5 mg/kg 14 hours prior, the other served as a loline naïve lamb.

Data were analysed in Genstat (18th edition) using a two sample t-test with tissues from LOL or CON fed lambs as factors and percentage establishment of larvae in abomasal tissue as the response variable.

6.2.4 *In-vivo* study 1: Loline effects against *T. circumcincta* and *T. colubriformis*

Loline seed extract was used to examine antimicrobial effects of L4 and adult parasite stages of *T. circumcincta* and *T. colubriformis* in four sets of twin lambs (eight lambs total) that were approximately six weeks old (Lincoln University Animal Ethics Committee #2018-34). Lambs were still suckling, and housed in four individual pens for each set of twins with their dam. Ewes and lambs were fed *ad-libitum* lucerne pellets and had free access to water. Lambs were treated with an anthelmintic at 2 mL per 15-20 kg (Zolvix Plus, PGG Wrightson, Christchurch NZ) and 10 days post treatment all lambs were infected with 10,000 L3 infective *T. colubriformis* and concurrently four lambs chosen at random were also dosed with 5,000 *T. circumcincta* L3 infective. There were two treatments, loline treated lambs, and non-loline treated lambs.

The four loline treatment lambs were trained to drink milk from a bottle with a teat to stimulate the oesophageal groove reflex delivering milk directly to the abomasum and bypassing the rumen. Two of the bottle trained lambs were infected both *T. circumcincta* and *T. colubriformis* and two infected with only *T. circumcincta*. At 10 days post dosing, two lambs (only one was infected with *T. circumcincta*) were offered 200 ml milk twice, 60 hours apart containing loline in a seed extract delivering 52.5 mg/kg LW (Gooneratne et al., 2012). This was to determine the effect of lolines against L4 larvae. Loline dose was based off calculations from Gooneratne et al. (2012), an estimate representing the daily loline consumption of a sheep grazing meadow fescue grass. The timing between dosing was chosen due to loline metabolites in sheep urine plateauing at 60 hours post-dosing (Gooneratne et al., 2012). The remaining two lambs were offered 200 ml milk twice 60 hours apart with the same loline rate at 23 days post larvae infection examining lolines effect on adult parasites.

Faecal samples were taken directly from the rectum at the start and the end of the experiment and analysed for concentration of nematode eggs. Faeces (1.7 g) were added to a jar with 5 mL water and allowed to soften overnight. Following the next day, 50 mL of saturated NaCL was added and samples were thoroughly mixed to ensure the faecal pellets were entirely broken up. A Pasteur pipette was used to sample faecal mixture and fill both chambers of a moistened McMaster slide. Slides were allowed to sit for a few minutes and faecal eggs were counted under a microscope. Number of eggs per gram (epg) was calculated by totalling both sides of the McMaster slide and multiplying by 100.

Approximately, 12 hours after the last loline dose lambs were slaughtered following stunning with a captive bolt, the abomasum and first 10 m of small intestine was ligated and removed, the number of worms present in abomasum and small intestine were enumerated. Worm burdens were determined separately for *T. circumcincta* in the abomasum and *T. colubriformis* in the small intestine. Following slaughter, the abomasum and small intestine contents were collected and tissues were washed with water removing any non-adhered worms. The contents and washings were transferred to a beaker;

water was added so each had a volume of 2 litres. Contents were thoroughly mixed and four 50 ml subsamples were taken and placed with 20 ml formalin representing 1/10th of the original sample. Washed tissues were then cut up into pieces and digested with 1% pepsin, 1% HCL solution at 38˚C for 16-20 hours (Herlich, 1956). After digestion material was passed through a 45 micron sieve, washed with water and fixed in a 5% formalin for storage until worm enumeration. Total digest and wash containers were made up to 100 mL with water and two, 10 ml samples were taken and counted under a microscope. As the wash represented 1/10th of the original sample number of worms were determined per mL, multiplied by the containers 100 mL and multiplied by 10 to yield a final total count. Digests were a total sample so were only multiplied by the dilution factor (10) to give a total number of worms established in the tissue. Percent establishment was calculated by adding the total number of worms in digest and wash divided by the number of worms dosed to the lambs times 100.

Worm count data of *T. circumcincta* and *T. colubriformis* was transformed logarithmically to base 10 and analysed using ANOVA in Genstat (18th edition) between LOL dosed at L4 or adult worm stage and CON fed lambs.

6.2.5 *In-vivo* study 2: Loline effects against *H. contortus*

Loline seed extract was used to examine antimicrobial effects of L4 and adult parasite stage of the nematode *H. contortus* in lambs (seven lambs total) approximately 12-16 weeks old (Lincoln University Animal Ethics Committee #2019-07). Lambs were still suckling, and group pens were used to house the lambs with their dams. Ewes and lambs were fed *ad-libitum* lucerne pellets and had free access to water. A creep feeder for the lambs was also set up for ad libitum feed of a lamb muesli. Lambs were treated with an anthelmintic at 2 mL per 15-20 kg (Zolvix Plus, PGG Wrightson, Christchurch NZ) and 10 days later were infected with 20,000 L3 infective *H. contortus*. There were three treatments, loline treated lambs fed via milk bottle (LOL milk, n=2), loline treated lambs dosed orally (LOL feed, n=2), and non-loline treated lambs (CON, n=3).

Similar to *in-vivo* study 1 (Section 6.2.4) lambs were trained to drink milk from a bottle with a teat stimulating the oesophageal groove reflex. At 10 days post parasite infection, two lambs were offered 100 ml milk twice, 60 hours apart containing loline seed extract to supply 52.5 mg/kg LW (Gooneratne et al., 2012) of loline. The same two lambs were offered loline laced milk 23 days post larvae infection (LOL milk), and two additional lambs received loline orally dosed with water (LOL feed).

Faecal samples were also taken at the beginning prior to parasite infection, 14 days and every 3 days after until the end of the experiment. Number of eggs per gram (epg) was determined as described in *in-vivo* study 1 (Section 6.2.4).

Approximately, 12 hours after the last loline dose lambs were slaughtered following stunning with a captive bolt, and the abomasum was ligated and removed and the number of worms present were counted to determine efficacy using the procedures previously described by *in-vivo* study 1 (Section 6.2.4).

All data was analysed using Genstat (18th edition). Worm count data of *H. contortus* was transformed logarithmically to base 10 (log_{10}) and analysed using ANOVA between LOL given through milk or orally dosed and CON fed lambs. *H. contortus* faecal egg counts were also transformed to log10 and analysed using REML.

6.2.6 *In-vivo* study 3: Oral dosing loline and its effects against mixed infection of L4 *T. circumcincta*, *T. colubriformis*, and adult *H. contortus*

Following the outcome from the above mentioned pilot studies loline seed extract was used to examine antimicrobial effects of L4 stage of *T. circumcincta*, *T. colubriformis* and adult parasite stage of the nematode *H. contortus* in weaned lambs (sixteen coopworth lambs total) approximately 6-8 week old (Lincoln University Animal Ethics Committee #2018-34A). Lambs were removed from pasture and housed in individual pens and offered *ad-libitum* lucerne pellets and had free access to water. Feed intake and refusals were recorded daily. Lambs were treated upon housing with an anthelmintic at 2 mL per 15-20 kg (Zolvix Plus, PGG Wrightson, Christchurch NZ) and 10 days later

were infected with 20,000 L3 infective *H. contortus* at day 0 and 10,000 *T. circumcincta*, and 10,000 *T. colubriformis* at day 14. Treatments were, loline treated lambs (LOL) fed orally, and non-loline treated lambs (CON). Additionally two of the non-loline treated lambs were fed NIL seed extract orally (NIL) as a control for the seed extract.

Loline was delivered at a rate of 52.5 mg/kg LW (Gooneratne et al., 2012), this was mixed with water (30 mL) and dosed on day 13, 15, 17, 20, 22, 24, and 26. Day 13 dose was one day prior to *T. circumcincta*, *T. colubriformis* infection (Figure 6.1). The two lambs receiving NIL seed extract were administered at the same amount as lambs receiving LOL treatment.

Faecal samples were taken prior to parasite infection, and at each day of loline doses (Figure 6.1) and at the day of slaughter. Number of eggs per gram (epg) was determined as described *in-vivo* study 1 (Section 6.2.4).

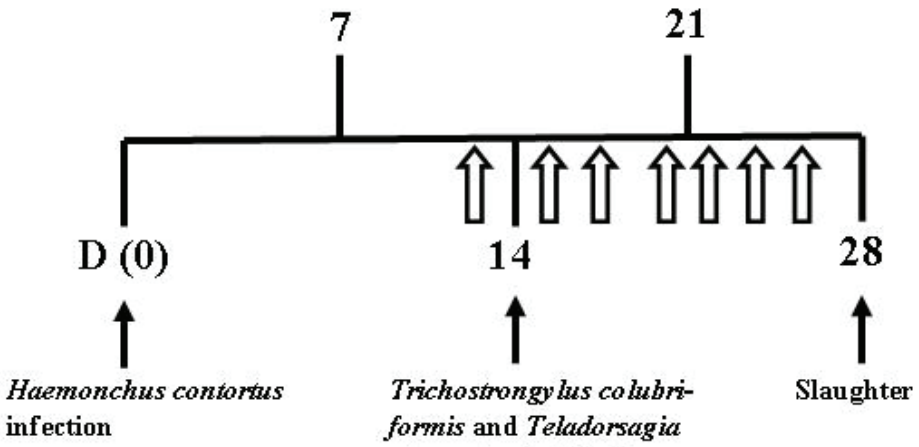


Figure 6.1. Timeline for lambs infected *Haemonchus contortus*, *Trichostrongylus colubriformis*, and *Teladorsagia circumcincta* and dosed loline/faecal sampled ⇧

Approximately, 12 hours after the last loline dose lambs were slaughtered following stunning with a captive bolt, and the abomasum was removed and the number of worms present counted to determine efficacy using the procedures previously described by *in-vitro* study 1 (Section 6.2.4).

Genstat (18th edition) was used to analyse all data. Worm count data was logarithmically transformed to base 10 for *H. contortus*, *T. colubriformis*, and *T. circumcincta* and analysed using ANOVA. Faecal egg count data was also $\log_{10}$ transformed and was analysed using REML. Lamb weight, gain, and feed intake was analysed using two way ANOVA. Significance was declared at $P<0.05$ and trends at $0.05<P<0.10$.

6.3 Results

6.3.1 *In-vitro* experiments

Loline effects on larval migration of *T. colubriformis* in either LOL or NIL extracts are displayed in Table 6.1. Percent migration was not affected by concentration in either LOL or NIL seed extracts (P=0.29), nor was there an extract x concentration interaction (P=0.52).

Table 6.1. *In-vitro* effects of different concentrations of loline on larval migration (LM) of *Trichostrongylus colubriformis*

	Concentration, ppm			
Sample	16000	8000	4000	2000
LOL, % LM	87.2 ± 9.0	85.5 ± 6.1	88.8 ± 6.8	91.2 ± 5.8
NIL, % LM	59.7 ± 20.6	85.0 ± 3.5	81.2	93.2

Establishment of *T. circumcincta* or *H. contortus* in lambs fed loline is displayed in Table 6.2. *T. circumcincta* was decreased (P<0.01) by 59% in the abomasal tissues of a lamb fed loline laced milk (LOL) compared with control (CON). However, establishment of *H. contortus* was unaffected (P=0.49) by loline feeding (Table 6.2).

Table 6.2. *In-vitro* percent establishment of *Teladorsagia circumcincta* or *Haemonchus contortus* in lambs treated with or without loline

	Average % Establishment	
	LOL	CON
Teladorsagia circumcincta	30.8 ± 2.4[a]	74.8 ± 2.3[b]
Haemonchus contortus	56.5 ± 4.4	50.5 ± 8.3

[a,b] Means within rows with unlike superscripts differ, P < 0.05

6.3.2 *In-vivo* studies 1 and 2: Loline effects against *T. circumcincta* and *T. colubriformis, or H. contortus* establishment

Average worm count of *T. circumcincta*, *T. colubriformis*, or *H. contortus* of CON lambs or lambs treated with loline at L4, or adult worm stage through feed or milk is displayed in Table 6.3. Percent establishment of *T. circumcincta* was 78%, 75%, and 60% for CON, and lambs treated with loline at adult or L4 worm stage, respectively. However, no statistics were calculated, as there was only one lamb per treatment.

Establishment of *T. colubriformis* was 45%, 80.5%, and 11% for CON, and lambs treated with loline at adult or L4 worm stage, respectively. Lambs treated at L4 was significantly reduced (P=0.02) compared with CON or lambs treated loline at an adult larvae.

No treatment difference between control and loline dosed in feed or milk was observed for lambs infected with *H. contortus* (P=0.68).

Table 6.3. Arthritic mean worm count of *Trichostrongylus colubriformis*, *Teladorsagia circumcincta*, or *Haemonchus contortus* of CON lambs or lambs treated loline at L4, or adult worm stage through feed or milk

	Average worm count			
	LOL, L4 dose	LOL, adult dose		CON
		Milk fed	Feed fed	
[1]*Teladorsagia circumcincta*	2985	3765		3890
[1]*Trichostrongylus colubriformis*	1137 (589-1687)[a]	8050 (7810-8290)[b]		4473 (2933-4180)[b]
[2]*Haemonchus contortus*		18,750 (16875-20625)	19,250 (12750-25750)	29,125 (16875-49875)

[a,b] Means within rows with unlike superscripts differ, P < 0.05
[1]*In-vivo* study 1 (Section 6.2.4)

Prior to the infection of a mix infection of *T. circumcincta*, *T. colubriformis* faecal egg counts were zero. At the end of the experiment, faecal egg counts were similar (P= 0.17) with an average of 100, 500, and 225 faecal eggs per gram of faeces for lambs treated with loline at L4, or adult worm stage, and CON, respectively.

Faecal egg counts for *H. contortus* infection throughout the *in-vivo* trial 2 are displayed in Figure 6.2. Overall, faecal egg counts increased over time (P=0.01) however there was no treatment difference (P=0.19) between CON, milk LOL, and feed LOL nor a treatment by day interaction (P=0.82).

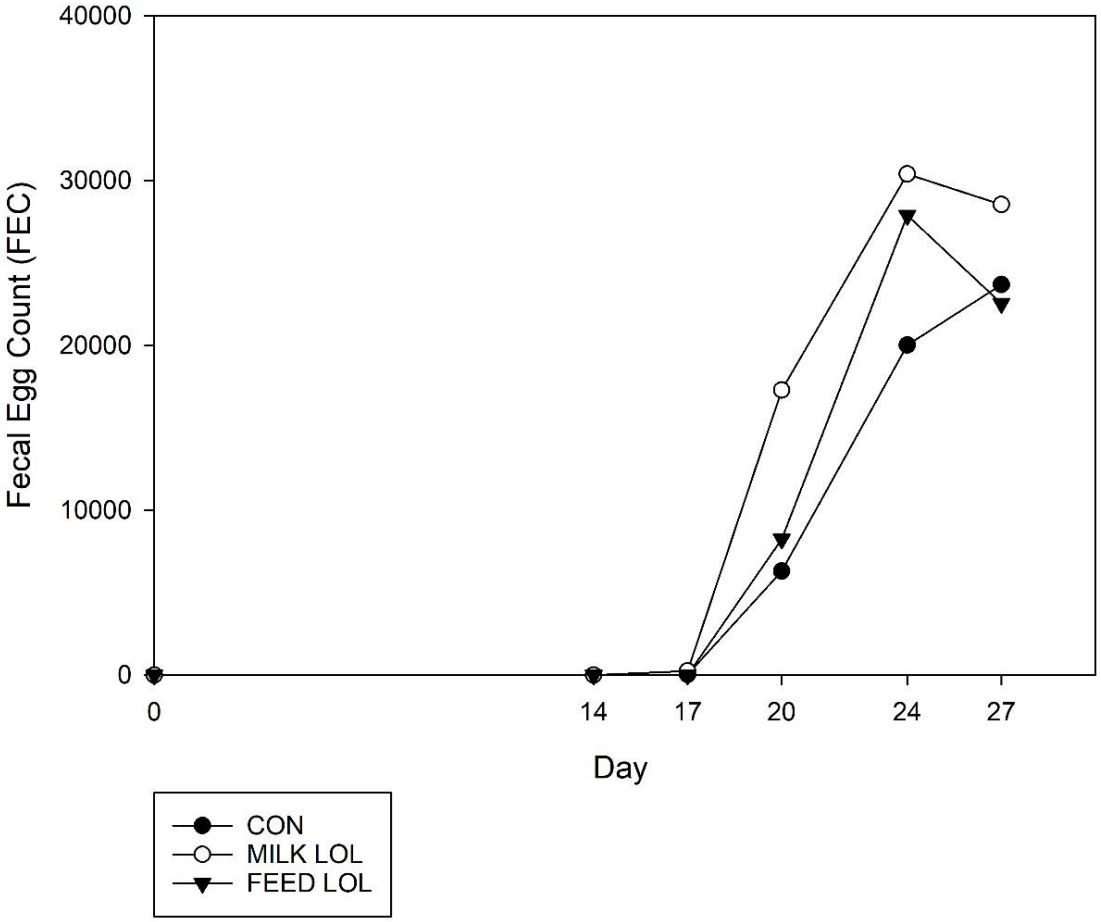


Figure 6.2. Arthritic means of faecal egg counts of lambs infected with *Haemonchus contortus* treated with loline either orally or through milk or without

6.3.3 *In-vivo* study 3: Oral dosing loline and its effects against mixed infection of L4 *T. circumcincta*, *T. colubriformis*, and adult *H. contortus*

Feed intake increased over time (P=0.01, Figure 6.3) but no treatment differences were observed between LOL, CON and NIL (P=0.18). No treatment x day interaction (P=0.9) occured.

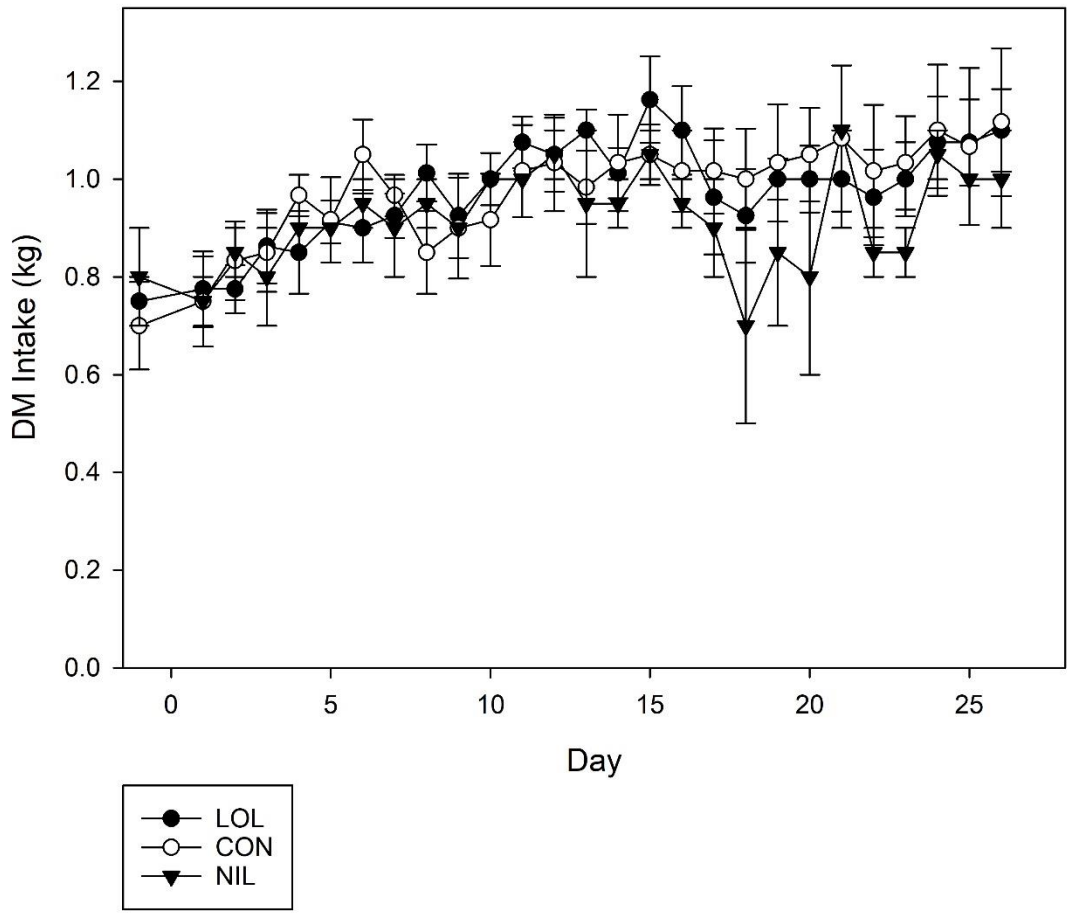


Figure 6.3 Dry matter intake of lambs with a mix parasite infection treated with a meadow fescue seed extract with (LOL) or without loline (NIL), or non-treated (CON)

Average lamb weight gains increased over time (P=0.01, Figure 6.4) there were no treatment differences between NIL, CON and LOL (P=0.51) nor was there a treatment by day interaction (P=0.75). Average lamb weight gains were 3.9 ± 0.6, 3.5 ± 0.5, and 2.8 ± 0.6 kg for LOL, NIL and CON, respectively, throughout the trial (P =0.49).

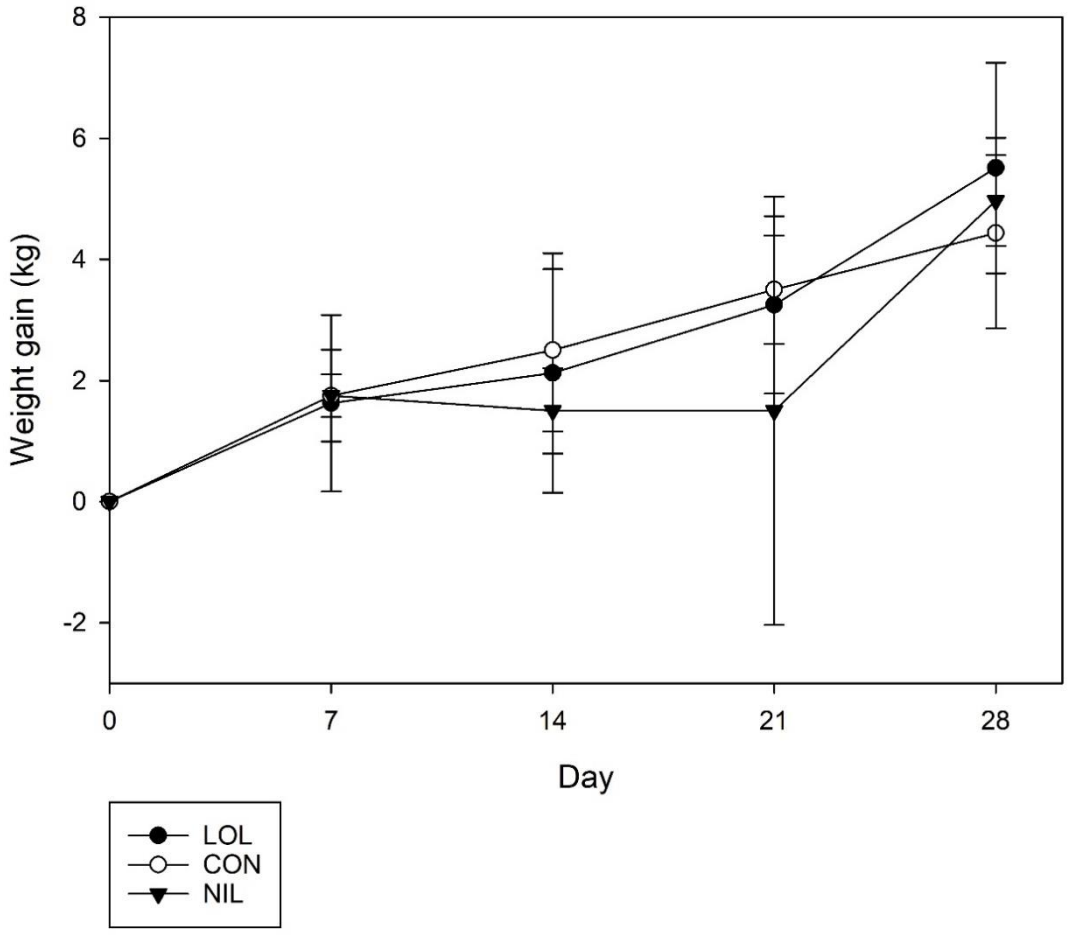


Figure 6.4. Average lamb weight of lambs with a mix parasite infection treated with a meadow fescue seed extract with (LOL) or without loline (NIL), or non-treated (CON)

Growth efficiency measured as gain per feed (kg LWG/ kg DM intake) varied throughout the trial (P=0.01, Figure 6.5). Overall, average growth efficiency was 0.18 in CON treated lambs which was less (P=0.01) than LOL (0.24) or NIL (0.23) treated lambs. Day by treatment was significant (P=0.01), day 0-7 was similar. Day 8-14 and 15-21 NIL was less than CON or LOL. Day 22-28 treatments varied with CON being greater than LOL or NIL.

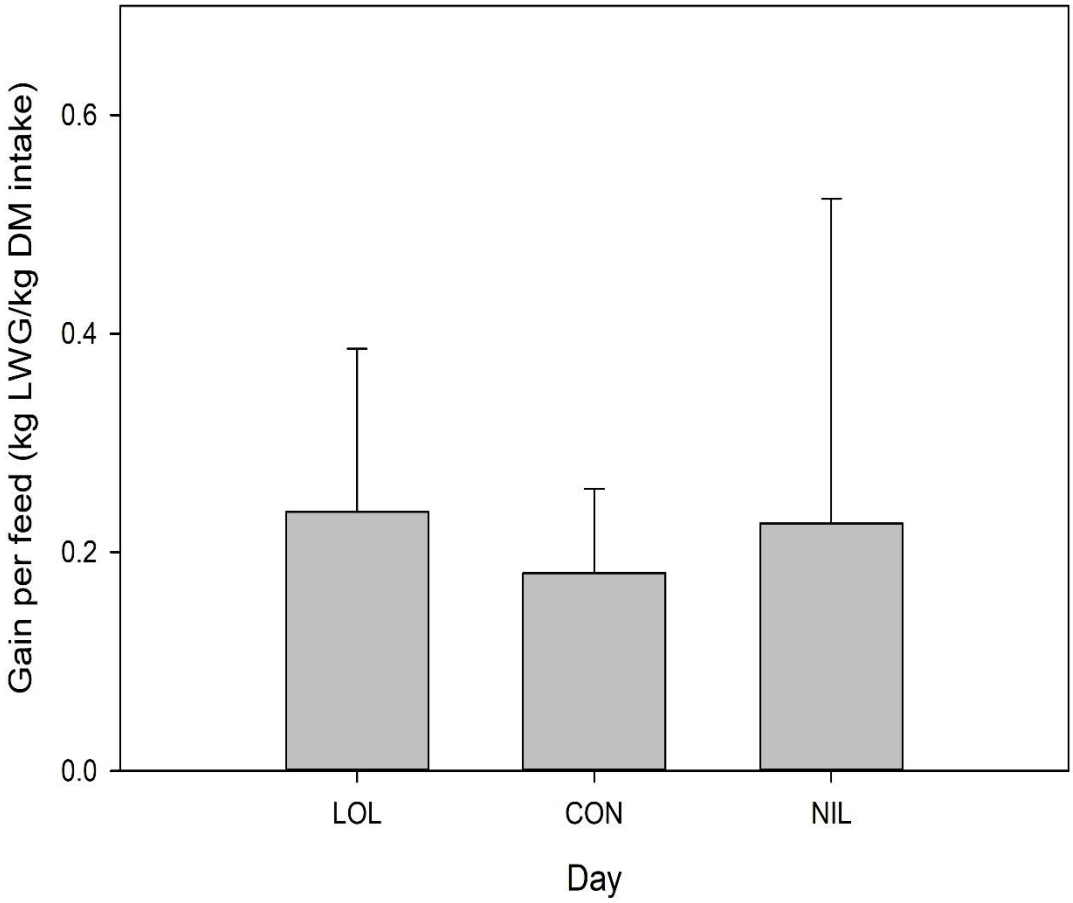


Figure 6.5. Average growth efficiency over the 28 day trial measured as gain per feed (kg LWG/kg DM intake) of lambs with a mix parasite infection treated with a meadow fescue seed extract with (LOL) or without loline (NIL), or non-treated (CON)

Faecal egg counts were zero at the start of the trial and increased with time (P=0.01, Figure 6.6). Overall, there was no treatment difference (P = 0.39) between LOL, CON, or NIL nor a treatment by day interaction (P = 0.25). Average faecal egg count was 19,925, 15,917, and 37,800 for LOL, CON and NIL, respectively, at slaughter on day 27.

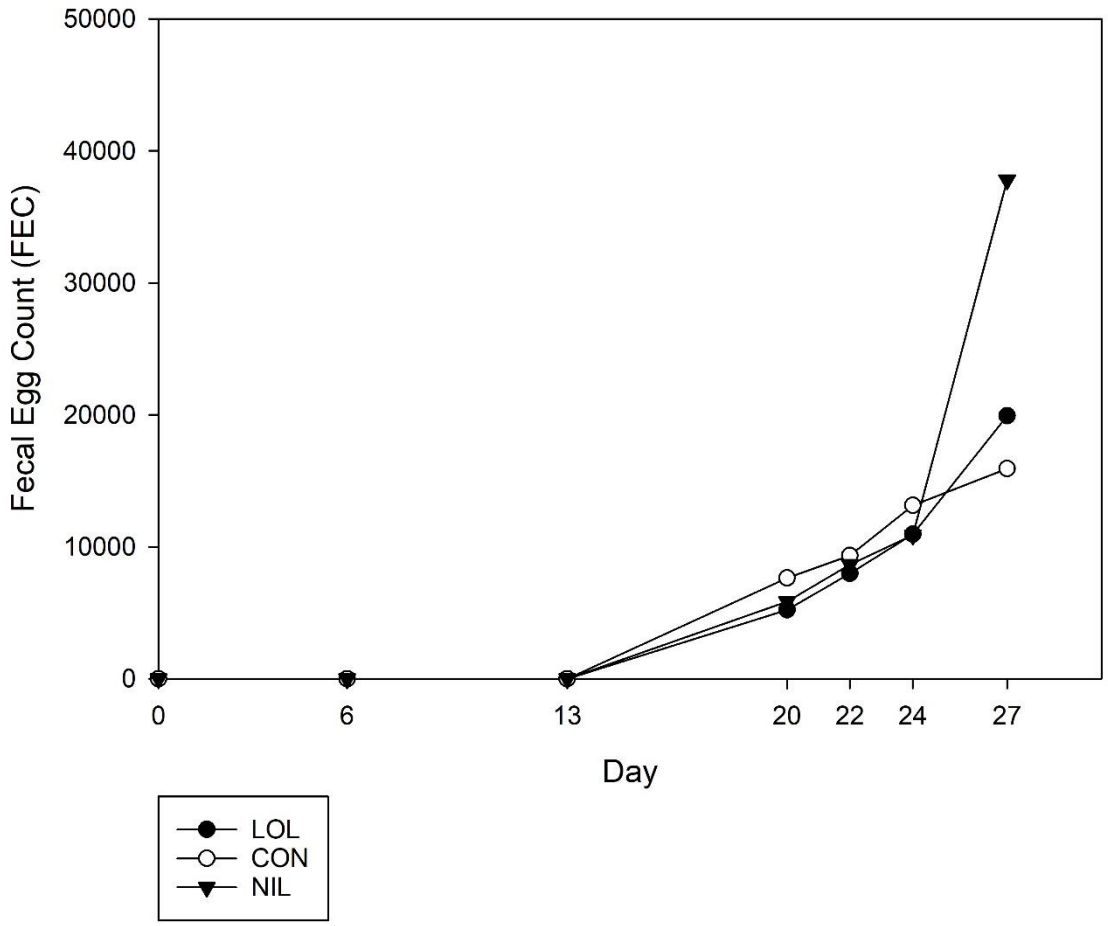


Figure 6.6. Arthritic means of faecal egg counts of lambs with a mix parasite infection treated with a meadow fescue seed extract with (LOL) or without loline (NIL), or non-treated (CON)

Average worm counts of CON or LOL treated lambs at L4 stage of *T. circumcincta*, and *T. colubriformis*, or adult *H. contortus* are displayed in Table 6.4.

Percent establishment of *T. circumcincta* was similar (P=0.96), being 69%, 69.5%, and 69.4% for LOL, CON and NIL, respectively. Similarly, no treatment differences were observed (P=0.43) for *T. colubriformis* infected lambs with an establishment rates of 24.2%, 27.2%, and 47.8% for LOL, CON, and NIL, respectively. Average worm counts of LOL, CON, or NIL lambs infected with *H. contortus* is displayed in Table 6.4 and was not significantly different from each other (P=0.15).

Table 6.4. Arthritic mean worm counts of lambs treated with a meadow fescue seed extract with (LOL) or without loline (NIL), or non-treated (CON) at L4 stage of *Teladorsagia circumcincta*, and *Trichostrongylus colubriformis*, or adult *Haemonchus contortus*

	Average Worm Counts		
	LOL	CON	NIL
Teladorsagia circumcincta	6,900 (2700-10000)	6,951.7 (2960-9810)	6,940 (6280-7600)
Trichostrongylus colubriformis	2,415 (100-4450)	2,720 (490-5590)	4,775 (3190-5590)
Haemonchus contortus	14,630 (6650-29090)	7,938 (440-11590)	29,750 (23810-35690)

6.4 Discussion

Loline had limited anti-parasitic effects, with the only suggestion of an anti-parasitic effect occurring when larvae are in close contact with gastric tissues and mucosal surfaces. Loline had no or minimal effects on *in-vitro* larval migration of L3 *T. colubriformis* and preliminary *in-vivo* results of adult *T. colubriformis* and *T. circumcincta*. For the larval migration assay this was unexpected as plant extracts containing loline has shown antimicrobial qualities (Bacetty et al., 2009, Muponda, 2014). Bacetty et al. (2007), found a combination of NAL, and NFL at 100 and 250 µg/mL was nematocidal to the plant nematode *Pratylenchus scribneri*. N-formyl loline and NAL are the principle natural loline alkaloids produced in meadow and tall fescue (Bush et al., 1993), with NFL being the predominant alkaloid (Yates et al., 1990). Combined NFL and NAL composed 86% of the seed extract used in this experiment, and every tested concentration in the larval migration assay would have been greater than 250 µg/mL. Furthermore, egg hatching and larval motility of *T. colubriformis* and *T. circumcincta* have been affected by a seed extract of meadow fescue and tall fescue in a high concentration (1:1 ratio of water to seed extract). Meadow fescue and tall fescue both contain lolines however, concentrations were not reported, and results appeared independent of the presence of alkaloid producing endophyte or endophyte type and identification of causing agent was not explored (Muponda, 2014). These reported concentrations are higher than the offered loline dose *in-vivo*. Lambs were offered a loline dose at 52.5 mg/kg which converted to µg/mL is the same concentration. It is possible the dose was too low to have any effect on adult *T. colubriformis* and *T. circumcincta* although the dose chosen was to reflect the likely consumption *in-situ* when grazing loline pastures (Goonernate et al., 2012).

When in close association with blood or tissues there was some indication of an anthelmintic effect. This was observed in the L4 mucosal browsers *T. colubriformis* and *T. circumcincta* and adult blood feeder *H. contortus in-vivo*. Preliminary results showed lambs treated with loline reduced establishment of L4 larvae of *T. circumcincta* and *T. colubriformis* by 75 and 23%, respectively and 35% in adult *H. contortus* compared with controls. Loline has a documented history of being a feed deterrent in grass grubs and *Pratylenchus scribneri* (Bush et al., 1997, Patchett et al., 2011c, Bacetty

et al., 2009) and it is possible the same mechanisms may be at play here. Furthermore, it is suggested that alkaloids mode of action against parasites is antagonizing receptors in the central nervous system causing death (Roy et al., 2010, Jain et al., 2013, Dubois et al., 2019). However, loline has no documented affinity to a variety of central nervous system receptors (Dannhardt & Steindl, 1985, Strickland et al., 1992, Larson et al., 1999) and unlikely to cause parasite death that way. Main routes of administration of any chemical to have an effect against a parasite is either through oral ingestion or diffusion through its external surfaces which is dependent on a molecule lipophilicity (Lifschitz et al., 2017, Alvarez et al., 2007). Based on lolines characteristics (hydrophilicity, neutral charge, and low molecular weight) diffusion through parasites external surfaces would be limiting, however, would be an ideal molecule for clearance to underlying mucous gel layers in the gastrointestinal tract (Lai et al., 2009). Loline is also found in the blood of horses and sheep orally dosed (Rudolph et al., 2018, and chapter 5). Meaning loline has the location availability (blood and gastric mucous layers) to be a possible feed deterrent. Although concentrations in mucous were not measured this could explain the decreased mucosal browsers *T. colubriformis* and *T. circumcincta* and adult blood feeder *H. contortus in-vivo* as well as the reduced *in-vitro* establishment of *T. circumcincta* more than 12 hours after the last loline dose in lamb tissues.

Unexpectedly, preliminary results (Section 6.2.4, 6.2.5) contrasted with further investigations (Section 6.2.6). This potentially reflects mode of ingestion or the length of time loline had contact with L4 mucosal browser or adult *H. contortus*. Orally dosed loline had minimal effects on worm counts and faecal egg counts of a mix of L4 *T. colubriformis*, *T. circumcincta* and adult *H. contortus*. Concentration and target availability influences maximum drug efficacy on parasites (Lifschitz et al., 2017, Alvarez et al., 2007). In the preliminary trials, loline was dosed in the abomasum through milk and esophageal groove closure. Based on chapter 3, and chapter 5 loline appears to be minimally destroyed and/or absorbed out of the rumen and was decided dosing via abomasum was not necessary. However, oral dosing could have resulted in loline becoming diluted or associated with rumen contents, reducing available concentration for parasites in the abomasum and small intestine. It is known that short fasting or temporary reduced feeding reduces gastric transit time, increasing

plasma availability and anthelmintic efficacy in calves and sheep (Hennessy, 1993, Ali & Hennessy, 1993, Sánchez et al., 1997). If loline became associated with rumen contents that could have sped up its excretion or made less available for absorption into the gastric mucous layer or blood, influencing its effect on parasites. In addition, it is possible target availability was affected by slaughtering animals too soon and loline did not have enough contact time with parasites. In the preliminary *in-vivo* study 1 (Section 6.2.4) *T. colubriformis* and *T. circumcincta* larvae were treated with loline at L4 and were slaughtered 16 days later whereas lambs in *in-vivo* study 3 (Section 6.2.6) were slaughtered 3 days after developing to L4. Loline is excreted in urine of sheep for up to 60 hours after dosing (Goonernate et al., 2012) and it is possible longer contact is needed to provide an anthelmintic and detect an effect on the *T. colubriformis* and *T. circumcincta*.

The effects observed, when they were observed, can be attributed to loline. *In-vivo* study 3 (Section 6.2.6) using a seed extract with (LOL) and without (NIL) loline to potentially identify and isolate loline effects on parasites showed the NIL extract appeared to have little effect on the average worm counts of *T. circumcincta*, *T. colubriformis*, or *H. contortus*, or the faecal egg count. Plants naturally contain a variety of compounds (Ahmed Wani et al., 2015) and a disadvantage of crude plant extracts is purification and isolating compounds effects. Loline is no different as its isolation from plants and creation of synthetic loline is difficult (Cakmak et al., 2011). From the study, NIL extract had little effect on parasites, suggesting any observed anti-parasitic effect was due to loline. However, many secondary metabolites and plant compounds have anti-parasitic effects (Githiori et al., 2006) and can work in synergy with each other (Santos et al., 2019). In one case, investigating effects of quinolizidine and piperidine alkaloids on *H. contortus* and *T. circumcincta* some of the non-alkaloid seed extract was better suggesting non-alkaloid components having an effect (Dubois et al., 2019). As plant breeding can change primary and secondary metabolites in the plant (Joost, 1995, Bush et al., 1997, Easton, 1999), future research may need to isolate lolines effects in meadow fescue.

The form of loline may be important. Based on previous chapters loline metabolites are generally unconverted in ruminal fluid (chapter 3), but of the loline forms only loline base and NFL were

passively transported across all gastrointestinal epithelial (chapter 5). Furthermore, although all five loline metabolites are excreted in lamb urine, excretion peaks at 2 hours post dosing after which the predominate metabolites are loline base and NFL (Gooneratne et al., 2012). Similar, only NFL and loline base has been found in the blood plasma of lambs (chapter 5) and some NFL and NAL in horse serum (Rudolph et al., 2018). This may be expected as NFL is the predominant metabolite found in meadow fescue and tall fescue (Bush et al., 1997, Yates et al., 1990) and has been noted as a strong insecticide to several orders of insects (Dahlman et al., 1997, Yates et al., 1989). However, some research has pointed to NAL (Bultman et al., 1997) or a combination of NAL and NFL (Bacetty et al., 2007) to have some insecticidal and plant parasitic effects, respectively. It is possible the other loline forms may be effective but have not been reported/studied due to low natural concentrations. From the combination of this thesis studies it could be proposed that NFL is most likely the most effective form, assuming simple loline base is an inactive loline form in which metabolites degrade to. NFL is the metabolite of greatest concentration in seed extract (Table 5.1, chapter 5) and would have the greatest bioavailability to effect parasites. As it is slowly excreted in urine (Gooneratne et al., 2012), and is found in blood hours after dosing (chapter 5) compared with other loline metabolites.

In conclusion, there was limited evidence to support an anti-parasitic effect of loline, but it remains possible loline may have some feed deterrent behaviour to parasites that are in close contact with the gastric mucous layers or blood. Preliminary *in-vivo* results showed loline to reduce establishment of L4 mucosal *T. colubriformis* and *T. circumcincta*, and adult blood feeder *H. contortus* when dosed via milk. However, this was contrasted when loline was orally dosed, possibly a reflection of loline concentration being diluted by rumen contents and target availability decreased, an effect which may have reflected the early ending of the trial resulting in lower deterrence.

Chapter 7

General Discussion- Animal physiology, and metabolism of loline alkaloids and their effect on gastrointestinal nematodes

7.1 Introduction

Loline is a secondary plant metabolite classified as an alkaloid with promising effects on parasites. It is thought secondary plant metabolites, like loline, may provide an alternative, cost-effective treatment to gastrointestinal parasites limiting both drug use, and development of anthelmintic resistance (Githiori et al., 2006, Williams et al., 2015). Specifically, loline has been suggested to have antimicrobial, and insecticidal (Schardl et al., 2007, Bacetty et al., 2009) properties. Both egg hatching and larval motility of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* nematodes, as well as the plant parasitic nematode *Pratylenchus scribneri* have been shown to be affected by loline (Muponda, 2014, Bacetty et al., 2007). Many secondary plant metabolites, specifically alkaloids, have shown promising antimicrobial properties to a variety of gastrointestinal parasites (Perrett & Whitfield, 1995, Satou et al., 2002, Ayers et al., 2007, Simon et al., 2012, Fomum & Nsahlai, 2017). However, either the alkaloids have exhibited cytotoxic properties (Satou et al., 2002, Ayers et al., 2007, Simon et al., 2012), toxic properties were unknown (Perrett & Whitfield, 1995, Fomum & Nsahlai, 2017) or antimicrobial effects were confounded with other alkaloids or non-alkaloid component (Westendorf et al., 1993, Muponda, 2014, Dubois et al., 2019). Although, loline is considered non-toxic to mammals (Bush et al., 1993, Gooneratne et al., 2012), information is limited on the effects of lolines and metabolism in ruminants. The potential of loline to alleviate gastrointestinal parasites without the reliance of anthelmintic to control gastrointestinal parasites warrants further investigation on where and what forms loline is metabolized, and if any active forms will reach the intestine to affect microflora or gastrointestinal nematodes.

Overall, these results investigate lolines bioavailability through surviving digestion and passive absorption in the gastrointestinal tract and lolines metabolism through looking at distribution of

different loline metabolites *in-vivo*. Lastly assessing lolines anti-microbial effects on ruminal microbes and internal parasites of sheep.

7.2 Bioavailability

One objective of this study was to explore the fate of lolines during digestion and determine the bioavailability. Loline is a water-soluble alkaloid thought to be readily available to ruminants, through *in-vitro* studies was found to survive digestion (chapter 3) and have limited passive absorption (chapter 5) in the gastrointestinal tract. This contrasts previous *in-vivo* data suggesting loline to be ruminally destroyed/ rapidly absorbed in the foregut. Earlier loline studies found low abomasal recovery (5%) (Westendorf et al., 1993), rapid appearance (<15 minutes) in sheep urine with excretion peaking at 2 hours post dosing (Gooneratne et al., 2012), and less than 5% eliminated through faeces (Gooneratne et al., 2012). Limited fecal excretion limits lolines path to either being metabolized in the digestive tract or absorbed through the gastrointestinal tract. These results show upon consumption, solubilisation of loline from meadow fescue seeds occurs relatively quickly but is pH dependent. The bioavailability of loline from plant material in digestive fluid is relatively high. Seeds digested for 2 hours in simulated abomasal fluid at a pH of 2 or 3, ruminal fluid (pH 6.8), or water (pH 7) had a recovery of 76%, 70%, 65%, and 63%, respectively (chapter 3). Rumen microbes have an influence on loline bioavailability with some degradation appearing after 12 hours incubation. At 12 hours, incubation of loline in simulated ruminal fluid with and without viable ruminal microbes equated to 79% and 95% recovery, respectively (chapter 3). All loline forms (NFL, NANL, NML, and loline base) were greater except NAL, which had no difference in ruminal fluid without viable ruminal microbes. Greater recovery in ruminal fluid without viable microbes indicates microbes degraded some loline although this degradation is far from complete.

Ruminal recovery rates however, contrasts Westendorf et al (1993) who concluded loline is extensively metabolised in the rumen. Over 90% of NFL and NAL was unaccounted for prior to the abomasum in sheep fed NFL and NAL orally (Westendorf et al 1993). *In-vitro* versus and *in-vivo* experiment could attribute the differences. One being a closed system and the other not and opened

to various factors such as absorption across the rumen wall. Surprisingly, less than 2% of loline was passively absorbed in ruminal or abomasal tissues, and only 5% in ileum tissues (chapter 5). Indicating the majority of loline is not passively absorbed (chapter 5), although the lack of absorption of the caffeine as a positive control may reflect a weakness in this assay. Alkaloid absorption across gastrointestinal epithelia is structural dependent with compounds in similar classes not guaranteeing similar absorption (Peck et al., 2014). Loline base and NFL were the predominant metabolites passively absorbed in all tissues and follow a similar trend *in-vivo*.

7.3 Loline metabolism

Although limited in scope, these studies shed light on the metabolism of loline. Loline base was a predominant metabolite found in all experiments potentially reflecting loline derivatives metabolizing to the simple loline base. It is known that loline catabolizing microbes exist on the surface of plant leaves with the ability to live exclusively on loline (NFL) as its main carbon and nitrogen source and consume little loline base (Roberts & Lindow, 2014). Loline derivatives are suggested to be metabolized to the simple loline base *in-vivo* (Westendorf et al., 1993, TePaske & Powell, 1993, Gooneratne et al., 2012). Specifically, in ruminal fluid with and without viable microbes loline base is the only metabolite not plateauing after 48 hours suggesting conversions can occur (seeds were not sterile) independent of rumen microbes (chapter 3). In the Ussing chamber experiment (chapter 5) recovery of loline after 2 hours resulted in the majority of total loline being recovered (>85%) suggesting loline is not degraded in a physiological solution. However, *in-vivo* the predominant metabolite found was loline base and NFL (Gooneratne et al., 2012, chapter 5). This may simply reflect those metabolites being the highest form in plants (Justus et al., 1997) therefore most likely the only ones detected or potential metabolism of loline moving through the body (Figure 7.1). It is known that saturated pyrrolizidines are metabolized in the liver by the oxidative hepatic cytochrome P450 enzymes to a water-soluble dehydropyrrolizidine carboxylic acid (Ruan et al., 2014a). These P450 enzymes are found in multiple body sites including the gastrointestinal epithelium, blood, liver, and kidneys (Pond & Tozer, 1984, Mclean & Duncan, 2006). It is possible metabolism of loline could occur in these sites and would help explain why *in-vivo* the predominant

metabolite was loline base. Although loline metabolism is thought to be rapid (appearing in urine of lambs within 15 minutes), recovery of total loline in urine is low (6%), and less than 5% recovered in the abomasum (Westendorf et al., 1993) of sheep suggesting a majority is metabolized *in-vivo*. Furthermore, as discussed earlier loline remains intact during digestion (>80%), and some loline is passively absorbed (<5%). Loline is found in small amounts in blood serum of horses (Rudolf et al., 2018) and, for the first time in the plasma of lambs (chapter 5). In lambs, 15 hours after dosing the predominant metabolites found in blood plasma are NFL and loline base and only loline base is detected in liver and kidneys (chapter 5). This could reflect loline metabolism, and suggest limited metabolism occurs in the gastrointestinal epithelium. Urine data had a similar trend, for two hours after dosing lambs with loline, predominant metabolites were NANL and NFL, changing to mostly loline base and NFL 20 hours after. Further metabolism results in only loline base after 36 hours in urine of lambs (Gooneratne et al., 2012), and after 15 hours is the only metabolite found in liver and kidneys of lambs (chapter 5). While urine data from a previous study and liver/kidney data from this study do not completely agree, differences could be attributed to dosing, one being orally the other being through the abomasum speeding up drug metabolism. These data suggest loline derivatives are metabolized to loline base in perhaps blood, liver and/or kidneys explaining why it is the predominant metabolite found *in-vivo* hours after dosing.

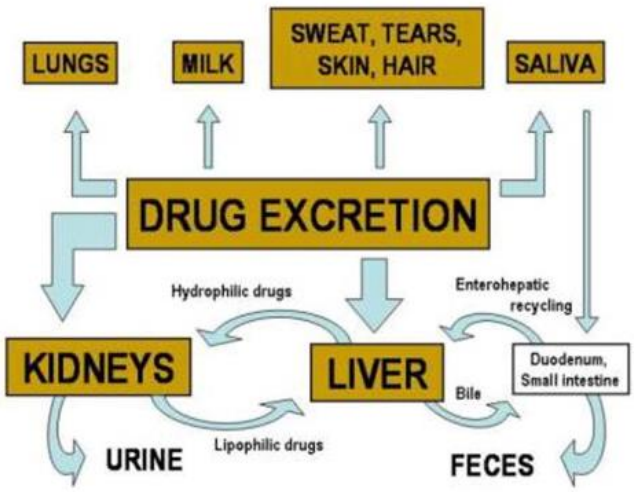


Figure 7.1. Potential drug excretion pathways

7.4 Antimicrobial properties

One of the major objectives of these studies was to explore the anti-microbial effects of loline. Through a series of *in-vitro* and *in-vivo* studies loline appears to have limited evidence to support an anti-microbial effect on either ruminal microbes (chapter 3 & 4) or gastrointestinal parasites of sheep (chapter 6). Specifically, chapters 3 & 4 were able to evaluate lolines effects on 2 (short chained fatty acids & gas) of the 3 rumen products; 1) short chained fatty acids, 2) fermentative gases, and 3) microbial mass production that are indicative of the microbial health (Blümmel et al., 1997). Of the short-chained fatty acids, loline seed extracts produced more propionate than NIL seed extracts, and there was a tendency for more ammonia production in loline seed extracts compared with NIL seed (chapter 3). Loline seeds also had a tendency to reduce total gas production compared with NIL seeds. Conversely, LOL seed extract added to a ryegrass substrate did not affect gas production however, slowed rate of degradation compared with NIL seed extract. Despite those slight differences, apparent DMD was similar in both *in-vitro* experimental chapters (3 & 4). Dry matter digestibility is highly correlated with gas and VFA production (Blümmel et al., 1997, Wolin 1960); indicating that despite observed tendencies, loline has minimal effects on rumen microbes. Similarly, loline antimicrobial effects explored in chapter 6 on *H. contortus*, *T. colubriformis* and *T. circumcincta* parasites were limited. *In-vitro* loline had no effect on *T. colubriformis* larval migration but did significantly decreased establishment of *T. circumcincta* by 58% in tissues excised from lambs fed loline 12 hours prior. Preliminary *in-vivo* results showed significant reductions in *T. colubriformis* worm burdens and hinted at a decline in *T. circumcincta* worm burdens and *H. contortus* faecal egg counts with lambs dosed loline in abomasum (via milk). However, these observations were not repeated *in-vivo* when lambs were orally dosed loline (chapter 6). These results were slightly surprising, as loline has suggested antimicrobial, insecticidal, and anthelmintic properties (Schardl et al. 2007; Bacetty et al. 2009; Muponda 2014). However, many studies are confounded with un-pure extracts, as was this study.

Loline appears to have few detrimental effects on the host. As described above despite loline having mixed antimicrobial effects there was limited effects on ruminal microbes by way of VFA, gas, and

dry matter digestibility of feedstuff (chapter 3 &4). Furthermore, loline appears to have no effect on weight gain, or dry matter intake of lambs (chapter 6). In general, this is in agreement with literature accepting loline as being non-toxic (Schardl et al., 2007 & Bush et al., 1993, Gooneratne et al., 2012). Perhaps, given in a greater dose the results may have been different. Intravenous injection of loline base into mice is lethal at 400 mg/kg BW (Aasen & Culvenor, 1969). However, given orally at 415 mg/kg BW, feed intake, and weight gain of mice is only slightly depressed at the start of trials (Jackson et al., 1996, Finch et al., 2016) but quickly recovers, and in some cases loline has a suggested growth-stimulating factor (Jackson et al., 1996, Strickland et al., 1996). Similarly, increased feed conversion efficiency was observed between control and loline fed lambs (chapter 6). Overall, feeding loline has no detrimental effect on the growth of lambs but may increase efficiency.

7.5 Future Research

Potential future research could examine creating a purified loline extract. Currently, synthetic loline is difficult (Cakmak et al., 2011) and therefore expensive to recreate. One difficulty of these studies was determining whether effects were the result of loline or other compounds within the seeds. Chapter 3, 4, & 6 used meadow fescue seeds or extracts with and without loline to explore differences in an attempt to isolate loline effects. However, crude extracts/plant parts can contain multiples of different secondary metabolites that can vary with plant part used and season. Furthermore, compounding the problem is these secondary plant metabolites can contribute to synergistic or superimposing effects (Wangchuk et al., 2016). This has been observed in research examining alkaloids antimicrobial properties, with different methods of extraction (water, methanol, etc.) resulting in differences in effect and some effects resulting from different plant compounds (Dubois et al., 2019) and promising *in-vitro* results not replicated *in-vivo* (Santos et al., 2019). As a result, creation of a pure extract and further investigation could help elucidate some of the mixed and inconsistent results found in this study. Furthermore, creation of a pure extract would allow the study of loline in higher concentrations. The doses selected in these studies were based on average ability of a lamb to consume loline containing pasture in a day. Although mixed, earlier research has successfully dosed loline orally in mice at 1000 mg/kg BW without any toxic effects (Yates & Tookey,

1965). This concentration is significantly higher than the 52.5 mg/kg BW given in this thesis studies and as concluded in chapter 6, given sufficient contact time and concentration loline may have an effect on reducing mucosal browser or blood sucking parasites. Therefore, examining a higher concentration and/or increasing contact time such as dosing every day or multiple times a day like an animal would receive if they were grazing a loline containing pasture, perhaps loline would have more of an anti-parasitic effect.

The studies in this thesis was a mix of lolines and potentially there could be one loline form more active than another requiring future research to tease out individual effects of forms. This could get around just increasing loline concentrations, as it is unknown whether a higher concentration could have an effect on ruminal microbes or the animal or if it would be possible to increase loline concentrations in plants. In the previous murine study, examining loline toxicity, loline base was orally dosed and is considered the inactive loline form or the form other metabolites are broken down into as the detoxification process. NFL is a predominant alkaloid in meadow and tall fescue grasses (Yates et al., 1990) and has been identified as a contact and ingested broad-spectrum insecticide to a variety of insects (Dahlman et al., 1997), and is more toxic than other alkaloids (Yates et al., 1989), potentially being a more effective anti-parasitic compound.

In addition to researching increased concentrations or different loline forms, researching the availability and delivery of loline to gastrointestinal mucous or inside parasites could give useful insight on delivering loline in sufficient quantities or prolonging contact time. This would hopefully allow consistent anthelmintic results through direct targeting with limited or no detrimental effects to the animal.

7.6 Conclusions

In conclusion, rumen and abomasal digestion have a small effect on the form and concentration of loline. Surprisingly, little loline was passively absorbed across gastrointestinal epithelia indicating the majority of loline is not passively absorbed. Of loline forms, there were suggestions that loline derivatives are metabolized to the simple loline base. Loline base was present in rumen fluid that previously had none, and was the predominant metabolite found in the liver, kidney, and blood plasma of lambs fed loline. Loline had limited evidence to support an antimicrobial effect to rumen microbes or gastrointestinal parasites. However, may have potential to reduce establishment of parasites that are in close contact with the gastric mucous layers or blood if given sufficient contact time and concentration. Overall, loline appears to have few detrimental effects to host with similar weight gain, and feed intake however, may promote greater feed conversion efficiency compared with controls.

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