HARM AND BENEFIT OF PLANT AND FUNGAL SECONDARY METABOLITES IN FOOD ANIMAL PRODUCTION

EDITED BY: Michael D. Flythe, Glen Eris Aiken and Arthur Louis Goetsch

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HARM AND BENEFIT OF PLANT AND FUNGAL SECONDARY METABOLITES IN FOOD ANIMAL PRODUCTION

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Livestock species are either herbivores or omnivores that are maintained largely on plant-based diets. We have long appreciated the importance of understanding dietary plants from both nutritional and agronomic perspectives. However, it is increasingly clear that the fungi, bacteria and other microorganisms that live in the plants and animals are also significant factors in the ecology of agricultural animals. Many of the effects exerted on animals by dietary plants are attributable to secondary metabolites produced by the plants themselves or commensal microorganisms. Some fungal and plant secondary metabolites have multiple biological effects. We must be careful not to categorize a plant as strictly beneficial or harmful. Furthermore, we must be careful not to categorize even a particular plant or fungal compound as strictly beneficial or harmful. Rather, the harm or benefit of secondary metabolites are often dependent on the metabolic status of the animal, the interaction with other dietary factors including other secondary metabolites, and the dose received through the diet. This collection examines a range of agriculturally important plant and fungal products including essential oils, alkaloids, isoflavones and nitrates.

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EDITORIAL
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Editorial: Harm and Benefit of Plant and Fungal Secondary Metabolites in Food Animal Production

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Keywords: plant secondary metabolites, food, agriculture, natural products, plant toxins, animal nutrition, ergotism, essential oils

Editorial on the Research Topic

Harm and Benefit of Plant and Fungal Secondary Metabolites in Food Animal Production

The diets of agricultural animals provide energy and components for anabolism, as well as vitamins and minerals. Other impacts of the diet are caused by plant and fungal secondary metabolites. Toxins have always been a concern in animal agriculture. Ergot alkaloids are central to five articles in this *Research Topic* (Jackson et al.; Jia et al.; Shappell et al.; Coufal-Majewski et al.; Aiken et al.). Coufal-Majewski et al. state that as much as 20% of the wheat produced in Canada is infected with ergot-producing *Claviceps* species. The effects of ergot alkaloids on livestock include tremors, vasoconstriction, and gangrene, and less obvious impacts on growth and reproduction. The review covers impacts on livestock as well as the general information on *Claviceps* fungi. Importantly, the analytical methods we need to detect and quantify ergot alkaloids in feeds are also described (Coufal-Majewski et al.).

Ergot alkaloids are also the toxins involved in fescue toxicosis, which impacts forage-based livestock production in the USA, Australia, and New Zealand (Coufal-Majewski et al.). The tall fescue endophyte, *Epichloë coenophiala*, produces ergot alkaloids, which cause the toxicosis in grazing animals. The primary symptom of fescue toxicosis is vasoconstriction, which causes heat stress and gangrenous lameness. Jackson et al. conducted a tall fescue grazing experiment with steers and examined blood parameters. Depressed prolactin is diagnostic of fescue toxicosis, but they found that albumin, cholesterol, and red blood cell number were also altered throughout the trial, while other parameters changed transiently or only after prolonged exposure. Many of the negative effects of fescue toxicosis are associated with poor blood flow from vasoconstriction. Two of the contributions examined vasoconstriction (Jia et al.; Aiken et al.). Both dealt with the interaction of ergot alkaloids and another category of secondary metabolites, isoflavones from red clover. Aiken et al. examined the effects of ergot alkaloids and clover isoflavones on two arteries in goats *in vivo*. The other study by Jia et al. employed ergot alkaloids and specific isoflavones in an *in vitro* assay with the mesenteric artery and vein. In the *in vivo* study (Aiken et al.), the isoflavones reversed vasoconstriction caused by the ergot alkaloids, but in the *in vitro* (Jia et al.) study, they did not. The differences between the experiments are many, but notably include peripheral versus visceral vasculature and the metabolism of the compounds that occurs *in vivo*. If isoflavones do, in fact, reverse vasoconstriction in fescue toxicosis, it would explain the long-standing observation that cattle performance on tall fescue improves when there is clover in the pasture.

The investigations into clover isoflavones by our research group were originally initiated to explore isoflavones as an alternative to feed antibiotic growth promoters. The antimicrobial action of the isoflavones in these forage plants is similar to those of the secondary metabolites of

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a horticultural crop, the hops plant. The *Research Topic* includes a review of hops bitter acids as antimicrobial ruminant feed additives (Flythe et al.). The bitter acids are potent antimicrobials, and, depending on the diet, the benefits include decreased rumen ammonia, acidity, methane production, and improved average daily gain.

There is a great need for antimicrobials in modern animal agriculture, and many other non-forage plants also contain antimicrobial compounds that are beneficial in terms of animal growth, waste product reduction, or the suppression of pathogens. It is common for compounds to be extracted to produce essential oils, which are the subject of review by O'Bryan et al. Animal industries are challenged with ensuring the safety of their food products, and antimicrobials play a role in food safety strategies. The plethora of plant essential oils offers alternatives to clinically important antibiotics. However, the mechanisms of action must be understood to maximize the utility of compounds. O'Bryan et al. describe common physiological targets of essential oils, such as the cell envelope and the respiratory chain, in important food-borne pathogens.

We must be careful not to categorize a plant as strictly beneficial or harmful. Red clover offers a potential mitigation strategy for the harmful effects of tall fescue alkaloids, but red clover can also contain harmful alkaloids (Kagan). Kagan describes the historical and current work on *Rhizoctonia leguminicola*, a fungal pathogen that causes blackpatch in red clover. Infected plants can contain the alkaloids slaframine, which causes profuse salivation, and swainsonine, which causes neurological problems. The review offers several risk-reduction strategies for blackpatch and slobbers, but little is known about *R. leguminicola* and the associated toxicosis (Kagan).

Furthermore, we must be careful not to categorize even a particular plant or a fungal compound as strictly beneficial or harmful. Some fungal and plant secondary metabolites have multiple biological effects. Isoflavones, for example, are antimicrobial, antioxidant, and improve blood flow, but they are also estrogenic (Shappell et al.). The estrogenic activity is beneficial to ruminant growth and blood flow. The isoflavones in soy hulls fed to steers led to an increased estrogenic activity in the blood and improved performance beyond estradiol-implanted steers (Shappell et al.). However, decreased reproductive success has long been noted in animals on high-isoflavone diets. Thus, different recommendations regarding isoflavones might be made for stocker calves versus cow herds.

One of the most well-known harmful plant metabolites is nitrate. Forages can accumulate nitrate, which is reduced to nitrite by rumen bacteria (Anderson et al.). The nitrite is absorbed by the host and binds hemoglobin to form methemoglobin and decreases its ability to carry oxygen in the blood. Anderson et al. point out that even ingested nitrate might have desirable outcomes. It was previously determined that exogenous nitrate or nitro-compounds could reduce rumen methane, a major greenhouse gas. Anderson et al. showed that forages that naturally contained nitrate or nitro-compounds reduced methane production by rumen microorganisms by as much as 87%. It is unclear if nitrate and nitro-rich forages can be applied as a methane mitigation technology without risk to the animals.

It has long been clear that the impacts of plants on food animals go beyond nutrition *sensu stricto*. The harm or benefit of a plant or a fungal secondary metabolite depends on many factors.

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REVIEW
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Hops (*Humulus lupulus* L.) Bitter Acids: Modulation of Rumen Fermentation and Potential As an Alternative Growth Promoter

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Antibiotics can improve ruminant growth and efficiency by altering rumen fermentation *via* selective inhibition of microorganisms. However, antibiotic use is increasingly restricted due to concerns about the spread of antibiotic-resistance. Plant-based antimicrobials are alternatives to antibiotics in animal production. The hops plant (*Humulus lupulus* L.) produces a range of bioactive secondary metabolites, including antimicrobial prenylated phloroglucinols, which are commonly called alpha- and beta-acids. These latter compounds can be considered phyto-ionophores, phytochemicals with a similar antimicrobial mechanism of action to ionophore antibiotics (e.g., monensin, lasalocid). Like ionophores, the hop beta-acids inhibit rumen bacteria possessing a classical Gram-positive cell envelope. This selective inhibition causes several effects on rumen fermentation that are beneficial to finishing cattle, such as decreased proteolysis, ammonia production, acetate: propionate ratio, and methane production. This article reviews the effects of hops and hop secondary metabolites on rumen fermentation, including the physiological mechanisms on specific rumen microorganisms, and consequences for the ruminant host and ruminant production. Further, we propose that hop beta-acids are useful model natural products for ruminants because of (1) the ionophore-like mechanism of action and spectrum of activity and (2) the literature available on the plant due to its use in brewing.

Keywords: antimicrobial growth promoter, phytochemicals, plant secondary metabolites, rumen microbiology, feed efficiency, alternatives to antibiotics

The purpose of this review is to collect and reexamine experiments that evaluated bitter acids from the hops plant (*Humulus lupulus* L.) as modifiers of rumen microbiology. These experiments were largely performed and reported over the last decade. However, historical work is drawn upon for context and for the origins of hypotheses. The thesis of the review is that the effects of bitter acids on rumen bacteria are similar to the effects of ionophore antibiotics, which have been used in ruminant nutrition for many years. This similarity and the vast body of current and historical literature on the hops plant make it an ideal model among rumen-active plant secondary metabolites. We have encountered a number of natural products researchers interested in microbiological uses of the bitter acids, but unfamiliar with rumen microbiology and its role in ruminant nutrition. Likewise, there are many ruminant scientists who are unfamiliar with the plant and its biochemistry. Both of these

groups are the intended audience. Therefore, the review includes introductions to rumen microbiology and the hops plant.

INTRODUCTION TO RUMEN FUNCTION

The rumen is the distinguishing adaptation of the ruminant animal. This first chamber of the digestive tract is, in some respects, more like an intestine than a gastric stomach (1). The ideal pH of the rumen is neutral, and it does not make the copious mucus produced by a gastric stomach to protect it from pepsin and acidic conditions. Like an intestine, the rumen epithelium absorbs certain nutrients, and it also serves as habit for a dense community of phylogenetically diverse microorganisms (2, 3). Unlike the microorganisms in the lower digestive tract, those found in the rumen gain metabolic access to the feed before the animal host. The trait of rumen microorganisms having first access to the feed has adaptive value because they in turn give the host metabolic access to fiber (1). Ruminants, like other mammals, do not make enzymes to catabolize cellulose and hemicellulose. However, the fibrolytic bacteria and fungi break down these fibers to the constituent sugars. The sugars are fermented and the fermentation acids can be absorbed through the rumen epithelium.

Fiber digestion is only one lifestyle of rumen microorganisms. Each feed component is a possible growth substrate, and thus, an ecological niche. A group of related niches are collectively called a *guild*, and it is in terms guilds or functional groups that we usually consider rumen microorganisms. In addition to the fibrolytic or cellulolytic bacteria, there are also those that utilize starch, pectin, or simple sugars to produce fermentation acids (3). Amylolytic bacteria can cause rumen acidosis when the dietary concentration of starch or water-soluble carbohydrates is too great (2). Many predominant amylolytic bacteria, such as *Streptococcus bovis*, exhibit homolactic fermentation. An excess of starch results in accumulation of lactic acid; the rumen pH declines; fiber digestion slows, and the animals develop problems ranging from feed refusal to rumen ulceration and death. Sub-acute rumen acidosis is a major problem in modern dairy operations.

Under normal conditions lactate production plays an important role in the rumen ecosystem. Some bacteria specialize in the utilization of lactic or succinic acid. A notable member of the lactate-utilizing guild is *Megasphaera elsdenii*, which converts lactic acid to propionic acid. Lactate and succinate fermentation to propionate is another essential function of the rumen microbiota because the propionate is absorbed and converted to glucose by the host (2–4). It is now known that *M. elsdenii* is also involved in the bioconversion of dietary fats, including production of conjugated linoleic acid (5, 6). However, *Anerovibrio lipolytica* and other bacteria are the major lipolytic species (7).

Protein catalysis by rumen bacteria can be compared and contrasted with fiber catalysis. Like cellulose or starch, proteins are polymers. Protozoa consume intact proteins in feed particles, but bacteria must depolymerize protein and transport the resulting peptides or amino acids. Like all organisms, some form of nitrogen is required for anabolism by bacteria, and many rumen microorganisms express proteinases to this end. *S. bovis* is proteolytic and the growth rate is fastest when free amino acids are available, even though it can also assimilate ammonia (8).

There are also rumen microorganisms that catabolize amino acids for energy (**Figure 1**). Protozoa and some Gram-negative bacteria, like *M. elsdenii*, can utilize amino acids and produce ammonia. However, a particular group of rumen bacteria are known for exceptional rates of ammonia production *via* fermentation (i.e., deamination of peptides and free amino acids). This guild of amino acid-fermenters is termed the hyper-ammonia-producing bacteria, HAP or HAB (9). Some of the best-studied HAB are non-proteolytic. Like the saccharolytic bacteria, which metabolize sugars only after other bacteria degrade the polymer, many of the HAB ferment amino acids only after other members of the microbial community depolymerize protein (10). A major difference between catalysis of fiber and protein is that the ruminant host does not need the rumen microflora to utilize protein. Feed protein that is not depolymerized and deaminated is digested in the abomasum and absorbed in the small intestine. Such feed protein that escapes ruminal degradation has been termed *bypass protein*, and it is associated with increased weight gain and feed efficiency (11). Some of the ammonia produced by HAB and other microorganisms is assimilated into microbial protein that can be digested by the host. Excess ammonia is lost.

Certain characteristics are true of all fermentations regardless of the substrate. All fermentations have optimal temperatures, water availabilities, and pH values. The ruminant host helps to meet these needs for its fermentative rumen microbiota. Another requirement of all fermentation is one or more terminal electron acceptors so that reducing equivalents, *i.e.*, NAD^+ and $NADP^+$, can be recovered (12). Hydrogen gas (H_2) is one of the most important terminal electron acceptors in rumen bacteria. An example of an H_2-producting pathway is the so called *clostridial fermentation*, in which ATP is generated by acetate- or butyrate-kinases and NAD^+ is regenerated with hydrogenases (12). Bacterial hydrogenases are notoriously subject to end-product inhibition, but the H_2 is removed by a cross-feeding mechanism that was termed interspecies hydrogen transfer (3, 13). The hydrogen transfer is actually inter-domain because the H_2-utilizing guild is composed of methanogenic *Archaea*. These methanogens convert H_2 and CO_2 into CH_4. Other methanogens produce CH_4 from acetate. Eructation of the gases is the hosts' role in recovery of reducing equivalents (1). Like ammonia production, some methane production is necessary for rumen microbial ecology, but both products represent matter lost from the system.

RUMEN OPTIMIZATION HYPOTHESIS AND ANTIMICROBIAL GROWTH PROMOTERS

When we acknowledge that the microbial activity in the rumen constitutes a natural fermentation, we engender the hypothesis that it can be optimized like any industrial fermentation (3). Applied microbiology has been used since the mid-twentieth century to improve ruminant health and productivity *via* manipulation of the rumen fermentation. As mentioned above, adding starch can decrease the pH of the rumen. Conversely, pH can be increased

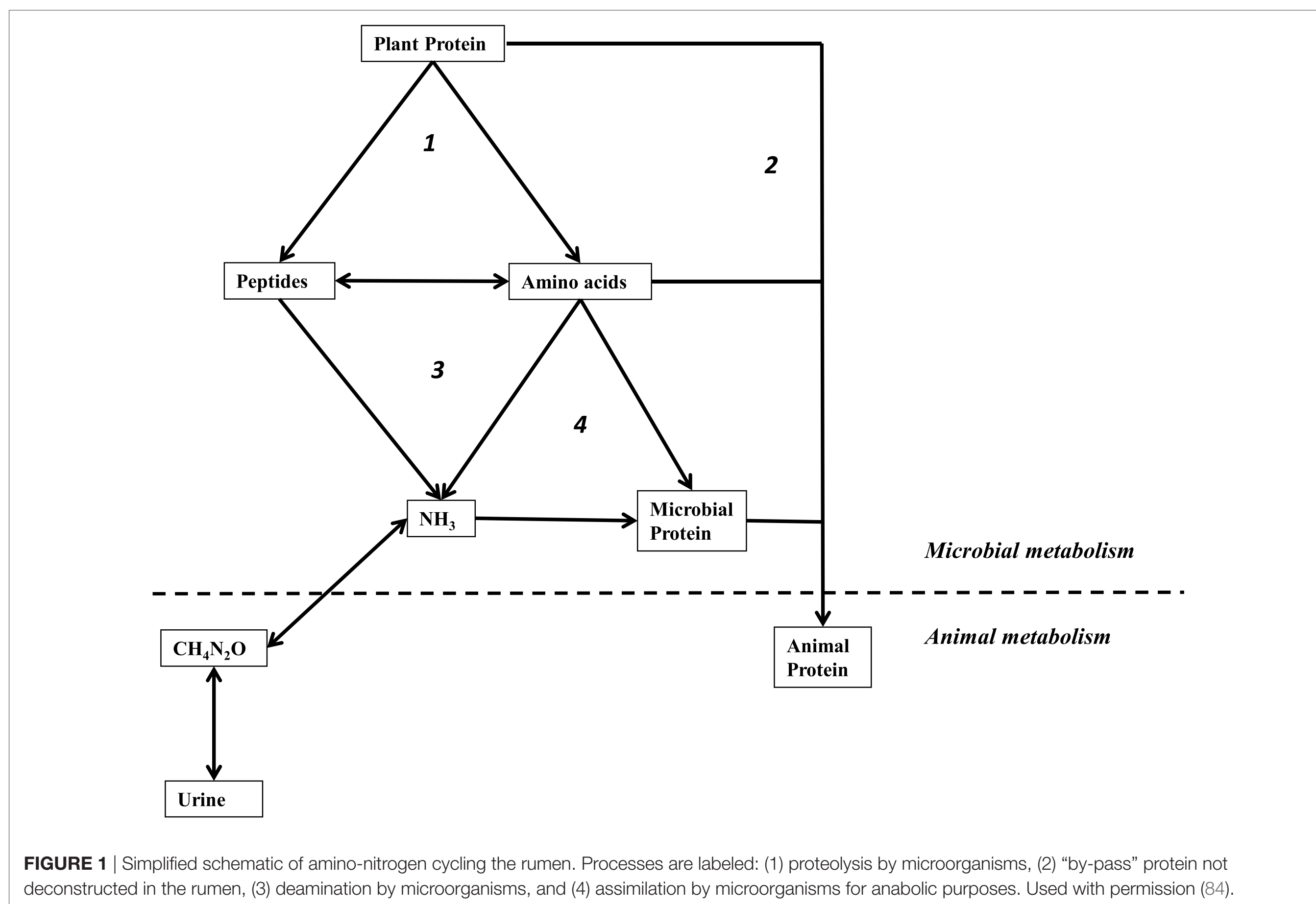

FIGURE 1 | Simplified schematic of amino-nitrogen cycling the rumen. Processes are labeled: (1) proteolysis by microorganisms, (2) "by-pass" protein not deconstructed in the rumen, (3) deamination by microorganisms, and (4) assimilation by microorganisms for anabolic purposes. Used with permission (84).

by adding buffers to the diet (2). Because the rumen is a complex, polymicrobial fermentation, it can also be influenced through the use of antimicrobials. Antimicrobial growth promoters that are administered as feed additives are among the greatest successes to date (14).

Consider the effects of antimicrobial growth promoters in terms of *selective inhibition* and *compensatory product formation*. Selective inhibition is inhibition of specific physiologies. Compensatory product formation is a change in the amount or composition of metabolic products due to selective inhibition. Compensatory product formation can occur in pure cultures as a result of altered physiology. An example outside of the rumen is the cellulolytic bacterium *Clostridium thermocellum*, which converts cellulose into acetate, formate, lactate, ethanol, and H_2 (15). When the hydrogenases are inhibited by methyl viologen, ethanol, produced by dehydrogenases as an alternative route of NAD^+ recovery, increases (16). In the case of a pure culture of *C. thermocellum*, it is only the physiology of the culture that is affected. However, the effects of selective inhibition can be ecological as well as physiological. Another example apart from the rumen is industrial ethanol production by yeast. *Saccharomyces cerevisiae* produces the ethanol, but lactic acid bacteria on the feedstock can also produce lactate. Adding an antibiotic, such as virginiamycin, selectively inhibits the growth of lactic acid bacteria, and ethanol production is enhanced. This can be considered an ecological effect because diversity within the fermenter decreases. The rumen and other gastrointestinal habitats are characterized by rapid and continuous flow of matter through the system. Selective inhibition of a metabolic pathway can cause a rapid change in the fitness of the affected organisms. Thus, physiological- and ecological-selective inhibitions are effectively synonymous in the case of the rumen.

The points for optimization of rumen fermentation are end products that exit the rumen. Products leaving the rumen can be divided into two categories according to the usefulness to the host, waste products, and nutrients. Waste products are potential targets for selective inhibition and nutrients are the desired end products of compensatory product formation. An antimicrobial that has a favorable impact on rumen fermentation should selectively inhibit the waste products and compensate in nutrient production. The most widely used and most thoroughly studied ruminant antibiotic growth promoter is the polyether antibiotic, monensin. Ionophores, such as monensin, selectively inhibit members of several guilds of microorganisms and metabolic processes that they carry out (17).

Monensin is known in the ruminant industries as a coccidiostat, but it is also an inhibitor of methanogenesis (18). The gas eructated by a ruminant is composed of waste products, CO_2 and CH_4. While these are necessary end products of the rumen fermentation, they also represent mass lost from the system, and are obvious targets for optimization. Additionally, CO_2 and CH_4 are greenhouse gases, and the US Environmental Protection Agency

indicates that CH_4 has a global warming potential as much as 36 times greater than CO_2 over a 100-year period (19). Including monensin in the diet decreases CH_4 production by as much as 25% (20). Methanogens vary in their sensitivity to monensin (18), and one well-studied species, *Methanobrevibacter ruminantium*, is not sensitive (21). However, CH_4 production can also be decreased by inhibition of monensin-sensitive H_2-producing bacteria and protozoa because less H_2 is available for interspecies hydrogen transfer (17, 18).

Early research identified that monensin and other ionophores increased the ratio of propionic to acetic acid (22). All of the major VFA and amino acids can serve as energy sources, but propionate is the most rapidly utilized by the liver for either oxidation or gluconeogenesis (4). Lactate and succinate are the substrates for propionate production, and both metabolic pathways involve dehydrogenases and the reduction of reducing equivalents (12). Thus, propionate is an alternative electron sink and a compensatory product of CH_4 inhibition. It has been proposed that the shift from a methanogenic to a propionic electron sink is governed by the sensitivity of rumen methanogens to acidic pH, which would explain the shift in acetate: propionate when cattle are switched from a forage-based to a grain concentrate-based diet (22). The pH-based explanation of compensatory propionate production is consistent with the mechanism of action of ionophores (described below). However, it is also important to note that known propionate-producing bacteria, such as *M. elsdenii* and *Selenomonas ruminantium*, are members of Class *Negativicutes*, known for their outer membranes (23). The outer membrane of these Gram-negative species confers insensitivity to ionophores (24). We would expect ionophores to select for these propionate-producers even if reducing equivalent disposal were not considered.

Nitrogenous waste is another target for selective inhibition (**Figure 1**). It has long been recognized that ionophores also inhibit rumen amino acid degradation (18). However, prior to the discovery of the HAB, all known amino acid-fermenting bacteria (e.g., *M. elsdenii*) were Gram-negative and ionophore-insensitive (9, 24). Most of the characterized HAB are members of Order *Clostridiales* with classical, Gram-positive cell envelopes that render them susceptible to ionophores (9, 25–28). The ciliates, the other major ammonia producers, are also inhibited, but there is evidence that they adapt to monensin (29). When HAB and other ammonia producers are inhibited, the rate of free amino acid and peptide catabolism is decreased, and more amino-nitrogen is available for the host to absorb in the small intestine (9). As previously mentioned, protein, peptides, and amino acids that escape rumen degradation have been called *bypass protein*, and are associated with increased weight gain and feed efficiency.

Antibiotic growth promoters have been very important tools in ruminant production for decades. In 1989, Russell and Strobel (17) estimated that ionophores alone were responsible for a feed savings of 560,000,000 USD. A more recent estimate by Capper and Hayes (30) indicates abolishing antibiotics and other growth promoting technologies would increase production costs by 9.1%. Moreover, they and others point to the environmental benefits (i.e., decreased carbon and nitrogen emissions) when growth promoters are used (18, 30). However, a considerable body of evidence now indicates that growth promoting and veterinary uses of antibiotics contributes to antibiotic-resistant bacteria in food animals (31–33). These concerns are compounded by the spread of antibiotic-resistant food borne pathogens and the presence of antibiotic residues in compost and fertilizer from animal operations (34, 35). Clearly, it is in our best interest to minimize the use of clinically important antimicrobials while maintaining, or even expanding, the benefits of growth promoting technologies.

Researchers have proposed a variety of alternative antimicrobials as ruminant growth promoters. The candidate compound should have an antimicrobial mechanism of action dissimilar to clinically important antibiotics. Ionophores fit the mechanism of action criterion, and there is evidence that ionophore-resistant bacteria are not typically resistant to other classes of antibiotics (36). A study by Simjee and co-workers (37) also indicated that monensin-resistance is not highly heritable. However, the acute toxicity of monensin to humans and horses makes it a perennial concern (36, 38). The purpose of this review is to consider secondary metabolites from the hops plant (*Humulus lupulus* L.), as feed antimicrobial growth promoters. A variety of rumen-active phytochemicals have been considered as feed additives, and many have merits (39). We believe that hops secondary metabolites, particularly the beta-acids (lupulone and its derivatives; see **Figure 2**, structure 2a–e), have a special role as model rumen-active phytochemicals. The basis for this assertion is the considerable body of literature available on the plant and compounds and the spectrum of activity and mechanism of action, which are similar to feed ionophores. Information on hops essential oils and prenylated flavonoids is provided as well in the next section to illustrate the diversity present in the plant.

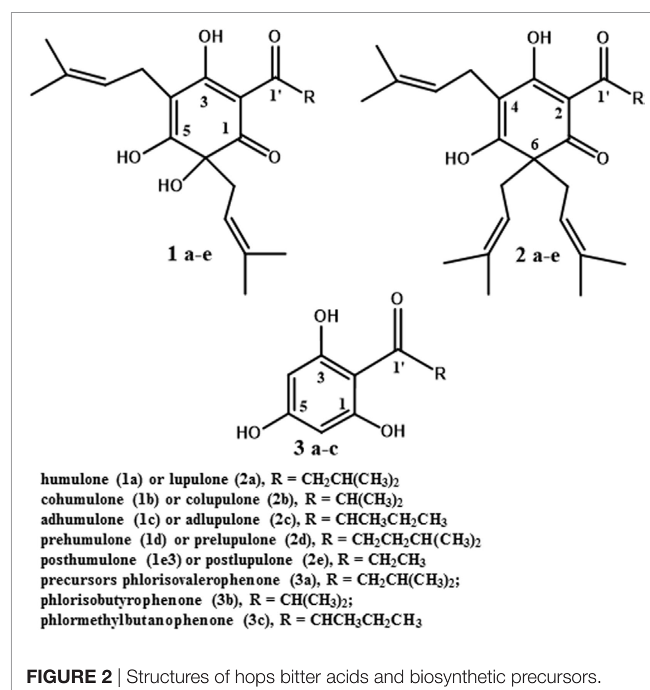

FIGURE 2 | Structures of hops bitter acids and biosynthetic precursors.

TAXONOMY AND DISTRIBUTION OF HOPS

Humulus lupulus L. is a member of the family Cannabaceae, which also contains the genus *Cannabis* (40). It is a dioecious vine, with male and female flowers growing on separate plants (40, 41). Hops is indigenous to the Northern hemisphere, although it is grown in both hemispheres (42). The species variety native to Europe is *H. lupulus* var. *lupulus*, and four other species varieties have been described based on native distribution and morphology: *H. lupulus* var. *cordifolius* (Miquel) Maximowicz, a native of Japan and possibly of parts of mainland Asia; *H. lupulus* var. *neomexicanus* Nelson and Cockerell (native to western North America); *H. lupulus* var. *lupuloides* E. Small (native to eastern and central North America); and *H. lupulus* var. *pubescens* E. Small (native to the midwestern US) (42). Some commercial cultivars are the results of crosses between native plants from different continents (42), and some wild populations may be the result of introducing plants into an area and letting them grow wild (43). Wild populations in various regions have been studied because of their potential value as a source of germplasm for commercial hops cultivation (44–46), or because comparisons of morphological or chemical traits can provide information on relationships among populations (47, 48).

LOCALIZATION AND ACCUMULATION OF HOPS SECONDARY METABOLITES

Only the mature female inflorescences (cones) of hops are used in the beer brewing industry (40). The female cones, which develop over a few weeks after flowering, consist of clusters of bracts subtended by bracteoles, all grouped around a central axis (49), also referred to as a central rachis (50). On these bracts and bracteoles are glandular trichomes (51), which have been described both as distinct from lupulin glands (52) and as including both lupulin glands (also called peltate trichomes) and the smaller bulbous trichomes (53). Trichomes are present on leaves as well (53). They contain secondary metabolites such as prenylated flavonoids (54), essential oils (mono- and sesquiterpenes; 53), and bitter acids (55). This latter class of secondary metabolites includes the α-acids (humulone and derivatives, **Figure 2**, structures *1a–e*) and the β-acids (lupulone and derivatives, **Figure 2**, structures *2a–e*). Bitter acids and prenylated flavonoids have been found at lower concentrations in male than in female inflorescences (56). Some of the secondary metabolites of hops are found in other tissues of female plants besides trichomes. A couple of sesquiterpenes are found in leaves and flowers (51), and prenylated flavonoids xanthohumol and desmethylxanthohumol (**Figure 3**, structures *4a* and *4b*) are at low concentrations in leaves and immature cones (55). Lipophilic compounds like mono- and sesquiterpenes (51), and bitter acids and prenylated flavonoids (55), are most abundant in trichomes.

The changing concentrations of prenylated flavonoids, essential oils, and bitter acids in mature trichomes indicate that hops secondary metabolite content is closely related to trichome maturity. Extraction of bitter acids and essential oils from hop cones over a few weeks revealed that both types of compounds increased during this period (52). Extraction of bitter acids from individual trichomes revealed a similar trend of increased bitter acid concentration with increasing maturity (57). In those studies, electron microscopy of trichomes has demonstrated that while trichomes are initially concave, they gradually fill out as bitter acid concentrations increase (52, 57), and the alpha-acid content of trichomes is positively correlated with trichome volume (50).

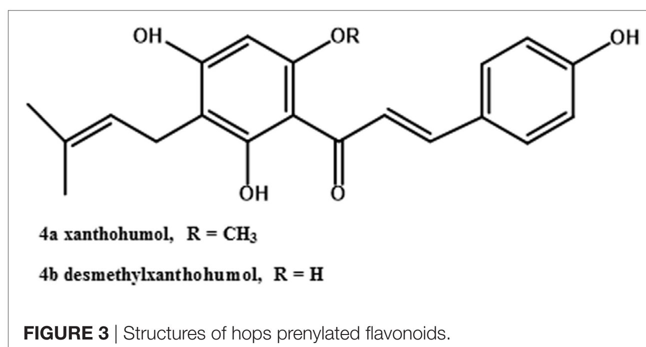

FIGURE 3 | Structures of hops prenylated flavonoids.

BIOSYNTHESIS OF BITTER ACIDS AND PRENYLATED FLAVONOIDS

The genes, enzymes, and intermediates of hop bitter acid and prenylated flavonoid biosynthesis have been studied extensively, due to interest in manipulating their production for brewing purposes. Precursors of the moieties comprising bitter acids, and the associated enzymes that have been identified, are listed in **Table 1**. Some of the earlier studies (58) demonstrated that feeding ^{14}C-labeled acetic acid to cone-bearing hops plants led to isolation of ^{14}C-labeled humulone, lupulone, and colupulone (**Figure 2**, structures *1a*, *2a*, and *2b*, respectively), indicating an acetate precursor. Because acetate can be converted into acetyl-CoA, which can react with carbon dioxide to form malonyl-CoA, a precursor of some aromatic compounds (59), a role for acetate agrees with later findings (60) that the 6-carbon ring and acyl side chain at C-2 (**Figure 2**, structures *1* and *2*) are formed through the biosynthesis of an acylphloroglucinol nucleus (**Figure 2**, structures *3a* to *3c*). A crude enzyme extract from flowers or cones, incubated with malonyl-CoA (likely precursor of the 6-carbon ring) and isovaleryl-CoA or isobutyryl-CoA (likely precursors of the acyl side chain at C-2 in **Figure 2**), catalyzed the reactions producing the acylphloroglucinol compounds phlorisovalerophenone (**Figure 2**, structure *3a*), and phlorisobutyrophenone (**Figure 2**, structure *3b*) (60). Structure *3a* is a likely precursor of lupulone and humulone (**Figure 2**, structures *2a* and *1a*, respectively), and structure *3b* is a likely precursor of colupulone and cohumulone (**Figure 2**, structures *2b* and *1b*, respectively) (60). The purification of the biosynthetic enzyme, phlorisovalerophenone synthase (VPS), from trichomes has been described (61), as have the cloning of the gene and its trichome-specific expression (62). *In situ* hybridization of *VPS* RNA in trichomes demonstrated that *VPS* gene expression occurs only

TABLE 1 | Biosynthetic precursors and enzymes confirmed for lupulone or humulone and their derivatives.

Moiety	Precursor	Enzyme(s) involved	Reference
6-carbon ring (structures *1* to *3*, **Figure 2**)	Malonyl-CoA	VPS (valerophenone synthase)	(61, 62)
C-2 acyl side chain to structure *3* (**Figure 2**)	Isovaleryl-, 2-methylbutyryl, or isobutyryl-CoA	HlCCL2 and HlCCL4 (carboxyl-CoA ligases)	(55)
Alkyl moiety on C-2 acyl side chain of humulone (structure *1a*, **Figure 2**)	Leucine	Enzymes not characterized in refs. 64 or 66; BCAT1 (branched-chain amino transferase) proposed to convert leucine into a precursor of isovaleryl-CoA	(64–66)
Prenyl group on C-4 of structures *1* to *3* (**Figure 2**)	Deoxyxylulose-5-phosphate in plastidial isoprenoid pathway	Enzymes not characterized in ref. 66; HlPT1 (prenyltransferase) prenylates C-4	(63, 66, 70)
Prenyl group on C-6 of humulone	Deoxyxylulose-5-phosphate in plastidial isoprenoid pathway	Enzymes not characterized in ref. 66; HlPT2 (prenyltransferase) prenylates C-6	(63, 66, 71)
Oxygen on C-6 in humulone and cohumulone (**Figure 2**, structures *1a* and *1b*)	Molecular oxygen	Oxygenase; enzyme not characterized	(72)

*These have not been confirmed for all the bitter acids listed in **Figure 2**.*

during a late stage of trichome development (53), supporting the relationships between bitter acid accumulation and trichome maturity described above.

Work has been done to determine the precursors of the acyl side chain at C-2 of α- and β-acids. The alkyl moiety at C-1′ (the R group in structures *1* to *3* of **Figure 2**) appears to be derived from the carbon backbone of aliphatic amino acids, and feeding studies with ^{14}C-labeled leucine and isoleucine led to incorporation of isoleucine into 2-methylbutyrate (a likely precursor of compounds *1c* and *2c*), and of leucine into isovalerate and lupulone (64). Gene expression studies revealed that among the genes expressed strongly in hop trichomes were those encoding branched-chain aminotransferase enzymes for the biosynthesis and catabolism of branched-chain amino acids (leucine, valine, and isoleucine) (65). The trichomes were also the tissues highest in isovaleryl-, isobutyryl-, and 2-methylbutyryl-CoA, which are derived from the breakdown products of leucine, valine, and isoleucine, respectively (65). Xu et al. (55) identified and cloned some hops carboxyl-CoA ligase genes, including two (*HlCCL2* and *HlCCL4*) that were expressed most strongly in mature cones or trichomes. They encoded enzymes catalyzing conversion of metabolites of isovalerate, isobutyrate, and 2-methylbutyrate into their corresponding CoA esters (55). When *HlCCL2* was co-expressed in yeast with a *VPS* gene, structure *3a* was produced (**Figure 2**), and coexpression of *VPS* and *HlCCL4* produced structures *3b* and *3c* (**Figure 2**) (55). Structure *3c* is a probable precursor of adlupulone and adhumulone (**Figure 2**, structures *2c* and *1c*, respectively).

The prenyl side chains at carbons 4 and 6 of humulone (**Figure 2**, structure *1*) is synthesized from glucose *via* the plastidial isoprenoid pathway (66), in which deoxyxylulose-5-phosphate is the precursor of the isopentenyl-pyrophosphate (IPP) or dimethylallylpyrophosphate (DMAPP) building block of isoprenoids (67). The biosynthetic origin of lupulone prenyl side chains does not appear to have been determined. In agreement with the humulone labeling and NMR studies, expressed sequence tags (ESTs) of the plastidial isoprenoid pathway were found in cDNA libraries from hops trichomes (51, 54), but few ESTs (54) or none (51) were present from the cytosolic isoprenoid pathway, which is characterized by a mevalonate precursor to DMAPP and IPP (67).

The prenyl side chains are added to bitter acids by prenyltransferases. Incubation of structures *3a* and *3b* with DMAPP and a crude enzyme extract from hops trichomes led to formation of mono- and diprenylated versions of structures *3a* and *3b* (63), confirming the presence of prenyltransferase enzyme activity in the trichomes. Incubating this crude extract with deoxyhumulone (**Figure 2**, structure *1a* minus the -OH group at C-6) led to some production of humulone, indicating that deoxyhumulone might be a precursor (68). A prenyltransferase gene (*HlPT1*, catalyzing the transfer of a prenyl group to an aromatic nucleus) was identified in a hops trichome cDNA library and found to be expressed most strongly in the trichomes of young cones (69). Assays of the expressed prenyltransferase revealed that it was capable of only one prenylation step, namely the addition of the prenyl group at C-4 of the structures in **Figure 2** (70). Therefore, additional enzymes are needed to complete the biosynthesis of α-acids (two prenyl groups) and β-acids (three prenyl groups) (70). A similar gene (*HlPT1L*), as well as an additional gene (*HlPT2*, encoding the prenyltransferase catalyzing transfer of the additional prenyl groups to alpha- or β-acids), were cloned from hops trichomes (71). When both genes were expressed in yeast, along with the *HlCCL2*, *HlCCL4*, and *VPS* genes, β-acids were produced, as well as various other prenylated acylphloroglucinols (71). No α-acids were produced in this yeast expression system, indicating that additional enzymes were needed to convert deoxyhumulone or related compounds into humulone and its derivatives (71). A late-stage hydroxylation of C-6 of structure *1* is supported by the determination that $^{18}O_2$, when fed to whole hop plants, is incorporated only into the oxygen atom bonded to C-6 of humulone and cohumulone (72).

Biosynthesis of xanthohumol (**Figure 3**, structure *4a*), the most abundant prenylated flavonoid in hops (73), involves both the phenylpropanoid and isoprenoid biosynthetic pathways. Many phenylpropanoid biosynthetic genes were present in a hop trichome cDNA library (54). A chalcone synthase cDNA from hop cones was incubated with *p*-coumaroyl CoA and produced naringenin (74). A prenyltransferase capable of prenylating

acylphloroglucinols was also capable of prenylating naringenin chalcone (the precursor of naringenin), producing desmethylxanthohumol (**Figure 3**, structure *4b*) (70). Methylation of desmethylxanthohumol to produce xanthohumol was achieved in the presence of an *O*-methyltransferase cloned from hop trichomes (54).

FACTORS AFFECTING CONCENTRATIONS OF SOME HOPS SECONDARY METABOLITES

Concentrations of bitter acids (44, 56, 75), prenylated flavonoids (56), and essential oils (44) can vary within cultivars or populations from year to year. The year-to-year variation may be due to differences in temperature or precipitation because De Keukeleire et al. (56) observed generally higher bitter acid concentrations in a year with a wet summer, and lower concentrations in a year with a hot summer. Despite environmentally influenced fluctuations, secondary metabolite concentrations tend to stay within certain ranges for a given genotype, indicating genetic as well as environmental effects. Cultivated hops are categorized in the brewing industry by their bitter acid and essential oil content. Aroma hops contain a maximum of 5–7% w/w α-acids and <1% w/w essential oil, while bitter hops contain 7–10% w/w α-acids and 1–2% w/w essential oil, and high-α hops contain over 10% w/w bitter acids and 1.5–3% w/w essential oil (75). The ratios of α- to β-acids in these cultivar classes tend to increase in that same order (<1–2 for aroma hops, 2 for bitter hops, and 2–3 for high-alpha hops) (75). Wild populations of hops tend to have bitter acid concentrations on the order of that observed for aroma or bitter hops (46). For example, in a survey of 22 wild Italian hops populations, the α-acid content was 1.7–7.3%, and the β-acid content was 1.2–3.9%, with only two populations having an α- to β-acid ratio greater than 2 (46). In a survey of wild hops populations from the Czech Republic, Switzerland, France, and Russia, the α- to β-acid ratio was below 1.5 for all (44).

Because secondary metabolite production is partly under genetic control, types and concentrations can also help to identify geographic origins. Wild European and North American hops can be distinguished from each other by their relative amounts of certain essential oils and bitter acids (45). Wild hops from the southwestern United States differ from other North American hops in lacking several 4′-*O*-methylchalcones structurally similar to xanthohumol, but with different placement or number of methyl groups (48). Types and amounts of secondary metabolites can also serve as a fingerprint to identify individual cultivars. Concentrations of essential oils (76, 77) or non-prenylated flavonoids (76) have been used for this purpose. A dichotomous key to seven Czech cultivars was constructed based on concentration or presence of selected essential oils, flavonoids, and other phenolic compounds (78). Another study of Czech hops cultivars (79) classified them according to bitter acid, prenylated flavonoid, total phenolic and flavonoid, essential oil, antioxidant, and proteinase inhibitory activities. This type of clustering based on multiple parameters may help to identify cultivars with qualities relevant to other industries besides brewing.

HOPS: A SOURCE OF "PHYTO-IONOPHORES"

It has long been recognized that the antimicrobial activity of hop secondary metabolites could have value as clinical antiseptics (80). However, the mechanism of action was first elucidated by Teuber and Schmalreck in the 1970s (81). They noted that the most hydrophobic hop resin components were also the most antimicrobial, and hypothesized that those components must be active against the bacterial cell membrane. *Bacillus subtilis* cells were treated with lupulone (a β-acid; **Figure 2**, structure 2a), humulone (an α-acid; **Figure 2**, structure 1a), isohumulone or humulinic acid fractions of hop resins. The secondary metabolites inhibited growth and prevented the transport of radioactively labeled α-methyl-D-glucopyranoside (a sugar) and a variety of amino acids. Those results alone could be the result of membrane leakage or other action on bioenergetics that would stop energy-dependent transport. However, lupulone also caused serine efflux from *B. subtilis* membrane vesicles that were loaded with ^{14}C-serine, which was indicative of membrane leakage.

Some ionophores are highly specific in terms of ion selectivity. Protonophores, such as 3,3′,4′,5-tetrachlorosalicylanilide, transport only protons. Monensin is less selective, but it is generally considered a proton/potassium antiporter because those are the two major monovalent cations in disequilibrium across an energized cell membrane (17). Membrane perturbation induced by hop constituents is even less specific than monensin, but the effects of hop secondary metabolites and feed ionophores were similar enough to warrant comparison.

A RELEVANT SPECTRUM OF ACTIVITY

Feed ionophores and hop bitter acids have similar spectra of activity; i.e., they inhibit the same microorganisms (17, 81). They both inhibit bacteria with a classical Gram-positive cell envelope. *B. subtilis*, which Teuber and Schmalreck employed to determine the mechanism action, is a long-standing Gram-positive model. Hops β-acid, purportedly lupulone, also inhibits important Gram-positive pathogens, such as *C. perfringens* (82). As mentioned above, HAB and other Gram-positive rumen bacteria are sensitive to monensin, but Gram-negative rumen species are not. The Gram-positive spectrum of activity for a mixture of β-acids, consisting of compounds 2a through 2c (**Figure 2**) was shown in pure culture growth experiments (**Figure 4**). The Gram-positive, *S. bovis* was inhibited by β-acids, as it is by monensin (83). When an inhibitor suppresses Gram-positive bacteria, like *S. bovis,* lactic acid production is limited. Decreasing lactate production can ameliorate lactic rumen acidosis (2). *S. ruminantium* and *M. elsdenii* belong to Class *Negativicutes*, and have a Gram-negative cell envelope. The growth of these two bacteria was not inhibited by β-acids [**Figure 4**; (83)]. The propionate-producing *Negativicutes,* like *M. elsdenii,* are not sensitive to monensin, and it is thought that this resistance to ecological perturbation by feed ionophores is responsible for the increased proportion of propionate when ionophores are fed. β-acids, like ionophores, decreased acetate

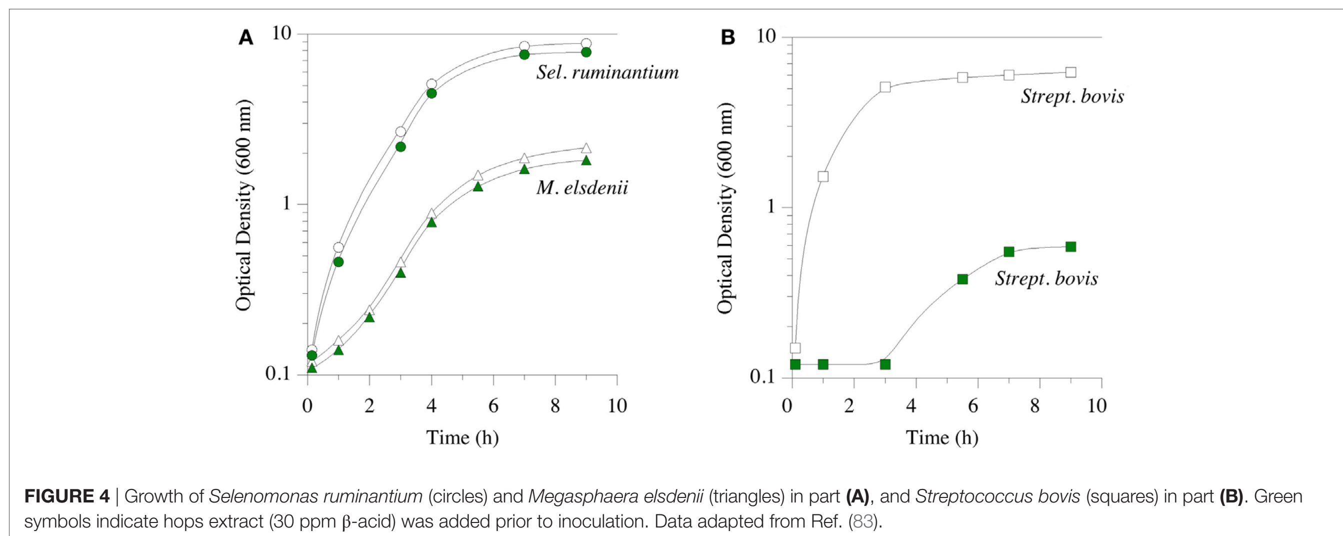

FIGURE 4 | Growth of *Selenomonas ruminantium* (circles) and *Megasphaera elsdenii* (triangles) in part **(A)**, and *Streptococcus bovis* (squares) in part **(B)**. Green symbols indicate hops extract (30 ppm β-acid) was added prior to inoculation. Data adapted from Ref. (83).

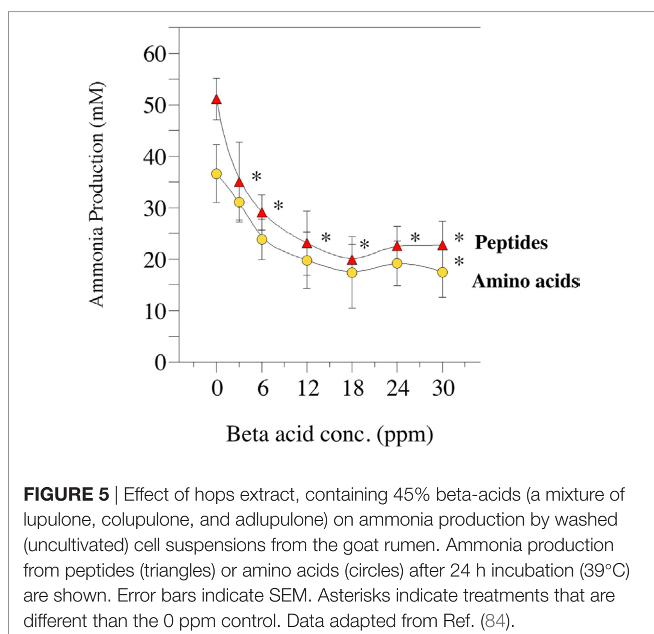

FIGURE 5 | Effect of hops extract, containing 45% beta-acids (a mixture of lupulone, colupulone, and adlupulone) on ammonia production by washed (uncultivated) cell suspensions from the goat rumen. Ammonia production from peptides (triangles) or amino acids (circles) after 24 h incubation (39°C) are shown. Error bars indicate SEM. Asterisks indicate treatments that are different than the 0 ppm control. Data adapted from Ref. (84).

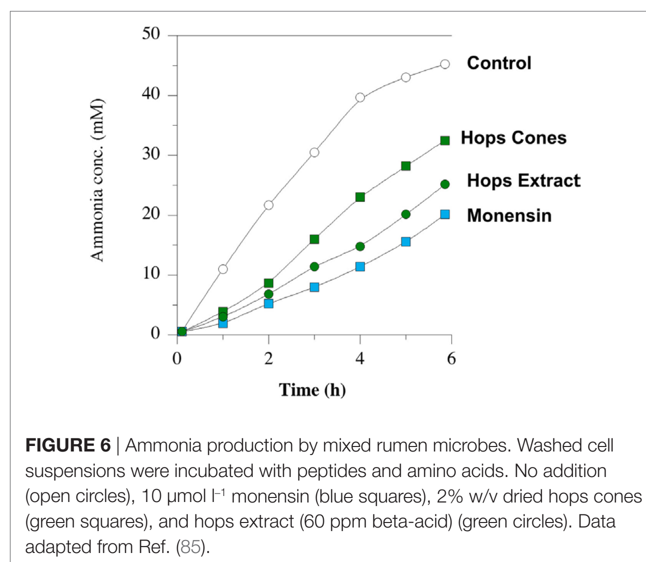

FIGURE 6 | Ammonia production by mixed rumen microbes. Washed cell suspensions were incubated with peptides and amino acids. No addition (open circles), 10 μmol l^{-1} monensin (blue squares), 2% w/v dried hops cones (green squares), and hops extract (60 ppm beta-acid) (green circles). Data adapted from Ref. (85).

production by washed cell suspensions of uncultivated, mixed rumen microorganisms (hereafter called *washed cell suspensions*) without decreasing propionate (83).

Amino acid degradation can also be evaluated using washed cell suspensions. **Figure 5** shows the effect of β-acid concentration on ammonia production from either free amino acids or peptides (84). **Figure 6**, on the other hand, compares inhibition of ammonia production by a β-acid-rich extract to unprocessed hops cones and monensin (85). It is important to note that the substrates in these experiments were free amino acids and peptides, not protein. However, Lavrenčič and colleagues (86) used similar *in vitro* assays to show that two different hops varieties could also decrease proteolysis by rumen microorganisms, which could include proteolytic bacteria or ciliate protozoa. Hops secondary metabolites, like feed ionophores, inhibit both the proteolysis and amino acid fermentation stages of rumen protein degradation.

The advantage of washed cell suspension and similar *in vitro* fermentations is that the microorganisms can include the full diversity that is in the rumen, not just a few laboratory models. However, absorption by the host does not confound the measurements. Thus, washed cell suspensions show the net metabolic outputs of the rumen microbial community. Van Nevel and Demeyer first discovered the effects of monensin on rumen microbiology using *in vitro* mixed rumen microorganisms (18).

Three varieties of hops cones have also been tested in a continuous *in vitro* rumen fermentation system (87, 88). Like washed cell suspensions, this system starts with uncultivated rumen microorganisms, but continuous systems allow adaptation over time. In this case, Gram-positive bacteria, such as *S. bovis*, were inhibited and the proportion of propionate to acetate increased. The number of methanogens and methane production decreased in the presence of hops, which would be an expected result with feed ionophores. Indeed, similar results were observed when monensin was used in the same fermentation system (88).

We can see that the spectrum of activity of hop β-acids is like that of feed ionophores because: (1) Gram-positive rumen bacteria are inhibited, (2) Gram-negative rumen bacteria are insensitive, (3) the shift in fermentation acid production is like ionophores, and (4) proteolysis, ammonia production, and methane production decrease.

RELATIONSHIP TO pH AND IMPACT ON TRANSMEMBRANE GRADIENTS

The most important feature for a putative antimicrobial rumen modifier is the spectrum of activity, but the spectrum is largely dictated by the mechanism of action. Based on the early work with *B. subtilis*, it was a reasonable hypothesis that β-acids would disrupt the membrane integrity of Gram-positive rumen bacteria (85). A key feature of a proton-transporting ionophore's effect on cell membranes is that the ionophore becomes more potent as the pH decreases (89). There are several ways to determine increased efficacy at acidic pH including lower minimum inhibitory concentrations or steeper time-kill curves. **Figure 7** simply shows the effect of increasing concentrations of the previously mentioned β-acid mixture on the viable number of three HAB pure cultures at neutral and acidic pH [**Figure 7**; (85)]. These three Gram-positive bacteria were all sensitive at neutral pH at β-acid concentrations between 3 and 30 ppm. Decreasing the pH from 6.7 to 5.6 decreased the viable numbers in all cases. In some cases, pH made the difference between a bacteriostatic effect, in which the bacteria simply do not grow, and a bactericidal effect, in which the cells are killed.

The effects of a putative ionophore on membrane bioenergetic parameters can also be measured. The effects of mixed β-acids on intracellular pH and intracellular potassium are shown in **Figure 8** (85). The test organism was *Clostridium sticklandii*, and it was maintaining a transmembrane pH (ΔpH) gradient of approximately 1.0 pH units (intracellular pH 7.6). The ΔpH collapsed within 2 min when β-acids were added, and the intracellular pH was equal to the extracellular pH (6.7). The loss of ΔpH interferes with bacterial physiology in two ways. First, ΔpH along with the difference in charge across the membrane (ΔΨ) comprise protonmotive force, which is utilized for transport and the establishment of other gradients (90). When the membrane depolarizes and protonmotive force dissipates, other concentration gradients, such as valuable ATP, must be used for transport. Second, the cytoplasmic pH could fall out of the optimal range for the cell's enzymes. It is noteworthy that cytoplasmic acidification is independent of the effects of fermentation acids. It was once thought that fermentation acids were metabolic "uncouplers" of protonmotive force, like ionophores. However, it has been shown that intracellular anion accumulation is the primary cause of growth inhibition by fermentation acids (91).

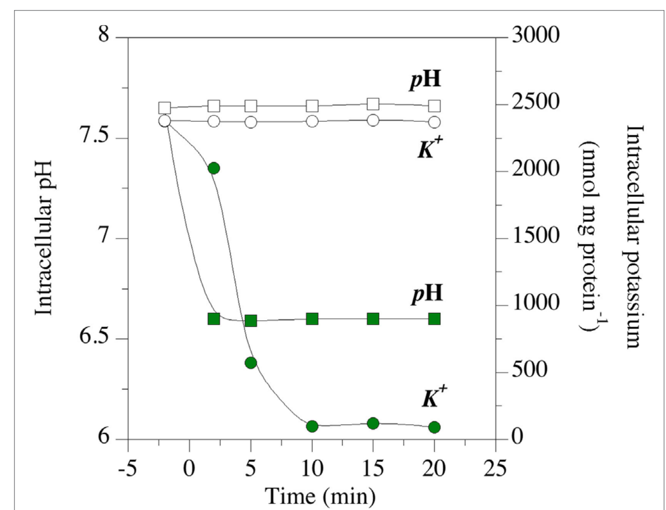

FIGURE 8 | Effect of hops β-acids on transmembrane monovalent cation gradients of the HAB, *Clostridium sticklandii*. The intracellular pH (squares) and intracellular potassium (circles) of energized cell suspensions. Open symbols are controls. Green symbols indicate suspensions to which β-acids were added at 0 min. Data adapted from Ref. (85).

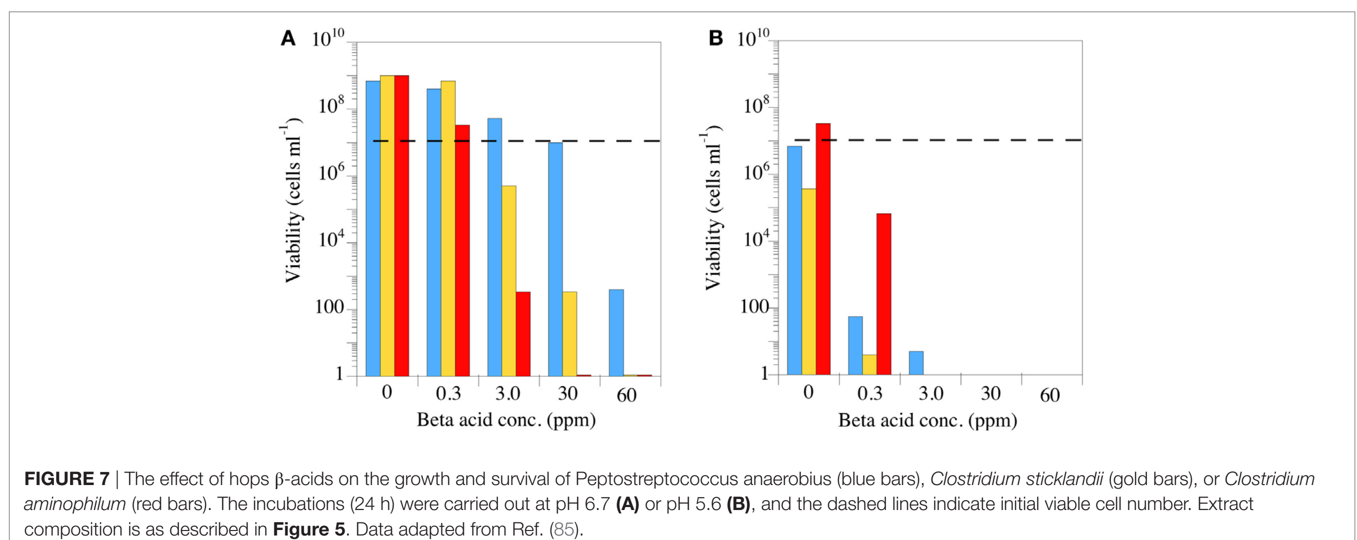

FIGURE 7 | The effect of hops β-acids on the growth and survival of Peptostreptococcus anaerobius (blue bars), *Clostridium sticklandii* (gold bars), or *Clostridium aminophilum* (red bars). The incubations (24 h) were carried out at pH 6.7 **(A)** or pH 5.6 **(B)**, and the dashed lines indicate initial viable cell number. Extract composition is as described in **Figure 5**. Data adapted from Ref. (85).

Clostridium sticklandii also lost intracellular potassium $\left(K_I^+\right)$ when β-acids were added [**Figure 8**; (85)]. Like many growing bacteria, *C. sticklandii* cells maintain a K_I^+ of more than 2 μmol/mg cell protein. When the cells were treated with β-acids, K_I^+ was negligible in 10 min. A homeostatic concentration of potassium is necessary for the function of ribosomes, many enzymes and maintenance of the sodium gradient. However, intracellular turgor is the key function of K_I^+ (92). Potassium maintains water inside the cell, and turgor associated with water (as much as 20 atmospheres in Gram-positive cells) provides the kinetic energy for cytokinesis in cell division. When K_I^+ is lost, plasmolysis occurs and the cells cannot divide.

The foundational work with *B. subtilis* shows that hop α- and β-acids are less specific than ionophores like monensin because they allow the efflux of larger molecules, like serine (81). However, the impact on the transmembrane gradients of monovalent cations is much like the proton/potassium antiporter, monensin. Furthermore, the hop spectrum of activity against rumen micro-organisms impacts rumen microbial ecology in the same way that we expect from feed ionophores.

TABLE 2 | Demonstrated effects of hops and hops bitter acids or ionophores.

Effect	Hops (or hops bitter acid)	Ionophores
Animal performance		
Increased average daily gain	Yes (96), no (93)	Yes (14, 17, 22)[a]
Increased gain:feed	No (93, 96)	Yes (14, 17, 98)
Increased carcass weight	No (96)	Yes (14, 17, 98)
Rumen metabolism		
Increased pH	Yes (83, 93), No (93, 94)	Yes (14, 17, 18, 83, 98)
Decreased A:P	Yes (83, 96, 97), No (93)	Yes (14, 17, 18, 83, 98)
Decreased NH_4^+	Yes (84, 85, 97)	Yes (14, 17, 18, 98)
Decreased CH_4	Yes (93, 97)	Yes (14, 17, 18, 98)
Other benefits		
Decreased coccidia	Not reported	Yes (14, 17)

References discussing the effects listed in the first column are given in parentheses, with yes references supporting the increase or decrease following hops or ionophore treatment and no references not supporting the listed increase/decrease.
Gain:feed, amount of weight gained relative to the amount of feed given; A:P, acetate-to-propionate ratio; NH_4^+, ammonium or ammonia; CH_4, methane.
[a]References (14, 17, 98) are reviews of ionophore research, rather than discrete studies.

HOPS AND RUMINANTS *IN VIVO*

Few studies have been published on the effects of hops or hop extracts on nutrient digestion/metabolism, or on productive performance of ruminant animals. In a meeting abstract, Schmidt et al. (93) described the effect of β-acids on *in vivo* ruminal fermentation of a concentrate diet containing 90% corn and 10% alfalfa haylage. The study used four ruminally cannulated steers that were supplemented with 0, 16.5, or 33 mg β-acid/kg diet. Intake and methane emission decreased, while ruminal pH and lactic acid concentration increased linearly with β- acids addition. However, the acetate: propionate ratio, and digestibilities of fiber, starch, and protein were not affected by β-acids addition. Additionally, β-acids increased total protozoa and *Entodinium* spp. The results indicated that addition of β-acids at the dietary concentration of 16.5–33 mg/kg diet resulted in more efficient ruminal fermentation and starch digestion. In contrast, Uwituze et al. (94) reported that supplementing 1, 8, 16, 24, and 30 mg β-acids/kg DM to steers fed a diet containing 64.8% corn, 10% alfalfa hay, and 15% dried corn distillers grains had no effect on rumen pH, concentrations of VFA or lactic acid, or on acetic acid to propionic acid ratio. Supplementation of β-acids up to 24 mg/kg DM also did not affect feed intake or total tract digestibilities of DM, OM, protein, starch, or crude fat. These results seem to indicate that addition of hop β-acids up to 30 mg/kg DM had little effect on nutrient digestibility of cattle fed a concentrate diet. In a more recent study, Axman et al. (95) found that adding 25 and 50 mg/kg DM of β-acid extracts to a corn based concentrate diet did not affect heifers' growth performance (feed intake, growth rate, and feed efficiency), which was similar to that observed when feeding 33 mg/kg DM of monensin. Please note that the trials by Uwituze et al. and Axman et al. were reported in a reputable, but non-peer-reviewed forum.

To the best of our knowledge, Wang and colleagues (96) conducted the only peer-reviewed study evaluating the potential of hops as a feed additive for cattle. In this study, hops were added to a barley-based growing diet at levels of 0, 119, 238, and 476 mg/kg DM, and to the finishing diet at levels of 0, 238, 476, and 952 mg/kg DM. The hops used in this study contained 84 g of β-acids/kg DM, which resulted in dietary concentrations of β-acids up to 40 and 80 mg/kg DM in the growing and finishing diets, respectively. The results showed that inclusion of hops in growing or finishing diets at these rates did not affect the feed intake, growth, feed efficiency, carcass characteristics, or fatty acid composition of diaphragm tissue of steers. However, growth rate of steers supplemented with the highest level of hops during the growing and finishing period was 6% higher than the growth rate of the control group. These results suggest that higher concentrations of hops in the diet may be required to improve feed utilization and growth in feedlot cattle. Further research is needed to evaluate the applicability of hops and hop β-acids extract as a feed additive in the cattle industry.

Table 2 summarizes demonstrated effects of hops or hops compounds on ruminants and rumen metabolism (83–85, 93, 94, 97). Known effects of feed ionophores are also included. There have been many experiments that included ionophores, and the reviews cited here cover three decades. Please see these and other references for a complete review of feed ionophores (14, 17, 98).

ECONOMICS OF HOPS FOR RUMINANTS AND FUTURE DIRECTIONS

The hops plant is a high value food ingredient, not an inexpensive feedstock or co-product like those typically fed to animals. The situation is also complicated by the variations of the hops market price (99). For example, the average 5-year cost (US Dollars/kilogram) of producing hops varied from 5.00 for Simcoe variety to 12.10 for US Northern Brewer variety. The demand for hops has increased with the popularity of craft beer, but production has also increased. In the United States alone, planted acreage has increased from approximately 29, 000 in 2012 to 54,000 in 2016

(100). The studies reviewed here all tested hops cones, that would otherwise be used to brew beer, or food-grade extracts. The *in vivo* animal trials showed positive results on animal performance only at the highest β-acid inclusion rates tested (94, 96), which gives little hope for optimization of lower dosing rates.

Many phytochemicals, like vanillin, can be synthesized more cost effectively than they can be grown and processed, but the practicality of producing synthetic α- and β-acids is unclear. Synthetic routes to humulone (101) and lupulone (102) have been published, but industrial-scale production of these compounds does not seem readily available. The desirability of synthesizing a single compound is uncertain because the hop extracts used in many of the aforementioned studies contain a mixture of compounds, and these may act synergistically to provide a greater benefit than would be derived from a large amount of a single α- or β-acid. Another option is utilization of byproducts from breweries. Brewery byproducts have long been fed to livestock (103), but they have not been evaluated in terms of residual biologically active plant secondary metabolites. Bryant and Cohen (104) recently identified spent yeast from American craft breweries that had combined α- and β-acid concentrations in excess of 2.5 mg/g. *In vitro* experiments with rumen microorganisms revealed that the spent brewers' yeast contained enough hops secondary metabolites to suppress ammonia production (105) and methane production (106). These results suggest that brewery waste streams could be used to provide hops phytochemicals for ruminants and other livestock.

Beyond the direct benefit to ruminant industries, we believe that hops secondary metabolites, particularly the β-acids, are useful as model phytochemical antimicrobial growth promoters. Other phytochemicals act as antimicrobial growth promoters in ruminants. For example, red clover (*Trifolium pratense*) isoflavones promote growth through antimicrobial action on the rumen HAB (107). However, isoflavones do not have an ionophore-like mechanism of action (108). When the mechanism of action and spectrum of activity against rumen bacteria are considered, lupulone and related compounds distinctly resemble feed ionophores. These hop compounds could be thought of as "phyto-ionophores" for biological points of comparison.

This review focused on the bitter acids, particularly the β-acids, but other secondary metabolites are known to be biologically active. In particular, xanthohumol has been shown to inhibit methanogens and reduce methanogenesis by mixed rumen microorganisms (109). Xanthohumol did not alter ammonia or pH, and it appeared to selectively inhibit methanogens. Xanthohumol is a prenylated flavonoid, rather than a bitter acid (73). The antimicrobial mechanism of action on rumen microorganisms has not been elucidated, but the spectrum of activity appears to be distinct from ionophores because there was no effect on ammonia concentration or pH (109). Other flavonoids are known to inhibit rumen HAB and amylolytic bacteria (84, 110). Thus, xanthohumol might have a mechanism of action that is different from either bitter acids or other flavonoids. The differences between plant secondary metabolites, even within the hops plant, and the interactions between these compounds require further investigation.

AUTHOR CONTRIBUTIONS

MF was primarily responsible for the manuscript, wrote sections of the manuscript, and asked the other authors to participate. IK, YW, and NN wrote sections of the manuscript.

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Ruminal Fermentation of Anti-Methanogenic Nitrate- and Nitro-Containing Forages *In Vitro*

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Nitrate, 3-nitro-1-propionic acid (NPA) and 3-nitro-1-propanol (NPOH) can accumulate in forages and be poisonous to animals if consumed in high enough amounts. These chemicals are also recognized as potent anti-methanogenic compounds, but plants naturally containing these chemicals have been studied little in this regard. Presently, we found that nitrate-, NPA-, or NPOH-containing forages effectively decreased methane production, by 35–87%, during *in vitro* fermentation by mixed cultures of ruminal microbes compared to fermentation by cultures incubated similarly with alfalfa. Methane production was further decreased during the incubation of mixed cultures also inoculated with *Denitrobacterium detoxificans*, a ruminal bacterium known to metabolize nitrate, NPA, and NPOH. Inhibition of methanogens within the mixed cultures was greatest with the NPA- and NPOH-containing forages. Hydrogen accumulated in all the mixed cultures incubated with forages containing nitrate, NPA or NPOH and was dramatically higher, exceeding 40 μmol hydrogen/mL, in mixed cultures incubated with NPA-containing forage but not inoculated with *D. detoxificans*. This possibly reflects the inhibition of hydrogenase-catalyzed uptake of hydrogen produced *via* conversion of 50 μmol added formate per milliliter to hydrogen. Accumulations of volatile fatty acids revealed compensatory changes in fermentation in mixed cultures incubated with the nitrate-, NPA-, and NPOH-containing forages as evidenced by lower accumulations of acetate, and in some cases, higher accumulations of butyrate and lower accumulations of ammonia, iso-buytrate, and iso-valerate compared to cultures incubated with alfalfa. Results reveal that nitrate, NPA, and NPOH that accumulate naturally in forages can be made available within ruminal incubations to inhibit methanogenesis. Further research is warranted to determine if diets can be formulated with nitrate-, NPA-, and NPOH-containing forages to achieve efficacious mitigation in ruminant methane emissions without adversely affecting fermentative efficiency or risking toxicity to animals.

Keywords: alfalfa, anti-methanogenic, barley, nitrate, nitrocompound, milkvetch, rumen

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INTRODUCTION

Nitrate and the naturally occurring nitrocompounds, 3-nitro-1-propionic acid (NPA) and 3-nitro-1-propanol (NPOH), are oxidized nitrogen compounds that can accumulate to toxic levels in certain forages and feedstuffs. Toxicologically, nitrate exerts its effects by first being biologically reduced to the toxic intermediate nitrite by bacteria within the rumen, and upon subsequent absorption, the nitrite

complexes with the host's hemoglobin to form methemoglobin, which thus loses its oxygen carrying capacity. Consequently, severely poisoned animals suffer asphyxiation (1). Poisoning by NPA and NPOH occurs differently, *via* inactivation of cellular succinate dehydrogenase activity, which thus inhibits cellular respiration (2). Nitrate, NPA, and NPOH are also recognized as potent inhibitors of ruminal methane production, a microbiological process that can result in the loss of 4–12% of the gross energy consumed by concentrate- or forage-fed cattle, respectively (3). Production of methane from ruminant sources also contributes nearly 20% of the total U.S. emissions of methane, which is a potent greenhouse gas (4), and strategies are sought to reduce the economic and environmental impact of this digestive inefficiency.

A number of recent reviews have been published on the methane-inhibiting potential and the toxicity of nitrate, its reduced intermediate, nitrite (1, 5, 6), as have reviews on similar aspects of NPA, NPOH, and a number of other nitroalkanes (1, 2, 6, 7). Most of the research findings discussed in these reviews have been based on studies investigating effects of the specific chemicals themselves on the rumen ecosystem and the host with fewer studies investigating the effects of plants containing the compounds.

In the case of nitrate, for instance, the use of nitrate salts as feed supplements to reduce methane emissions from ruminants has been investigated in a number of studies, as recently reviewed (1, 5, 6). Mechanistically, nitrate is attractive as a methane-inhibitor because of its conversion to nitrite, which is further converted to ammonia by a process that consumes reducing equivalents that otherwise would be used to reduce carbon dioxide to produce methane. However, concerns persist that the potential accumulation of the toxic intermediate nitrite, which if occurring too rapidly and at too high a concentration within the rumen, may inhibit microbes that are important for fiber digestion, and if absorption is sufficient to cause methemoglobinemia can risk poisoning of the host (8). This may be problematic when using sodium or potassium salts as these are very rapidly converted to nitrite, which may accumulate to toxic levels before it can be further metabolized to ammonia. Potential approaches to lessen the rate of nitrite accumulation within the rumen are to use more resistant salts, such as calcium salts, or to use encapsulated sources (9), but these may add cost to their commercial application.

With respect to the effects of nitrocompounds on rumen methane production, only a few studies have examined the natural compounds, NPA and NPOH (10, 11), with most reporting results from studies examining the xenobiotic nitroalkanes (12–22) and nitroxy compounds (23–30). Mechanistically, the natural and synthetic nitroalkanes have been suggested to inhibit ruminal metabolism of hydrogen and formate, substrates used for rumen methanogenesis, although the mechanisms have not been defined (11). The nitrooxy compounds are reported to inhibit methyl-coenzyme M reductase of methanogenic bacteria (24, 25).

From a practical standpoint, the xenobiotic nitrocompounds will likely require extensive testing to address toxicity and safety concerns. Moreover, the known or presumed microbial metabolic by-products of nitroalkanes, such as nitroethane, 2-nitroethanol, and 2-nitro-1-propanol (aminoethane, ethanolamine, and aminopropanol), are anticipated to be of little nutritional value for the ruminant host. We are not aware of reports on the fate of the nitrooxy nitrocompounds. On the other hand, NPA is known to be metabolized by ruminal microbes to β-alanine (31), a non-essential amino acid that may be metabolized in the rumen to sources of carbon, nitrogen, and energy (32). Thus, it is likely that when occurring in their natural state as secondary plant compounds, forages containing NPA or NPOH may be viewed more favorably by regulatory agencies. While the toxicity of NPOH may limit its application as a methane-inhibitor, NPA is not as toxic and has been safely fed to ruminants in various feedstuffs, most notably as cured Crownvetch (*Coronilla varia*) hay (33), indicating that its toxicity may be managed by controlled feeding. Other leguminous forages can accumulate NPA, and these include *Indigofera* and a number of different species and varieties of *Astragalus*, commonly named milkvetches, some which may also accumulate NPOH (2), but little is known about how these forages may affect ruminal fermentation. The objectives of the present studies were to assess the methane-inhibiting activity of forages available to us containing nitrate, NPA, or NPOH and to examine their effects of ruminal fermentation and methanogen numbers *in vitro*.

MATERIALS AND METHODS

Forage Sources

Alfalfa (*Medicago sativa*) used as a control was grown and harvested by a farmer in College Station, TX, USA and was purchased locally. The low and high nitrate-containing barley (*Hordeum vulgare*) containing 0.23 and 1.69% nitrate, respectively, were provided by Dr. Jan G. P. Bowman and have been studied for their potential genetic effects on forage quality (34). *Astragalus canadensis* containing predominantly the tri- and di-NPA glucopyranose esters karakin (1,2,6-tri-*O*-[3-nitropropanyl]-β-D-glucopyranose) and cibarian (1,6-di-*O*-[3-nitropropanyl]-β-D-glucopyranose) at approximately 1.6% of plant dry matter and *Astragalus miser* containing ether glycosides of NPOH, predominantly as miserotoxin (3-nitro-1-propyl-β-D-glucopyranoside) at approximately 2–5% plant dry matter, were graciously provided by Dr. Walter Majak (Agriculture and Agrifood Canada, Kamploops, BC, Canada). Procedures for the collection and measurement of conjugated nitrocompounds in the forages have been described (35, 36). For the present study, chemical composition and nitrate concentration for each of the forages was determined by the Soil, Water and Forage Testing Laboratory at the Texas AgriLife Extension Services' Department of Soil and Crop Sciences (37), and these data are presented in **Table 1**.

Mixed Culture of Ruminal Microbes

Two separate *in vitro* rumen incubation experiments were conducted using freshly collected ruminal fluid obtained at 1000 hours (2 h after morning feeding) from a rumen-cannulated Holstein cow (approximately 660 kg) maintained on 50:50 corn-based concentrate:alfalfa diet, supplemented with a commercially available mineral mix (Producers CO-OP, Bryan, TX, USA). All procedures with the cow were conducted in accordance with the Southern Plains Agricultural Research Center's approved Animal Care and Use protocol. The ruminal fluid was strained through

TABLE 1 | Composition of alfalfa, high and low nitrate-containing barley, and NPA- and NPOH-containing milkvetches.

	Alfalfa	High nitrate-containing barley	Low nitrate-containing barley	NPA-containing milkvetch	NPOH-containing milkvetch
Crude protein (%)	17.3	13.8	6.4	10.0	9.6
Digestible crude protein (%)	12.6	9.4	2.5	5.8	5.4
Acid detergent fiber (%)	35.5	35.4	31.4	32.4	34.8
Neutral detergent fiber (%)	38.8	45.0	46.9	38.3	37.8
Total digestible nutrients (%)	60.2	59.1	59.5	60.0	58.1
Net energy lactation (Mcal/kg)	1.36	1.32	1.34	1.34	1.30
Net energy maintenance (Mcal/kg)	1.45	1.43	1.43	1.45	1.39
Net energy gain (Mcal/kg)	0.73	0.70	0.70	0.73	0.68
In vitro true digestibility (%)	75.7	80.4	68.5	85.3	82.3
Ash (%)	10.2	9.3	5.4	7.4	7.9
Relative feed value	146.7	126.8	127.7	154.7	152.0
Nitrate[a] (%)	0.16	1.69	0.23	0.15	0.03
Mineral analysis (NIR)					
Calcium	1.22	0.69	0.48	0.72	0.77
Magnesium	0.29	0.15	0.04	0.11	0.11
Phosphorus	0.39	0.29	0.16	0.25	0.23
Potassium	2.92	2.23	1.52	1.64	1.50

[a]*Amounts of nitrate potentially available in each tube for mixed cultures of ruminal microbes incubated without being inoculated with D. detoxificans (experiment 1) are 1.3, 13.6, 1.8, 1.2, and 0.2 μmol/mL of incubation fluid for alfalfa, high nitrate-containing barley, low nitrate-containing barley, NPA-containing milkvetch, and NPOH-containing milkvetch, respectively. Amounts of nitrate potentially available in each tube for mixed cultures of ruminal microbes in experiment 2 are estimated to be 16.7% less to account for additional 2 mL volume with D. detoxificans inoculation.*

a nylon paint strainer during collection into insulated containers until completely full, then capped and returned to the laboratory within 30 min of collection for immediate use. At the laboratory, the ruminal fluid was amended to achieve 50 mM sodium formate and then distributed (within 30 min of collection) in 10 mL volumes to two sets of 18 × 150-mm crimp-top culture tubes preloaded in triplicate with 0.5 g (92–96% dry matter) of each test forage previously ground to pass a 4 mm Willey Mill screen. The ruminal fluid was kept under a 100% carbon dioxide atmosphere during preparation and transfer at the laboratory to maintain anaerobiosis, and tubes were immediately closed with rubber stoppers and crimped to prevent leakage during subsequent 24-h incubation at 39°C in upright position without agitation. In experiment 1, no further additions were made. In experiment 2, which was conducted concurrently with experiment 1, the loaded and capped tubes were inoculated with approximately 2 × 100 cells of a 72-h old culture of the NPA-, NPOH-, and nitrate-metabolizing ruminal bacterium *Denitrobacterium detoxificans* strain NPOH1, grown previously in 50-mL nitro-supplemented Medium B as described by Anderson and Rasmussen (10). Inoculations were accomplished *via* injection of 2 mL culture volume into each tube through the rubber stopper using a needle just prior to the start of incubation. At the end of the incubation period, 1 mL of atmosphere from the headspace of each tube was collected *via* a 1-mL glass syringe and injected into a Gow-Mac series 580 gas chromatograph (Gow-Mac Instrument, Bridgewater, NJ, USA) equipped with a HaySep Q column heated to 60°C and operated with Argon as the carrier gas flowing at 25 mL/min. Methane and hydrogen were measured with a thermal conductivity detector. Gas volumes were measured *via* volume displacement using a 30-cc lubricated glass syringe. Molar concentrations of hydrogen and methane were calculated using the Idea Gas Laws and are expressed as micromole per milliliter of incubation fluid. Fluid samples collected at the end of incubation were used for colorimetric measurement of ammonia, nitrate, nitrite, NPA, and NPOH (38–41) and for gas chromatographic measurement of volatile fatty acids (42). Most probable numbers (MPN) of methanogens, expressed as $\log_{10}$ cells/mL incubation fluid, at the end of the incubations were determined as described by Saengkerdsub et al. (43) except using an Association of Official Analytical Chemists' 3-tube MPN table (44).

Statistical Analysis

Statistical comparisons between mixed cultures incubated with the different forages were made within experiment to avoid confounding effects of volume differences between the two experiments. Because each resultant population had the opportunity to respond independently, each was considered an independent experimental unit. Tests for effects of forage type on accumulations of hydrogen, methane, ammonia, and volatile fatty acids after 24-h incubation of mixed cultures were conducted using a completely randomized analysis of variance (n = 3/forage type) with an LSD separation of means (Statistix 10 Analytical Software, Tallahassee, FL, USA). MPN estimates were similarly analyzed for effects of forage type within experiments 1 and 2 using a completely randomized analysis of variance with an LSD separation of means except using measurements made from only two of the three replicate tubes per each forage type (n = 2/forage type) incubated with mixed cultures of ruminal microbes.

RESULTS

Experiment 1 (Incubation of Mixed Cultures without *D. detoxificans* Inoculation)

Total volume of gas produced tended to differ between mixed cultures of ruminal microbes incubated with the different forages (**Table 2**). Methane accumulations were 79, 85, and 35%

TABLE 2 | Fermentation characteristics of alfalfa and select nitrate- and nitro-containing forages during incubation with mixed cultures of ruminal microbes in experiment 1 with 50 mM added sodium formate, but without inoculation with *Denitrobacterium detoxificans*.

	Alfalfa	High nitrate-containing barley	Low nitrate-containing barley	NPA-containing milkvetch	NPOH-containing milkvetch	*P* value	SEM
Headspace measurements							
Total gas, mL	46.0^{b}	41.3^{b}	54.0^{a}	46.3^{a,b}	46.7^{a,b}	0.0529	2.463
Hydrogen, μmol/mL (kPa)	0.66^{c} (1.0)	3.06^{b,c} (4.7)	0.88^{c} (1.4)	40.15^{a} (61.6)	5.50^{b} (8.5)	<0.0001	0.956
Methane, μmol/mL	62.36^{a}	13.12^{c}	76.35^{a}	9.52^{c}	40.72^{b}	<0.0001	4.680
Fluid measurements							
Acetate, μmol/mL	178.68^{a}	138.06^{c}	151.60^{b}	125.61^{d}	129.63^{d}	<0.0001	2.617
Propionate, μmol/mL	63.74^{a}	63.81^{a}	60.35^{b}	50.06^{c}	66.96^{a}	<0.0001	1.030
Butyrate, μmol/mL	38.66^{c}	45.18^{b}	55.42^{a}	52.48^{a}	53.97^{a}	<0.0001	1.372
Iso-butyrate, μmol/mL	6.38^{b}	7.81^{a}	5.84^{c}	4.83^{d}	6.04^{c}	<0.0001	0.089
Iso-valerate, μmol/mL	5.71^{b}	6.44^{a}	5.11^{c}	4.04^{d}	5.70^{b}	<0.0001	0.066
Valerate, μmol/mL	8.45	11.95	8.65	8.82	9.5	0.4441	1.428
Total VFA, μmol/mL	301.63^{a}	273.24^{b}	286.96^{a,b}	245.83^{c}	271.86^{b}	0.0003	5.211
Ratio of acetate to propionate	2.80^{a}	2.16^{c}	2.51^{b}	2.51^{b}	1.94^{d}	<0.0001	0.030
Ammonia, μmol/mL	58.08^{a,b}	63.25^{a}	42.26^{c,d}	38.54^{d}	49.96^{b,c}	0.0003	2.628
Numbers of methanogens, log_{10} cells/mL	3.01^{a}	2.17^{b,c}	2.36^{a,b}	1.45^{c}	2.80^{a,b}	0.0196	0.209

[a,b,c,d]*Means within rows with unlike superscripts differ at P < 0.05.*

lower after 24 h in mixed cultures incubated with the high nitrate-containing barley and the NPA- and NPOH-containing milkvetches, respectively, when compared to accumulations in mixed cultures incubated with alfalfa (**Table 2**). Hydrogen accumulations were dramatically higher in mixed cultures incubated with the NPA-containing milkvetch and were lowest in mixed cultures incubated with alfalfa or the low nitrate-containing barley, with accumulations in mixed cultures incubated with the high nitrate-containing barley and NPOH-containing milkvetch being intermediate (**Table 2**). Differences in accumulations of all volatile fatty acids except valerate were observed, and these data are presented in **Table 2**. Differences in ratios of acetate to propionate were also observed, being highest with mixed cultures incubated with alfalfa and 10–31% lower in mixed cultures incubated with the other forages (**Table 2**). Ammonia accumulations differed between the mixed cultures incubated with concentrations being highest in the mixed cultures incubated with high nitrate-containing barley and lowest in mixed cultures incubated with NPA-containing milkvetch. Residual concentrations of nitrate, nitrite, and the nitrocompounds at the end of the 24-h incubations were not tested for differences between the different forages because initial concentrations were not the same. However, measurements revealed that residual concentrations of nitrate and nitrite in fluids from all incubations were below 1.3 and 0.04 μmol/mL, respectively. Residual concentrations of NPA and NPOH were 4.4 and 8.7 μmol/mL, respectively. MPN of methanogens were highest in mixed cultures incubated with alfalfa, lowest in mixed cultures incubated with the NPA-containing milkvetch, and intermediate in mixed cultures incubated with NPOH-containing milkvetch and the high and low nitrate-containing barley.

Experiment 2 (Incubation of Mixed Cultures with *D. detoxificans* Inoculation)

Total gas volumes after 24-h incubation of mixed cultures of ruminal microbes that had been inoculated with *D. detoxificans* differed, with amounts produced being higher in mixed cultures incubated with alfalfa than in the mixed cultures incubated with the other forages (**Table 3**). Accumulations of methane and hydrogen also differed, with more methane accumulating in mixed cultures incubated with alfalfa than with the other forages, and with more hydrogen accumulating in mixed cultures incubated with the NPA-containing milkvetch than with the other forages (**Table 3**). Differences in accumulations of all volatile fatty acids except propionate were observed, and these data are presented in **Table 3**. Differences in total volatile fatty acid accumulations were observed, with lower accumulations observed for mixed cultures incubated with the NPA- and NPOH-containing milkvetches than in the other forages, mainly due to lower accumulations of acetate (**Table 3**). Lower accumulations of acetate observed in mixed cultures incubated with the NPA-, NPOH-, and nitrate-containing forages, than in mixed cultures incubated with alfalfa, are also reflected in the ratios of acetate to propionate (**Table 3**). Accumulations of ammonia were highest in mixed cultures incubated with alfalfa, lowest in mixed cultures incubated with the NPA- and NPOH-containing milkvetches, and intermediate in mixed cultures incubated with the high and low nitrate-containing barley (**Table 3**). Residual concentrations of nitrate and nitrite in fluids from all incubations were below 0.82 and 0.02 μmol/mL, respectively. Residual concentrations of NPA and NPOH were 3.6 and 7.2 μmol/mL, respectively. MPN of methanogens were higher in mixed cultures incubated with alfalfa and the high nitrate-containing barley than in mixed cultures incubated with the NPA- and NPOH-containing milkvetches and the low nitrate-containing barley (**Table 3**).

DISCUSSION

As reviewed recently by Latham et al. (1), nitrate and certain short-chain nitrocompounds are known to be potent methane-inhibiting compounds, although their use in research studies has almost exclusively been done with commercially available

TABLE 3 | Fermentation characteristics of alfalfa and select nitrate- and nitro-containing forages during incubation with mixed cultures of ruminal microbes in experiment 2 with 50 mM added sodium formate and inoculation with *Denitrobacterium detoxificans*.

	Alfalfa	High nitrate-containing barley	Low nitrate-containing barley	NPA-containing milkvetch	NPOH-containing milkvetch	*P* value	SEM
Headspace measurements							
Total gas, mL	48.7[a]	30.3[b]	27.3[b]	19.3[b]	27.3[b]	0.0062	4.145
Hydrogen, μmol/mL (kPa)	1.07[b] (1.7)	0.95[b] (1.5)	0.92[b] (1.4)	6.58[a] (10.1)	4.18[a,b] (6.4)	0.0392	1.308
Methane, μmol/mL	54.90[a]	2.35[b]	6.48[b]	0.80[b]	4.57[b]	<0.0001	1.951
Fluid measurements							
Acetate, μmol/mL	145.19[a]	123.08[b]	130.55[b]	97.99[c]	106.77[c]	<0.0001	2.967
Propionate, μmol/mL	54.54	57.30	55.35	53.54	59.70	0.3856	2.270
Butyrate, μmol/mL	34.34[b]	37.73[b]	48.07[a]	46.76[a]	48.98[a]	0.0093	2.698
Iso-butyrate, μmol/mL	6.07[b]	7.14[a]	5.97[b]	4.86[d]	5.40[c]	<0.0001	0.119
Iso-valerate, μmol/mL	5.37[b]	5.92[a]	5.41[b]	4.56[c]	5.28[b]	0.0013	0.150
Valerate, μmol/mL	7.69[b]	7.68[b]	8.30[a,b]	9.33[a]	9.14[a]	0.0138	0.335
Total VFA, μmol/mL	253.19[a]	238.85[a,b]	253.66[a]	217.04[c]	235.26[b]	0.0037	5.333
Ratio of acetate to propionate	2.66[a]	2.15[b,c]	2.40[a,b]	1.83[c]	1.79[c]	0.0020	0.121
Ammonia, μmol/mL	51.60[b]	60.87[a]	42.02[c]	34.73[d]	42.06[c]	<0.0001	1.479
Numbers of methanogens, $\log_{10}$ cells/mL	2.36[a]	2.38[a]	1.80[c]	1.27[c]	1.36[c]	0.0006	0.086

[a,b,c,d]*Means within rows with unlike superscripts differ at P < 0.05.*

chemical sources using various nitrate salts or chemically synthesized nitrocompounds. Results from the present study confirm that rumen methanogensis can be lowered with forages containing nitrate, NPA, and NPOH, thus indicating that these compounds were readily solubilized or otherwise made available within the incubation fluids to inhibit methanogenesis. Forage quality and digestibility can also affect ruminal methanogenesis (3), and thus quality differences between the forages in the present study may have contributed to differences in amounts of methane produced. However, the neutral detergent fiber, acid detergent fiber content, and the *in vitro* true digestibility of the nitrate- and nitro-containing forages differed no more than 10% and in some cases were higher than that of the alfalfa (**Table 1**). Accordingly, it is seems likely the differences in methane production were due largely to nitrate and the nitrocompounds contained in the barley and milkvetch forages.

An attractive aspect of using nitrate as a methane-inhibitor is that it can act as an energetically favorable alternative electron acceptor that consumes electrons at the expense of methanogenesis, thereby preserving energetic efficiencies of interspecies-hydrogen transfer thought to be beneficial for rumen digestive processes (1). In such cases, nitrate is reduced to nitrite, which in the rumen is predominantly reduced further to ammonia (45), with the process consuming eight electrons, the equivalent of 4 μmol of hydrogen, for each micromole of nitrate reduced to ammonia (1). The methane-inhibiting potential of nitrate, however, is largely dependent on sufficiently active nitrate-reducing ruminal microbes, such as those having been adapted to nitrate *via* prior exposure. For instance, Božic et al. (16) reported that methane production was not inhibited during an initial 24-h incubation of ruminal microbes with 16 μmol nitrate/mL but was inhibited upon subsequent transfer of this 24-h old population to a fresh nitrate-containing medium. Based on the amounts of nitrate in the forages used in the present study, initial concentrations of nitrate potentially available in the incubations in experiment 1 were estimated to be 1.3, 13.6, 1.8, 1.2, and 0.2 μmol/mL of incubation fluid for alfalfa, the high and low nitrate-containing barley, and the NPA- and NPOH-containing milkvetch, respectively. Potentially available nitrate concentrations for incubations in experiment 2 would be expected to be 16.7% less due to dilution of the incubation fluid that occurred with inoculation of *D. detoxificans*. Accordingly, except for the mixed cultures incubated with the high nitrate-containing barley, nitrate concentrations in incubations with the other forages would most likely have been too low to affect appreciable decreases in methane production. While nitrate *per se* is not particularly toxic to rumen methanogenic bacteria, the reduced intermediate, nitrite, is a potent inhibitor of methanogenesis, causing 50% decrease in methane-producing activity with concentrations as low as 0.5 μmol/mL (46). Thus, it is possible that nitrite may accumulate to concentrations directly inhibitory to methanogens when rates of nitrate reduction to nitrite exceed rates of nitrite reduction to ammonia. In experiment 1, the mixed cultures had no known prior exposure to nitrate, and therefore, rates of nitrate reduction to nitrite would be expected to proceed slowly at first but increase rapidly as a consequence of induction of nitrate-reducing activity and selection of nitrate-reducing microbes. Eventually, rates of nitrate reduction could far exceed the rates of nitrite reduction as the mixed cultures adapted, thus potentially allowing nitrite to accumulate to inhibitory concentrations in the mixed cultures in experiment 1, particularly for the mixed cultures incubated with the high nitrate-containing barley where higher nitrite accumulations could have persisted for a longer duration than in mixed cultures incubated with the other forages. However, nitrite concentrations, in all of the mixed cultures in both experiments 1 and 2, were below 0.04 μmol/mL in fluid samples measured at the end of the 24-h incubation period, which suggest that inhibitory concentrations of nitrite would have been temporary.

For mixed cultures in experiment 2 that had been inoculated with the competent nitrate-metabolizing bacterium *D. detoxificans*, rapid rates of nitrate and nitrite metabolism would be expected to have commenced sooner, and thus accumulations of

nitrite would be expected to be lower and to persist for a shorter duration than in cultures of experiment 1. The lower accumulation of methane observed with mixed cultures incubated with the low nitrate-containing barley in experiment 2 is not readily explained, as the available nitrate (and subsequently nitrite) would be expected to be too low to affect appreciable inhibition in methanogenesis. It is possible that there may have been some carry over of residual nitrocompound with the 2-mL inoculum, but this seems unlikely as a potential carry over effect would have manifested itself in all the mixed cultures. Differences in MPN of methanogens in the *D. detoxificans*-inoculated cultures in experiment 2 were observed, being lower in mixed cultures incubated with the low nitrate-containing barley cultures, as well as in those incubated with the milkvetches, than in cultures incubated with the high nitrate-containing barley. In the latter case, the lower methane production is consistent with competitive consumption of electrons rather than direct inhibition of methanogens. The high and low nitrate-containing barley forages were different genotypes sampled at different stages of maturity, with the high nitrate-containing forage sampled at flowering (plant anthesis) and the low nitrate-containing forage sampled at peak forage yield (34). It is possible that maturation of the low nitrate-containing barley may have caused accumulations of reactive nitrogen derivatives or accumulations of oxidized sulfur-containing compounds, such as oxidized cysteine residues in Rubisco (47), which could potentially be metabolized to yield suitable electron acceptors for *D. detoxificans*. *D. detoxificans* is known to be able to respire anaerobically, oxidizing hydrogen, formate, or lactate to reduce nitrate, NPA, NPOH, as well as various other oxidized compounds, such as trimethylamine oxide and dimethyl sulfoxide (48, 49), but its ability to use other naturally occurring electron acceptors has not been thoroughly investigated.

In the case of the nitro-containing milkvetches, greater reduction in methane production was achieved in mixed cultures incubated with the NPA-containing milkvetch than with the NPOH-containing milkvetch. This likely reflects a more potent methane-inhibiting potential of NPA compared to NPOH, considering that nearly twice as much nitrocompound was potentially available in the incubations supplemented with the NPOH-containing milkvetch (8.7 and 7.2 μmol/mL in experiments 1 and 2, respectively) than in the NPA-containing milkvetch (4.4 and 3.6 μmol/mL, in experiments 1 and 2, respectively). Earlier work also indicated that NPA inhibited methane production more effectively than 2-nitro-1-propanol, a structural isomer of NPOH (11). In support of these observations, MPN estimates of methanogens revealed a differential response within the mixed cultures incubated with the NPA- or NPOH-containing milkvetch in experiment 1. In this case, mixed cultures incubated with the NPA-containing milkvetch had lower methanogen numbers than mixed cultures incubated with alfalfa as well as those incubated with NPOH-containing milkvetch and low nitrate-containing barley. Conversely, methanogen numbers in experiment 2 were found to be equivalently lower in mixed cultures incubated with the NPA- and NPOH-containing milkvetches when compared to mixed cultures incubated with alfalfa. It is recognized that when *D. detoxificans* is present, NPA and NPOH may transition, at least partially, from being direct inhibitors of methanogenesis to being used as alternative electron acceptors to support the growth of *D. detoxificans* (1).

For the mixed cultures incubated with the NPOH-containing milkvetch, and to a lesser extent with the NPA-containing milkvetch, there is evidence that inoculation with *D. detoxificans* may have promoted consumption of electrons, but this would have had little impact in limiting availability of electrons for methanogenesis. For instance, residual concentrations of NPA and NPOH at the end of the 24-h incubations with the NPA- and NPOH-containing milkvetches were 86 and 87% of initial concentrations in experiment 1 indicating metabolism of about 0.5–1.1 μmol NPA or NPOH/mL, respectively. Conversely, residual NPA and NPOH were 61 and 64% of initial concentrations in the incubations with NPA- and NPOH-containing milkvetches in experiment 2, which corresponds to metabolism of about 1.4 and 2.6 μmol NPA or NPOH/mL, respectively. Assuming that each micromole of NPA or NPOH reduced consumes six electrons or the equivalent of 3 μmol of hydrogen, based on stoichiometric estimates for the reduction of nitroethane by a *Clostridium pasteurianum* hydrogenase/ferredoxin system (50), the NPA and NPOH metabolized in this study would have consumed at most only 4.2 and 7.8 μmol hydrogen equivalents/mL, respectively. The lesser amounts of NPA and NPOH metabolized within the mixed cultures incubated with NPA- and NPOH-containing milkvetch incubations in experiment 1, which were not inoculated with *D. detoxificans* is not surprising, considering that *D. detoxificans* is usually present at low concentrations (<10^4 cells/mL) in rumen populations having no prior nitrocompound exposure (51). Based on these considerations, the more potent methane-inhibiting effect observed in the mixed cultures inoculated with *D. detoxificans* and incubated with the NPOH-containing milkvetch cannot be explained solely by competitive consumption of reducing substrates for the reduction of NPOH. Thus, other modes of action must be operative, and this possibility warrants further investigation. It is also possible that populations of rumen microbes sufficiently adapted to higher concentrations of NPA or NPOH may be able to consume greater concentrations, reducing substrates to quantitatively impact methanogens. However, the toxicity of higher concentrations of NPA or NPOH may limit amounts of these nitrocompounds that can be fed in practical animal feeding situations.

In the present study, 50 μmol formate/mL was added to the incubations of both experiments to provide non-limiting amounts of reducing substrate to support the reduction of nitrate, NPA, or NPOH, which would be expected to yield ammonia, β-alanine, or 3-amino-1-propanol, respectively (1, 48–52). It was expected that most, if not all, of the added formate would be converted to hydrogen, which would subsequently serve as reducing substrate, as formate is usually converted to hydrogen *via* activity of microbial formate hydrogenlyase and formate dehydrogenase (53, 54). However, it is possible that some of the formate may have served as a reducing substrate itself as formate is a good substrate for methane production and for the reduction of nitrate and the nitrocompounds. In experiment 1, hydrogen accumulations in the mixed cultures incubated with the NPA- and NPOH-containing milkvetches were higher than in the mixed cultures incubated with alfalfa, thus indicating an effect of

the nitrocompounds on hydrogen utilization. In the case of the mixed cultures incubated with the NPA-containing milkvetch, the higher accumulation of hydrogen, exceeding 40 μmol/mL incubation fluid, supports our expectation that considerable amounts of the 50-μmol/mL added formate was biotransformed into hydrogen. The high accumulation of hydrogen in the mixed cultures incubated with the NPA-containing milkvetch also suggests subsequent inhibition of hydrogen oxidation. In an earlier study, ruminal populations treated with NPA and other short-chain nitrocompounds (2-nitro-1-propanol, 2-nitroethanol, and nitroethane) and incubated without added *D. detoxificans* were found to inhibit the oxidation of hydrogen and formate, but mechanistic aspects of this inhibition have yet to be resolved (11). Formate concentrations were not measured in the present experiments, and thus the possibility that some residual formate may have been retained in these incubations cannot be excluded.

Differences in accumulations of volatile fatty acids were observed in the mixed cultures incubated with the different forages, thus reflecting differences in digestibility of the different forages due in part to the inhibition of methanogenesis and its role in maintaining low hydrogen concentrations. In both experiments 1 and 2, accumulations of total volatile fatty acids were nearly 30% lower in mixed cultures incubated with both NPA- and NPOH-containing milkvetches than in cultures incubated with alfalfa. Earlier work had reported modest inhibitory effects of 21 μmol/mL NPA and NPOH on total culturable rumen anaerobes, with decreases in viable cell counts being 32% or less from untreated counts (1.8×10^9 colony-forming U/mL), although the specific microbes inhibited were not characterized (31). More severe inhibition of total culturable anaerobes was observed with 42 μmol/mL NPA and NPOH, with viable cell counts being decreased as much as 90% compared to untreated populations (31). In the case of the barley forages in the present study, only the mixed cultures incubated with the high nitrate-containing barley in experiment 1 had accumulated lower concentrations of total volatile fatty acids than the cultures incubated with alfalfa. These observations suggest that in mixed cultures in experiment 1, having not been adapted to nitrate or inoculated the with nitrate/nitrite metabolizing *D. detoxificans*, the rate of nitrate metabolism to nitrite may have exceeded the rate of nitrite metabolism to ammonia, thus allowing nitrite to temporarily accumulate to levels inhibitory to fiber degrading microbes.

Acetate production by mixed cultures of ruminal microbes is often decreased, and production of more reduced fatty acids, such as propionate and butyrate, are often increased when methane production is inhibited. In both of the present experiments, acetate concentrations in mixed cultures incubated with the nitrate- and nitro-containing forages were lower than in mixed cultures incubated with alfalfa, but this was not always associated with lower methane accumulations. Decreased acetate production in mixed cultures incubated with the NPA- and NPOH-containing forages is not surprising, considering that hydrogen accumulations in these incubations exceeded 1 kPa, which is sufficient to inhibit reoxidation of reduced nucleotides produced during glycolysis (55, 56). Microbial populations often compensate to the accumulation of reduced nucleotides resulting from methane inhibition by redirecting electrons to more reduced acids. Unexpectedly, however, concentrations of propionate were never higher in mixed cultures incubated with the nitrate- and nitro-containing forages than in the mixed cultures incubated with alfalfa. Ratios of acetate to propionate were lower in the mixed cultures incubated with the nitrate- and nitro-containing forages than mixed cultures incubated with alfalfa, due mainly to lower accumulations of acetate. Conversely, butyrate concentrations were almost always higher in the mixed cultures incubated with the nitrate- and nitro-containing forages than those incubated with alfalfa, the exception being mixed cultures inoculated with *D. detoxificans* in experiment 2 that were incubated with the high nitrate-containing barley. Thus, it seems reasonable to suspect that reductant was directed toward butyrate production. These results conflict with earlier results reporting that NPA concentrations as high as 20 μmol/mL had little negative effect on accumulations of acetate, propionate, and butyrate in ruminal populations incubated with or without *D. detoxificans* inoculation (10, 11). Experimental conditions differed between the present and the earlier experiments, however, which confound comparisons with the present experiments.

It is possible reductant was also directed toward the production of valerate in the mixed cultures incubated with the nitrate- and nitro-containing forages of the present experiments. Concentrations of this fatty acid, often associated with protein catabolism, were not higher in the mixed cultures incubated with alfalfa, which is contrary to that expected, due to the alfalfa forage having the higher crude protein content (**Table 1**). Concentrations of ammonia were higher in the mixed cultures incubated with alfalfa than most of the cultures incubated with the nitrate- and nitro-containing forages, which indicates that protein catabolism was indeed higher in mixed cultures incubated with alfalfa. The main exception being the high accumulations of ammonia observed in the mixed cultures in both experiments 1 and 2 that were incubated with the high nitrate-containing barley, but this is likely due to the near complete reduction of the more than 11 μmol nitrate/mL potentially available in these incubations to ammonia. Accumulations of iso-butyrate and iso-valerate, also associated as potential end products of protein catabolism, were higher in the mixed cultures incubated with alfalfa than in some but not all of the mixed cultures incubated with the nitro- and nitrate-containing forages.

CONCLUSION

Forages containing NPA, NPOH, or nitrate effectively decreased methane production during fermentation by mixed cultures of ruminal microbes compared with that produced by mixed cultures incubated with alfalfa, although under the conditions of this experiment these forages caused compensatory changes in fermentation. Inoculation of the mixed cultures with *D. detoxificans*, a ruminal bacterium known to metabolize nitrate, NPA, and NPOH, caused further decreases in methane production during with some but not all of the nitro- or nitrate-containing forages indicating that the *D. detoxificans* effect was not necessarily due to enhanced consumption of reducing substrates. These results will serve as a foundation for continued investigations regarding the inhibitory effects of nitrate and the nitrocompounds on rumen

methanogenesis which ultimately may allow formulation of anti-methanogenic diets containing safe amounts of nitrate-, NPA-, or possibly even NPOH-containing forages.

AUTHOR CONTRIBUTIONS

Drs. RA, LR, TC, KG, RH, and DN contributed to the design, to the conduct of the experiment as well as to the analysis and interpretation of the results and to writing the manuscript. Dr. RB conducted volatile fatty acid analysis and contributed to interpretation of results and writing the manuscript. Dr. JB contributed the barley forages and to the analysis and interpretation of the results and to writing the manuscript.

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Mitigation of Ergot Vasoconstriction by Clover Isoflavones in Goats (*Capra hircus*)

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Ergot alkaloids produced by a fungal endophyte (*Epichloë coenophiala*; formerly *Neotyphodium coenophialum*) that infects tall fescue (*Lolium arundinaceum*) can induce persistent constriction of the vasculature in ruminants, hindering their capability to thermo-regulate core body temperature. There is evidence that isoflavones produced by legumes can relax the vasculature, which suggests that they could relieve ergot alkaloid-induced vasoconstriction and mitigate the vulnerability to severe heat stress in ruminants that graze tall fescue. To test if isoflavones can relieve alkaloid-induced vasoconstriction, two pen experiments were conducted with rumen-fistulated goats (*Capra hircus*) to determine with ultrasonograpy if isoflavones can (1) promote vascular compliance by countering alkaloid-induced vasoconstriction and (2) relieve already imposed alkaloid-induced vasoconstriction. Goats were fed *ad libitum* chopped orchardgrass (*Dactylis glomerata*)–timothy (*Phleum pratense*) hay prior to conducting the experiments. Measures of carotid and interosseous luminal areas were obtained pre- (baseline) and post-ruminal infusions in both experiments with goats being fed the hay, and for blood flow rate in the carotid artery in Experiment 2. Responses to infusion treatments were evaluated as proportionate differences from baseline measures. Peak systolic velocity, pulsatility index, and heart rate were measured on the last day on treatment (DOT) in Experiment 1, and on all imaging sessions during Experiment 2. For Experiment 1, rumens were infused with ground toxic fescue seed and isoflavones in Phase A and with only the toxic seed in Phase B. The infusion treatments were switched between phases in Experiment 2, which employed a fescue seed extract having an ergot alkaloid composition equivalent to that of the ground seed used in Experiment 1. During Experiment 1, luminal areas of carotid and interosseous arteries in Phase A did not deviate ($P > 0.1$) from baselines over 1, 2, 3, and 4 DOT, but the areas of both declined linearly from baselines over 1, 2, 3, and 4 DOT in Phase B. By 6, 7, and 8 DOT in Experiment 2, luminal areas of the arteries and flow rate declined from baselines with infusions with the only seed extract in Phase A, but luminal areas and flow rate increased over 4, 5, and 6 DOT with the additional infusion of isoflavones. Peak systolic velocity and heart rate were not affected by treatment in either experiment, but were highest when infused with only ergot alkaloids in both experiments. Treatment with isoflavones was demonstrated to relax the carotid and interosseous arteries and reduce resistance to blood flow. Results indicate that isoflavones can relax persistent vasoconstriction in goats caused by consumption of ergot alkaloids, and mitigate the adverse effect that ergot alkaloids have on dry matter intake.

Keywords: ergot alkaloids, goats, isoflavones, tall fescue, vasoconstriction

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INTRODUCTION

Tall fescue (*Lolium arundinaceum*) is a cool-season perennial grass that covers ~15 million hectares in the eastern half of the USA and is extensively utilized for livestock grazing. The grass is productive and tolerant of environmental stresses, which has been attributed to the presence of a fungal endophyte (*Epichloë coenophiala*) that infects most plants of tall fescue (1). However, ergot alkaloid toxins are also produced by the endophyte, and they cause a toxicosis in grazing livestock. Signs of "fescue toxicosis" include poor weight gain and reproductive performances, elevated body temperature, labored respiration, and decreased serum prolactin (2, 3).

Ergot alkaloids bind biogenic amine receptors in the vasculature (4) to induce persistent vasoconstriction (5) that incapacitates the animal's ability to regulate core body temperature (6, 7). Cattle (5, 8), sheep (9), horses (10), and goats (11) have been reported to vasoconstrict when exposed to ergot alkaloids. Therefore, the vasculatures of these livestock species are vulnerable to ergot alkaloid-induced vasoconstriction. Ergot alkaloids also bind D2 receptors of the anterior pituitary gland to reduce secretion of prolactin (3, 4).

Isoflavones are phytoestrogens commonly found in legumes and can have estrogenic activity in mammals (12). Isoflavones have been used as a treatment for ameliorating hot flashes in postmenopausal women (13, 14). Cruz et al. (15) used *in vitro* techniques with small arteries collected from subcutaneous circulation of men with and without coronary heart disease to determine a vaso-relaxation response with exposure to phytoestrogens, resveratrol and genistein. Relaxation of the vasculature from exposure to isoflavones has been associated with agonist activity of isoflavones at β-adrenergic receptors in the vasculature endothelium to stimulate synthesis of nitric oxide that mediates vaso-relaxation (13–16). Recently, Shappell et al. (17) reported an additive effect of combining estradiol implants with feeding soybean hulls on the estrogenic activity of serum from steers grazing toxic tall fescue. Although it could not be concluded, the mitigation of fescue toxicosis observed with this treatment combination (18) could have been related to estrogenic activity with the treatment combinations being above a threshold for relief of vasoconstriction to occur. It could be possible that β-agonist activity of isoflavones could ameliorate the persistent vasoconstriction caused by ergot alkaloids. Therefore, a pen study was conducted using color Doppler and B-mode ultrasonography to determine if treatment of wether goats with isoflavones can mediate relaxation of their carotid and interosseous arteries during and after the occurrence of ergot alkaloid-induced vasoconstriction.

MATERIALS AND METHODS

Two independent experiments were conducted in the basement of the Garrigus Building on the University of Kentucky Campus using in-door pens maintained at ambient temperature of ~21°C. The same set of six Spanish, wether goats that were ~2-year old and with rumen fistulas were used in both experiments. Initial body weights for Experiments 1 and 2 averaged 36.3 ± 2.6 (SD) and 38.6 ± 5.0 kg, respectively. Goats were weighed initially and periodically to make sure that body weights of the mature goats were stable during the experiment. Handling of goats and data collection followed procedures approved by the University of Kentucky Institutional Animal Care and Use Committee (protocol number 2013-1152).

With the low number of fistulated goats available for the study and the possibility of amelioration of ergot alkaloid-induced vasoconstriction being subtle and over a long period of time, it was decided to keep all goats on the same infusion treatment within a phase for each experiment. Carry-over effects of ergot alkaloids are not understood, so the goats were kept on the non-toxic hay for 78 days between the two experiments. Furthermore, conduct of the experiments in a controlled environment (21 ± 1°C) minimized confounding effects of time and changes in environmental conditions.

Isoflavone Analysis

Promensil® (Natrol Inc., Chatsworth, CA, USA), an over-the-counter red clover dietary supplement that has been safely administered to humans in clinical studies (19) was infused trans-ruminally as a source of isoflavones.

For assaying isoflavones in the red clover tablets (~600 mg), tablets were incubated in 7.5 mL water in a 50-mL tube for about 30 min (including 10 min at 27–32°C) to dissolve other additives in the tablets. After a brief centrifugation (3 min, 25°C, 2200 × *g*), the supernatant was discarded. Loss of isoflavones in this first step was expected to be minimal because preliminary extractions in water had demonstrated that after 1–2 h of stirring or sonicating, ~2.3 mg of isoflavones (6% of the expected total) was recovered.

The pellet recovered from the above centrifugation was then extracted after the method of Flythe and Kagan (20), but with volumes modified for the larger samples, and with some heating to approximate changes that might occur in the rumen. The pellet remaining after the water incubation was sonicated for 30 min, 28–35°C, in 8.4 mL 85% MeOH in 0.5% aqueous acetic acid. This extraction solvent included an internal standard, consisting of 0.58 mM catechin (Sigma-Aldrich, St. Louis, MO, USA) prepared in MeOH. After addition of 3.6 mL water (final solvent composition of 60% MeOH in 0.35% aqueous acetic acid), samples were centrifuged (8 min, 25°C, 2200 × g,), and 5 mL supernatant was filtered through a 0.45-μm hydrophilic membrane (GH Polypropylene, Pall Life Sciences, Ann Arbor, MI, USA). A portion of filtrate was diluted threefold in the same methanol–acetic acid solvent (to be within the range of the standard curve) and used for high-performance liquid chromatography (HPLC) analysis. Diluted extracts were separated on an Agilent 1100 HPLC system equipped with a Merck LiChrospher RP-18 endcapped column (250 mm length × 4.6 mm i.d.), using separation conditions described previously (21). Isoflavones were monitored at 270 nm and quantified based on calibration curves of standards purchased from Sigma-Aldrich (biochanin A) or Indofine Chemical (genistein). Aglycones and their corresponding glycosides, if identifiable, were summed to give the total amounts of each isoflavone of interest. Formononetin was quantified based on a biochanin A calibration curve, which decreased the final formononetin concentrations by about 20%

and, thus, gave a slightly more conservative number. Genistein was quantified with a genistein calibration curve. Genistin, the genistein glucoside, was not included in the genistein quantification because it was not detectable at the concentrations injected. Similarly, daidzein was not detected.

The amounts of biochanin A, formononetin, and genistein (representing the sum of aglycones and corresponding glycosides if present) were corrected for recovery, based on the amount of catechin recovered in extracts. Recoveries were about 80%. When biochanin A, formononetin, and genistein content were averaged among six replicates, the mean concentrations per tablet, with the extraction and analytical methods used, were 5.7 ± 0.7 mg biochanin A, 5.2 ± 1.0 mg formononetin, and 0.42 ± 0.05 mg genistein. These results did not include the contribution from the biochanin A malonylglucoside, and they underestimated the amount of formononetin by about 20% because formononetin was quantified with a biochanin A calibration curve, which has a higher response factor.

Infusion Treatments

Rumen infusion treatments were evaluated in each experiment. Goats were penned as pairs and were adapted to *ad libitum* consumption of a chopped orchardgrass (*Dactylis glomerata*)–timothy (*Phleum pratense*) hay for a minimum of 35 days prior to initiation of the infusion treatments. Quantity of Promensil infused daily (~27 g) was set to provide 30 mg/L of biochanin A, the isoflavone of highest concentration in the red clover tablets. In Experiment 1, ground toxic endophyte-infected seed of "Kentucky 31" tall fescue and isoflavones were infused in combination into the rumen of each goat during the Phase A of the experiment. Seed was ground using a Wiley Mill with 5.0 mm screen. Quantities of ground seed infused daily were targeted to provide a diet concentration of ~0.8 μg/g of diet DM of ergovaline plus its epimer, ergovalinine. Amounts of seed were daily adjusted based on the previous day's consumption for each goat pair. In Experiment 2, toxic seed was replaced with an extract of toxic endophyte-infected tall fescue that was generated following procedures described by Foote et al. (22). The extract was wrapped in a soft tortilla for infusion in a quantity that targeted a diet concentration of 1.1 μg ergovaline/ergovalinine per g DM of hay. Ground seed used in Experiment 1 and seed extract used in Experiment 2 were from composite of seed samples from bags of the endophyte-infected cultivar, "Defiance." Seed and extract were analyzed for concentrations of the ergopeptine, ergovaline, and its epimer, ergovalinine, using procedures of Yates and Powell (23) and modified as described by Carter et al. (18). Concentrations of ergovaline/ergovalinine in the seed averaged 2.33 μg/g, and the amount in daily fed seed extract per goat was 722.0 μg.

Chopped orchardgrass–timothy hay was fed *ad libitum*. Seed that was previously coarse-ground using a Wiley Mill with a 5.0-mm screen was infused into each rumen using a funnel. Feeding of hay and rumen infusions were done at 1500 hours each day. During the experimental periods, the previous day's intake for each pen was used to estimate intake per goat and the ratio of fed chopped hay to rumenally infused seed for providing a daily diet concentration of 0.8 μg of ergovaline and ergovalinine, per gram of dry matter. Because a 30 ppm dose of biochanin A was found to decrease ammonia production in mixed goat rumen bacteria *in vitro* (24), the amount of the Promensil extract needed for each goat was based on the amount needed to achieve a biochanin A concentration of 30 mg per liter of rumen. The volume of the goat rumen was estimated at 8.5 L.

Experiment 1

The goats were fed chopped orchardgrass–timothy hay *ad libitum* for 35 days prior to start of infusion treatments. During Phase A of the experiment, goats were infused daily with endophyte-infected seed and isoflavones in amounts that were previously discussed. Phase B was conducted with infusion of only the toxic ground seed for a 5-day duration.

Color Doppler ultrasound images of the cross-sections of the left common carotid artery and left recurrent interosseous artery over the scapula-humerus joint were collected using a Classic Medical TeraVet 3000 Ultrasound Unit (Classic Universal Ultrasound, Tequesta, FL, USA) with a 12L5-VET (12 MHz) linear array transducer. Hair was kept clipped with surgical clippers at each site to maintain consistent transducer placement at each imaging session. Baseline measures for hay feeding with no rumen infusions were collected for each goat on the day before and the fourth and fifth days prior to start of infusions for the Phase A. Images were collected on days 1, 2, 3, and 4, and during Phase B on days 1, 2, 3, 4, and 5. Each imaging session was started at ~1100 hours and was done in 30–40 min. Five images were collected and stored for each artery using a pulse frequency of 5.0 MHz. Scan depth was set at 4 cm for the common carotid artery and 3 cm for the recurrent interosseous and caudal arteries. Following freezing of an individual scan, frames stored in the cine memory of the unit were searched to store the image exhibiting the maximum flow signal and assumed to be at peak systolic phase. The flow signal was traced to estimate lumen area (8).

On the final day on treatment (DOT) for each infusion treatment, five longitudinal images of the left common carotid arteries also were collected using Pulse Wave Doppler feature to measure peak systolic velocity, heart rate, and pulsatility index. Pulsatility index was used as an indicator of vascular resistance (25). Images were collected on the last DOT that cross-sectional images were collected for the two infusion treatments, but not for baseline measures. Doppler spectra were collected using a Doppler frequency of 5.0 MHz, a correction angle of 60°, and a sample volume size of 1.0 mm.

Experiment 2

The goats were fed chopped orchardgrass–timothy hay *ad libitum* for 77 days prior to start of infusion treatments. The seed extract was rumenally infused daily into each goat for 8 days during Phase A, and both the seed extract and isoflavones were infused for 6 days during Phase B.

Ultrasound B-mode images of the cross-sections of the left common carotid and left recurrent interosseous arteries were collected with a Z. One Ultra SP System (Zonare Medical Systems, Inc., Mountain View, CA, USA) using the L14-5W linear array transducer. Baseline measures for hay feeding with no rumen infusions were collected for each goat on the same days prior to initiation of the infusion treatment as for Experiment 1. Another

difference from Experiment 1 was that images were collected on latter days during each phase when, based on observations in Experiment 1, a response was assumed to be present. Images were collected during Phase A on days 6, 7, and 8, and during Phase B on days 4, 5, and 6. The B-mode images were collected using a frequency of 5.0 MHz. Five image clips were stored for each artery for freezing the still image exhibiting the maximum flow signal and assumed to be at peak systolic phase for measurement of lumen diameter and calculation of luminal area. The elastin tissue in the endothelial intima was used for tracing the lumen and estimating luminal area (8).

Similar to Experiment 1, five longitudinal images of the left common carotid arteries also were collected using the Pulse Wave Doppler feature to measure peak systolic velocity, mean velocity, heart rate, and pulsatility index; however, unlike Experiment 1, the images were collected when baseline cross-sectional images were collected, and after initiation of infusion treatments on DOT when cross-sectional images were collected. Instrument settings were the same between both experiments.

Statistical Analyses

Proportionate differences of luminal areas of arteries during the phases from baseline measures were analyzed using mixed models of SAS as repeated measures with the autoregressive covariance (26). The analysis used individual animals as the experimental unit. Infusion treatment, DOT, and the interaction between infusion treatment and DOT were analyzed as fixed effects for both experiments, and goat and goat × fusion treatment were evaluated as random effects. Measures from images collected when hay was fed with no infusions were averaged for each goat and subtracted from measures made during the two phases of each experiment, and divided by the average baseline measure to estimate the proportionate difference. Infusion treatment was analyzed as a discrete variable and DOT was evaluated as a continuous variable. In the presence of interaction between infusion treatment and DOT, least square means for luminal areas in both experiments and blood flow rate in the carotid artery measured in Experiment 2 were evaluated as being above, below, or similar ($P < 0.05$) to baseline measures. Linear trends over DOT for each infusion treatment were determined as described by Littell et al. (26) using mixed models.

Blood flow characteristics for the carotid were analyzed using the same statistical model as the artery luminal areas, with the exception that actual measures and not proportionate differences were analyzed. The PDIFF option was used for comparisons of least square means between baselines and the two infusion treatments in Experiment 2.

RESULTS

Experiment 1

Baseline measures of the carotid luminal areas averaged 12.1 ± 0.7 mm^2. There were no trends over DOT during Phase A with infusions of toxic seed and isoflavones. However, carotid luminal areas were similar ($P > 0.12$) to baselines for the first 2 days, but were greater ($P < 0.05$) than baselines on days 3 and 4 (**Figure 1**). During infusion with only the toxic seed in Phase B, there was a linear decline in carotid luminal area over DOT. Mean luminal area fell below the baseline by day 3 (26% decrease from the baseline measure) and remained proportionately less on day 4 (45% decrease from the baseline measure).

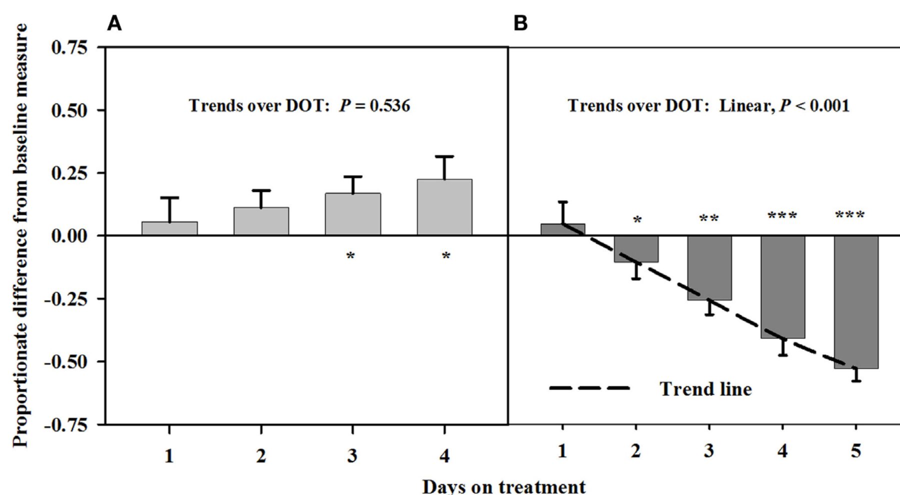

FIGURE 1 | Ultrasonic measures in Experiment 1 of proportionate differences from baseline measures for luminal area of the carotid artery in rumen-fistulated wether goats that were infused with (A) toxic endophyte-infected tall fescue seed and isoflavones in phase A, and (B) toxic seed only in phase B. Baseline measures were taken with goats receiving *ad libitum* chopped orchardgrass (*Dactylis glomerata*)–timothy (*Phleum pratense*) hay with no rumen infusions. Images for determining baseline measures were collected when goats were being adapted to the hay diet on the last, fourth, and fifth days prior to initiating the infusion treatments. Asterisks above the bars denote significant differences between treatment and baseline measures at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) levels of significance. A trend line is provided for Phase B that illustrates the linear relationship between proportionate differences from baseline and days on treatment.

Interosseous luminal areas (mean baseline measure = 2.7 ± 0.1 mm^2) with infusions of toxic seed and isoflavones during Phase A did not differ from baseline measures, but were increasingly less than the baseline over DOT during Phase B when only the toxic seed was infused (**Figure 2**). Mean luminal areas were proportionately less than baselines on 2, 3, and 4 DOT during Phase B, with ranges in areas relative to the baseline of 22% at 2 DOT to 45% at 4 DOT.

There were no differences between the infusion treatments (**Table 1**) on peak systolic velocity ($P = 0.47$) or heart rate ($P = 0.712$). A greater pulsatility index occurred at the conclusion of Phase B, during which only toxic seed was infused, which indicated greater resistance to blood flow with infusion of ergot alkaloids and less resistance to blood flow when isoflavones were combined with the toxic seed in the infusion.

Experiment 2

After being infused with the seed extract for 6 days in Phase A, mean carotid luminal areas were proportionately less than baseline measures (14.5 ± 1.0 mm^2) to the same extent over 6, 7, and 8 DOT (**Figure 3**), averaging ~41% decrease in area relative to the baseline. Carotid luminal area was still less than the baseline on day 4 after including the isoflavones with the seed extract; however, the areas increased linearly over DOT and were similar to baselines on 5 and 6 DOT.

Blood flow rate in the carotid artery responded similarly as luminal area of the artery (**Figure 4**). Flow rates were proportionately less than the baseline measures after fescue seed extract infusion (112 ± 10 mL/min.) over 6, 7, and 8 DOT, and the areas averaged ~47% of baseline areas. Flow rates increased linearly over 4, 5, and 6 days after isoflavones were included in the infusion treatment and were similar to baseline by 6 DOT.

Mean luminal area of the interosseous artery also was stable and less than baseline measures (0.7 ± 0.1 mm^2) over 6, 7, and 8 DOT when only the seed extract was infused (**Figure 5**). Decreases in interosseous luminal areas ranged from 34.2 to 41.3% of the

TABLE 1 | Mean responses (±SEM) of blood flow characteristics in rumen-fistulated wether goats in Experiment 1 when toxic tall fescue seed and isoflavones were rumenally infused in Phase A and only toxic tall fescue seed was infused in Phase B, and in Experiment 2 when toxic seed extract only was infused in Phase A and toxic seed extract and isoflavones were infused in Phase B.

Experiment	Measure	Baseline	Phase A	Phase B
1	Peak systolic velocity, cm/s		29.8 ± 3.6	33.2 ± 2.6
	Pulsatility index		1.41 ± 0.18[b]	3.39 ± 0.71[a]
	Heart rate, bpm		61 ± 4	63 ± 3
2	Peak systolic velocity, cm/s	23.4 ± 1.7	24.1 ± 2.3	20.4 ± 2.0
	Pulsatility index	1.24 ± 0.11[b]	1.63 ± 0.13[a]	1.42 ± 0.13[a,b]
	Heart rate, bpm	60 ± 3	52 ± 3	56 ± 3

The infusion treatments were reversed between the two phases in Experiment 2. Goats were fed ad libitum chopped orchardgrass–timothy grass hay and baseline measures for Experiment 2 were obtained during the adaptation period when the goats were fed the hay prior to initiating the infusion treatments.

[a,b]*Measures within rows with different superscripts are different ($P \leq 0.05$).*

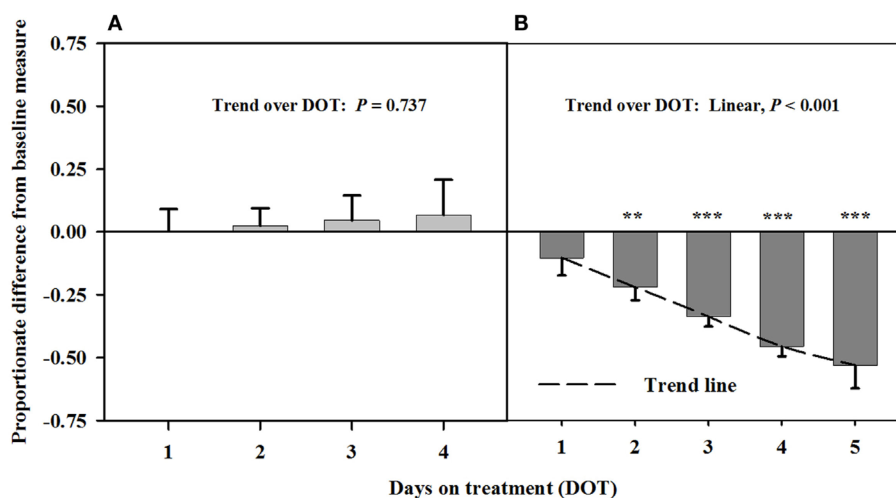

FIGURE 2 | Ultrasonic measures in Experiment 1 of proportionate differences from baseline measures for luminal area of the interosseous artery in rumen-fistulated wether goats that were infused with (A) toxic endophyte-infected tall fescue seed and isoflavones in phase A, and (B) toxic seed only in phase B. Baseline measures were taken with goats receiving *ad libitum* chopped orchardgrass (*Dactylis glomerata*)–timothy (*Phleum pratense*) hay with no rumen infusions. Images for determining baseline measures were collected when goats were being adapted to the hay diet on the last, fourth, and fifth days prior to initiating the infusion treatments. Asterisks above the bars denote significant differences between treatment and baseline measures at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) levels of significance. A trend line is provided for Phase B that illustrates the linear relationship between proportionate differences from baseline and days on treatment.

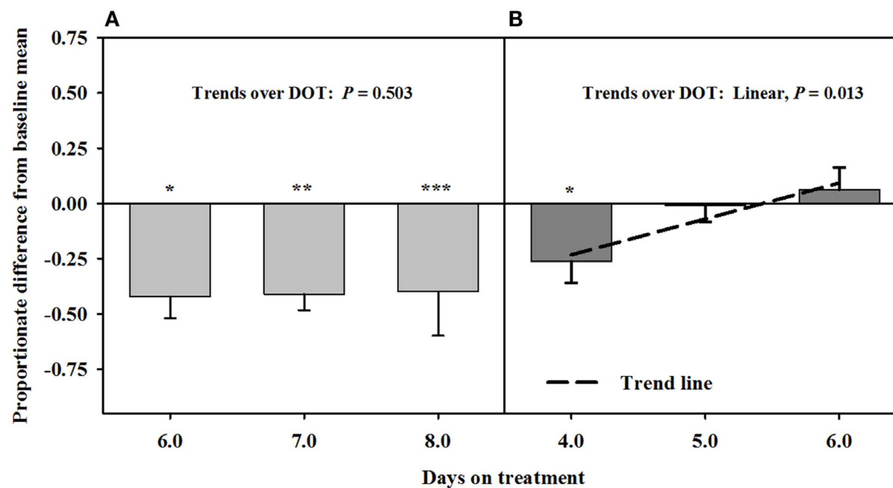

FIGURE 3 | Ultrasonic measures in Experiment 2 of proportionate differences from baseline measures for luminal area of the carotid artery in rumen-fistulated wether goats that were infused with (A) toxic seed extract only in phase A, and (B) toxic seed extract and isoflavones in phase B. Baseline measures were taken with goats receiving *ad libitum* chopped orchardgrass (*Dactylis glomerata*)–timothy (*Phleum pratense*) hay with no rumen infusions. Images for determining baseline measures were collected when goats were being adapted to the hay diet on the last, fourth, and fifth days prior to initiating the infusion treatments. Asterisks above the bars denote significant differences between treatment and baseline measures at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) levels of significance. A trend line is provided for Phase B that illustrates the linear relationship between proportionate differences from baseline and days on treatment.

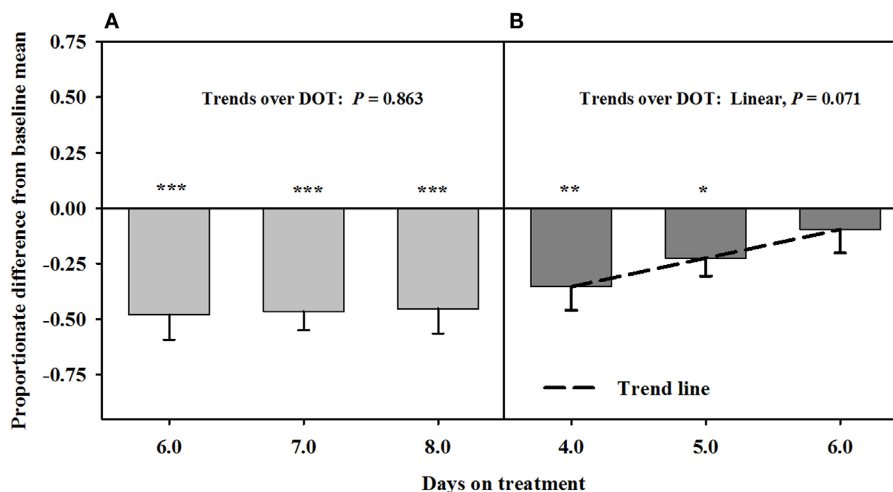

FIGURE 4 | Ultrasonic measures in Experiment 2 of proportionate differences from baseline measures for blood flow rate through the carotid artery in rumen-fistulated wether goats that were infused with (A) toxic seed extract only in phase A, and (B) toxic seed extract and isoflavones in phase B. Baseline measures were taken with goats receiving *ad libitum* chopped orchardgrass (*Dactylis glomerata*)–timothy (*Phleum pratense*) hay with no rumen infusions. Images for determining baseline measures were collected when goats were being adapted to the hay diet on the last, fourth, and fifth days prior to initiating the infusion treatments. Asterisks above the bars denote significant differences between treatment and baseline measures at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) levels of significance. A trend line is provided for Phase B that illustrates the linear relationship between proportionate differences from baseline and days on treatment.

baseline measures. By 4 DOT after isoflavones were included with the seed extract in the infusion, the luminal areas had increased and stabilized to areas that were similar to baseline measures.

Peak systolic and mean velocities in the carotid artery did not vary ($P = 0.889$) over DOT for the infusion treatments, and were similar ($P = 0.338$) between baseline measures and both infusion treatments (**Table 1**). Heart rate also did not differ ($P = 0.339$) between the baseline and infusion treatments. Pulsatility index with the seed extract infusion was greater ($P = 0.025$) than the baseline measure, but decreased back to indices that were similar

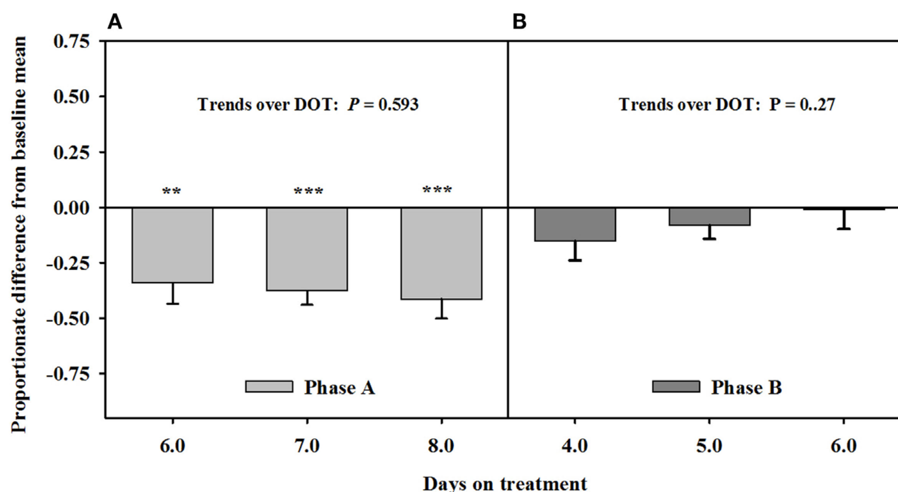

FIGURE 5 | Ultrasonic measures in Experiment 2 of proportionate differences from baseline measures for luminal area of the interosseous artery in rumen-fistulated wether goats that were infused with (A) toxic seed extract only in phase A, and (B) toxic seed extract and isoflavones in phase B. Baseline measures were taken with goats receiving *ad libitum* chopped orchardgrass (*Dactylis glomerata*)–timothy (*Phleum pratense*) hay with no rumen infusions. Images for determining baseline measures were collected when goats were being adapted to the hay diet on the last, fourth, and fifth days prior to initiating the infusion treatments. Asterisks above the bars denote significant differences between treatment and baseline measures at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) levels of significance.

($P = 0.25$) to the baseline average when isoflavones were included with seed extract in the infusion.

DISCUSSION

The focus of this research was to investigate if isoflavones can mitigate the adverse effects of ergot alkaloids on the hemodynamics of goats. Persistent constriction of blood flow to peripheral tissues incapacitates the animal's ability to thermoregulate their core body temperature. This causes animals being vulnerable to severe heat stress under moderate ambient temperatures. Recent research has reported ergot alkaloid-induced vasoconstriction of the ruminal artery and vein (27), and mesenteric vasculature (28) in cattle. These results provided strong evidence that exposure to ergot alkaloids can have a negative influence on nutrient efficiency.

Low prolactin concentrations are often used as an indicator that animals are suffering from fescue toxicosis. It would have been informative to monitor serum prolactin concentrations in the present experiment with goats; however, it was critical to control goat temperament and nervousness for accurate measurement artery lumen areas and blood flow characteristics. Although body temperature measures and collection of blood for assaying prolactin concentrations would have been informative, they would likely have caused extraneous error in the ultrasound measures that were necessary in meeting experimental objectives.

The diet concentrations of isoflavones used in the experiments were based on the concentration needed to decrease ammonia production *in vitro* (24). However, it should be mentioned that selectivity of clovers relative to grasses and other forages could be such that typical diet concentrations of isoflavones are much lower or higher than what is used in the present experiment. Dose–response experiments and grazing experiments with clover–grass mixtures will be needed to determine optimum diet concentrations of isoflavones for mediation of a response.

Experiment 1

Ergovaline is the ergopeptine that is consistently in the highest concentration in toxic endophyte-infected tall fescue (29) and also has demonstrated to be the most potent ergopeptine produced by the tall fescue endophyte in mediating a vasoconstrictive response in bovines (30, 31). Aiken et al. (32) reported a vasoconstrictive response of the caudal arteries of endophyte-naïve heifers in less than 48 h after initial consumption of diets containing ground tall seed in quantities that provided ergovaline concentrations of 0.8 μg/g DM.

Carotid and auricular arteries in goats were reported by Aiken and Flythe (11) to exhibit a vasoconstrictive response in 2 days after initial consumption of a diet containing 0.8 μg ergovaline + ergovalinine/g DM. Responses in Experiment 1 were similar between the carotid and interosseous arteries. A diet concentration similar to that used by Aiken and Flythe (11) was fed in the preset experiment, but alkaloid-induced vasoconstriction did not occur over the first 4 days when rumens were infused with both toxic seed and isoflavones. There did, however, appear to be vasodilation of the carotid artery at 3 and 4 DOT. The strong linear decline in luminal areas after isoflavones were removed from the infusion provided evidence that isoflavones mediated vaso-relaxation of the carotid artery. Similar to the findings of Aiken and Flythe (11), significant vasoconstriction occurred at 2 DOT when only the toxic seed was infused.

Higher pulsatility reflects greater resistance to blood flow; thus, greater blood flow resistance was observed on 4 DOT with infusions lacking isoflavones. Greater peak systolic velocity could also be expected with higher pulsatilities, but there can be considerable error in these measures due to between-animal and between-image variations. Aiken and Strickland (33) warned that placement of the sample gate within the vessel and animal tension could be major sources of variation between images.

Experiment 2

Based on reduced luminal areas of both arteries at 6, 7, and 8 DOT during phase A when only the seed extract was infused, the arteries appeared to have been saturated with ergot alkaloids. This is because the luminal areas at each of these latter DOT were less than the baselines and with no declining trends. Furthermore, blood flow rates in the carotid were similar to the responses for the luminal areas to seed extract over DOT. Luminal area and blood flow rate for the carotid were less than baselines on 4 DOT, but linear increases over the latter DOT indicated that the areas and blood flows were back to baseline levels by 5 DOT with inclusion of isoflavones in the infusion. Luminal areas of the interosseous artery did not exhibit increases relative to the baseline, but were similar to baseline at each of the three DOT; therefore, vaso-relaxation had occurred prior to 4 DOT.

The responses of the carotid arteries to isoflavones appeared to be gradual during the phase; however, measures were needed at earlier DOT to verify that trend. Again, saturation of the carotid was indicated to have occurred in Phase A, which could have been a factor in early mitigation of the vasoconstriction. The mitigation of vasoconstriction of the interosseous artery might have occurred earlier than 4 DOT, which suggests that there could be a greater density of β-adrenergic receptors in the interosseous than carotid artery.

It is curious that baseline measures of mean luminal area of the interosseous were almost fourfold greater in Experiment 1 than in Experiment 2. This was likely related more to the methods of measuring luminal area and not to differences in accuracy between the two ultrasound instruments, which was checked. Aiken et al. (8) concluded that tracing connective tissue in the intima with a B-mode image could overestimate luminal area and tracing the color Doppler signal could underestimate the signal. However, it was difficult without using the color Doppler signal as a guide to take the measures at the true peak systolic phase of the cardiac cycle because of a weaker pulse of the interosseous artery, as compared to the carotid artery. Tracing the color Doppler signal offers more accuracy in taking measures at the peak velocity during a cardiac cycle, whereas relying on weaker pulses without color Doppler could cause errors in detection of peak systolic velocity that would lead to underestimation of luminal area.

Peak systolic velocity and heart rate were not affected by either infusion treatment; however, the least square mean for pulsatility index was greater for the seed extract without than with isoflavones, and the baseline measure. Similar mean pulsability indices between the baseline and the seed extract and isoflavone infusion further indicated that isoflavones mitigated resistance to blood flow mediated by the alkaloid-induced vasoconstriction.

Jia et al. (34) examined the interaction of the isoflavones, formononetin and biochanin A, and tall fescue seed extract on the vasoactivity of bovine mesenteric arteries and veins. In these experiments, the two isoflavones tested did not mitigate vasoconstriction, which superficially appears to contradict the results presented here. However, comparison of the studies is informative. The most obvious difference is that the experiment performed by Jia and co-workers was *in vitro,* on a myograph, and with different blood vessels than those monitored in the current study. It is well established that β-adrenergic receptors are differentially expressed in tissue types across species (35, 36). Thus, isoflavones could have greater impact on some regions of the vascular system than others, which is consistent with the activity of known β-adrenergic agonists, such as ractopamine (37). Second, the mesenteric arteries and veins were exposed to the isoflavones for 2 h prior to the myograph measurements, while the goats received the isoflavones in the rumen daily. Microorganisms metabolize some isoflavones in the rumen (38). For example, formononetin is converted into daidzein and then equol, which undergoes renal clearance (39, 40). The detection of equol in the urine indicates that equol, rather than formononetin, was present in the blood.

When the results of the current study and the results of Jia and co-workers are considered together several plausible hypotheses emerge, including (1) clover isoflavones mitigate vasoconstriction in the carotid and interosseous arteries, but not in the mesenteric artery and vein; (2) exposure periods longer than 2 h are required; and (3) the biologically active forms are microbial metabolites of the clover isoflavones. Differences in animal species should also be considered, but previously demonstrated activities in a variety of animals (including humans and rodents) make differences between the two ruminant species the less likely possibility (16, 19, 12). It is also possible that the amounts of isoflavones in the fed tablets were underestimated. The amounts determined in this study were about 25% of the total isoflavone content reported on the product label. The discrepancy may reflect differences in the extraction or the quantitation methods used. It may also be due to the isoflavone extraction employing 30 min of sonication at ambient temperature instead of refluxing (15 min) at 85°C, the method used by Krenn et al. (41). Sonication for 30 min in acidified methanol has thoroughly extracted phenolic compounds from fruits and vegetables in other studies (42). However, it is possible that the sonication did not completely extract isoflavones from the clover tissue. In that case, the amount of isoflavones available to the animal may have exceeded the amounts determined by the tablet analysis, although it is difficult to determine how the amounts extracted by acidified methanol would compare to the amounts extracted by rumen microorganisms in an aqueous environment.

CONCLUSION

Results indicated that isoflavones in the diet of goats grazing toxic endophyte-infected tall fescue can counter and relieve ergot alkaloid-induced vasoconstriction. Although results from Experiment 2 indicated that relief with isoflavones can occur after goats are exposed to ergot alkaloids, it is uncertain if the

vasculature of the goats was saturated with ergot alkaloids, which could have had a bearing on efficacy of the treatment. More experiments are needed to verify the responses and evaluate dose–responses for determining threshold concentrations of isoflavones in the diet for mediating a positive response by the vasculature. Furthermore, the rapidness of the responses to both ergot alkaloids and isoflavones were such that valid interpretations and conclusions could be made using Latin square types of experimental designs, which will be necessary if environmental controls are not possible. Nonetheless, these results provide strong evidence that isoflavones produced by legumes can ameliorate the adverse effects of fescue toxicosis.

Studies with cattle, sheep, and horses should be conducted to determine if alkaloid-induced vasoconstriction can be relaxed with exposure to isoflavones. Given that isoflavones ameliorate alkaloid-induced vasoconstriction in grazing livestock, management approaches can be developed to utilize isoflavone containing clovers and other legumes in pastures or feed additives to alleviate or mitigate severe heat stress suffered by animals grazing toxic tall fescue in moderate air temperatures. Relaxation of alkaloid-induced vasoconstriction from consumption of isoflavones could alleviate or mitigate the adverse effects of ergot alkaloids on blood circulation and the ability to thermos-regulate core body temperature. This could ultimately enhance dry matter intake and improve overall animal performance and well-being. However, findings from the present experiments should be verified with replicated grazing trials that use a higher number of animals and with longer term exposure to ergot alkaloids.

AUTHOR CONTRIBUTIONS

GA conceived the research, designed and planned the experiment, conducted ultrasonography measures, analyzed data, and wrote the manuscript. MF assisted in the design and planning of experiment, performed the infusion treatments, and assisted in writing the manuscript. IK conducted analyses of biochanin A and determined the diet concentrations. Also, assisted in the writing of the manuscript. HJ conducted analyses of ergovaline in seed and seed extracts. LB provided the seed extracts and assisted in writing the manuscript.

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REVIEW
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Impacts of Cereal Ergot in Food Animal Production

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The negative impacts of ergot contamination of grain on the health of humans and animals were first documented during the fifth century AD. Although ergotism is now rare in humans, cleaning contaminated grain concentrates ergot bodies in screenings which are used as livestock feed. Ergot is found worldwide, with even low concentrations of alkaloids in the diet (<100 ppb total), reducing the growth efficiency of livestock. Extended periods of increased moisture and cold during flowering promote the development of ergot in cereal crops. Furthermore, the unpredictability of climate change may have detrimental impacts to important cereal crops, such as wheat, barley, and rye, favoring ergot production. Allowable limits for ergot in livestock feed are confusing as they may be determined by proportions of ergot bodies or by total levels of alkaloids, measurements that may differ widely in their estimation of toxicity. The proportion of individual alkaloids, including ergotamine, ergocristine, ergosine, ergocornine, and ergocryptine is extremely variable within ergot bodies and the relative toxicity of these alkaloids has yet to be determined. This raises concerns that current recommendations on safe levels of ergot in feeds may be unreliable. Furthermore, the total ergot alkaloid content is greatly dependent on the geographic region, harvest year, cereal species, variety, and genotype. Considerable animal-to-animal variation in the ability of the liver to detoxify ergot alkaloids also exists and the impacts of factors, such as pelleting of feeds or use of binders to reduce bioavailability of alkaloids require study. Accordingly, unknowns greatly outnumber the knowns for cereal ergot and further study to help better define allowable limits for livestock would be welcome.

Keywords: ergot alkaloids, sclerotia, *Claviceps purpurea*, toxicoses, animal performance

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INTRODUCTION

Mycotoxigenic fungi have the ability to inhabit grain cereals, leading to decreased grain yield and quality, mycotoxin production, and reduced animal performance (1, 2). Grain ergot is found worldwide and most commonly under conditions where flowering crops are exposed to extended cold and wet periods, as ergot infects the open floret (3, 4). Ergot alkaloids are produced by a group of fungi of the genus *Claviceps* and are one of the six major classes of mycotoxins (others being aflatoxins, trichothecenes, fumonsins, zearalenone, and ochratoxins) frequently found in cereal grains. Ergot

alkaloids are toxic to humans and animals if they are consumed in sufficient amounts, causing a disease called "Ergotism" (5). In most countries, grain that is contaminated with ergot is banned from human consumption and redirected for use as livestock feed (6). Consequently, ergot alkaloids continue to be a concern for livestock as allowable limits are less rigorous for feeds and the screenings containing ergot bodies are frequently used as feed. There is also a common misconception that livestock are less sensitive than humans to ergot alkaloids (7). The study of ergot toxicoses is further complicated due to climate-dependent fluctuations in fungal populations as well as genetic changes in fungi that can alter the concentration and types of alkaloids produced, potentially leading to previously uncharacterized alkaloids (7, 8). Therefore, increasing concentrations of ergot in feed grains pose a challenge for both grain and livestock industries. This review aims to describe the major ergot alkaloids currently identified in grain, how the alkaloids impact livestock and the technologies that can be used to measure alkaloids and reduce their impacts on livestock.

ERGOT AND ITS LIFECYCLE

Ergot found in grain crops arises from a parasitic fungus of the genera *Claviceps* with *Commiphora africana, Claviceps sorghi, Calotropis gigantea*, or *Claviceps purpurea*, being members and *C. purpurea* the predominant species (**Table 1**). The term "*purpurea*" originates from its ability to replace kernels in grain with hard purplish ergot bodies (sclerotia) that contain a diversity of alkaloids (9, 10).

Field and storage mycotoxins have become more abundant over the past 5 years in some areas of Canada because environmental conditions favored growth of mycotoxigenic fungi (11). For example, as much as 20% of the wheat produced in western Canada in 2011 was infected to some degree by ergot (12). With climate-change models predicting increased precipitation and prevalence of insects, concentrations of ergot in Canadian cereal grains are likely to increase in the future (13). Susceptibility of grains to ergot (from most to least) is ranked rye (*Secale cereale*), wheat (*Triticum spp.*), triticale (*Triticosecale*), barley (*Hordeum vulgare*), and oats [*Avena sativa*; (14)]. Rye, an open pollinator is more susceptible to ergot infection, whereas wheat and barley are self-pollinators. Ergot contamination typically reduces yield by 5–10% (rye and wheat, respectively), but the reduction in quality grade accounts for the majority of the economic loss associated with contaminated grain (14). Ergot alkaloids are also produced by the fungus *Neotyphodium coenophialum* in grasses, particularly fescues (15). Fescue toxicosis is prevalent in the costal and tableland regions of Australia and is estimated to cost ranchers in the USA more than $860 million per year (16, 17).

TABLE 1 | Species of Claviceps found on grain crops (10).

Claviceps species	Host crops
C. africana and *C. sorghi*	Sorghum
C. gigantea	Maize
C. purpurea	Barley, wheat, rye, oats

The life cycle of ergot has two stages, germination and the honeydew stage (9). While germination typically refers to the developmental stage from a seed to plant growth, ergot germination is defined by drumstick-shaped fruiting structures that develop from the sclerotia (9). These structures produce spores known as ascospores, which become wind-borne and easily infect the ovaries of flowering cereals (9). Contaminated grain heads can contain multiple ergot sclerotia that often require differing incubation periods to germinate. Generally, the sclerotia of *C. purpurea* require 4–8 weeks at 0–10°C to initiate germination, with higher temperatures (>25°C) prolonging germination (18). The optimal temperature range for germination of ergot in rye is thought to be 18–20°C (19), although germination in rye has also been documented between 9 and 15°C (20). Furthermore, it has been noted that germination can occur without a chilling period, but ergot body formation is enhanced during cool, wet weather, especially during the flowering stage (19).

The second stage involves the florets oozing a sticky conidia that is spread by insects and in moist environments. Following the honeydew stage, the infected ovary hardens and is replaced by an ergot body that either falls before or during harvest, contaminating the field or the harvested grain (21). However, if the flowers had fertilized prior to infection, they would have become resistant (10).

ERGOT ALKALOIDS

Although fescue toxicoses have been studied for over 50 years, the alkaloids prevalent in fescue differ from those in grain (22) and few studies have investigated the impact of grain ergot on livestock production (23). Cattle, sheep, and swine have a greater tolerance of mycotoxins produced by *Fusarium* spp. such as deoxynivalenol (DON) than for ergot alkaloids (24, 25). The FDA restricts the levels of DON in grains and grain by-products to 5 ppm for swine and 10 ppm for cattle as greater concentrations can adversely impact weight gain (26).

Concentrations of ergot alkaloids in the sclerotia of *Claviceps* can be as great as 0.75% DM (27). The concentration and the type of alkaloid produced can vary among fungal species, the type of cereal grain and with environmental conditions, with production being more pronounced in periods of heavy rainfall and with moist soils (10, 28). More than 50 different ergot alkaloids have been identified in grains infected with *Claviceps spp.*, which are divided into ergopeptine and ergoline alkaloid subfamilies. These are further divided into three biogenetically related classes: clavinet, simple sysergic acid derivatives, and peptide alkaloids [**Table 2**; (10, 29)]. However, new alkaloids are continually discovered further increasing the complexity of defining the toxicity of ergot (29).

The most dominant alkaloids in grain ergot bodies are ergotamine, ergocristine, ergosine, ergocornine, and ergocryptine (29). By contrast, ergovaline is the most common form of alkaloid present in forages infected by endophytic fungi, followed by ergine (3, 31, 32). Endophytic fungi produce alkaloid concentrations far lower than those found in the sclerotia of *Claviceps*, accounting for the differences in clinical symptoms between the two forms of toxicoses (7).

When describing ergot alkaloids, it is essential to identify their chemical structure [**Figure 1**; (27)] as the degree of toxicity may be dependent on the nature of the matrix and feed processing technique. The main ergot alkaloids, such as ergometrine, ergotamine, ergosine, ergocristine, ergocryptine, and ergocornine, are structurally similar, differing only in substitutions on C-8 (13). Moreover, alkaloids containing a C9 = C10 double bond easily epimerize depending on temperature and pH [**Figure 2**; (10, 33)] and it is possible that application of heat during pelleting may alter chemical bonds and the chemical composition of feed (34). However, minimal effects on ergot alkaloids have been observed at storage temperatures <5°C (33), but prolonged storage at higher temperatures can increase the amount of ergopeptinines that arise from natural right-hand rotation epimerization [C-8-(S); (10)].

The activation of "-ines" to "-inines" is rapid in acidic and alkaline solutions, increasing the challenge of ergot removal using extraction and cleaning processes. Avoiding the reactivation of -ines is important as this conversion appears to produce products that are more toxic to livestock (10, 37).

TABLE 2 | Limits of detection (LOD) and retention time of major ergot alkaloids and their epimers in wheat flour (30).

Ergot alkaloid	LOD (μg/g)	Retention time (min)
Ergometrine	0.0034	6.6
Ergometrinine	0.0017	7.2
Ergotamine	0.0093	8.2
Ergotaminine	0.012	9.8
Ergosine	0.0063	8.1
Ergosinine	0.0030	9.5
Ergocristine	0.017	9.1
Ergocristinine	0.021	10.5
Ergocryptine	0.0023	9.0
Ergocryptinine	0.0081	10.4
Ergocornine	0.0060	8.7
Ergocorinine	0.0055	10.1

DETERMINATION OF ERGOT AND ERGOT ALKALOIDS

Analytical methods to determine ergot alkaloids should aim to detect major alkaloids in combination with their corresponding biologically active metabolites. While some techniques are more sensitive than others, European Feed Standard Association (EFSA) determined that new validated methods are still required to quantify ergot alkaloids in feed materials to provide more reliable regulatory limits for each individual alkaloid in food and feed (27). All methods have detection limits, yet information concerning these limits for different alkaloid types is scarce.

Ergot Contamination by Visual Detection

Ergot is typically detected upon visual inspection, with dark sclerotia bodies being up to 10 times larger than grain kernels. However, ergot bodies may range in size from a few millimeters

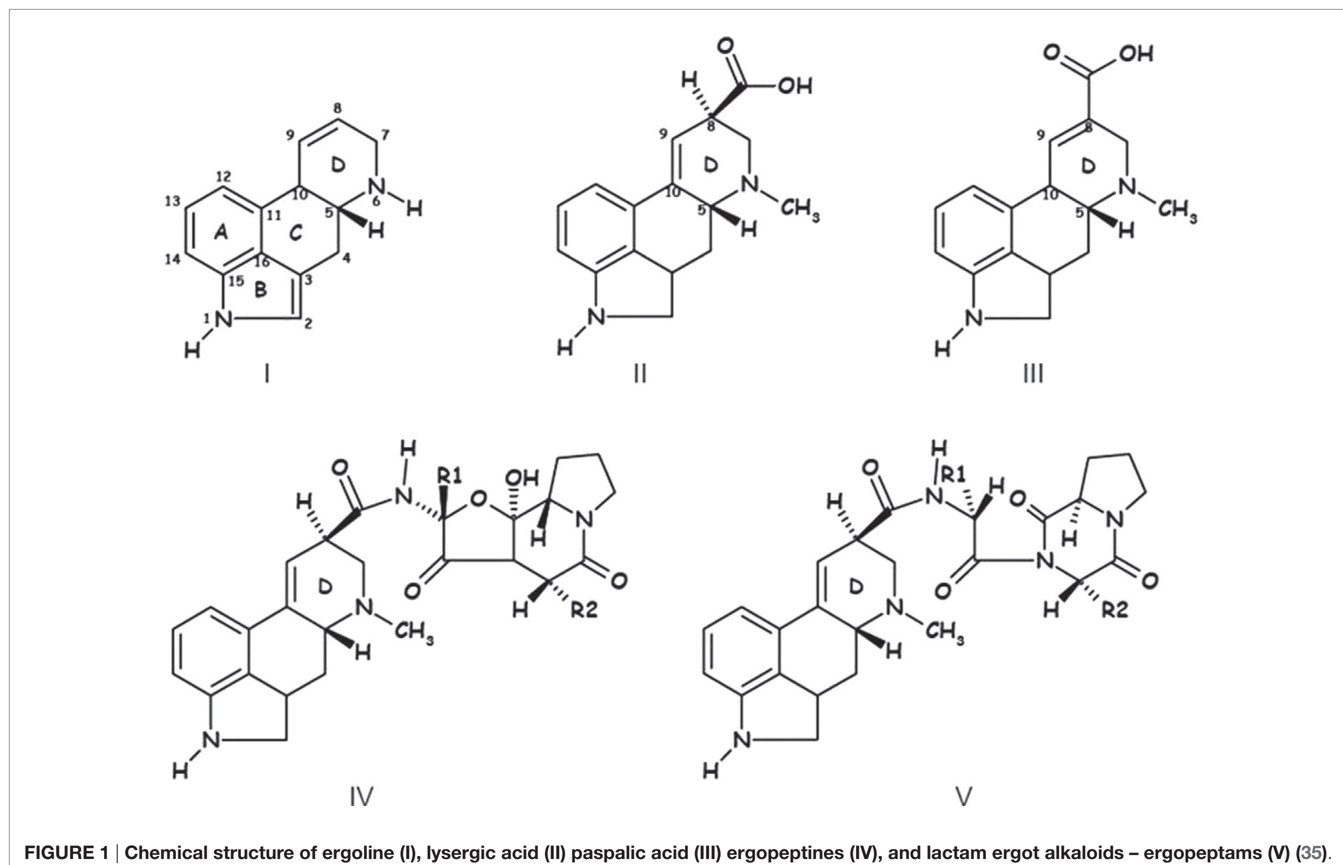

FIGURE 1 | Chemical structure of ergoline (I), lysergic acid (II) paspalic acid (III) ergopeptines (IV), and lactam ergot alkaloids – ergopeptams (V) (35).

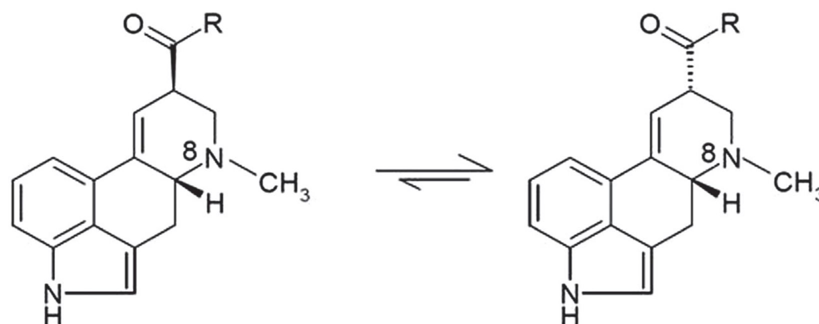

FIGURE 2 | **Ergot alkaloids containing C9 = C10 double bond readily epimerize at the center of symmetry C-8, adapted from Crews (36).**

to more than 4 cm depending on the size of the host plant (10). In some cases, sclerotia bodies are smaller (21), increasing the degree of difficulty in detecting them within grain screenings (25). Upon visual inspection, counting >5 sclerotia/L grain, or having sclerotia weighing 0.1–0.3% of grain DM is sufficient contamination that the grain should not be fed to pregnant or lactating livestock (38).

Thin-Layer Chromatography

This method uses a plate that is coated in a solid adsorbent (silica gel) in combination with a small amount of the mixed sample to be analyzed (39). The method is often used to identify a compound of interest in a mixture, as different components will vary in solubility and, therefore, migrate and be absorbed at different locations on the plate. Lobo et al. (40) found that it was difficult to separate the 12 main alkaloids in rye ergot, even using two-dimensional thin-layer chromatography (TLC), a result that likely reflects the low sensitivity of the method (7). Nevertheless, TLC may be valuable for separating individual alkaloids, particularly in developing countries (10, 35).

Liquid Chromatography and Mass Spectrometric Detection

Liquid chromatography (LC) is often used to analyze ergot alkaloids in combination with mass spectrometric detection (MS) for different matrixes in feed and foodstuffs (35). The benefit of this technique is that any known alkaloid can be determined in one run using solvent extraction, separation, detection, and quantification (10, 38).

Although only a few studies have used LC–MS–MS to detect ergot alkaloids, this technique is useful for structural confirmation and the identification of unknown alkaloids (10). Stahl and Naegele (41) reported that this technique can be used to reveal unknown ergot derivatives (semi-synthetically-derived alkaloids, such as lysergic acid diethylamide), emphasizing the importance of implementing such chemical analysis for future research. Blakley and Cowan (38) described quantification of four common ergot alkaloids using this method and recognized that combined alkaloid content should not exceed 100–200 ppb (60 g of grain is required for analysis). However, issues with the collection of representative samples, variation in kernel size, and crop type may produce inaccurate results with this method (38).

Byrd (30) determined the limits of detection (LOD) of six ergot alkaloids in wheat and their epimers, in combination with their corresponding retention time (**Table 2**). Moreover, Krska and Crews (10) validated the use of LC–MS as a means of reliable detection in determining certain alkaloids, yet today only six alkaloids and their isomers can be accurately identified using this method (**Figure 3**).

High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) uses a column to pump the sample mixture at great pressure in a solvent with chromatographic packing material, producing excitation wavelengths ranging between 235 and 250 nm as detected by UV absorption (43, 44). With the ability to detect compounds at concentrations as low as parts per trillion, HPLC is a common method currently used to identify ergot alkaloids. The most common alkaloids detected using this method are ergometrine, ergotamine, ergocornine, ergocryptine, ergocristine, ergosine, and their respective isomers, with the sum of these alkaloids equating to total alkaloid content (45).

Although alkaloid concentrations have been detected as low as 0.02–1.2 μg/kg using multi-analyte LC–MS/MS, extensive epimerization was noted, affecting the estimation of overall alkaloid content (10). Sulyok et al. (46) demonstrated that HPLC could detect concentrations as low as 0.17–2.78 μg/kg without epimerization, validating the prevalent use of HPLC for determining alkaloid content.

Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) involves combining antibodies with an enzyme-mediated color change (commonly alkaline phosphatase and glucose oxidase) to identify small quantities of targeted substances. The antigen is capable of binding to the specific antibody, which can be identified by a secondary antibody and revealed using fluorogenic substrates (47). This technique is attractive for ergot screenings in crops, but has difficulty in identifying a marker toxin to serve as a standard to determine the extent of alkaloid contamination (48).

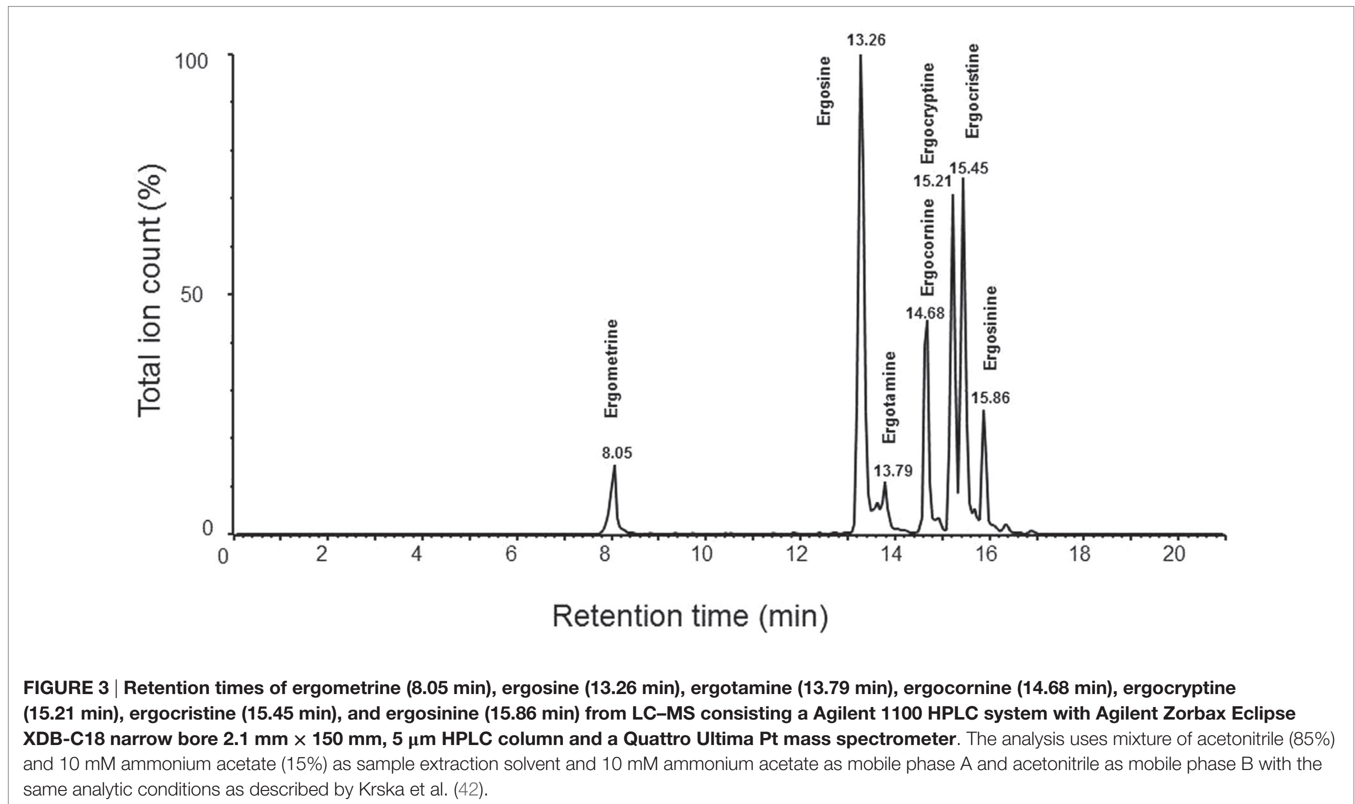

FIGURE 3 | Retention times of ergometrine (8.05 min), ergosine (13.26 min), ergotamine (13.79 min), ergocornine (14.68 min), ergocryptine (15.21 min), ergocristine (15.45 min), and ergosinine (15.86 min) from LC–MS consisting a Agilent 1100 HPLC system with Agilent Zorbax Eclipse XDB-C18 narrow bore 2.1 mm × 150 mm, 5 μm HPLC column and a Quattro Ultima Pt mass spectrometer. The analysis uses mixture of acetonitrile (85%) and 10 mM ammonium acetate (15%) as sample extraction solvent and 10 mM ammonium acetate as mobile phase A and acetonitrile as mobile phase B with the same analytic conditions as described by Krska et al. (42).

Furthermore, cross-reactivity can vary substantially depending on the nature of alkaloids being detected (10).

Near Infrared Spectroscopy

The near infrared spectroscopy (NIR) method is used to estimate total ergot alkaloid content, particularly in tall fescue, with calibrations based on measurement obtained through ELISA (35). This method can be employed with both grain and pelleted feeds; however, pelleted grain must be ground prior to measurement to improve the accuracy of estimates (2, 35). A great advantage of NIR is the speed of detection and its ability to analyze both large and small quantities of feeds, thereby avoiding errors associated with inconsistent sampling (2). The system can also make measurements in real time by placing sensors in grain augers or belt systems (100 kg grain can be analyzed in 1 h). However, the system is heavily dependent on the establishment of an accurate calibration in which alkaloids have been measured using the sensitive techniques described above. Variation in the types of alkaloids present in grains and feeds may make development of universal calibration equations difficult.

Detection in Animal Tissues

Alkaloids, such as ergocornine, can decrease pituitary prolactin release and counteract the stimulatory effect of estrogen on prolactin concentrations, significantly reducing milk production (49). Therefore, isolating serum from whole blood and conducting prolactin analysis may be useful in the detection of ergot alkaloids as a low prolactin concentration could be indicative of ergot alkaloid poisoning. Direct detection of ergot alkaloids and/or their derivatives in liver tissues is as yet, only at a preliminary stage (25).

Tissue accumulation of ergot alkaloids, while of concern, has been little studied, largely due to a lack of suitable assays. Dairy cattle fed 125 mg ergot alkaloids/kg dietary DM over a 2-week period led to a carry-over of toxins into milk, although less than 10% of ingested ergot alkaloids were detected (50). However, when swine were fed 1–10 g ergot/kg body weight, no evidence of ergot alkaloid residues was found in meat (45). Additional knowledge of the kinetics, metabolism, and tissue deposition of ergot alkaloids is required to determine whether the carry-over of alkaloids to livestock products other than milk occurs (35).

FEEDING ERGOT-CONTAMINATED GRAIN TO LIVESTOCK

Allowable Limits

The concentration of ergot alkaloids that are allowable for livestock consumption is presently contentious, as there are several different measurements in the literature that are not interchangeable. The toxicity of ergot alkaloids depends on both the type and the absolute concentration of the individual alkaloid as well as interactions with other mycotoxins that may be present in feed (27).

Individual countries have established specific tolerances for concentrations of ergot bodies in both cereal grains and animal feed (**Table 3**). Legislation is in place that sets the limits of ergot contamination in cereal grains for the human market at 0.05%

TABLE 3 | Allowable levels of ergot contamination (ppm) in cereal grains and feed in various regions of the world [T, triticale; W, wheat; R, rye; B, barley; O, oats; (51)].

Region	Ergot limit in cereal grains for humans (ppm)	Ergot limit in animal feed (ppm)	Other comments
Australia and New Zealand	0.05	N/A	0–0.1% (T)
Canada	0–0.05	0.10–0.33	Varies with grade of wheat
European Union	0.05	0.10	–
Switzerland	0.02	N/A	0.05 limit on cereals destined for milling
Japan	0.04	N/A	–
United Kingdom	Zero tolerance	0.001	–
United States	0.3 (W, R)	0.3 (W, R)	0.1% (B, O, T)

N/A, not available.

TABLE 4 | Recommended practical limits for ergot or ergot alkaloids in animal feeds to reduce negative effects on health and performance.

Animal	Recommended ergot alkaloids practical limits [ppm; (52)]			Maximum tolerance (allowable) level of ergot alkaloids [ppm; (5)]
	Low	Moderate	High	
Piglets/sows/gilts	0.5	1	2	4–6
Poultry broiler/layer	0.75	1.5	3	6–9
Dairy/beef cattle	0.5	1	2	2–3
Calf	0.25	0.5	1	2–3
Horses	0.25	0.5	1	2–3

in Australia and European Union (EU) and 0–0.05% in North America. The EU and the United States require grains destined for livestock feed to contain less than 0.1 and 0.3 ppm total ergot, respectively. The United Kingdom has a 0.001 ppm tolerance for total ergot in animal feeds. Grain exceeding these limits is banned from entering either the food or feed chain.

In Canada, maximum allowable levels of ergot alkaloids in cattle and swine feed have also been established and are 2–3 and 4–6 ppm, respectively (5). It is also recommended that feed contaminated with 250 ppb ergot alkaloids not be fed to pregnant or lactating animals due to a greater risk of abortion and agalactia syndrome. In general, 5–10 μg ergot alkaloids/kg body weight represents the general threshold dosage for all livestock (5), yet EFSA recommends doses as low as 0.6–1 μg of ergot alkaloids/kg body weight to avoid their vasoconstrictive effects (27).

Although legislation establishes tolerances for ergot alkaloids or ergot bodies in livestock feed, in most cases these concentrations have not been established through toxicological studies with livestock (2, 5, 51). For example, dietary concentrations of ergot alkaloids as low as 100–200 ppb (ergovaline) can have adverse impacts on livestock growth, especially livestock suffering from heat stress and interactions among alkaloids can lead to heightened toxicity (25). The concentration of alkaloids in the ergot bodies also varies between 0.01 and 0.21% (27). The great variation in reported impacts of ergot on animal performance has led to inconsistent recommendations of tolerable limits of ergot across countries (2). It is also evident that calves and horses are the most sensitive to ergotism, with poultry having the greatest tolerance [**Table 4**; (5)].

Impact of Feed Processing and Grain Storage on Ergot Alkaloids

Unlike other mycotoxins that are capable of forming post-harvest as a result of spoilage during storage, ergot only forms pre-harvest, with concentrations of alkaloids remaining relatively constant during storage (25). However, Krska and Crews found that extended storage of high-moisture grain that led to aerobic instability resulted in increased ergopeptinines by promoting ergot growth (10). Despite speculations that alkaloids may degrade over time, ergot stored at 15°C for 12 months still germinated, emphasizing the importance of screening techniques to avoid propagation of ergot in grain (18). Storage temperatures lower than 5°C had little effect on ergot alkaloids (33), although high-temperature storage has the potential to alter their chemical structure and biological activity.

Pelleted grain screenings are a popular low-cost feed for both sheep and cattle. Anecdotal observations suggest that pelleting or high-temperature processing of feed may increase the bioavailability of ergot alkaloids (25), but this possibility has not been investigated experimentally. Grain by-products used for ethanol production, such as distiller's grains, are frequently fed to livestock, although this product retains and concentrates ergot alkaloids through the production process (25). However, the effects of fermentation on the activity of ergot alkaloids and potential implication for animal health and productivity have not been fully studied. Further complications arise with grain after being processed and pelleted as ergot is then impossible to visually detect.

Effects of Ergot Alkaloids on Health and Productivity of Livestock

Clinical Symptoms of Ergot Poisoning in Livestock

Ergot toxicity was first described in the middle ages as a gangrenous outbreak in humans known as "St. Anthony's fire," responsible for disfigurement of people and deaths (3, 7). At present, ergot poisoning rarely occurs in humans due to advanced grain processing technology and strict legislation.

Clinical symptoms of ergot poisoning can be manifested in as little as a few hours or may require months to become observable. This variability reflects differences in physiological responses to the type and concentration of alkaloids and accounts for the frequent misdiagnosis of the condition (5). Furthermore, symptoms of ergot toxicosis often resemble other conditions, such as foot rot, frostbite, and respiratory disease, further complicating diagnosis (25, 53).

Generally, ergot toxicosis is manifested in three forms:

(1) *Convulsive:* convulsions, staggering, muscle spasms, and temporary paralysis occur. This condition is often confused with tremors associated with *Claviceps paspali* (which

TABLE 5 | Summary of ergot symptoms in mammals (8).

Form of ergotism	Species	Subfamily	Toxic alkaloid(s)	Symptoms
Convulsive ergotism	*C. purpurea*	Ergoline	Ergotoxin, ergometrine, ergotoxin (lysergic acid amines including lysergic acid, lysergol, ergine)	Writhing, tremors, twisted neck or head tilt (torticollis), confusion, hallucinations, tingling sensation underneath the skin (formication) and death
Gangrenous ergotism	*C. purpurea*	Ergopeptine (total dietary concentrations of >100–200 ppm can lead to death)	Ergotoxin, ergometrine, ergotoxin (lysergic acid amines), ergovaline, ergocryptine	Vasoconstriction, hot and cold feelings in the extremities, cold skin, spontaneous abortion, heat stress, severe lameness, reduced feed intake, reduced growth rate, agalactia, and gangrene. Ergocryptine affects prolactin levels and greatly reduces or eliminates milk production for lactation
Enteroergotism	*C. fusiformis*	Unknown	Clavine	Nausea, vomiting, somnolence, and giddiness
Hyperthermic ergotism	*A. coenophialum, C. africana, C. cyperi, C. purpurea, C. sorghi*	Unknown	Ergotamine, ergosine, and agroclavine	Fever, diarrhea, clear nasal discharge, weight loss, labored breathing, increased metabolic rate, excessive salivation, and low levels of prolactin

contains great amounts of lysergic acid). This type of poisoning is more common in sheep and horses but seldom seen in cattle [**Table 5**; (29)]. Upon slaughter, rigor mortis is never complete, leaving muscles flaccid.

(2) *Gangrenous:* this form results in lameness, followed by the loss of extremities, such as the ears, tail, hooves, and in severe conditions even limbs (7). This form results from impaired circulation and blood supply and is most common in cattle and pigs. The condition is more severe under hot or cold conditions where vasoconstriction or vasodilation is necessary for thermoregulation (28). Gangrenous ergotism can require up to 3 months to become clinically obvious, with early symptoms, including an elevated respiration rate, gradual weight loss, a reduction in milk production, and reduced reproductive performance.

(3) *Other:* these symptoms can be less severe and include vomiting (enteroergotism), fever (hyperthermic ergotism), and alterations in endocrine function. Long-term exposure to ergot, intensified during hot and humid conditions, favors hyperthermic ergotism (54). Heifers injected with ergotamine and ergonovine exhibited a combination of symptoms, such as lower skin temperature, heart rate, and blood prolactin concentrations, with an increase in respiration rate and blood pressure (55). Chronic exposure to alkaloids can result in the greatest economic losses due to decreased reproductive performance and increased abortions (3).

Ergot toxicosis can often be misdiagnosed as other forms of syndromes associated with feed refusal such as those associated with vomitoxin (56, 57).

Effects on Health and Performance of Livestock Animals

Consumption of ergot-contaminated grains can have negative effects on feed intake, growth, and reproduction, but factors such as livestock species, age, and the presence of other stressors such as heat or cold can influence the extent of negative health outcomes (58). Low concentrations of ergot alkaloids (<2 ppm) in feed can depress animal performance and result in intoxication, especially if feeds are administered for a prolonged period of time.

Animal Growth

Cattle fed diets containing 1.6% ergot (12.7 g ergot intake/day) exhibited a lower average daily gain (0.55 vs. 0.83 kg/day) and lower feed intake (6.36 vs. 10.1 kg/day) as compared to those fed uncontaminated grain (59). The study also showed that ergot intake from 1.14 to 8.17 g/day had little effect but at 12.7 g/day significantly decreased feed intake. By contrast, growth rate was linearly decreased with ergot intakes from 0 to 12.7 g/day. This observation suggests that ergot alkaloids have a direct negative impact on energy metabolism and feed efficiency when ergot intake exceeds certain limit.

Reproductive Performance

Cattle consuming endophytic fescue have consistently lower prolactin concentrations in plasma, with minimal changes in plasma luteinizing hormone or growth hormone [GH; (60, 61)]. Prolactin concentrations sharply declined and plateaued in cattle intravenously injected with 7 mg of ergotamine tartrate in saline over 240 min [average dosage of ergot alkaloids was 28.8 μg/kg body weight; (62)]. This decline in prolactin secretion is due to activation of D2-dopamine receptors in pituitary lactotrophs (10). Furthermore, lysergic acid derivatives are structurally similar to noradrenaline transmitters, including dopamine and serotonin, enabling ergot to disrupt the endocrine system (10).

By contrast, plasma GH concentrations in steers exhibited a transient increase after ergot alkaloid administration (23.8 μg/kg body weight) through i.v. injections (62). While ergot derivatives increased human GH concentrations, ergotamine had no impact on GH secretion from rat pituitary cells (63, 64). Accordingly, Browning et al. (62) found that cattle fed endophytic fescue displayed greater GH concentrations compared to steers grazing fescue with low endophyte content. A suppression of luteinizing hormone when ergotamine was injected suggests that this alkaloid alters the activity of the hypothalamic–pituitary–gonadal axis. By contrast, Christopher et al. (65) demonstrated that tall fescue has suppressive effects on GH secretion in ovariectomized heifers. Consequently, with acute exposure to alkaloids, particularly

ergotamine or ergonovine, noticeable alterations to plasma concentrations of prolactin, GH, and luteinizing hormone become apparent (62). Similar endocrine impacts from grain ergot alkaloids are also likely, although have yet to be studied.

Pregnancy Rates

The alkaloids that promote vasoconstriction and lead to gangrene can also promote developmental and reproductive toxicity, such as abortions by restricting blood supply to the uterus. Duckett et al. (17) documented that ewes fed endophyte-infected tall fescue seed had shorter gestation lengths (up to 5-day difference), leading to a 2 kg reduction in lamb birth weights. During pregnancy, consumption of ergot alkaloids can impact maternal lipid metabolism, mammary growth and reduce milk production and secretion from the inhibition of prolactin release (17, 66). Similarly, compared to cows consuming endophyte-free fescue, Watson et al. (67) observed a 15% reduction in birth weight of calves delivered from cows consuming endophyte-infected fescue. However, both occurred under high ambient temperatures conditions where alkaloid consumption has the greatest impact on reproductive function. As umbilical blood flow increases throughout pregnancy, the vasoconstrictive response to ergot alkaloids can restrict blood flow to the fetus and impair fetal development (17). Moreover, Dyer (68) also observed that ergovaline induced contraction in the uterus further altering fetal development.

Abortions and premature births have been noted in sows fed grain ergot (69). Similarly, supplementing ewes with 0.1, 0.5, or 0.7% ergot-contaminated feed decreased lambing by 20% (70). However, because there were no data on the type of ergot and the quantities of alkaloids in these studies, it is difficult to determine whether the reduced pregnancy rate was due to ergot or other mycotoxins. In a later study, Burfening (59) reported that lambing rate increased to 0.87 lambs/ewe when ewes were fed diet containing 0.5% ergot. This was contrasted to the observation that the lambing rate declined from 1.02 to 0.78 lambs/ewe when ewes were fed diets containing 0.1% ergot. This may demonstrate adaptability of the ewes to the toxin throughout pregnancy or differences in relative concentrations of alkaloids in the two studies. Furthermore, lambs fed a 0.5 or 0.7% ergot-contaminated diet demonstrated a greater susceptibility to lameness with 21% of lambs showing signs of impaired movement. However, no abortions were observed in either of the above trials, although reduced body condition from grain ergot ingestion was noted (70).

Agalactia refers to the absence or failure to secrete milk, displaying irreversible effects for pregnant livestock during late gestation, with greatest susceptibility in sows (71). A direct correlation with a decrease in prolactin secretion and the inhibition of milk production was first identified by Zeilmaker and Carlsen (72) in rats injected with 1 mg of ergocornine, a condition that could be reversed by continuous administration of prolactin. Similarly, it has been shown that feeding 0.5–1.0% ergot to gestating sows impaired udder development (73). Yaremcio (74) proposed that estrogen concentrations in ergot can cause abortions, along with temporary sterility resulting in lowered subsequent conception rates. Hence, even low concentrations of ergot should be avoided in the feed of pregnant or lactating animals to avoid the risk of underdeveloped neonates and reduced mammary tissue development (71). However, even though several studies have showed that prolactin concentrations decrease upon exposure to ergot alkaloids (49, 75), milk production in ewes fed diets containing 0.5–0.7% grain ergot did not decrease (70).

Sperm Motility

Some ergot alkaloids can negatively affect sperm and uterine motility in mammals through agonistic interactions with dopaminergic, alpha-adrenergic, and serotonergic receptors (76, 77). Such membrane receptors are involved in the regulation of mammalian sperm function and increases in intracellular cAMP and calcium concentrations can negatively impact the motility of bovine spermatozoa (77). Moreover, ingestion of ergot alkaloids by growing bulls depressed growth rate, serum prolactin concentration, scrotal circumference, and sperm motility (78). Treating sperm with ergonovine (20 mg/mL) resulted in the greatest reduction in sperm motility and the percentage of intact acrosomes as compared to treatment with phenylephrine, oxytocin, and norepinephrine (76). Ultimately, sperm motility is affected by grain ergot, whereas both cortisol and testosterone concentrations are not impaired when bulls were fed toxic endophyte-infected and novel endophyte-infected feed (78). It has been shown that the interaction of ergot alkaloids with membrane receptors is complex and different alkaloids affect different receptors in different types of tissues (77).

Species Differences

In addition to noticeable differences in tolerance levels between species, variation in absorption rate and ability to detoxify toxins is extremely diverse. Although poultry is regarded as a group for recommended allowable limits, dependent on species, they can either be quite tolerant or extremely sensitive (ducks) to ergot alkaloids. This demonstrates the need to develop recommended allowable limits of alkaloids for all species of livestock and poultry.

Compared to mammals, poultry appear to have a greater ability to detoxify alkaloids (27). Mainka et al. (45) reported that ergot did not cause changes in weight gain of 28-day-old chickens fed *ad libitum* with an ergot content of 0, 0.5, 1, 2, and 4 g/kg diet. The same levels of ergot reduced weight gain in piglets. Chickens rapidly turn over epithelial cells (within 48 h) that may explain their rapid detoxification of ergot (79, 80).

However, even for poultry long-term exposure to alkaloids may lead to loss of appetite, increased thirst, diarrhea, vomiting, and weakness (81). Similarly, Dänicke (82) exposed Peking ducks to four different diets containing 1, 10, 15, and 20 g ergot/kg diet, respectively. This corresponded to total ergot alkaloid contents of 0.0, 0.6, 7.0, 11.4, and 16.4 mg/kg. They found that feed intake decreased up to 47% with the high ergot diets. While Mainka et al. (45) identified no adverse effects on weight gain of chickens, Dänicke (82) observed a significant growth reduction after 2 weeks, suggesting that existing ergot alkaloid limits for poultry(1 g ergot/kg unground cereal grains in EFSA regulations) may not offer sufficient protection for ducks. Furthermore, Dänicke (82) detected alkaloid residues in edible tissue (5 ng/g) of Peking ducks that also had ergonovine in bile (40 ng/g). Thus, the negative performance of ducks when exposed to 0.6 mg/kg of ergot alkaloids indicates that not all species of poultry are equally tolerant of dietary ergot.

Impact on the Plant and Animal Industries

Most mycotoxins that infect growing crops and stored feed will be detected based on the type of symptoms shown by livestock (83). However, with ergot displaying broad symptoms, such as heat stress, reduced growth, and feed refusals, producers are challenged to identify the occurrence of ergot toxicosis before it has already had a negative impact on the economics of livestock production. With no universal standard for the safe concentration of ergot in feed, producers must exercise caution when introducing potentially contaminated feed sources such as grain screenings into their feeding programs.

While some livestock can tolerate greater concentrations of ergot in feed, the potential for residual toxins to remain in tissues of animals could cause detrimental effects to the human population (1, 45, 50). More importantly, by-products, such as screenings for livestock feed may be highly contaminated with mycotoxins and, moreover, have a greater potential of harming livestock (57). With the prevalence of ergot increasing from 0.01% in 2002 to 0.025% in 2014 in western Canada (84), it is evident that monitoring ergot is becoming more essential for the safety of both livestock and humans (23).

The need to produce cereal varieties that are capable of withstanding ever-changing climatic conditions has seen an increased use of hybrid varieties of rye and perennial rye breeds in the last 10 years, particularly in European countries such as Germany (10). However, today with grain-cleaning procedures now capable of removing up to 82% of ergot bodies from unprocessed grain (broken ergot sclerotia are less reliably removed as the particle size is similar to the grain), it is evident that improvements are being made, though often at substantial cost to the producer (10, 27).

The European Food Safety Authority (27) suggested that in order to successfully reduce the risk of ergotism in livestock, contaminated cereal grains should undergo seed cleaning, in combination with the adoption of certain husbandry measures such as crop rotation and grazing during summer months to reduce the establishment of flower-heads. However, when considering the level of contamination in cereal crops, it is important to determine alkaloid epimers, as these could alter the toxicity of ergot and cause more harm to livestock than anticipated (10, 37).

Economic impacts surrounding reproductive losses and lowered growth performance are detrimental both on a domestic basis and a global basis. Moreover, with no current treatment marketed to improve symptoms of ergot toxicity and the difficulty of diagnoses, the only available response is to remove the contaminated feed from the diet and allow the liver to detoxify consumed alkaloids (28). It is evident that further investigations are needed to develop effective measures to prevent ergot toxicity in livestock and reduce the economic impact of ergot on agricultural commodities.

DETOXIFICATION AND ABSORPTION OF ERGOT ALKALOIDS

Livestock and poultry have the capacity to detoxify ergot alkaloids in the liver. However, given the diversity of ergot alkaloids, it is impractical to estimate the length of time required for detoxification and clearance of all alkaloids from the liver. Moubarak et al. (85) characterized the role of cytochrome P450 3A (CYP3A) subfamily in the metabolism of ergot alkaloids, in beef liver microsomes. Ergotamine was metabolized by CYP3A after 60 min of incubation; however, other alkaloids, such as ergocryptine and ergocornine, inhibited CYP3A activity. Cattle intravenously administered ergopeptine rapidly cleared it from the blood through biliary excretion, whereas lower molecular weight alkaloids, such as ergovaline, were excreted in urine (29).

If absorption varies among alkaloids, it may be possible that not all ergot alkaloids are harmful to livestock. Schumann et al. (58) identified that while ruminants have the potential to detoxify mycotoxins in the rumen, microbes are influenced by the passage rate of feed. Increased feed intake reduces feed retention time in the rumen and increases passage rate, impacting digestion and metabolism. Increasing ergovaline in feed from 0, 1.5 to 3 mg/kg diet depressed feed intake, in addition to reducing ruminal and total tract organic matter and neutral detergent fiber (NDF) digestibilities in sheep. This may have lowered the metabolism of ergot alkaloids in the rumen (58, 86). Westendorf et al. (87) reported that feeding 945 mg/d ergovaline (16 mg/kg body weight) decreased DM and NDF ruminal digestibilities, while exposing sheep to 2,346 mg/day ergovaline increased DM and NDF ruminal digestibilities, also possibly due to reduced intake and a longer retention time of feed in the rumen.

Absorption of alkaloids occurs primarily in the ruminant forestomach, with rumen tissue having the greatest transportation rate [25% more than the omasum; (88)]. Extensive excretion of toxins via the urine was noted in steers exposed to infested tall fescue as measured by ELISA (89). In comparison, fecal excretion was limited to 5% of alkaloids fed to sheep, emphasizing the high level of absorption that occurs in ruminants (87). Furthermore, varying differences in liver enzyme function and individual rumen microorganisms will alter an individual animal's capability of detoxifying alkaloids, leading to varying levels of tolerance (90, 91).

Veterinary recommendations suggest that ergotism can be controlled through an immediate change to an ergot-free diet. However, for pregnant livestock and in particular for sows in late gestation (<1 week prior to parturition), agalactia syndrome cannot be corrected (71). Agalactia syndrome from fescue sources can be corrected in horses through administration of dopamine D2 antagonist domperidone (1.1 mg/kg for 10–14 days). In cases where livestock have been clinically diagnosed with peripheral gangrene, the removal of ergot-contaminated feed will not lead to recovery.

TECHNOLOGIES AND PRACTICAL MEASURES DESIGNED TO REDUCE THE IMPACT OF ERGOT ON LIVESTOCK

Genetic Engineering Strategies

It is possible to select for genetic resistance to ergot among grain crops, although genetic engineering strategies and the selection

of hybrids naturally resistant to molds could be a means of controlling ergot in wheat (92). Though minimal information is known on the role of insects in ergot epidemiology, there is future potential for plants to be selected that deter insects and reduce the spread of mycotoxins (57, 93).

Development of Vaccines and/or Alkaloid Binders to Allow the Animal to Systemically Bind the Toxic Alkaloids

The development of vaccines against ergot alkaloids is a possible long-term solution. Filipov et al. (94) observed a greater average daily gain (13.0 g/day) when rabbits were vaccinated with 50 μg lysergol-human serum albumin compared to non-treated rabbits (12.1 g/day). While this study evaluated a vaccine against the effects of alkaloids from tall fescue (total dietary alkaloids 340 ppb), development of a vaccine associated with grain alkaloids should also be possible.

Deoxynivalenol is a mycotoxin causing similar symptoms to ergot in livestock, such as reduced feed intake and body weight gain (57). Although ergot alkaloids and DON differ in chemical structure, studies conducted using DON have relevance for ergot. Young et al. (95) revealed that feeding swine corn contaminated with 7.2 mg DON/kg resulted in a reduction in feed intake. When corn was treated with sodium bisulfite, impacts of DON decreased 10-folds. Treatment with sodium bisulfite appeared to remove short-term toxic effects on pigs due to the presence of DON in their diet. Accordingly, there is a possibility that chemical treatments could be developed to reduce the toxicity of ergot alkaloids in feed.

It is also plausible that using alkaloid binders will decrease bioavailability of ergot alkaloids. However, studies of alkaloid binders are limited and in the one published study, Friend et al. (96) evaluated a chemical binding agent, polyvinylpyrrolidone (Antitox Vana®) and ammonia carbonate and noted that these binders did not reduce the negative impacts of DON on swine production. Further investigations using alkaloid binders to reduce the toxicity of ergot-contaminated grain are required, but care must also be taken to ensure that such binders do not reduce overall nutrient availability (24, 97).

Isolation of Anaerobic Bacteria to Degrade Ergot Alkaloids before Systemic Absorption

Anaerobic microbes present in the rumen of sheep and cattle are capable of detoxifying some ergot alkaloids and inoculating other microbes into the rumen might be beneficial in this regard. Anaerobic microbes in the gut of the red wiggler earthworm, *Eisenia fetida,* degraded over 60% of ergovaline, with the flora responsible for this degradation from four major phyla: *Plantomyce, Chloroflexi, Bacteroides,* and *Proteobacteria* (16). Further research to isolate and characterize microorganisms that are capable of detoxifying ergot alkaloids may allow their use as a direct-fed microbial to minimize the impact of feed ergot on animals.

Hydrothermal Treatment Effects on Ergot Alkaloid Content in Contaminated Grain

Hydrothermal treatments are often incorporated to improve the digestibility of nutrients and feed value, particularly for non-ruminant species (45). Treating ergot-contaminated grain with steam for 2 min at 95°C at 17% moisture, followed by 5 s at 120°C at 18% moisture decreased total alkaloid content by 10%, with reductions becoming more marked with increasing levels of alkaloids (45). This method could be employed during the feed processing stage to further reduce alkaloids, although impacts on alkaloid toxicity would require investigation prior to use in livestock feeds.

Other On-Farm Prevention Measures

Irrespective of advanced technologies that can be potentially implemented on-farm to minimize negative impacts, ergot toxicoses are mainly controlled by limiting ergot presence at all levels of production, including storage, milling, and delivery (57). Chemical treatments used to clean the grain kernels can be implemented to significantly reduce the toxin level if the ergot contamination is not too severe. Removal of grain dust and lighter, shriveled kernels through density segregation can also reduce the risk of ergot poisoning (5, 23). Other procedures, such as soaking, dehulling, roasting, or high velocity air cleaning of grain can be used to remove surface ergot contaminants (27).

A variety of prevention measures have been identified to help producers minimize ergot establishment and growth in cereal crops (5) including:

(a) Limiting the number of damaged kernels from birds and insects, as molds thrive on kernels where the pericarp or hull has been compromised.
(b) Harvesting grain as soon as practically possible, especially when ergot is visually detected. Areas on-farm highly susceptible to ergot should be harvested as forage prior to the heading stage in order to avoid the formation of ergot bodies.
(c) Correctly storing and drying grain. With high moisture content, conditions remain anaerobic, increasing the likelihood of mycotoxin contamination.
(d) Rotating crops to avoid the carry-over of molds, as sclerotia are capable of remaining viable prolonged periods. Increasing seedling vigor and using seeds treated with fungicides will reduce seed-borne inoculum.

CONCLUSION

Minimizing the economic loss of producers due to ergot contamination in grains and subsequent ergot toxicoses in livestock is challenging. The diversity of fungal species and ergot alkaloids, their interactions with the surrounding environments for different crops and their varying toxicities in different tissues and/or livestock and poultry add to the complexity of the issue. As the climate is changing to favor ergot-producing fungi in some parts of the world and as regulations for human food become stricter, the frequency of ergot-contaminated grains will likely increase

in the future. Accordingly, strategies to reduce risks of ergot toxicoses are required to support the livestock industry. Although regulations and recommendations for the ergot alkaloid level in animal feed exist, a scientific basis for these recommendations is generally lacking.

While eliminating the threat of ergot toxicoses in livestock is likely impossible, application of some practical measures, including chemical cleaning grain, would minimize their impact, but the process is costly and may leave toxic residues. Devising methods to combat toxicoses could be aided by a better understanding of the physiological pathways impacted by ergot alkaloids. Moreover, experiments incorporating individual alkaloids in *in vitro* and *in vivo* animal studies would benefit this effort. Alkaloid binders and the use of antioxidants to lessen the effects of ergot poisoning would be valuable if effective binders could be identified. With grain contamination by ergot increasing annually and globally, effective new technologies are required to either reduce the occurrence of the ergot in grains or reduce the toxicity of alkaloids for livestock.

AUTHOR CONTRIBUTIONS

SC-M collected literature and wrote the manuscript; TM, KS, and YW perceived the concept and co-wrote the manuscript; BB, JM, and AC performed the critical review and co-wrote the manuscript.

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REVIEW
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Blackpatch of Clover, Cause of Slobbers Syndrome: A Review of the Disease and the Pathogen, *Rhizoctonia leguminicola*

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Rhizoctonia leguminicola Gough and Elliott is a widely used name for the causal agent of blackpatch disease of red clover (*Trifolium pratense* L.). This fungal pathogen produces alkaloids (slaframine and swainsonine) that affect grazing mammals. Slaframine causes livestock to salivate profusely, and swainsonine causes neurological problems. Although the blackpatch fungus was classified as a *Rhizoctonia* species (phylum Basidiomycota), morphological studies have indicated that it is in the phylum Ascomycota, and sequencing data have indicated that it may be a new genus of ascomycete. The effects of the alkaloids on grazing mammals and their biosynthetic pathways have been extensively studied. In contrast, few studies have been done on management of the disease, which requires a greater understanding of the pathogen. Methods of disease management have included seed treatments and fungicides, but these have not been investigated since the 1950s. Searches for resistant cultivars have been limited. This review summarizes the biological effects and biosynthetic precursors of slaframine and swainsonine. Emphasis is placed on current knowledge about the epidemiology of blackpatch disease and the ecology and taxonomy of the pathogen. Possibilities for future research and disease management efforts are suggested.

Keywords: ***Rhizoctonia leguminicola*, *Trifolium pratense*, slobbers, slaframine, swainsonine**

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INTRODUCTION

Rhizoctonia leguminicola Gough and Elliott is a widely used name for the causal agent of blackpatch or black spot disease of red clover (*Trifolium pratense* L.) (1). This fungal pathogen is currently considered most likely to be *Botrytis fabae* Sardiña, according to the database of the U.S. Department of Agriculture (USDA) Systematic Mycology and Microbiology Laboratory (2). Recent work (3) may lead to another reclassification of this pathogen. Blackpatch was first identified on red and white (*Trifolium repens* L.) clover (4–7), but it can also infect other legumes (7–10). On red clover, the disease has caused large losses for producers in the past (5, 6). In addition, alkaloids produced by the pathogen can be harmful to livestock consuming clover hay or pasture, due to excessive salivation (slobbering) and resulting dehydration (11). Few methods exist to manage blackpatch in the field or to mitigate the onset of physiological symptoms of alkaloid consumption. This review briefly discusses the alkaloids of the blackpatch pathogen and the associated problems for livestock. The alkaloids and their effects have been extensively reviewed (11–14), but they provide a context

for understanding the characteristics of the pathogen and studies of pathogenicity and disease management. Possible future approaches to disease management are suggested.

TAXONOMY

R. leguminicola traits in common with other *Rhizoctonia* species include branched hyphae with septa dividing the hyphae, and an absence of spores (15). Gough and Elliott (1), when naming this fungus, listed the lack of spores, dichotomous branching of hyphae, and constrictions at the branch points (see **Figure 1**) as reasons for classifying it as a *Rhizoctonia*. Parasitism on roots, detected by Kilpatrick et al. (16) and commonly associated with *Rhizoctonia* species (15), was not detected by Gough and Elliott (1) but not considered critical in the latter's decision to classify blackpatch as a *Rhizoctonia*. The taxonomy of *R. leguminicola* has been questioned at various times (3, 8, 17). Andersen and Stalpers (17), in a broad study of putative *Rhizoctonia* species in herbaria, examined hyphal morphology and general appearance under a dissecting microscope and sclerotia and infected tissue under a light microscope. The study indicated that the pathogen was *B. fabae* Sardiña, a reclassification making the pathogen an ascomycete instead of a basidiomycete. This is the current name given for the pathogen in the database of the USDA Systematic Mycology and Microbiology Laboratory (SMML) (2). Recent sequencing studies (3) indicate that *R. leguminicola* is indeed an ascomycete, but that it is most closely related to the genera *Pleochaeta* and *Mycocentrospora*. Reclassification as a unique genus, named *Slafractonia*, has been proposed (3). Because neither the genus name nor Mycobank accession number (809882) yield search results on Mycobank at the time of the writing of this review, and the name has not been revised in the SMML database, the pathogen is referred to in this manuscript as "the pathogen," "blackpatch," "the blackpatch pathogen," or "*R. leguminicola*."

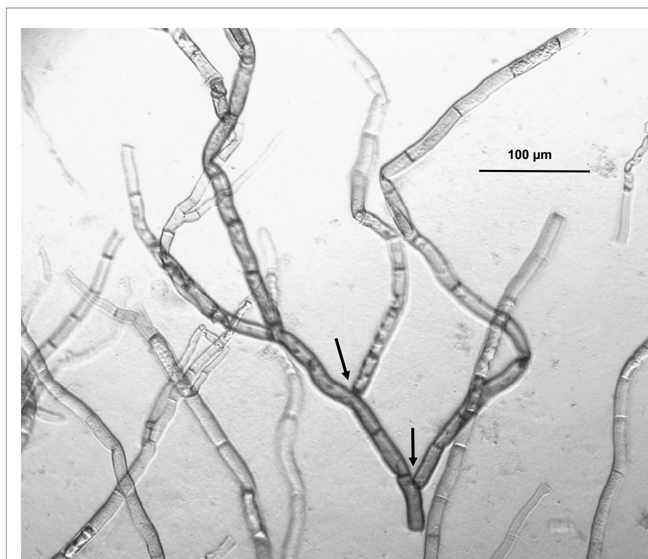

FIGURE 1 | Light micrograph of *Rhizoctonia leguminicola* mycelium, 100× magnification, grown on red clover agar (120 g/L red clover soaked 4 h in water). The dichotomous branching of hyphae and constrictions at the branch points (indicated by arrows) are considered typical of this pathogen.

ALKALOID PRODUCTION AND PHYSIOLOGICAL EFFECTS

The presence of the blackpatch pathogen is often signaled by excessive salivation (slobbering) observed in livestock consuming red clover forage (**Table 1**). The pathogen produces two indolizidine alkaloids: slaframine [(1*S*, 6*S*, 8a*S*)-1-acetoxy-6-aminooctahydroindolizine, **Figure 2A**] and swainsonine [(1*S*, 2*S*, 8*S*, 8a*R*)-1,2,8-trihydroxyoctahydroindolizine, **Figure 2B**]. Slaframine, a name derived by Aust et al. (18) from the Old Norse *slafra* (to slaver, a synonym for slobber), is thought to be primarily responsible for the slobbering seen in livestock after consuming infected forages. In forages causing slobbers syndrome, slaframine concentrations varied from 1.5 ppm (9) to 50–100 ppm (19). Broquist (11) reviews the process by which the link was made between slobbering and blackpatch, as well as the work done to purify and characterize the molecule responsible for slobbering. Slaframine has to be converted into an active form in order to have a physiological effect. This requirement was suspected due to the delay between injection of slaframine into the body cavity of rodents and start of salivation (18), suggesting that time was needed to convert slaframine by the liver into an active form. The active form was determined to be a ketoimine (20), with a proposed structure as shown in **Figure 2C** (the position of the C–N double bond was not confirmed in that study). Slobbering is induced only by the pathogen's mycelium, and not by the medium in which the pathogen is grown (21). However, legumes in the medium may provide a signal molecule for slaframine production, because slaframine in one study was maximal when cultures were grown on a cold-water extract of red clover (18).

TABLE 1 | Some published reports of outbreaks of "slobbers syndrome."

Reference	Location	Animals affected	Associated forage
(19)	North Carolina, USA	Horses	Mixture of second-cutting red clover and orchardgrass hay
(22)	Wisconsin, USA	Dairy cows	Freshly chopped red clover fodder
(23)	Minnesota, USA	Horses	Red clover and alfalfa in pastures (timothy and bromegrass also present)
(24)	Missouri, USA, various counties from 1949 to 1958	Cattle, horses, and sheep	Hay containing red clover (5 of 15 cases involved second-cutting red clover)
(25)	Oklahoma, USA	Horses	Red clover in bermudagrass pasture
(9)	Brazil	Horses	Alfalfa hay
(26)	Brazil	Horse	Red clover in pasture
(27)	Netherlands	Horses	Red clover in pasture

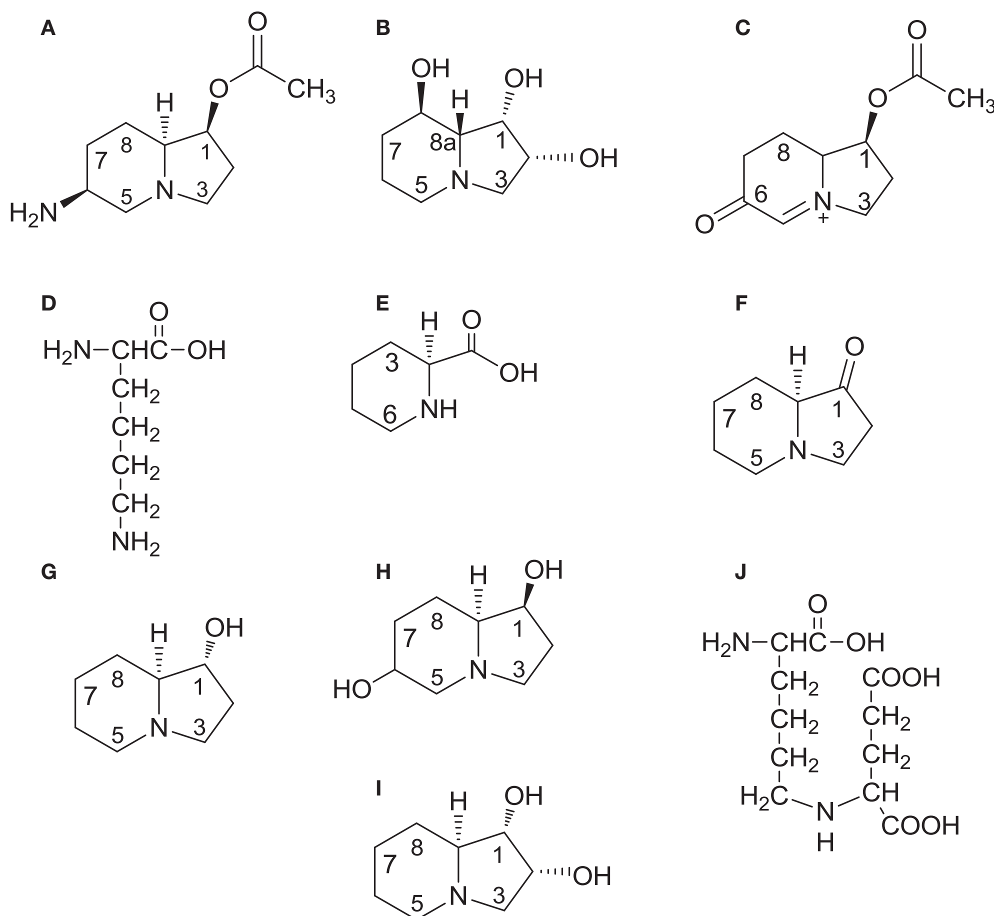

FIGURE 2 | Structures of slaframine (A), swainsonine (B), the biologically active form of slaframine (C), intermediates [lysine (D); pipecolic acid (E); and 1-oxoindolizidine (F) common to the biosynthetic pathways of both slaframine and swainsonine, swainsonine intermediate *trans*-1-hydroxyindolizidine (G), slaframine intermediate 1,6-dihydroxyindolizidine (H); configuration at C-6 undetermined], swainsonine intermediate *trans*-1,2-dihydroxyindolizidine (I), and common intermediate saccharopine (J). On compounds 1G and 1I, *trans* refers to the positions of the hydrogen atoms relative to each other at carbons 1 and 8a.

Swainsonine, so called because it was structurally characterized after isolation from the legume *Swainsona canescens* (Darling pea) (28), is the other alkaloid produced by the blackpatch pathogen (29). Swainsonine is also produced by the fungus *Metarhizium anisopliae* (30). It has been identified in other *Swainsona* species (31, 32), as well as from various species of *Ipomoea* (Convolvulaceae) and the legumes *Astragalus* and *Oxytropis*, known as locoweeds (13, 14, 31). Other plant species in which swainsonine has been identified are *Turbina cordata* (Convolvulaceae) and *Sida carpinifolia* (Malvaceae) (31). Swainsonine production in many of these species is strongly correlated with the presence of a fungal endophyte of the genus *Undifilum* in *Astragalus*, *Oxytropis*, and *Swainsona* species (31), and of the order Chaetothyriales in *Ipomoea carnea* (33). The *I. carnea* endophyte (33) and some of the *Undifilum* species (34–36) produce swainsonine in culture, indicating that the presence of swainsonine in plants may be entirely due to production by the plant endophytes.

Biosynthetic studies, conducted by feeding ^{14}C-lysine to *R. leguminicola* cultures and recovering ^{14}C-slaframine, have demonstrated that slaframine is derived from lysine (**Figure 2D**) (18, 37), which is converted into pipecolic acid (**Figure 2E**) (37). The incorporation of ^{14}C into slaframine decreased if non-radioactive pipecolic acid was fed along with ^{14}C-lysine, an indication that pipecolic acid was a more immediate precursor of slaframine than lysine (37, 38). Pipecolic acid is a precursor of various microbial secondary metabolites (39). Deuterated and tritiated pipecolic acid were incorporated into swainsonine as well as slaframine (40), and ^{14}C-acetate and ^{14}C-malonate were incorporated into both alkaloids at positions 2 and 3 on the piperidine ring (41). These results indicate that biosynthesis of slaframine and swainsonine proceeds along the same pathway initially, and that the piperidine ring of slaframine and swainsonine is probably formed by nucleophilic attack of malonyl-CoA on the carboxyl carbon of 2E, with subsequent cyclization. The roles of pipecolic acid and malonate in swainsonine biosynthesis are supported by

the increased yields of swainsonine obtained in root cultures of *Swainsona galegifolia* fed malonate and pipecolic acid (42).

Feeding tritiated 1-oxoindolizidine (**Figure 2F**) to *R. leguminicola* cultures yielded tritiated slaframine (37), suggesting that compound 2F is an intermediate. Compound 2F is thought to be the point at which the slaframine and swainsonine biosynthetic pathways diverge because of the opposite configurations of slaframine and swainsonine about carbon 8a (*S* and *R*, respectively) (40). Ketone 2F could conceivably be reduced to *trans*-1-hydroxyindolizidine (**Figure 2G**), or to *cis*-1-hydroxyindolizidine, with *cis* and *trans* referring to the positions of the hydrogens at carbons 1 and 8a (43). Incubating 1-oxoindolizidine with a crude enzyme extract from *R. leguminicola* resulted in recovery of *cis* and *trans*-1-hydroxyindolizidine, although less of the *trans* form (the likely swainsonine precursor) than of the *cis* form was produced in the incubation (37). When deuterated forms of those hydroxyindolizidines were fed to *R. leguminicola* cultures, the *cis* form was incorporated most efficiently into slaframine, and the *trans* form (**Figure 2G**) was incorporated most efficiently into swainsonine (43).

In addition to the abovementioned hydroxyindolizidines, some intermediates specific to slaframine or swainsonine biosynthesis have been identified. A 1,6-dihydroxyindolizidine (**Figure 2H**) was identified in *R. leguminicola* cultures fed deuterated *cis*-1-hydroxyindolizidine (43). The configuration about carbon 6 was not determined (43). Compound 2H was proposed to be a slaframine precursor whose 6-hydroxy group could be oxidized to a carbonyl group and converted into an amino group by transamination (43). Subsequent acetylation at carbon 1 would result in slaframine (43). A tritiated diol (**Figure 2I**), in addition to tritiated swainsonine, was isolated from separate feeding studies with tritiated *cis* and *trans*-1-hydroxyindolizidine, and it seemed a likely intermediate between compound 2G and swainsonine, given the structure and efficient incorporation into swainsonine when fed (44). Feeding tritiated pipecolic acid to *Astragalus oxyphysus* shoots revealed the presence of tritiated swainsonine and tritiated compounds 2G and 2I, indicating that swainsonine biosynthesis in *A. oxyphysus* shares some of the intermediates of swainsonine biosynthesis in *R. leguminicola* (45).

Subsequent biosynthetic studies with *R. leguminicola* have explored the steps of slaframine and swainsonine biosynthesis between lysine and pipecolic acid. Wickwire et al. (46) demonstrated that the nitrogen in pipecolic acid is derived from the alpha-nitrogen of ʟ-lysine in *R. leguminicola*, and that saccharopine (**Figure 2J**) is cleaved by a saccharopine oxidase to form delta-1,6-piperideine carboxylic acid (compound 2E with a double bond between N and C-6), a precursor of pipecolic acid. The saccharopine oxidase was purified (47). In *Undifilum oxytropis*, an endophyte of *Oxytropis sericea*, a saccharopine reductase gene was identified (48). Saccharopine reductase catalyzes conversion of alpha-aminoadipic semialdehyde, a lysine precursor, into saccharopine (39). *O. sericea* mutants lacking the saccharopine reductase gene produced more swainsonine and pipecolic acid, and less saccharopine, than wild-type cultures (48). These studies on saccharopine reductase may provide insight on the conversion of saccharopine into slaframine and swainsonine in *R. leguminicola*. The development of a method for proteomics analysis of *R. leguminicola* (49) may facilitate identifying enzymes of slaframine and swainsonine biosynthesis in the blackpatch pathogen.

Because swainsonine causes neurological problems (e.g., staggering, nervousness, and lack of coordination) in livestock that are referred to as locoism (12–14, 50), the first report of swainsonine detection in blackpatch-infected red clover hay suggested that it might contribute to the "slobber syndrome," a combination of slobbering, feed refusal, bloating, stiffness, diarrhea, weight loss, decreased milk production in dairy cattle, and abortion (51). Violent behavior and lacrimation are sometimes included as symptoms of the slobbers syndrome (12). The role of swainsonine in this array of symptoms is uncertain (12, 52). Croom et al. (12) compared clinical signs in ruminants and horses that were diagnosed with locoism or slobber syndrome, or that were fed purified slaframine and swainsonine. Stiffness, weight loss, and violent behavior were common to locoism, slobber syndrome, and swainsonine ingestion, suggesting that if those clinical signs are observed in slobbering animals, swainsonine may be responsible (12). The presence of unidentified active metabolites may contribute to the slobber syndrome as well, and synergistic effects of swainsonine and slaframine may be involved (12).

The effects of slaframine and swainsonine on livestock depend partly on their stability in blackpatch-infected clover hay and fresh clover. Stability in fresh clover over time does not appear to have been studied. In infected red clover hay stored for 10 months at room temperature, the slaframine content decreased from an initial 50–100 to 7 ppm, a 7- to 14-fold change in concentration (19). Initial swainsonine concentrations were not determined, but the fact that swainsonine (2.5 ppm) was recovered 3 years later from the same hay suggests that it is quite stable, although storage at −50°C may have improved the stability (51). Additional evidence for stability of swainsonine in plants lies in the reportedly stable swainsonine content found over 8 months in root cultures of *S. galegifolia* (42). Also, swainsonine was present in *O. sericea* plants sampled from four geographic locations over a 5-month period, spanning the vegetative to the senescent stages of growth (53). In rangelands, dead locoweed stalks are toxic, demonstrating that swainsonine can persist in dead tissue and continue to be a hazard to grazing livestock (54). In *M. anisopliae* cultures, swainsonine was stable up to 100°C over a pH range of 2–10 (30). Swainsonine reportedly resists autoclaving (55).

Treatment of affected livestock may include removing the infected feed (9, 22, 51) or access to the infected pasture (23, 26). Subsequently, slobbering may cease in 24 h (9, 22, 26, 52) to 3–4 days (23).

GEOGRAPHIC DISTRIBUTION AND PLANT HOSTS OF BLACKPATCH

The first report of blackpatch is in a 1933 report of the Kentucky Agricultural Experiment Station. The disease was named for its similarity to brown patch of grasses (4). Leach and Elliott (6) specify that because the mycelium spreads among plants, a "patch of black diseased plants" can develop, as documented by Elliott (56) (**Figure 3**).

Blackpatch identification methods include isolation of the pathogen from infected plant tissue, with histological analysis

FIGURE 3 | Blackpatch infection (wilted plants) in a red clover field. Photo from reference (56), courtesy of West Virginia University Agricultural Extension.

(8, 19, 22, 23), administration of infected plant tissue or its extracts to animals to look for slobbering (8, 9, 19), or chromatography to quantify slaframine in extracts (9, 19). Blackpatch has been identified in Canada (8), the midwestern United States (7, 22–24), and the southeastern United States (4–6, 10, 19). It has also been identified in Brazil (9, 26), Japan (12), and the Netherlands (27).

Early reports of blackpatch were based on symptoms on red and white clover (7). In the field, blackpatch also occurs on sainfoin (*Onobrychis viciifolia* Scop.), cicer milkvetch (*Astragalus cicer* Scop.) (8), soybean (*Glycine max* L.) (10), and alfalfa (*Medicago sativa* L.) (9, 57). Host range tests have demonstrated that the pathogen can infect several species of sweet clover (*Melilotus*), and other *Trifolium* species (7, 8). Birdsfoot trefoil (*Lotus corniculatus* L.) has been infected experimentally (8), as have kudzu (*Pueraria thunbergiana* Benth.) and blue lupine (*Lupinus angustifolius* L.) (10).

Because slobbering sometimes occurs in mixed pastures of legumes and grasses (23, 25), and one outbreak was linked to a mixture of red clover and orchardgrass hay (19), it seems possible that the blackpatch pathogen may also infect grasses. Sanderson (22) tested the susceptibility of 11 grass species to four blackpatch isolates. In a humid greenhouse, lesions formed on inoculated *Dactylis glomerata*, *Bromus inermis*, *Panicum virgatum*, *Festuca arundinacea, Phalaris arundinacea*, *Phleum pratense*, *Lolium perenne*, and *Agrostis alba*, and the pathogen was reisolated from each of these grasses. However, lesions were small, unlike the large, coalescing lesions observed on legumes (22). Therefore, it is possible that the pathogen can infect grasses, but that growth is so restricted that no significant amount of inoculum builds up.

DISEASE SYMPTOMS AND MEANS OF PATHOGEN SPREAD

Symptoms associated with blackpatch on red clover include dark brown, often concentric lesions on leaves (7, 8). The size and color of the lesions vary with the host plant (8). On red clover, lesions may also be gray or tan (8). Other symptoms are stem lesions and growth of aerial mycelium over the plant (1, 4). The aerial mycelium is typical of this pathogen (7, 8). It is sometimes difficult to distinguish from red clover pubescence unless examined under a magnifying glass, with the result that the disease may be difficult to detect in the field before it has spread and killed plants (5). Other reports, however, indicate that the disease may be detected from lesions on leaves (7, 23, 26, 27). Blackpatch may be difficult to detect on legume hay, because the mycelial color and texture are sometimes similar to those of cured hay (58). However, Borges et al. (9) found distinct, bright yellow lesions on alfalfa hay. Possibly, the difficulty in detecting the disease depends on the stage at which disease scouting is done. If red clover fields are watched less closely when leaves are emerging than when plants are flowering, early signs of infection on leaves may be overlooked.

The blackpatch pathogen is seedborne but can infect other parts of the plant, such as emerging hypocotyls, and then grow over stems, leaves, and flowers (5). It has been isolated from red clover roots in at least one study (16) but was unable to colonize roots in another (1). Because it does not produce spores, its spread to other red clover plants occurs by the spread of the aerial mycelium to other leaves and stems, which can be quite effective if the healthy and infected seedlings are in close proximity (5, 6). Elliott (56) found that in humid conditions in West Virginia, blackpatch could spread until the majority of a clover field was infected. Transportation of seed can contribute to long-distance dissemination (1).

The ability of blackpatch to serve as field inoculum depends on its ability to overwinter on seed, in soil, or on plant debris. Blackpatch mycelium was viable on seed kept 2 years under "seed storage conditions" (56), presumably at low temperature and humidity. Mycelium on red clover stems was viable after a year of storage at room temperature (1). It survived desiccation for up to 6 months at 20°C, indicating that it might persist in relatively dry regions with moisture from dew (1). The blackpatch pathogen is somewhat sensitive to low temperatures. Mycelium was viable after 30 but not 60 days at −20°C (1), suggesting that prolonged cold temperatures in winter may be detrimental to the pathogen. Because viable mycelium was difficult to find in the field in winter but easily found in the spring, it was suggested

that only small amounts of mycelium survive the winter, possibly by overwintering in the crown tissue of red clover (1). Mycelium may also overwinter on seed in the soil (56).

Weather and agricultural practices both play a role in blackpatch outbreaks. Decreases in red clover seed production, indicative of the presence of blackpatch, have been noticed in wet seasons (1). High humidity, which is favorable for red clover growth (56), is sometimes associated with outbreaks of slobbering in livestock (23, 27). An outbreak of slobbers among horses in Brazil was traced to alfalfa hay, which had been harvested at 78% humidity (9). The second cutting of red clover seems to be the biggest source of infected hay (11, 19, 24). Because the second cutting of red clover typically occurs in midsummer, more extensive fungal colonization seems to be associated with higher temperatures, at least in the humid regions in which blackpatch is historically a problem.

COMPARISON OF FUNGAL ISOLATES

Comparing different isolates of a pathogen can give insight into the diversity of the species and possibly help to determine if isolates should be assigned to different species. Sanderson (22) compared four isolates from Canada, North Carolina, and two different Wisconsin counties, based on dry weight accumulation, optimal growth temperature, alkaloid production, type of septa dividing the hyphae, and anastomosis among isolates. Optimal growth temperature did not differ among isolates. Only the two Wisconsin isolates anastomosed with each other (22). The Canadian isolate differed from the other isolates in that it produced no slaframine, accumulated the least dry matter, and had a different type of septa. It also did not anastomose with the other three isolates. Swainsonine was produced by this isolate, although two out of five laboratory cultures of the isolate produced none. These differences suggested that the Canadian isolate might be a different species (22), although it is also possible that this isolate illustrates considerable diversity within the species. DNA sequence information from these four isolates would facilitate determining if they were indeed all the same species.

BLACKPATCH PATHOGENICITY STUDIES AND THEIR POTENTIAL ROLE IN DISEASE MANAGEMENT

Controlled pathogenicity studies can give insights into potential strategies for managing the pathogen. A culture of the blackpatch pathogen can be obtained from the American Type Culture Collection (ATCC). The pathogen can grow on various types of media. The "salivation factor" (slaframine) was first isolated from fungal cultures grown on medium containing soybean meal, dextrose, calcium carbonate, and corn steep liquor (59). The pathogen has also been grown in stationary liquid culture on red clover infusion media, made by soaking chopped second-cutting red clover hay (200 g/L) in water for 4 h and filtering the infusion through cheesecloth (18). Agar medium made with 100 g/L red clover hay has been used as well (43, 44). Other media used to grow the pathogen include potato-dextrose agar (PDA) (7, 22, 23), malt-yeast broth (8), water agar (5); and soil-extract, oatmeal, and cornmeal agar (7). Cultures on PDA can be stored at −80°C as small squares of mycelium in a 40% glycerol solution of commercial potato dextrose broth. Because mycelium grown on PDA is quite brittle, it can easily be sectioned and deposited into tubes of glycerol media. Such cryostored cultures begin to grow a few days after being briefly thawed in a 25–30°C water bath and transferred to PDA.

In culture, mycelium is hyaline when young and darkens as it ages, becoming green to brown or black (7). Morphology varies on different media. More aerial mycelium was formed on soil extract agar and PDA than on oatmeal agar, and no aerial mycelium was formed on cornmeal agar (7).

Because the blackpatch pathogen does not produce spores, plants in pathogenicity studies have to be inoculated with the mycelium. Infection can be caused by mycelial plugs set on detached leaves (7, 8), as shown in **Figure 4A**. Suspensions of mycelial homogenates in water can be sprayed onto whole plants (8) or pipetted onto detached leaves (**Figure 4B**). Mycelium can also be grown on grain and then dried, crumbled, and sprinkled over plants (22). Measurement of inoculum density is not mentioned in the published descriptions of inoculation methods. Such a measurement could be valuable in pathogenicity studies because the observed symptoms on inoculated plants may depend partly on the inoculum density (60). Concentration of a dried, crumbled grain inoculum could be determined by plating and counting the number of colonies produced from a known

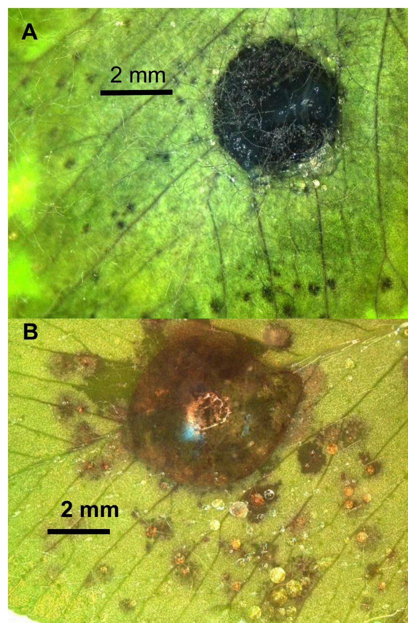

FIGURE 4 | Symptoms after inoculating a leaflet of the "Kenland" cultivar of red clover with (A) a plug of *R. leguminicola* mycelium from a 13-day-old culture on potato-dextrose agar (PDA), or (B) a 50-µL droplet of homogenized mycelium from a 12-day-old culture on PDA. Leaflet **(A)** was photographed 66 h after inoculation, and leaflet **(B)** was photographed 49 h after inoculation.

mass of inoculum, and a mycelial suspension could be quantified by optical density.

With a reproducible method of inoculating plants and eliciting symptoms, it becomes possible to evaluate cultivars for differences in susceptibility to a pathogen, based on symptom severity. If resistant or partially resistant clover cultivars could be identified in pathogenicity studies, those could be targeted for field trials to determine if the severity of blackpatch, and hence of slobbers outbreaks, could be mitigated with more resistant clover cultivars in pastures. Little information exists on differences in cultivar susceptibility to blackpatch. Susceptibility has been compared among red clover plants collected from areas with a high incidence of blackpatch (1). No resistant cultivars were found in that study, but criteria for resistance were not given. Therefore, it is uncertain if any variations existed in symptom severity. A disease rating scale that evaluates degrees of symptom severity, instead of looking at the presence or absence of symptoms, may facilitate identifying more desirable (less susceptible) cultivars. Differences in percentage of infected leaf area may translate into differences in the extent to which blackpatch spreads in a field. Sanderson (22) compared symptoms on seven red clover cultivars (Norlac, Prosper I, Arlington, Chesapeake, Redman, Redland II, and Pennscott) inoculated in a Wisconsin field with a dried grain inoculum. Symptom severity was scored based on the percentage of necrotic leaf area. One season of data indicated that the Norlac cultivar was more susceptible than the others (22). However, results were inconclusive because no symptoms were observed in the field in the following season, when plants were not reinoculated. These variable field results may indicate that in an initial approach to determining cultivar susceptibility, tightly controlled inoculum applications and environmental conditions (possibly in a growth chamber) are needed.

Reproducible methods of inoculation and symptom elicitation may also permit comparison of alkaloid accumulation in different red clover cultivars. The abovementioned study by Sanderson (22) determined that the Norlac cultivar of red clover, besides being the most susceptible to blackpatch during the season in which symptoms were elicited, had the highest slaframine and swainsonine concentrations in infected tissue. These results suggest that alkaloid production by blackpatch may be affected by host genotype, and that susceptibility may be correlated with alkaloid content in infected plants. In such a case, alkaloid content of cultivars infected by the same isolate may help to identify differences in susceptibility to blackpatch.

OTHER APPROACHES TO DISEASE MANAGEMENT

Fungicide treatments and disease scouting are other potential approaches to blackpatch management. Since blackpatch is seedborne, seed treatments have been studied as a means of control. Benomyl was ineffective in the field (1). Thiram prevented or slowed development of disease in greenhouse studies (5), but it was less effective in the field (1). Several other fungicides (quinolin-8-ol, captan, and zineb) appeared to provide some protection in greenhouse studies, but field data were not provided (1). These studies do not seem to have been continued since the 1950s, suggesting that (a) results were too poor to be worth pursuing, or (b) slobbers syndrome is managed adequately by taking livestock off the suspect hay or pasture, without incurring the expense of fungicide treatments. In the former case, it may be worthwhile to study the efficacy of more recent fungicides, possibly employing foliar sprays or soil treatments with sterol biosynthesis inhibitors like prochloraz, which can inhibit growth of some soilborne red clover fungal pathogens without greatly diminishing growth (61).

A greater understanding of the environmental conditions favoring blackpatch outbreaks may be useful in disease management. Blackpatch is generally associated with humidity, but this understanding is sometimes applied in hindsight, to aid in diagnosing an outbreak of slobbering in livestock (9, 19). If enough information were gathered about locations of infected clover and climatic conditions prior to the finding of the infected clover, such data could possibly be used to model and predict the likelihood of blackpatch outbreaks. Such models are sometimes used to determine when to spray fungicides on certain crops (62), but prediction of blackpatch outbreaks could be used to determine when to keep horses off pasture or look more carefully at red clover hay purchased from areas at risk for an outbreak.

In order to gather information about the distribution and proliferation of blackpatch under different environmental conditions, methods are needed for rapid scouting of blackpatch in the field. Given the difficulty sometimes reported for seeing the pathogen on legumes (5), molecular techniques could facilitate the detection process. Information on the taxonomy and key DNA sequences of the blackpatch pathogen (3) may facilitate designing molecular tools to aid in diagnosing the disease from randomly selected field samples. The specificity of DNA amplification by the polymerase chain reaction (PCR), if done with adequately specific primers, could permit detecting the pathogen in infected material and distinguishing it from other legume pathogens (63). Compared to the standard methods of isolating and detecting slaframine from plant tissue, DNA isolation and PCR would possibly require less time and use fewer hazardous reagents. Slaframine is extracted from plant tissue in methanol (57) or 95% ethanol (19, 22). After concentration and resuspension in water, sometimes followed by partitioning with an organic solvent to remove lipids (22, 57), the aqueous solution is basified and partitioned with chloroform (19, 22) or methylene chloride (57). The organic layer, containing slaframine, is dried, and slaframine is analyzed by thin-layer chromatography (57) or derivatized for gas chromatography (9, 19, 22). High-performance liquid chromatography has been used to analyze slaframine as well (64). Fungal DNA in plants can sometimes be extracted simply by incubating infected plant tissue in microliter volumes of the appropriate extraction and neutralization buffers, due to the specificity of PCR primers for fungal DNA in the extract (65).

DISCUSSION

Although the blackpatch alkaloids have been studied extensively due to their effects on mammals, the pathogen and methods for its management have been less studied, and little has been done on the latter over the past 30 years. Slobbers syndrome can be managed by removing suspect hay or aspect to a suspect pasture,

which may make investigation of the blackpatch pathogen seem unnecessary. However, a better understanding of how to manage blackpatch could be cost-effective for livestock producers who have difficulty affording the cost of replacing hay or finding alternate grazing sites. Red clover producers could benefit from raising cultivars more resistant to blackpatch. Searching for cultivars with resistance to blackpatch and developing a means to predict the likelihood of blackpatch outbreaks, could benefit livestock, livestock owners, and forage producers.

AUTHOR CONTRIBUTIONS

IK reviewed the literature and wrote the manuscript.

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Summer-Long Grazing of High vs. Low Endophyte (*Neotyphodium coenophialum*)-Infected Tall Fescue by Growing Beef Steers Results in Distinct Temporal Blood Analyte Response Patterns, with Poor Correlation to Serum Prolactin Levels

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Previously, we reported the effects of fescue toxicosis on developing Angus-cross steer growth, carcass, hepatic mRNA, and protein expression profiles of selected serum proteins, and blood clinical and chemical profiles, after summer-long grazing (85 days) of high endophyte (HE)- vs. low endophyte (LE)-infected fescue pastures. We now report the temporal development of acute, intermediate, and chronic responses of biochemical and clinical blood analytes determined at specified time intervals (period 1, day 0–36; period 2, day 37–58; and period 3, day 59–85). Throughout the trial, the alkaloid concentrations of the HE forage was consistently 19–25 times greater ($P \leq 0.002$) than the concentration in the LE forage, and HE vs. LE steers had continuously lower ($P \leq 0.049$) serum prolactin (85%), cholesterol (27%), and albumin (5%), but greater red blood cells (7%). The HE steers had decreased ($P = 0.003$) ADG only during period 1 (–0.05 vs. 0.4 kg/day). For period 1, HE steers had reduced ($P \leq 0.090$) numbers of eosinophils (55%) and lymphocytes (18%), serum triglyceride (27%), and an albumin/globulin ratio (9%), but an increased bilirubin concentration (20%). During period 2, serum LDH activities were 18% lower ($P = 0.022$) for HE vs. LE steers. During period 3, serum levels of ALP (32%), ALT (16%), AST (15%), creatine kinase (35%), glucose (10%), and LDH (23%) were lower ($P \leq 0.040$) for HE steers. Correlation analysis of serum prolactin and other blood analytes revealed that triglycerides ($P = 0.042$) and creatinine ($P = 0.021$) were moderately correlated ($r \leq 0.433$) with HE serum prolactin. In conclusion, three HE-induced blood analyte response patterns were identified: continually altered, initially altered, and subsequently "recovered," or altered only after long-term exposure. Blood

Abbreviations: ADG, average daily gain; ALP, alkaline phosphatase: ALT, alanine transaminase; AST, aspartate transaminase; BW, body weight; HE, high toxic endophyte-infected tall fescue; LE, low toxic endophyte tall fescue-mixed grass; LDH, lactate dehydrogenase; RBC, red blood cell.

analytes affected by length of grazing HE vs. LE forages were either not or poorly correlated with serum prolactin. These data reveal important, temporal, data about how young cattle respond to the challenge of consuming HE pasture.

Keywords: ***Neotyphodium coenophialum*****, ergot alkaloids, blood metabolites, blood cells, cattle**

INTRODUCTION

Tall fescue (*Lolium arundinaceum*) is a forage grass that is commonly used by many livestock producers in the southeastern United States. The hardiness of the grass is primarily due to the fact that the majority of the fescue in the southeast is infected with the endophytic fungus, *Neotyphodium coenophialum*. Unfortunately, cattle consuming endophytic fescue manifest clinical symptoms of fescue toxicosis ("summer slump"), such as decreased body weight (BW) gain (1–3), reduced feed intake (4, 5), lowered milk production (6), retained winter hair coat during the summer (7), increased body temperature (6), and increased respiration rate (4).

The consumption of endophyte-infected tall fescue also is known to alter the blood parameter profiles of cattle and other livestock, with decreased plasma prolactin being the most commonly observed indicator of fescue toxicosis (8). Previously, we reported (9) the effects of fescue toxicosis on developing Angus-cross steer growth, carcass, hepatic mRNA, and protein expression profiles of selected serum proteins, and blood clinical and chemical profiles that were induced by whole-summer-long grazing of forages containing either high or low amounts of endophyte-infected tall fescue. Therefore, the primary objectives of the current research were to (a) characterize the temporal changes in blood clinical and chemical profiles of steers grazing forages containing either high or low amounts of endophyte-infected tall fescue over the course of the summer and then (b) evaluate the potential relationships between serum prolactin and measured blood analytes.

MATERIALS AND METHODS

Animals and Experimental Periods

All experimental procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee (protocol no. 01012A2006). Nineteen predominately Angus crossbred beef steers were randomly allotted (day 0) to graze low-toxic endophyte tall fescue-mixed grass (LE, $n = 9$, 5.7 ha; BW = 266 ± 10.9 kg) or a high-toxic endophyte-infected tall fescue (HE, $n = 10$, 5.7 ha; BW = 267 ± 14.5 kg) for 85 days. Pastures were within 500 m of each other and are part of the University of Kentucky Agricultural Research Center, located in Woodford County, KY, USA. All steers had *ad libitum* access to fresh water and mineral supplement (Ca min. 13.0 – max. 15.0%; *P* 6.2%; NaCl min. 17.0 – max. 19.5%; Mg 3.0%; S 1.0%; K 0.8%; Zn 2300 μg/g; Mn 2200 μg/g; Cu 1450 μg/g; I 45 μg/g; Co 15 μg/g; Se 29 μg/g; Vit. A 661 IU/g; Vit. E 0.276 IU/g; as-fed). Shrunk (denied access to feed and water for 14 h) BW were determined on day 0 and 86 to determine overall experiment average daily gain (ADG). Full BW were taken on day 36 and 57 and shrunk BW calculated based on the mean percentage shrink of 4.04% from weight measurements taken on day 0 and 85. Experimental periods were as per weigh days: period 1 = day 0–36, period 2 = day 37–58, and period 3 = day 59–85.

Pasture Sampling and Analysis

On day 37, 59, and 88 of the study, leaf blades suitable for grazing were detached from each fescue plant in pasture for ergot alkaloid (ergovaline, ergovalinine, lysergic acid, and isolysergic acid) determination, and proximate analysis (9). Briefly, samples were obtained systematically from approximately 30 sites in each pasture, using a knife to cut the forage at approximately 2 cm above soil level. Samples were immediately placed into individual plastic bags, and then stored on ice during transportation to our laboratory. All samples were frozen and stored at −20°C. Analysis of ergot alkaloids was performed, as previously described (10), and isolysergic acid was quantified with a lysergic acid standard. Proximate analysis and mineral content were determined by a commercial laboratory (Dairy One Forage Lab, Ithaca, NY, USA).

Blood Collection and Analyses

Jugular venous blood samples were collected by venipuncture on day 36, 58, and 85. For plasma, 16 mL of blood was collected in EDTA-containing (0.9375 mg/mL) blood collection tubes (Becton, Dickinson and Company, Franklin, Lakes, NJ, USA). For serum, 16 mL of blood was collected in serum blood collection tubes without an anticoagulant. For whole blood, 2 mL of blood was collected in EDTA-containing (2.7 mg/mL) blood collection tubes (Becton Dickerson). Plasma and sera were recovered by refrigerated centrifugation at 3 000 × *g* for 10 min at 4°C and stored at −80°C. Plasma samples were analyzed for ammonia-N by modifications of the L-Glu dehydrogenase enzyme assay (11) using a Konelab 20XTi analyzer (Thermo Electron Corp., Finland). Serum prolactin analysis (12) was conducted (Dr. F. N. Schrick Laboratory, Johnson Animal Research and Teaching Unit, University of Tennessee-Knoxville). All other serum enzymes and analytes, and blood cell types, were analyzed by the American Association for Veterinary Laboratory Diagnosticians approved – University of Kentucky Livestock Disease Diagnostic Center (Lexington, KY, USA). For serum analytes, activities of alkaline phosphatase (ALP), E.C. 3.1.3.1; alanine transaminase (ALT), E. C. 2.6.1.2; aspartate transaminase (AST), E. C. 2.6.1.1; γ-glutamyltransferase, E. C. 2.3.22; creatine kinase, E. C. 2.7.3.2; and lactate dehydrogenase (LDH), E. C. 1.1.1.27 were determined as per the manufacturer of the reagent kits (Alfa Wassermann, Diagnostic Technologies, West Caldwell) using a VET-EX Chemical Analyzer (Alfa-Wassermann), as were the other serum analytes. The concentration of red blood cells (RBCs), white blood cells, packed cell volume, and hemoglobin in whole blood was determined using a Hemavet HV 950S cell

analyzer (Drew Scientific Inc., Miami Lakes, FL, USA). The whole blood concentration of neutrophils, lymphocytes, monocytes, and eosinophils were determined by manual identification and counting of cells (13).

Statistical Methods

Data are presented as least square means (±SEM). Individual steers were the experimental units. The effect of grazing HE vs. LE pastures on all measured experimental parameters was evaluated by ANOVA, using the MIXED procedure of SAS (v 8.01, SAS Inst. Inc., Cary, NC, USA). The statistical model used fescue toxicosis, the experimental period, and their interaction as fixed effects. Class variables were fescue toxicosis and steer, with steer included in the random statement. The Kenward–Roger adjustment was used to calculate the denominator df (14). Linear and quadratic non-orthogonal polynomial contrasts were used to characterize the effect of treatment over time using the imi procedure of SAS. Partial correlations between prolactin concentrations and serum analytes were determined by using ANOVA (PROC GLM) by using the MANOVA/PRINTE statement of SAS. For all data, significance was declared when $P \leq 0.05$ and tendency to differ was declared when $0.10 \geq P > 0.05$.

RESULTS

Nutrient Profiles of HE and LE Forages

Across-periods, the % compositions of DM, ADF, and crude fat did not differ ($P \geq 0.205$) between HE and LE pastures (**Table 1**). However, the HE forage contained less ($P \leq 0.001$) lignin (24%) and NDF (4.3%), and more ($P \leq 0.001$) TDN (3.8%), CP (7.6%), and ash (7.3%) than did LE.

Ergot Alkaloid Profiles of Forages

Across-period analysis of ergot alkaloid levels between the two forages revealed that the HE steers were exposed to 19 and 25 times more ($P \leq 0.002$) ergovaline/ergovalinine and lysergic acid/isolysergic acid, respectively, than LE steers (**Table 2**). No period or treatment by period interactions was observed for lysergic and isolysergic acids indicating that the differences in the concentration between HE and LE forages were similar throughout the trial. For ergovaline and ergovalinine, however, period ($P \leq 0.004$) and treatment by period ($P \leq 0.008$) were observed, perhaps reflecting the exceptionally low values for LE forages during period 1.

Steer Growth

Across-periods, BW tended to be lower ($P = 0.097$), and ADG was lower ($P = 0.014$), for HE vs. LE steers (**Table 3**). For BW, a period effect was found ($P \leq 0.001$) reflecting the greater growth of HE and LE steers during period 3. Similarly, the ADG for period 3 appeared greater for both groups of steers than for the other periods. Moreover, a treatment by period interaction was found ($P = 0.026$) for ADG. That is, although the ADG of LE and HE steers did not differ ($P \geq 0.595$) in periods 2 and 3, during period 1 LE steers achieved an ADG of 0.4 kg, whereas HE steers lost ($P = 0.003$) BW (0.05 kg/day). Overall, from day 0 to 85, the ADG of HE steers was 0.18 kg more ($P = 0.014$) than for LE steers.

Prolactin

Across all three periods, serum prolactin levels of HE steers ($P \leq 0.001$) were 15% that of LE steers (**Table 4**). Steers grazing HE forage had serum prolactin values that were 14, 19, and 10% that of LE steers in period 1, 2, and 3, respectively. The level of prolactin decreased in a quadratic manner ($P \leq 0.012$) over time for both LE and HE steers (**Table 5**), with the concentration of prolactin in the steers grazing LE pasture decreasing 58.3 ng/mL from periods 1 to 3 (**Table 4**). Steers grazing HE pasture exhibited a smaller decrease in prolactin (9.9 ng/mL) across-periods, likely due to the fact that HE steer prolactin levels had already been suppressed severely in period 1. A treatment by period interaction likely reflects this time-dependent differential decrease in serum prolactin values.

Biochemical and Clinical Blood Profiles

As shown in **Table 4**, the mean value across-periods for 9 of the biochemical and clinical blood profile measurements did not change ($P \geq 0.212$), whereas ALP, ALT, AST, ammonia, albumin, albumin:globulin ratio, creatine kinase, glucose, LDH, and cholesterol were influenced ($P < 0.10$) by grazing treatment. The trend across time for each biochemical and blood parameter was also determined for each treatment group (**Table 5**).

Serum ALP activity for HE steers tended ($P = 0.079$) to exhibit 26% less activity than LE steers (**Table 4**). In period 3, ALP was reduced ($P = 0.006$) by 32% for HE steers. Furthermore, there was a significant ($P < 0.001$) period interaction (**Table 4**), which showed quadratic trends ($P = 0.024$) (**Table 5**) for ALP for LE steers that decreased 13% from period 1 to 2 and increased 97% from period 2 to 3. For HE steers, there was also a quadratic trend ($P = 0.080$) (**Table 5**), which reduced ALP by 7.1% from periods 1 to 2 and increased 64% from periods 2 to 3. The period by treatment interaction ($P = 0.009$) most likely was due to the large increase in ALP by the LE steers from periods 2 to 3 (**Table 4**).

Across-periods, ALT activity was reduced ($P = 0.040$) by 9.5% in HE consuming steers compared to the LE consuming steers (**Table 4**). In period 3, ALT also was found to be depressed 16% ($P = 0.009$) in HE steers (**Table 4**). The period effect ($P = 0.001$) (**Table 5**) can be explained by the quadratic response to time ($P \leq 0.035$) (**Table 5**) for both treatments, with activity depressed during period 2 compared to periods 1 and 3. ALT activity for steers consuming LE pastures decreased by 24% from periods 1 to 2 and increased by 28% from periods 2 to 3, whereas ALT activity for HE consuming steers decreased by 17% from periods 1 to 2 and increased by 8.8% from period 2 to 3 (**Table 4**).

Across-periods, HE steer serum AST activity decreased ($P = 0.092$) 9.1% (**Table 4**). Within-periods, AST was decreased ($P = 0.014$) by 15% in HE vs. LE steers in period 3 and demonstrated no period or period by treatment interaction (**Table 4**). Across-periods, there was a linear decrease ($P = 0.031$) (**Table 5**) of 4.9% for HE steers across-periods but no trend for LE steers. For AST, the mean differed but there was no period or treatment by period interaction unlike prolactin and ALT, despite the fact that LE appeared to increase and HE to decrease (**Table 4**).

The serum albumin content of HE steers was 5% lower ($P \leq 0.009$) than LE steers across-periods (**Table 4**). Within-periods 1, 2, and 3, serum albumin of the HE steers was 4.6, 6.1,

TABLE 1 | Proximate analysis of low endophyte (LE) and high endophyte (HE) pasture samples[a].

Item (%)	Pasture treatment			*P*-value		
	LE	HE	SEM	TRT	Period	TRT by period
Across-periods						
DM	25.5	26.1	0.46	0.356	<0.001	0.273
CP	17.10	18.40	0.195	0.001	0.002	<0.001
TDN	58.56	60.78	0.377	0.001	0.001	0.093
ADF	32.12	31.83	0.370	0.591	0.782	0.069
NDF	60.41	57.82	0.387	0.001	0.008	0.004
Crude fat	3.46	3.61	0.082	0.205	0.006	0.179
Lignin	6.23	4.76	0.247	0.001	0.012	0.215
Ash	8.20	8.80	0.09	0.001	0.077	0.007
Within-periods						
DM						
Period 1	28.5	27.8	0.79	0.544		
Period 2	26.8	28.8	0.79	0.100		
Period 3	21.1	21.6	0.79	0.623		
CP						
Period 1	16.50	20.83	0.34	<0.001		
Period 2	16.70	17.93	0.34	0.024		
Period 3	18.10	16.43	0.34	0.004		
TDN						
Period 1	56.00	60.00	0.653	0.001		
Period 2	58.67	60.33	0.653	0.096		
Period 3	61.00	62.00	0.653	0.300		
ADF						
Period 1	32.87	30.93	0.641	0.054		
Period 2	32.40	32.07	0.641	0.720		
Period 3	31.10	32.50	0.641	0.149		
NDF						
Period 1	62.83	57.47	0.670	<0.001		
Period 2	60.87	58.17	0.670	0.015		
Period 3	57.53	57.83	0.670	0.757		
Crude fat						
Period 1	3.07	3.50	0.142	0.052		
Period 2	3.77	3.93	0.142	0.423		
Period 3	3.53	3.40	0.142	0.520		
Lignin						
Period 1	7.33	5.03	0.428	0.003		
Period 2	6.37	4.93	0.428	0.035		
Period 3	5.00	4.30	0.428	0.270		
Ash						
Period 1	7.9	9.1	0.16	0.001		
Period 2	8.4	9.1	0.16	0.008		
Period 3	8.4	8.3	0.16	0.864		

[a]*Proximate analysis values are presented on a DM basis. Samples were obtained systematically from approximately 30 sites in each pasture, using a knife to cut the forage at approximately 2 cm above soil level. Data are presented as least square means (±SEM).*

and 5.7% less ($P \leq 0.032$), respectively, than that of LE steers. The period effect could be explained by the fact that LE and HE steers decreased linearly ($P < 0.001$) (**Table 5**) in a parallel manner by 15 and 16%, respectively (**Table 4**). Additionally, the albumin:globulin ratio in HE steers was decreased ($P = 0.037$) by 8.5% across-periods when compared to LE steers (**Table 4**). In period 1, the albumin:globulin ratio was decreased by 9.5% in HE when compared to LE steers. The period effect ($P < 0.001$) was evident in that both treatment groups decreased over time with LE steers decreasing linearly ($P < 0.001$), whereas HE steers displayed a quadratic ($P = 0.071$) decrease across-periods (**Table 5**).

Across treatments, the creatine kinase activity that was 24% less ($P = 0.032$) in HE vs. LE steers (**Table 4**). In period 3, creatine kinase activity in HE steers was ($P = 0.001$) 35% less than that of LE steers. From periods 1 to 2, LE and HE steers decrease in a parallel manner. Nonetheless, from periods 2 to 3, LE increased by 75% while HE consuming steers creatine kinase activity increased by only 27%, likely reflecting the period and period by treatment interaction.

TABLE 2 | Alkaloid analysis of composited low endophyte (LE)- and high endophyte (HE)-infected forage samples[a].

Alkaloid (μg/g)	Pasture treatments			*P*-value		
	LE	HE	SEM	TRT	Period	TRT by period
Across-periods						
Ergovaline	0.018	0.276	0.016	<0.001	0.004	0.008
Ergovalinine	0.004	0.152	0.005	<0.001	<0.001	<0.001
Lysergic acid	0.002	0.064	0.011	0.002	0.731	0.908
Isolysergic acid	0.007	0.156	0.019	0.001	0.821	0.893
Within-periods						
Ergovaline						
Period 1	<0.001	0.260	0.028	<0.001		
Period 2	0.023	0.173	0.028	0.002		
Period 3	0.030	0.393	0.028	<0.001		
Ergovalinine						
Period 1	<0.001	0.087	0.008	<0.001		
Period 2	0.010	0.100	0.008	<0.001		
Period 3	0.003	0.270	0.008	<0.001		
Lysergic acid						
Period 1	<0.001	0.053	0.019	0.071		
Period 2	0.007	0.077	0.019	0.023		
Period 3	<0.001	0.063	0.019	0.037		
Isolysergic acid						
Period 1	<0.001	0.140	0.032	0.010		
Period 2	0.020	0.160	0.032	0.010		
Period 3	<0.001	0.167	0.032	0.032		

[a]*Across- and within-periods proximate analysis values are presented on a DM basis. Samples were obtained systematically from approximately 30 sites in each pasture, using a knife to cut the forage at approximately 2 cm above soil level. Data are presented as least square means (±SEM).*

Glucose concentrations were 8.5% lower ($P = 0.068$) in HE vs. LE steers across-periods (**Table 4**). In period 3, HE steers exhibited ($P = 0.040$) a 10% lower concentration of glucose. The period effect could be explained by a slight parallel decrease in serum glucose from periods 1 to 2 for LE and HE steers of 4.3 and 1.6% and increased from periods 2 to 3 by 18 and 11%, respectively. LE steers showed a quadratic response ($P = 0.036$), whereas HE steers demonstrated no linear or quadratic response (**Table 5**).

Across-periods, LDH activity was reduced ($P = 0.007$) 16% of HE steers vs. LE steers (**Table 4**), with LDH activity decreased ($P \leq 0.022$) by 18 and 23%, respectively, in periods 2 and 3. Similar to AST, the means that for LDH differed but there was no period or treatment by period interaction, even though the LE steer mean appeared to increase and HE steer mean decreased across-periods (**Table 5**).

There was no significant change ($P = 0.212$) in triglycerides across-periods between the two treatment groups (**Table 4**). Nonetheless, serum triglyceride concentration was reduced ($P = 0.017$) by 27% during period 1 in HE steers. A treatment by period interaction ($P = 0.003$) reflects a decreased triglyceride concentration from periods 1 to 2 for HE steers and increased concentrations from periods 2 to 3.

Across-periods, HE steers exhibited a 27% decrease ($P < 0.001$) in cholesterol as compared to LE steers (**Table 4**), with serum cholesterol concentrations that were 25, 29, and 29% less ($P < 0.001$) that of LE steers in periods 1, 2, and 3, respectively. The period effect ($P < 0.001$) can be explained by the fact that both treatments responded in a parallel quadratic manner ($P \leq 0.005$) (**Table 5**). From periods 1 to 2, there was a decrease of 13 and 17%; and from periods 2 to 3, there was an increase of 29 and 29% for LE and HE, respectively (**Table 4**), which was consistent with the lack of a treatment by period interaction ($P = 0.354$).

In contrast to other biochemical parameters, ammonia plasma concentrations tended ($P = 0.077$) to be increased in HE steers (**Table 4**). Across-periods, ammonia levels were 19% greater for HE steers. Within-periods 2 and 3, the ammonia concentration of HE steers was 34 and 38% greater ($P \leq 0.035$), respectively, than that of LE steers. The period effect ($P < 0.001$) could be explained by the fact that over time both treatment groups exhibited a decrease. The treatment by time interaction ($P = 0.052$) was apparent as LE steers exhibited a quadratic trend ($P = 0.096$) (**Table 5**), decreasing 32% from periods 1 to 2 and then 15% from periods 2 to 3. Nonetheless, the HE steers exhibited a linear ($P = 0.001$) decrease of 21% in plasma ammonia (**Table 5**).

Blood Cell Types

Across-periods, the amount of RBCs was increased ($P = 0.049$) 6.9% in HE vs. LE steers (**Table 6**). By contrast, other types of blood cells were not affected ($P \geq 0.228$). Within-periods, RBC concentrations of HE steers were increased ($P \leq 0.10$) by 6.3, 7.2, and 7.1% that of LE steers in periods 1, 2, and 3, respectively. The period effect ($P = 0.092$) was due to the fact that both LE and HE steers displayed a decrease in RBC during period 2.

Period affected blood cell abundance ($P \leq 0.044$) with the exception of eosinophils, which demonstrated a treatment by period interaction ($P = 0.087$) (**Table 6**). This interaction appears to reflect the linear ($P = 0.016$) (**Table 7**) decrease with time of LE

TABLE 3 | Body weight and ADG gain of steers grazing forages containing low endophyte (LE)- and high endophyte (HE)-infected forages for 85 days.

Item	P-value		
	TRT	Period	TRT by period
Across-periods			
BW (kg)[a]	0.097	<0.001	0.861
ADG (kg)	0.014	<0.001	0.026

Within-periods

Item	Pasture treatment[b]		SEM	P-value
	LE	HE		
No. of steers	9	10		
BW (kg)[a]				
Day 36	280	265	6.1	0.091
Day 58	287	273	6.1	0.094
Day 85	314	301	6.1	0.132
ADG (kg)				
Period 1	0.40	–0.05	0.10	0.003
Period 2	0.26	0.27	0.10	0.971
Period 3	1.3	1.4	0.10	0.595
Overall	0.58	0.40	0.05	0.014

[a]*BW are calculated, or measured shrunk weights, as described in text.*
[b]*Steers LE (ergovaline and ergovalinine 0.02 μg; lysergic acids 0.01 μg) or HE (ergovaline and ergovalinine 0.52 μg; lysergic acids 0.22 μg) endophyte-infected tall fescue pastures.*

eosinophils, whereas HE steer eosinophils appeared to increase in period 3. Lymphocytes were decreased ($P = 0.090$) in HE steers by 18% in period 1, and monocytes were decreased ($P = 0.070$) by 45% in period 3. The LE steers show a linear ($P = 0.060$) (**Table 7**) decline across-period for lymphocytes and the means of the HE steers appears to parallel that of the LE steers. The decline across-periods reflects a significant ($P = 0.044$) period effect for lymphocytes, but the parallel pattern did not allow for a treatment by period interaction ($P = 0.240$). The LE steers displayed a quadratic ($P = 0.016$) (**Table 7**) response for monocytes across-period with period 2 being depressed the most. The HE steers exhibited a linear ($P \leq 0.001$) decline for monocytes across-periods. The overall decline in monocytes across time was enough to account for a period effect, but the differences in the trends of each treatment group was not enough to allow for a significant treatment by period interaction ($P = 0.243$) (**Table 6**).

Correlation of Serum Prolactin with Blood Analytes

Correlation analyses were performed to determine if a relationship existed between prolactin levels and serum and plasma (**Table 8**) and blood cell types (**Table 9**). Across treatments, the relative abundance of prolactin was weakly ($0.321 \geq r \geq 0.236$) correlated with ALT ($P = 0.022$), blood urea nitrogen ($P = 0.092$), triglycerides ($P = 0.021$), and hemoglobin ($P = 0.043$). Within treatments, correlation coefficients for ALT ($r = 0.424$), blood urea nitrogen ($r = 0.405$), and hemoglobin ($r = 0.536$) were significant ($P \leq 0.045$) and stronger only for LE steers, whereas the correlation coefficient for triglycerides ($r = 0.388$) was significant ($P = 0.042$) and stronger only for HE steers. Within treatment prolactin by analyte correlation analysis also revealed a significant ($P = 0.021$) moderately strong correlation ($r = 0.433$) between prolactin and creatinine in HE but not LE steers, whereas correlations between prolactin and blood urea nitrogen:creatinine ($r = 0.377$; $P = 0.064$) and packed cell volume ($r = 0.375$; $P = 0.065$) were identified in LE but not HE steers.

DISCUSSION

Trial-Long (Across All Periods) Responses

The primary objectives of this research were to characterize the temporal changes in blood clinical and chemical profiles of beef steers grazing forages containing either high or low amounts of endophyte-infected tall fescue over the course of the summer. Forage analysis revealed that the high alkaloid concentration in HE vs. LE pastures was maintained throughout the trial. Of the 19 blood analytes and 8 blood cell type HE vs. LE treatment responses measured across all 3 experimental periods of the trial (**Tables 4** and **6**), 6 were decreased ($P \leq 0.049$; prolactin, ALT, albumin, creatine kinase, LDH, cholesterol, RBC) in HE steers, whereas 3 (ALP, AST, glucose) tended ($0.10 \geq P > 0.05.$) to be decreased and 1 (ammonia) increased ($P \leq 0.077$). However, only prolactin, albumin, and cholesterol were consistently affected in each of the three periods. Decreased serum prolactin is the most consistently observed, and accepted, indicator of cattle suffering from fescue toxicosis (2, 8, 17, 18). The accompanying decreased serum cholesterol also is consistent with that found by others for cattle suffering from fescue toxicosis and is thought to be caused by elevated body temperatures (19–21). Although shown to be consistently decreased through all periods of the current trial, albumin response to the consumption of HE forages can vary. For example, a number of studies report lower levels of albumin in the serum of cattle consuming HE and suggest that this response could be due to the reduced dietary intake, decreased uptake by the gut of AA for the biosynthesis of albumin, damaged tissue, or increased catabolism of albumin (9, 22, 23). Alternatively, others report increased albumin levels in cattle grazing HE forages, potentially due to dehydration (18).

As just described, of the nine analytes and cell types measured that had across-period treatment effects, only three were consistently affected for each of the three periods. To more discretely determine when metabolic alterations were concomitant with grazing HE forages (rather than a total across-period treatment effect after a whole summer of grazing), serum enzymes, and blood constituents were measured and analyzed within the three time periods over the 85-day trial. This analysis was critical to identify potential treatment effects that may have been dependent on the length of grazing, thus potentially identifying metabolic adaptations to the length of HE forage consumption. Of the blood analytes and cell types evaluated (**Tables 4** and **5**), 9/19 analytes and 5/8 had period-specific changes, putatively indicating period-dependent shifts in metabolic capacity as detailed below.

Period 1

Although the cohort groups was relatively small ($n = 9–10$), the large reduction in ADG of HE steers occurred only during the

TABLE 4 | Serum and plasma analytes of steers grazing low endophyte (LE)- or high endophyte (HE)-infected forages[a].

Item[b]	Pasture treatment			*P*-value			Reference range
	LE	HE	SEM[c]	TRT	Period	TRT by period	
Across-periods							
Prolactin (ng/mL)	70.8	10.8	3.09	<0.001	<0.001	<0.001	–
ALP (U/L)	111.3	82.4	7.08	0.079	<0.001	0.009	100.0–500.0[d]
ALT (U/L)	30.4	27.5	0.96	0.040	<0.001	0.149	11–40[e]
AST (U/L)	68.1	61.9	2.53	0.092	0.495	0.106	0–160[e]
AST/ALT ratio	2.30	2.33	0.115	0.823	0.007	0.961	–
Ammonia[f] (mM)	0.059	0.070	0.005	0.077	<0.001	0.052	–
Blood urea nitrogen (mg/100 mL)	17.0	17.7	0.60	0.459	<0.001	0.680	5.0–27.0[d]
Albumin (g/100 mL)	3.68	3.48	0.034	0.009	<0.001	0.860	2.30–3.70[d]
Globulin (g/100 mL)	3.27	3.34	0.062	0.556	<0.001	0.719	3.0–3.5[d]
Albumin/globulin ratio	1.17	1.07	0.025	0.037	<0.001	0.731	0.8–1.00[d]
γ-Glutamyltransferase (U/L)	12.1	11.3	0.43	0.276	<0.001	0.735	2.0–20.0[d]
Total bilirubin (mg/100 mL)	0.2	0.2	0.01	0.453	0.166	0.166	0.0–0.5[d]
Total protein (g/100 mL)	6.95	6.82	0.067	0.318	<0.001	0.569	6.50–7.50[d]
Creatinine (mg/100 mL)	1.29	1.28	0.049	0.857	<0.001	0.578	1.00–2.00[d]
Blood urea nitrogen:creatinine	13.33	13.90	0.387	0.305	0.424	0.489	40–100[d]
Creatine kinase (U/L)	165.0	126.0	8.70	0.032	<0.001	0.010	100.0–650[d]
Glucose (mg/100 mL)	71	65	1.6	0.068	0.001	0.530	40–100[d]
LDH (U/L)	1037	870	34.1	0.007	0.322	0.224	692–1445[e]
Triglycerides (g/100 mL)	30	26	1.4	0.212	0.002	0.003	–
Cholesterol (mg/100 mL)	95	69	2.2	<0.001	<0.001	0.354	62–193[d]
Within-periods							
Prolactin (ng/mL)							
Period 1	94.3	13.5	3.94	<0.001			
Period 2	82.1	15.2	3.94	<0.001			
Period 3	36.0	3.6	3.94	<0.001			
ALP (U/L)							
Period 1	93.0	71.6	12.27	0.218			
Period 2	81.2	66.5	12.27	0.393			
Period 3	159.8	109.1	12.27	0.006			
ALT (U/L)							
Period 1	33.3	30.1	1.41	0.102			
Period 2	25.4	25.1	1.41	0.860			
Period 3	32.6	27.3	1.41	0.009			
AST (U/L)							
Period 1	66.8	63.7	2.94	0.454			
Period 2	66.3	61.3	2.94	0.225			
Period 3	71.2	60.6	2.94	0.014			
AST/ALT ratio							
Period 1	2.05	2.14	0.180	0.723			
Period 2	2.63	2.63	0.180	0.995			
Period 3	2.21	2.23	0.180	0.934			
Ammonia[e] (mM)							
Period 1	0.078	0.078	0.0055	0.940			
Period 2	0.053	0.071	0.0055	0.026			
Period 3	0.045	0.062	0.0055	0.035			
Blood urea nitrogen (mg/100 mL)							
Period 1	18.8	19.8	0.73	0.314			
Period 2	16.1	16.8	0.73	0.496			
Period 3	16.2	16.4	0.73	0.860			
Albumin (g/100 mL)							
Period 1	3.94	3.76	0.060	0.032			
Period 2	3.77	3.54	0.060	0.009			
Period 3	3.33	3.14	0.060	0.025			
Globulin (g/100 mL)							
Period 1	2.93	3.08	0.107	0.327			
Period 2	3.04	3.07	0.107	0.864			

(Continued)

TABLE 4 | Continued

Item[b]	Pasture treatment		SEM[c]	*P*-value			Reference range
	LE	HE		TRT	Period	TRT by period	
Period 3	3.83	3.86	0.107	0.858			
Albumin/globulin							
Period 1	1.37	1.24	0.043	0.039			
Period 2	1.24	1.15	0.043	0.119			
Period 3	0.90	0.83	0.043	0.245			
γ-Glutamyltransferase (U/L)							
Period 1	12.0	11.6	0.75	0.699			
Period 2	13.8	13.0	0.75	0.454			
Period 3	10.6	9.2	0.75	0.195			
Total bilirubin (mg/100 mL)							
Period 1	0.20	0.24	0.01	0.055			
Period 2	0.23	0.22	0.01	0.515			
Period 3	0.20	0.20	0.01	1.000			
Total protein (g/100 mL)							
Period 1	6.88	6.84	0.117	0.816			
Period 2	6.81	6.61	0.117	0.220			
Period 3	7.17	7.00	0.117	0.308			
Creatinine (mg/100 mL)							
Period 1	1.44	1.39	0.057	0.496			
Period 2	1.19	1.21	0.057	0.791			
Period 3	1.23	1.23	0.057	0.967			
Blood urea nitrogen:creatinine							
Period 1	13.17	14.24	0.494	0.126			
Period 2	13.60	14.00	0.494	0.554			
Period 3	13.23	13.45	0.494	0.750			
Creatine kinase (U/L)							
Period 1	144.0	119.3	15.06	0.242			
Period 2	127.7	113.8	15.06	0.509			
Period 3	223.4	144.8	15.06	0.001			
Glucose (mg/100 mL)							
Period 1	69	64	2.8	0.181			
Period 2	66	63	2.8	0.455			
Period 3	78	70	2.8	0.040			
LDH (U/L)							
Period 1	952	889	59.0	0.440			
Period 2	1095	901	59.0	0.022			
Period 3	1063	821	59.0	0.004			
Triglycerides (g/100 mL)							
Period 1	33	24	2.4	0.017			
Period 2	27	23	2.4	0.204			
Period 3	30	31	2.4	0.695			
Cholesterol (mg/100 mL)							
Period 1	95	71	3.75	<0.001			
Period 2	83	59	3.75	<0.001			
Period 3	107	76	3.75	<0.001			

[a]*Data are presented as least squares means (±SEM) of LE (n = 9) and HE (n = 10) treatments.*
[b]*ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.*
[c]*Most conservative error of the mean.*
[d]*Taken from the University of Kentucky Livestock Disease Diagnostic Laboratory (Lexington, KY, USA).*
[e]*Taken from Kaneko et al. (15).*
[f]*Plasma.*

first period. In the latter two periods, the growth response of the HE steers paralleled the LE steers. Thus, the HE steers showed an adjustment in rate of BW gain to the alkaloid challenge but without a compensatory growth response. Concomitantly, there was a decrease in blood concentrations of triglycerides, albumin/globulin ratio, eosinophils, and lymphocytes but an increase in bilirubin. It was only during the first period that the concentrations of both serum cholesterol (27%) and triglycerides (25%) were depressed. The initial more than sixfold (14% of LE steers) depression of the classic endophytic fescue identifier, prolactin, in

TABLE 5 | Linear vs. quadratic time effects on serum and plasma analytes of steers grazing low endophyte (LE)- or high endophyte (HE)-infected forages[a,b].

Item[b]	LE		HE	
	Linear	Quadratic	Linear	Quadratic
Prolactin	<0.001	0.010	0.001	0.012
ALP	0.002	0.024	0.009	0.080
ALT	0.863	<0.001	0.194	0.035
AST	0.204	0.432	0.031	0.383
AST/ALT ratio	0.341	<0.001	0.822	0.066
Ammonia	<0.001	0.0960	0.001	0.954
Blood urea nitrogen	<0.001	0.002	0.001	0.050
Albumin	<0.001	0.180	<0.001	0.425
Globulin	<0.001	0.033	<0.001	0.008
Albumin/globulin ratio	<0.001	0.112	<0.001	0.071
γ-Glutamyltransferase	0.110	0.008	0.023	0.013
Total bilirubin	0.907	0.057	0.069	0.912
Total protein	0.062	0.156	0.283	0.044
Creatinine	0.001	0.002	0.011	0.032
Blood urea nitrogen:creatinine	0.952	0.335	0.170	0.809
Creatine kinase	0.010	0.055	0.003	0.024
Glucose	0.031	0.036	0.141	0.255
LDH	0.343	0.337	0.151	0.308
Triglycerides	0.482	0.253	0.015	0.075
Cholesterol	0.056	0.005	0.124	0.001

[a]*Data are P-values associated with linear and quadratic non-orthogonal polynomial contrasts used to characterize the effect of treatment over time on concentrations of blood analytes.*
[b]*ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.*

HE steers is consistent with reduced triglyceride synthesis in the presence of ergot peptides (24) and with the overall correlation found between serum triglyceride and prolactin concentrations (**Table 8**). This evidence of altered lipid metabolism has been associated with reduced forage intake of high endophyte fescue (25, 26). Although we did not measure these parameters, DiMarco et al. (25) found steers that had been fasted, and then refed, recovered their initially depressed cholesterol levels. With regard to correlating prolactin and triglyceride levels, cattle consuming high levels of endophyte forages have been shown to possess more saturated fatty acids in subcutaneous and perinephric fat than those consuming low levels of endophyte (27, 28). In the current study, a decreased in ADG and triglycerides was found in the first period, whereas ADG and triglyceride levels had recovered by the second and third period. By contrast, consumption of alkaloids is thought to be the cause of the loss of stimulation for insulin receptors that are involved in lipid metabolism (24), consistent with the trial-long depressed cholesterol levels of HE steers.

The reduced serum albumin and the albumin/globulin ratio in HE steers vs. LE steers would signify that albumin synthesis within the liver was impaired. For the HE steers, the 20% increased bilirubin also observed in period 1 signifies that excretory function may have been partially curtailed. The 6.3% increase in RBC concentration for HE steers indicates that the senescence of erythrocytes would not be the major contributor to the increased bilirubin (23). Moreover, increased RBC in HE steers is inconsistent with the understanding that prolactin increases erythropoiesis (29), as prolactin is decreased in HE steers. The unaltered value of total protein and γ-glutamyltransferase would suggest that even though some protein synthesis and excretory function may have been abridged, liver function was not uniformly impaired. The unaltered ammonia, blood urea nitrogen, creatinine, blood urea nitrogen:creatinine ratio, and creatine kinase between treatment groups indicates that nitrogen metabolism, urea synthesis, renal glomerular filtration function, and skeletal muscles were not influenced during period 1.

Eosinophils (55.2%) and lymphocytes (17.6%) numbers were decreased in HE steers in period 1, suggesting that immune function may have been impaired (30–32). The reduced level of eosinophils in these young HE steers also has been found in mature beef steers grazing endophyte-infected tall fescue (18).

Period 2

In contrast to the severe reduction in BW gains of HE steers found during period 1, the ADG by HE steers (0.26 kg/day) did not differ from that of LE steers (0.27 kg/day). Although moderate, this rate of gain suggests that the HE steers seemed to have adjusted to the physiological challenge of consuming forages that contained about 20 times more ergot alkaloids than LE steers. However, despite an equal rate of BW gain, prolactin, albumin, and cholesterol levels remained suppressed, and RBC increased in HE steers during period 2. By contrast, the albumin:globulin ratio and bilirubin levels no longer differed, indicating that hepatic albumin synthesis was still impaired, but possibly not to the overall extent as in period 1. Between treatment groups, similar values for bilirubin, total protein, and γ-glutamyltransferase would implied that hepatic excretory capacity was not affected during this period and that liver damage did not occur.

Some aspects of energy metabolism also appeared to have been altered in period 2 as compared to period 1. Although HE steers no longer possessed reduced levels of triglycerides relative to LE steers, they continued to have lower serum cholesterol, levels that were below the clinical reference range (9, 15). This suggests that the HE steers may be utilizing their cholesterol to supplement the altered energy metabolism. In addition, the reduced LDH of HE steers during period 2 could be indicative of a reduction in the transformation of pyruvate to lactate or a reduced "shuttling" of lactate between skeletal muscles and the liver. In rats, the sub-chronic exposure of rats to environmental toxins caused hepatotoxicity and a decrease in the serum levels of LDH (33). The increased duration of exposure to the high concentration of ergot alkaloids may have led to the reduced LDH as well for the HE steers.

Also occurring during period 2, increased (34%) plasma ammonia in HE vs. LE steers suggests that ammonia capturing and recycling mechanisms were impaired in the HE steers (34–36). However, because blood urea nitrogen did not differ, urea synthesis likely was not altered in HE vs. LE steers. Moreover, the unaffected creatinine, blood urea nitrogen:creatinine ratio, and creatine kinase between treatment groups suggests that renal glomerular filtration function and skeletal muscle turnover also were similar in HE and LE steers during period 2.

TABLE 6 | Blood cell types of steers grazing low endophyte (LE)- or high endophyte (HE)-infected forages[a].

Item	Pasture treatment			*P*-value			Reference range
	LE	HE	SEM[b]	TRT	Period	TRT × period	
Across-periods							
RBC[c] (1 × 10⁶/μL)	8.43	9.01	0.134	0.049	0.092	0.974	5.0–10[e]
Hemoglobin (g/dL)	10.8	10.9	0.17	0.811	0.001	0.539	8.0–15[e]
Packed cell volume (%)	30.1	30.5	0.50	0.701	<0.001	0.783	24–46[e]
WBC[d] (1 × 10³/μL)	9.26	8.89	0.606	0.663	0.010	0.296	4.0–12.0[e]
Neutrophils (1 × 10³/μL)	3.13	3.18	0.331	0.917	0.004	0.900	0.06–4.00[f]
Lymphocytes (1 × 10³/μL)	5.65	5.23	0.373	0.430	0.044	0.240	2.5–7.5[f]
Monocytes (1 × 10³/μL)	0.43	0.35	0.046	0.228	<0.001	0.243	0.0–0.9[f]
Eosinophils (1 × 10³/μL)	0.22	0.17	0.046	0.373	0.183	0.087	0.0–2.4[f]
Within-periods							
RBC[c] (1 × 10⁶/μL)							
Period 1	8.57	9.11	0.231	0.100			
Period 2	8.24	8.83	0.231	0.077			
Period 3	8.49	9.09	0.231	0.067			
Hemoglobin (g/dL)							
Period 1	10.8	11.1	0.29	0.472			
Period 2	10.5	10.5	0.29	0.989			
Period 3	11.1	11.1	0.29	0.945			
Packed cell volume (%)							
Period 1	29.5	30.2	0.87	0.580			
Period 2	28.6	29.2	0.87	0.657			
Period 3	32.3	32.3	0.87	0.973			
WBC[d] (1 × 10³/μL)							
Period 1	10.43	9.12	0.743	0.209			
Period 2	8.11	8.19	0.743	0.942			
Period 3	9.23	9.35	0.743	0.904			
Neutrophils (1 × 10³/μL)							
Period 1	3.14	3.03	0.411	0.857			
Period 2	2.62	2.72	0.411	0.864			
Period 3	3.65	3.80	0.411	0.792			
Lymphocytes (1 × 10³/μL)							
Period 1	6.58	5.42	0.483	0.090			
Period 2	5.11	5.09	0.483	0.976			
Period 3	5.25	5.18	0.507	0.924			
Monocytes (1 × 10³/μL)							
Period 1	0.64	0.53	0.066	0.263			
Period 2	0.27	0.31	0.066	0.723			
Period 3	0.38	0.21	0.070	0.081			
Eosinophils (1 × 10³/μL)							
Period 1	0.38	0.17	0.087	0.056			
Period 2	0.18	0.13	0.077	0.625			
Period 3	0.11	0.21	0.065	0.245			

[a]*Data are presented as least squares means (±SEM) of LE (n = 9) and HE (n = 10) treatments.*
[b]*Most conservative error of the mean.*
[c]*Red blood cells.*
[d]*White blood cells.*
[e]*Taken from the University of Kentucky Livestock Disease Diagnostic Laboratory (Lexington, KY, USA).*
[f]*Taken from Duncan et al. (16).*

Period 3

In the final period, ADG of the steers consuming the HE forage was the same as for LE steers. However, for both groups of steers, the ADG achieved was about four times greater than during period 2 (1.4 vs. 0.27 and 1.3 vs. 0.26, respectively). Despite these treatments, similar and good rates of gain for grazing steers, prolactin, albumin, and cholesterol levels remained suppressed, and ammonia and RBC levels remained higher in HE vs. LE steers. The prolonged high endophyte exposure allowed for further adaption by the HE steers. During period 3, the activities of creatinine kinase, AST, ALT, ALP, LDH, glucose concentration, and number of monocytes decreased.

Unlike periods 1 or 2, creatinine kinase in period 3 was depressed by 35% for steers grazing the HE pasture. The decreased level of creatine kinase was presumably the result of suppressed expression in striated muscle and possibly cardiac tissue or reduced tissue mass (9). Also, unique to period 3 was the serum reductions of clinical markers (ALP, ALT, and AST) for impaired metabolism. A reduction in serum ALP, a common indicator of high endophyte exposure (7, 37, 38), is related to decreased

bone and intestinal isoenzymes activities (39). The decreased ALP could also be the result of reduced intakes and absorption of nutrients vital for ALP function (18). The reduced serum ALT presumably represents reduced cytosolic ALT in hepatocytes (40, 41), whereas reduced AST typically is interpreted as increased mitochondrial turnover in hepatocytes (41–43) and striated muscles (44). The lack of change in the AST:ALT ratio and decreased content of both enzymes is indicative of a relative constant rate of tissue turnover with either decreased concentrations of AST and ALT in tissue or diminished tissue mass (9), again indicating a "healthy" but metabolically altered liver in HE vs. LE steers.

TABLE 7 | Linear vs. quadratic time effects on blood cell type of steers grazing low endophyte (LE)- or high endophyte (HE)-infected forages[a].

Item	LE		HE	
	Linear	Quadratic	Linear	Quadratic
RBC[b]	0.808	0.195	0.994	0.388
Hemoglobin	0.347	0.124	0.979	0.119
Packed cell volume	0.008	0.025	0.090	0.095
WBC[c]	0.224	0.029	0.617	0.059
Neutrophils	0.260	0.093	0.029	0.039
Lymphocytes	0.060	0.117	0.639	0.581
Monocytes	0.033	0.016	<0.001	0.161
Eosinophils	0.016	0.307	0.599	0.473

[a]*Data are P-values associated with linear and quadratic non-orthogonal polynomial contrasts used to characterize the effect of treatment over time on blood cell type.*
[b]*Red blood cells.*
[c]*White blood cells.*

During period 3, the concentration of RBC continued to be greater in HE vs. LE steers by 7.1%, whereas the monocytes were 44.7% lower in HE vs. LE steers. Monocytes are vital for immune response, wound healing, and tissue homeostasis (31, 45, 46). This decreased concentration of monocytes is similar to the report of Saker et al. (31) and could be due to the accumulated stress from the effects of grazing HE vs. LE pastures.

Serum Prolactin was Poorly Correlated with Blood Analytes

Prolactin is reported to affect many physiological processes besides lactation (47). Therefore, the second objective of this study is to evaluate the potential relationships between serum prolactin and measured blood analytes. In general, little evidence was found to indicate that HE-decreased serum prolactin concentrations were associated with other assessed blood analytes. This lack of correlation was unexpected given that prolactin is a multifunctional hormone known to exert metabolic effects on many tissues due to the ubiquitous expression of prolactin receptors (48). In this regard, it is interesting to note that prolactin levels were lowest for both LE and HE steers in period 3, the period of greatest ADG for both steer groups. However, the correlation between triglycerides and prolactin levels in HE steers could prove to be very important as an indicator of altered energy metabolism (48, 49).

CONCLUSION

In summary, growth, clinical, and biochemical parameters that changed in response to grazing HE vs. LE pastures can be

TABLE 8 | Correlation of serum and plasma analytes with serum prolactin of steers grazing low endophyte (LE)- or high endophyte (HE)-infected forages.

Item[d]	Overall[a]		LE[b]		HE[c]	
	Coefficient	*P*-value	Coefficient	*P*-value	Coefficient	*P*-value
ALP	0.176	0.212	0.155	0.4617	0.272	0.161
ALT	0.317	0.022	0.424	0.035	0.268	0.168
AST	0.161	0.256	0.154	0.462	0.202	0.302
AST/ALT ratio	−0.119	0.398	−0.144	0.492	−0.140	0.477
Ammonia	−0.146	0.301	−0.231	0.267	0.088	0.657
Blood urea nitrogen	0.236	0.092	0.405	0.045	0.204	0.297
Albumin	−0.111	0.434	−0.237	0.254	0.116	0.558
Globulin	0.092	0.517	0.214	0.305	−0.149	0.449
Albumin/globulin ratio	−0.159	0.262	−0.342	0.094	0.215	0.272
γ-Glutamyltransferase	0.100	0.479	0.240	0.247	−0.136	0.490
Total bilirubin	0.122	0.389	0.220	0.290	−0.024	0.904
Total protein	0.028	0.845	0.083	0.695	−0.074	0.709
Creatinine	0.110	0.438	−0.019	0.927	0.433	0.021
Blood urea nitrogen:creatinine	0.121	0.392	0.377	0.064	−0.237	0.226
Creatine kinase	0.023	0.870	0.014	0.948	0.109	0.614
Glucose	−0.057	0.689	−0.145	0.490	0.120	0.542
LDH	0.018	0.898	0.046	0.827	−0.120	0.542
Triglycerides	0.321	0.021	0.314	0.126	0.388	0.042
Cholesterol	0.046	0.748	0.054	0.799	0.019	0.923

[a]*(LE + HE) analytes vs. (LE + HE) prolactin.*
[b]*LE analytes vs. LE prolactin.*
[c]*HE analytes vs. HE prolactin.*
[d]*ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.*

TABLE 9 | Correlation of blood cell type with serum prolactin of steers grazing low endophyte (LE)- or high endophyte (HE)-infected forages.

Item	Overall[a]		LE[b]		HE[c]	
	Coefficient	*P*-value	Coefficient	*P*-value	Coefficient	*P*-value
RBC[d]	−0.144	0.307	−0.322	0.116	0.039	0.842
Hemoglobin	0.282	0.043	0.536	0.006	0.013	0.949
Packed cell volume	0.197	0.162	0.375	0.065	−0.005	0.982
WBC[e]	0.112	0.430	0.099	0.639	0.177	0.368
Neutrophils	0.193	0.170	0.277	0.180	0.127	0.519
Lymphocytes	−0.033	0.818	−0.103	0.633	0.151	0.445
Monocytes	0.062	0.669	0.055	0.799	0.094	0.643
Eosinophils	−0.078	0.649	−0.142	0.627	−0.051	0.816

[a]*(LE + HE) analytes vs. (LE + HE) prolactin.*
[b]*LE analytes vs. LE prolactin.*
[c]*HE analytes vs. HE prolactin.*
[d]*Red blood cells.*
[e]*White blood cells.*

divided into three groups: those that were continually altered (serum prolactin, albumin, cholesterol, and RBCs), those that were initially altered and then "recovered" (ADG, triglycerides, albumin/globulin ratio, bilirubin, lymphocytes, and eosinophils), and, subsequently, those that developed after long-term exposure (AST, ALT, ammonia, creatine kinase, LDH glucose, and monocytes). These findings suggest that the differences in blood analyte profiles may be indicative of whether and when grazing young steers are adjusting, metabolically, to consumption of HE pastures. However, mechanisms responsible for initial loss of BW gain of HE steers that occurred in period 1, and the subsequent parallel rates of gain to LE steers, but not compensatory growth, awaits discovery. Unexpectedly, with the exception of triglycerides and creatine, blood analytes were poorly correlated with serum prolactin levels.

AUTHOR CONTRIBUTIONS

JJ, JB, and JM were responsible for the design and acquisition of the data. All authors (JJ, ML, JB, and JM) were responsible for the analysis and interpretation of the data, drafting and/or critical revision of the intellectual content, and are accountable for the accuracy and integrity of this work.

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Interaction of isoflavones and endophyte-infected tall fescue seed extract on vasoactivity of bovine mesenteric vasculature

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It was hypothesized that isoflavones may attenuate ergot alkaloid-induced vasoconstriction and possibly alleviate diminished contractility of vasculature after exposure to ergot alkaloids. The objective of this study was to determine if prior incubation of bovine mesenteric vasculature with the isoflavones formononetin (*F*), biochanin A (*B*), or an ergovaline-containing tall fescue seed extract (EXT) and their combinations affect ergotamine (ERT)-induced contractility. Multiple segments of mesenteric artery and vein supporting the ileal flange of the small intestine were collected from Angus heifers at slaughter ($n = 5$, bodyweight = 639 ± 39 kg). Duplicates of each vessel type were incubated in tissue culture flasks at 37°C with a 50-mL volume of Krebs–Henseleit buffer containing: only buffer (control); or 1×10^{-6} M EXT; *F*; or *B*; and combinations of 1×10^{-6} M EXT + *F*; 1×10^{-6} M EXT + *B*; 1×10^{-6} M *F* + *B*; or 1×10^{-6} M EXT + *F* + *B*. After incubation for 2 h, sections were mounted in a multimyograph chamber. The ERT dose responses were normalized to 0.12 M KCl. Pretreatment with *F*, *B*, and *F* + *B* without EXT resulted in similar contractile responses to ERT in mesenteric artery and all incubations containing EXT resulted in a complete loss of vasoactivity to ERT. In mesenteric artery pretreated with EXT, treatments that contained *B* had higher contractile responses ($P < 0.05$) at ERT concentrations of 1×10^{-7} and 5×10^{-7} M. Also, treatments containing *B* tended ($P < 0.1$) to have greater responses than treatments without *B* at ERT concentrations of 1×10^{-6}, 5×10^{-6}, and 5×10^{-5} M. In mesenteric vein pretreated with EXT, treatments containing *F* had greater contractile responses to ERT at 1×10^{-5}, 5×10^{-5}, and 1×10^{-4} M ($P < 0.05$). These data indicated that *F* and *B* at 1×10^{-6} M and their combination did not impact the overall contractile response to ERT in mesenteric vasculature. However, *F* and *B* may offset some of the vasoconstriction caused by prior exposure to ergot alkaloids.

Keywords: ergot alkaloids, isoflavones, mesenteric vasculature, vasoconstriction

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INTRODUCTION

As a symbiotic endophyte of tall fescue (*Lolium arundinaceum*) (1–4), *Epichloë coenophiala* produces a variety of ergot alkaloids (5, 6), which have been identified as causative agents of vasoconstriction and symptoms of fescue toxicosis in grazing animals (7, 8). Ergovaline and ergotamine commonly draw more attention of researchers. Lyons et al. (5) reported that ergovaline

was the predominant (84–97%) alkaloid of all the five detected ergopeptine alkaloids from tall fescue (*Lolium arundinaceum*) pasture. Numerous studies have reported that ergot alkaloids (ergovaline and ergotamine) induce vasoconstriction in peripheral blood vessel models such as the caudal artery (9, 10), dorsal pedal vein (11), and lateral saphenous vein (12), and also core blood vessel models like the right ruminal artery and vein (13), bovine uterine and umbilical arteries (14), and mesenteric artery and vein (15). Consistent with findings in the bovine lateral saphenous vein (16, 17), Egert et al. (15) demonstrated that previous dietary exposure to ergot alkaloids reduced the vasoactivity in bovine mesenteric vasculature.

Isoflavones are almost exclusively found in the legume (*Leguminosae/Fabaceae*) family, such as soybean, chickpeas, and red clover (18, 19). A variety of isoflavones, such as genistein, daidzein, biochanin A (*B*), and formononetin (*F*), have been shown to elicit different beneficial effects on humans in many different ways, for example, improvements to the cardiovascular system, osteoporosis, anti-breast, and prostate cancer (20–23). Substantial evidence has been published on the vasodilative effects of isoflavones and their metabolites in different vessel types in humans (24, 25) and rats (26–29). However, based on our knowledge, no studies have investigated the vasodilative effects of isoflavones on bovine vessels.

Ergot alkaloids share some structural similarities with biogenic amines (i.e., dopamine, epinephrine, norepinephrine, serotonin) and thus can cause vasoconstriction by binding biogenic amine receptors (30) found throughout the body. On the other hand, isoflavones have estrogenic activities and could cause endothelium-dependent or -independent vasorelaxation. Although the precise mechanisms behind the vascular bioactivity of both ergot alkaloids and isoflavones have not been fully defined, current knowledge suggests that they are triggered by different mechanisms. Nevala et al. (31) reported that isoflavones relax noradrenaline precontracted rat mesenteric arteries. Likewise, Egert et al. (15) demonstrated that ergot alkaloids were vasoconstrictive in bovine mesenteric vasculature, whereas dietary exposure to ergot alkaloids decreased the contractility of mesenteric vasculature. Thus, it was hypothesized that isoflavones may attenuate ergot alkaloid-induced vasoconstriction and possibly alleviate the diminished contractility of mesenteric vasculature after preliminary exposure to ergot alkaloids. The objective of this study was to determine if an incubation of bovine mesenteric vasculature with *F*, *B*, or ergovaline-containing tall fescue seed extract (EXT) and their combinations affect ergotamine (ERT)-induced contractility.

MATERIALS AND METHODS

No live animals were involved this study, so approval from the University of Kentucky Animal Care and Use Committee was not required.

Animals and Tissue Collection

Five Angus heifers (Bodyweight = 639 ± 39 kg) were slaughtered and tissues were collected at the University of Kentucky abattoir. As originally described by Klotz and Barnes (32), the gastrointestinal tract was removed from the carcass, and the cecum, ileocecal fold, and the ileal flange were identified as landmarks. Within the mesentery supporting the ileal flange, multiple branches of the mesenteric artery and vein bundles were dissected and submerged in oxygenated Krebs–Henseleit buffer (95% O_2/5% CO_2; pH = 7.4; 11.1 mM D-glucose; 1.2 mM $MgSO_4$; 1.2 mM KH_2PO_4; 4.7 mM KCl; 118.1 mM NaCl; 3.4 mM $CaCl_2$; 24.9 mM $NaHCO_3$; Sigma Chemical Co., St. Louis, MO, USA) for transport to the laboratory. Samples were stored on ice until cleaned. At the time of cleaning, surrounding fat and connective tissues were carefully removed, and mesentery artery and vein were separated under a magnifying lamp (2.5 to 5.0× magnification). Cleaned vessels were sliced into 2-mm segments and examined under a dissecting scope (Semi 2000-C, Carl Zeiss Inc., Oberkochen, Germany) at 12.5× magnification to ensure the usability of the vessels. Cross-sections with abnormalities (branches, valves, or structural damage) were replaced with structurally integral ones.

Pre-Myograph Incubations

A tall fescue seed extract was prepared as described by Foote et al. (33) to contain a 1×10^{-6} M working concentration of ergovaline. Duplicates of each vessel type (from each animal) were incubated in tissue culture flasks with a 50-mL volume of Krebs–Henseleit buffer containing: only buffer (control); 1×10^{-6} M EXT; 1×10^{-6} M *F* (≥99.0%; 47752-5MG-*F*; Sigma Chemical Co., St. Louis, MO, USA); or 1×10^{-6} M *B* (D2016; Sigma Chemical Co., St. Louis, MO, USA); and combinations of 1×10^{-6} M EXT + *F*; 1×10^{-6} M EXT + *B*; 1×10^{-6} M *F* + *B*; or 1×10^{-6} M EXT + *F* + *B*. All buffer solutions were prewarmed for 30 min in a CO_2 incubator (95% O_2/5% CO_2; 37°C; Nu-8500, NUAIRE, Inc., Plymouth, MN, USA) prior to blood vessel addition. Duplicate blood vessel segments were randomly placed into each treatment flask and incubated in the same conditions for 2 h. Immediately after the 2-h incubation, dimensional measurements of cross-sections were recorded only for mesentery artery using Axiovision (version 20, Carl Zeiss, Inc.).

Experimental Myograph Protocol

Following the 2-h incubations, an ERT concentration response experiment was conducted using the procedures described by Klotz and Barnes (32). ERT (ergotamine D-tartrate; 97%; 45510; Aldrich, Milwaukee, WI, USA) standards were prepared by diluting a stock solution (0.0201 M) with dimethyl sulfoxide to working concentrations that resulted in a concentration range of 5×10^{-9} to 1×10^{-4} M in the myograph chamber (contained 5 mL of Krebs–Henseleit buffer).

Artery and vein cross-sections were mounted on the myograph (Multichamber myograph; DMT 610M, Danish Myo Technology, Atlanta, GA, USA) by inserting the supports through the lumen in individual myograph chambers containing 5 mL modified Krebs–Henseleit buffer and continuously gassed (95% O_2/5% CO_2; pH = 7.4; 37°C). The incubation buffer was modified from transport Krebs–Henseleit buffer by adding desipramine (3×10^{-5} M; D3900; Sigma Chemical Co.) to inhibit the reuptake mechanisms of biogenic amines and propranolol (1×10^{-6} M; P0844; Sigma Chemical Co.) to block the non-specific binding

of ERT to β-adrenergic receptors. A 90-min equilibration period with buffer replacement occurring every 15 min was completed based on the conditions above to achieve a stable resting tension of approximately 1 g. Following the equilibration period, the blood vessels were exposed to 120 mM KCl for 15 min to evaluate tissue viability and to normalize treatment data. Following the KCl addition, the incubation buffer was replaced every 15 min until vessel tension returned to the 1 g baseline. Once the vessel returned to baseline, addition of ERT standards was initiated in order to increase the concentration. ERT additions were added in 15-min intervals consisting of a 9-min-incubation period, two 2.5-min buffer washes, and a third, final buffer replacement that was followed by 1-min recovery before the next ERT addition. This 15 min cycle was repeated for the rest of the nine remaining ERT additions. Following the 1-min recovery after the final ERT addition, vessels were again exposed to 120 mM KCl to confirm the vessel viability at the end of the experiment.

Data Collection

The isometric contractions of the different preincubated mesenteric vessels to KCl and ERT additions were digitized and recorded as grams of tension using a PowerLab/8sp and Chart software (version 7.3, ADInstruments, Colorado Springs, Co.). Baseline tension was measured immediately before the addition of 120 mM KCl. For all contractile response data, the maximum observed tension (in grams) in the 9 min-incubation period was recorded as the contractile response. Contractile response data were corrected for baseline tension and normalized as a percentage of the reference compound KCl induced maximum contractile response. The differences of tissue response due to the variations of vessel size and across individual animal were minimized by this data normalization. The contractile response to ERT was determined and presented as percentage means ± SEM. A measurement of potency or the half-maximal effective concentration (EC_{50}) was calculated using GraphPad Prism (version 5; GraphPad Software Inc., La Jolla, CA, USA) using a non-linear regression with fixed slope. The sigmoidal concentration response curves of pretreated mesentery artery and vein to ERT was plotted by using a three-parameter equation:

$$y = \text{bottom} + \left(\frac{\text{top-bottom}}{\left[1 + 10^{\left(\log EC_{50} - x \right)} \right]} \right),$$

where y represents contractile response, and x denotes the agonist concentration, top and bottom are the plateaus of contractile response as percentage of 120 mM KCl maximum response. The EC_{50} is the molar concentration of ERT inducing 50% of the KCl maximum response.

Statistics

All data were analyzed using the MIXED model of SAS (SAS 9.4, SAS Inst. Inc., Cary, NC, USA). Contractile response data of mesentery artery and vein were analyzed separately as two datasets for treatments with and without EXT. Data for contractile response (within each ERT concentration), KCl maximal response, inside and outside diameters for mesenteric artery (these data could not be obtained for mesenteric vein samples due to the pliable nature of this vessel) were analyzed as a completely randomized design with a factorial treatment arrangement. The fixed effects included the effects of F, B, and the interaction of $F \times B$ in the presence or absence of EXT. Due to the shape of the response curve, EC_{50} data were analyzed only in treatments without EXT from mesentery artery using a completely randomized design with treatment as fixed variable. For all data, pair-wise comparisons of least square means (±SEM) were only performed if the probability of a greater F-statistic from the analysis of variance was significant for the tested effect and interaction. Mean separation was performed with the LSD feature of SAS. Differences are denoted as significant at $P < 0.05$, unless specifically reported otherwise.

RESULTS

The F and B incubation pretreatment did not impact ($P > 0.05$) the maximum contractile response of mesenteric artery or mesenteric vein to 120 mM KCl either without EXT (**Table 1**) or with EXT (**Table 2**). The pretreatments with EXT did not compromise the vessel viability, which was indicated by the ending response to KCl for either the artery or vein (**Figure 1**). In mesenteric artery, the inside and outside diameters after the 2 h incubation were not affected ($P > 0.05$) by F or B in both treatment groups with (**Table 2**) or without EXT (**Table 1**). However, there was a tendency ($P = 0.07$) for F treated vessels to have a larger inside diameter for those pretreated with EXT (**Table 2**), and a smaller outside diameter ($P = 0.09$) for those not pretreated with EXT (**Table 1**). Interestingly, mesenteric artery pretreated with B tended ($P = 0.06$) to have a smaller inside diameter when incubated with EXT (**Table 2**).

In the mesenteric artery, ERT induced similar contractile responses in all treatments (**Figure 2**) with $-\log^{EC50}$ values (5.99 ± 0.14, 5.80 ± 0.14, 5.88 ± 0.14, 5.74 ± 0.14 M, respectively) that did not differ with each other ($P = 0.63$). Within each ERT concentration, tendencies for $F \times B$ interactions in mesenteric artery were observed at ERT 5×10^{-9}, 1×10^{-8}, 5×10^{-8} M ($P = 0.09$, $P = 0.07$, $P = 0.08$, respectively; **Table 3**) for treatments without EXT. Contractile responses of $F + B$ treated mesentery artery were greater than B at ERT concentrations 1×10^{-8} and 5×10^{-8} M ($P < 0.05$). In mesenteric vein, ERT-induced contractile responses reached the maximum response at 1×10^{-6} M for Control, B, F, $F + B$ treatments (**Figure 3**), and then relaxed to negative response values with increases in ERT concentration.

For the blood vessels incubated with EXT, the contractile responses decreased as the concentration of ERT increased in both mesenteric artery (**Figure 4**) and mesenteric vein (**Figure 5**). In mesenteric artery, a main effect of B was observed at ERT concentrations of 1×10^{-7} and 5×10^{-7} M (**Table 4**), with treatments that contained B having higher contractile responses ($P < 0.05$). Also, treatments containing B tended ($P < 0.1$) to have greater responses than treatments without B at ERT concentrations of 1×10^{-6}, 5×10^{-6}, and 5×10^{-5} M (**Table 4**).

In the mesenteric vein, the contractile response of all treatments with EXT to ERT decreased and remained below zero from the second ERT addition (1×10^{-8} M) to the last addition at

TABLE 1 | Inside diameter, outside diameter, and SEM of mesenteric artery, and the mean KCl maximum response of mesenteric artery and vein to pretreatment without tall fescue extract: only Krebs–Henseleit buffer (control); 1×10^{-6} M treatments of formononetin (*F*), biochanin A (*B*), and combination of *F* and *B* (*F* + *B*).

Item	Control	*F*	*B*	*F* + *B*	SEM	*P*-value		
						F	*B*	*F* × *B*
Mesenteric artery								
KCl maximum response (g)	4.36	4.42	4.22	3.66	0.46	0.58	0.33	0.50
Inside diameter (mm)	0.81	0.79	0.86	0.77	0.06	0.38	0.77	0.61
Outside diameter (mm)	1.72	1.64	1.75	1.57	0.07	0.09	0.74	0.47
Mesenteric vein[a]								
KCl maximum response (g)	1.70	1.81	1.89	2.22	0.21	0.31	0.18	0.62

[a]*Due to the elasticity of the mesenteric vein, measurements of vascular dimensions were not possible.*

TABLE 2 | Inside diameter, outside diameter, and SEM of mesenteric artery, and the mean KCl maximum response of mesenteric artery and vein to pretreatment with tall fescue seed extract: 1×10^{-6} M ergovaline-containing tall fescue seed extract (EXT); combinations of 1×10^{-6} M EXT and formononetin (*F*), EXT and biochanin A (*B*), and the combination of EXT + *F* + *B*.

Item	EXT	EXT + *F*	EXT + *B*	EXT + *F* + *B*	SEM	*P*-value		
						F	*B*	*F* × *B*
Mesenteric artery								
KCl maximum response (g)	3.43	2.68	3.08	2.84	0.37	0.19	0.80	0.50
Inside diameter (mm)	0.72	0.78	0.60	0.72	0.05	0.07	0.06	0.50
Outside diameter (mm)	1.64	1.57	1.51	1.59	0.07	0.92	0.43	0.29
Mesenteric vein[a]								
KCl maximum response (g)	1.90	2.20	2.09	2.49	0.21	0.11	0.26	0.81

[a]*Due to the elasticity of the mesenteric vein, measurements of vascular dimensions were not possible.*

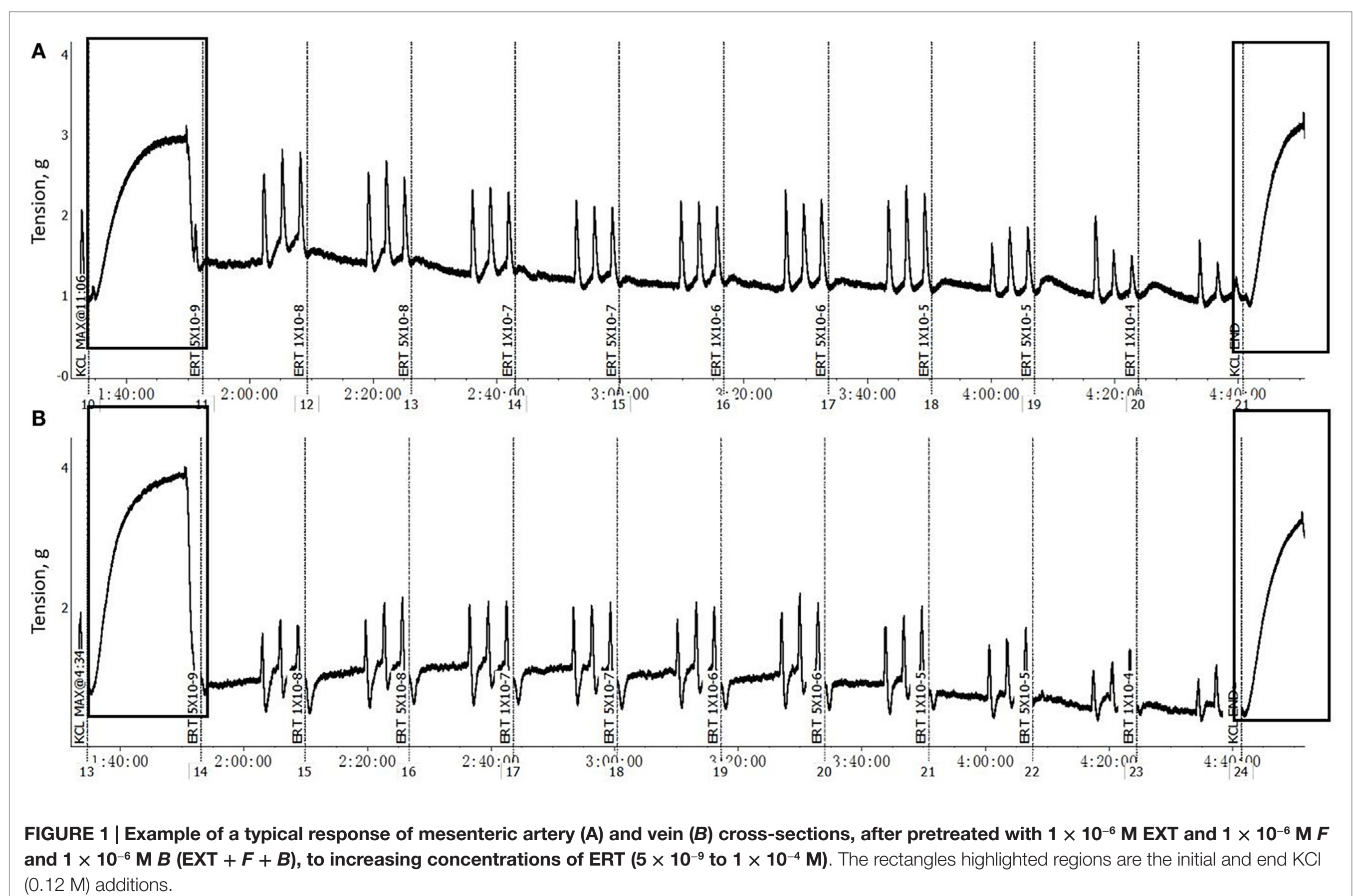

FIGURE 1 | Example of a typical response of mesenteric artery (A) and vein (*B*) cross-sections, after pretreated with 1×10^{-6} M EXT and 1×10^{-6} M *F* and 1×10^{-6} M *B* (EXT + *F* + *B*), to increasing concentrations of ERT (5×10^{-9} to 1×10^{-4} M). The rectangles highlighted regions are the initial and end KCl (0.12 M) additions.

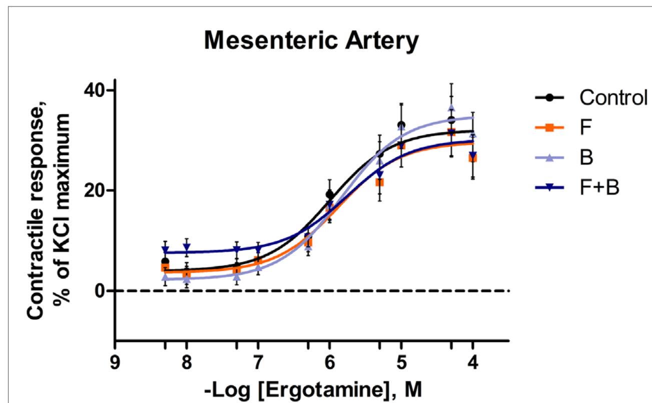

FIGURE 2 | Mean contractile response, as % KCl maximum of mesenteric artery to increasing concentrations of ergotamine for pretreatments without tall fescue seed extract: only Krebs–Henseleit buffer (control); 1×10^{-6} M formononetin (*F*); or 1×10^{-6} M biochanin A (*B*); and combination of 1×10^{-6} M *F* and 1×10^{-6} M *B* (*F* + *B*). The regression lines were plotted for each treatment using a non-linear regression with fixed slope, and the sigmoidal concentration response curves were calculated by the following equation: $y = \text{bottom} + \left[(\text{top-bottom})/(1+10^{(\log EC_{50}-x)})\right]$ where top and bottom are the plateaus of contractile response as percentage of 120 mM KCl maximum response. EC_{50} is the molar concentration of ergotamine inducing 50% of the KCl maximum response.

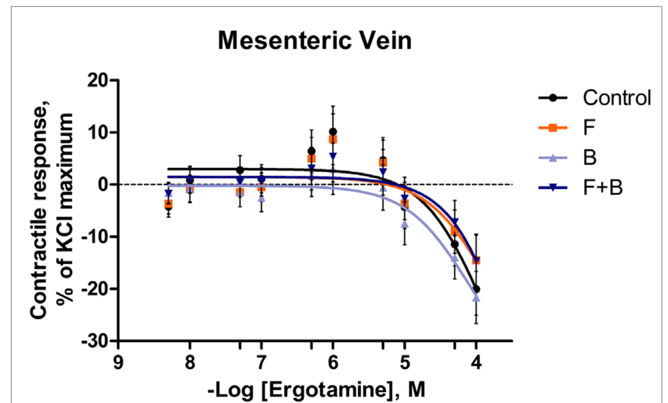

FIGURE 3 | Mean contractile response, as % KCl maximum of mesenteric vein to increasing concentrations of ergotamine for pretreatments without tall fescue seed extract: only Krebs–Henseleit buffer (control); 1×10^{-6} M formononetin (*F*); or 1×10^{-6} M biochanin A (*B*); and combination of 1×10^{-6} M *F* and 1×10^{-6} M *B* (*F* + *B*). The regression lines were plotted for each treatment using a non-linear regression with fixed slope, and the sigmoidal concentration response curves were calculated by the following equation: $y = \text{bottom} + \left[(\text{top-bottom})/(1+10^{(\log EC_{50}-x)})\right]$ where top and bottom are the plateaus of contractile response as percentage of 120 mM KCl maximum response. EC_{50} is the molar concentration of ergotamine inducing 50% of the KCl maximum response.

TABLE 3 | The analysis of variance and *P*-values of main effect of formononetin (*F*), biochanin A (*B*), and the interaction of formononetin and biochanin A (*F* × *B*) for pretreatments without tall fescue extract: only Krebs–Henseleit buffer; 1×10^{-6} M *F*, *B*, and combination of *F* and *B* on every ergotamine concentration.

Ergotamine concentration (M)	*P*-value		
	F	*B*	*F* × *B*
Mesenteric artery			
5×10^{-9}	0.23	0.91	0.09
1×10^{-8}	0.14	0.27	0.07
5×10^{-8}	0.20	0.51	0.08
1×10^{-7}	0.29	0.83	0.31
5×10^{-7}	0.88	0.79	0.43
1×10^{-6}	0.68	0.78	0.64
5×10^{-6}	0.27	0.99	0.72
1×10^{-5}	0.38	0.97	0.97
5×10^{-5}	0.43	0.81	0.77
1×10^{-4}	0.61	0.54	0.59
Mesenteric vein			
5×10^{-9}	0.92	0.27	0.85
1×10^{-8}	1.00	0.96	0.50
5×10^{-8}	0.67	0.67	0.28
1×10^{-7}	0.80	0.64	0.41
5×10^{-7}	0.99	0.43	0.74
1×10^{-6}	0.93	0.30	0.70
5×10^{-6}	0.78	0.43	0.70
1×10^{-5}	0.50	0.80	0.62
5×10^{-5}	0.29	0.93	0.61
1×10^{-4}	0.23	0.86	0.89

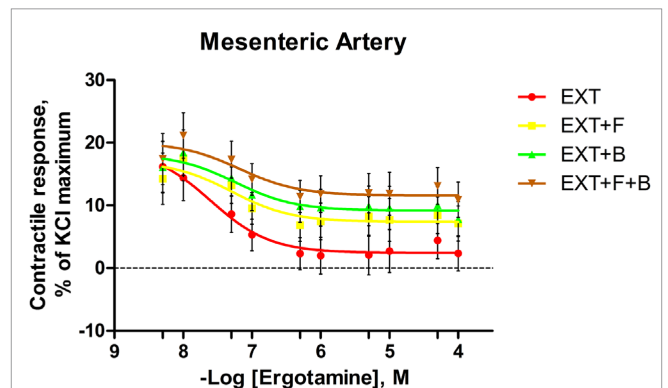

FIGURE 4 | Mean contractile response, as % KCl maximum of mesenteric artery to increasing concentrations of ergotamine for pretreatments with tall fescue seed extract: 1×10^{-6} M ergovaline-containing tall fescue seed extract (EXT); combinations of 1×10^{-6} M EXT and 1×10^{-6} M *F* (EXT + *F*); 1×10^{-6} M EXT and 1×10^{-6} M *B* (EXT + *B*); or 1×10^{-6} M EXT and 1×10^{-6} M *F* and 1×10^{-6} M *B* (EXT + *F* + *B*). The regression lines were plotted for each treatment using a non-linear regression with fixed slope, and the sigmoidal concentration response curves were calculated by the following equation: $y = \text{bottom} + \left[(\text{top-bottom})/(1+10^{(\log EC_{50}-x)})\right]$ where top and bottom are the plateaus of contractile response as percentage of 120 mM KCl maximum response. EC_{50} is the molar concentration of ergotamine inducing 50% of the KCl maximum response.

1×10^{-4} M. Tendencies for main effects of *B* ($P = 0.06$) and *F* × *B* interaction ($P = 0.09$) were observed at ERT concentrations of 5×10^{-9} M, where EXT treatment had a lower contractile response than EXT + *B* ($P < 0.05$; **Table 4**). Treatments containing *F* had greater contractile responses to ERT at 1×10^{-5}, 5×10^{-5}, and 1×10^{-4} M in mesenteric vein (main effect of *F*; $P < 0.05$; **Table 4**). Within the 5×10^{-5} M ERT concentration, the contractile response of EXT was the lowest ($P = 0.03$).

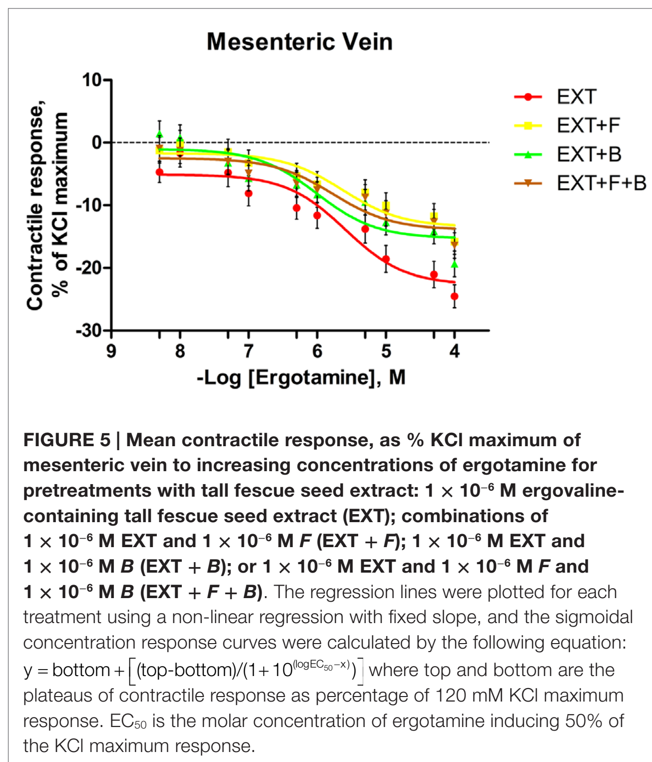

FIGURE 5 | Mean contractile response, as % KCl maximum of mesenteric vein to increasing concentrations of ergotamine for pretreatments with tall fescue seed extract: 1×10^{-6} M ergovaline-containing tall fescue seed extract (EXT); combinations of 1×10^{-6} M EXT and 1×10^{-6} M *F* (EXT + *F*); 1×10^{-6} M EXT and 1×10^{-6} M *B* (EXT + *B*); or 1×10^{-6} M EXT and 1×10^{-6} M *F* and 1×10^{-6} M *B* (EXT + *F* + *B*). The regression lines were plotted for each treatment using a non-linear regression with fixed slope, and the sigmoidal concentration response curves were calculated by the following equation: $y = \text{bottom} + \left[(\text{top-bottom})/(1+10^{(\log EC_{50}-x)})\right]$ where top and bottom are the plateaus of contractile response as percentage of 120 mM KCl maximum response. EC_{50} is the molar concentration of ergotamine inducing 50% of the KCl maximum response.

TABLE 4 | The analysis of variance and *P*-values of main effect of formononetin (*F*), biochanin A (*B*), and the interaction of formononetin and biochanin A (*F* × *B*) for pretreatments with tall fescue seed extract (EXT): 1×10^{-6} M (EXT); combinations of 1×10^{-6} M EXT and *F*, EXT and *B*, or 1×10^{-6} M EXT + *B* + *F* on every ergotamine concentration.

Ergotamine concentration (M)	*P*-value		
	F	*B*	*F* × *B*
Mesenteric artery			
5×10^{-9}	0.94	0.71	0.70
1×10^{-8}	0.43	0.31	0.96
5×10^{-8}	0.22	0.11	0.77
1×10^{-7}	0.20	0.04	0.73
5×10^{-7}	0.25	0.03	0.57
1×10^{-6}	0.21	0.06	0.58
5×10^{-6}	0.21	0.09	0.52
1×10^{-5}	0.31	0.13	0.70
5×10^{-5}	0.24	0.10	0.87
1×10^{-4}	0.18	0.12	0.77
Mesenteric vein			
5×10^{-9}	0.75	0.07	0.10
1×10^{-8}	0.91	0.77	0.41
5×10^{-8}	0.45	0.98	0.50
1×10^{-7}	0.18	0.85	0.32
5×10^{-7}	0.15	0.48	0.19
1×10^{-6}	0.19	0.58	0.32
5×10^{-6}	0.16	0.44	0.27
1×10^{-5}	0.03	0.30	0.12
5×10^{-5}	0.02	0.17	0.08
1×10^{-4}	0.01	0.24	0.14

DISCUSSION

This is the first study to investigate the interaction of ergot alkaloids and isoflavones on bovine mesenteric vasculature. Physiological effects of isoflavones and their metabolites on vasculature have been extensively studied on different vessel types in humans (24, 25) and rat models (26–29). The concentrations of isoflavones (1×10^{-6} M formononetin and biochanin A) used in the current experiment were based on a study that reported dose-dependent formononetin- and biochanin A-induced relaxations of rat-isolated thoracic aorta precontracted with phenylephrine (29). At 1×10^{-6} M, formononetin and biochanin A were capable of inducing about 35 and 25% relaxation, respectively. Since the current study was the first to investigate interactions of isoflavones and ergot alkaloids, our rationale was to pick an intermediate concentration (1×10^{-6} M) to avoid extreme scenarios and one that was equimolar with the concentration of ergovaline. The concentration of ergovaline-containing tall fescue seed extract that was chosen (1×10^{-6} M) is based on the findings of Egert et al. (15), where 1×10^{-6} M of ergovaline-containing extract was observed to induce contractile response (about 40% of KCl maximum) of bovine mesenteric artery and vein.

Several studies have demonstrated that cattle grazing endophyte-infected tall fescue had reduced contractile responses to 5-hydroxytryptamine (5-HT) and as well as ergot alkaloids in the lateral saphenous vein (16, 17). Recently, a study using mesenteric artery and vein from steers that had been ruminally dosed with endophyte-infected tall fescue seed observed a decreased or completely absent constrictive response to ergot alkaloids (15). In the current study, an *in vitro* incubation of bovine mesenteric vasculature in a medium containing EXT was used to achieve an ergot alkaloid pretreatment. The pretreatment of blood vessels with EXT (33) and isoflavones (*F* and *B*) were conducted at equimolar final concentrations of 1×10^{-6} M. It has been reported that *F* and *B* both induced vasorelaxation in phenylephrine-precontracted rat-isolated thoracic aorta at 1×10^{-6} M (29). This latter study identified both nitric oxide from endothelial nitric oxide synthase (NOS) and potassium efflux from endothelial cells as putative mechanisms of action. However, the 1×10^{-6} M ergovaline in the EXT may be considered a high dose compared to the physiological levels encountered by cattle grazing endophyte-infected tall fescue (34). Nevertheless, the viability of mesenteric artery and vein was not compromised by concentration of ergot alkaloids used in the current study, as evidenced by the fact that both artery and vein were responsive to the final KCl addition.

Studies have reported that many isoflavones and their metabolites can reduce the vasoconstriction induced by KCl in several different vessel types using rat models (28, 35). The contractile response to KCl of endothelium-denuded rat aortic rings was inhibited by pretreatment with genistein or daidzein at both 3×10^{-5} and 1×10^{-4} M for 30 min (36). However, in the same study, pretreatment with genistein or daidzein at 1×10^{-5} M did not relax the KCl-induced vasoconstriction. The similar dose-dependent inhibition of *F* (1×10^{-5}, 3×10^{-5}, and 1×10^{-4} M) on the contractile response to KCl was observed in rat mesenteric arteries without endothelium (37), where *F* again failed to inhibit KCl-initiated contraction at 1×10^{-5} M. However, in endothelium-intact rat aortic rings, Zhao et al. (38) reported that pretreatment with 1×10^{-5}, 3×10^{-5}, and 5×10^{-5} M of *F* all significantly inhibited the contractile response to KCl in a

non-competitive manner. In the current study, the prior exposure to *F* (1×10^{-6} M), biochanin A (1×10^{-6} M), and their combination did not have an impact on maximum contractile response of mesenteric artery and vein to KCl in treatments either with or without EXT. Since previous studies have indicated that the inhibition effects were dose-dependent (36, 37) and non-competitive (38), it is possible that the isoflavone concentration (1×10^{-6} M) in the current study was not high enough to elicit any inhibitory effects on the KCl response. However, in the current study, maximum KCl responses were unaffected by *F* and *B*, which validate the usage of KCl as a reference compound to normalize the contractile responses.

There is limited information about isoflavones' impact on blood vessel morphology in regards to vessel inside and outside diameters. In the current study, there was no significant evidence of an *F* or *B* effect on mesenteric artery inside or outside diameter regardless of EXT treatment. However, a tendency for a smaller inside diameter induced by *F* treatment was observed in mesentery artery pretreated without EXT. One possible explanation for this could be the inhibitory effect of *F* on vascular smooth muscle cells. Previously, estrogens [tamoxifen (39), estradiol (40)] and isoflavones [*F* and *B* (41)] have been shown to inhibit mitogen-induced proliferation, migration, and extracellular matrix synthesis of smooth muscle cells. On the other hand, the inside diameter of bovine lateral saphenous veins collected from steers grazing high-endophyte tall fescue pasture were smaller than those from steers grazing low-endophyte mixed-grass pasture (16). Likewise, Egert et al. (15) observed a smaller outside diameter of bovine mesenteric artery from endophyte-infected tall fescue seed-dosed steers than control steers. One possible explanation is the prolonged vasoconstriction induced by ergot alkaloids results in decreased inside or outside diameter. Additionally, the morphological changes of blood vessels, especially the expansion of the tunica media smooth muscle layer, could also lead to a smaller vessel insider diameter. It has been observed that calves given ethanolic extracts of tall fescue hay had symptoms of fescue foot and concomitant thickening of vessel walls and smaller vessel lumens in blood vessels of the coronary bands and tail tips (42). Similarly, Garner and Cornell (43) reported a thickening of the smooth muscle layer of peripheral blood vessels after consumption of endophyte-infected tall fescue. Although the exact mechanism associated with thickening of smooth muscle layer is unclear, evidence suggests hyperplasia over hypertrophy. Strickland et al. (44) reported that ergonovine, ergovaline, and α-ergocryptine stimulate the growth and mitosis of quiescent bovine vascular smooth muscle cells *in vitro*. Although, in the present study, there was no significant impact of *F* and *B* pretreatment on mesenteric artery inside or outside diameters, the tendency of larger inside diameter caused by *F* may indicate an alleviative effect on ergot alkaloids induced thickening of vessel walls and smaller lumens.

In the current study for pretreatments without EXT, ERT induced similar contractile response curves and $-\log^{EC50}$ values (Control, *F*, *B*, and *F*+*B*) in mesenteric arteries. The shape of contractile responses were similar with Egert et al. (15), who reported ERT-induced contractile responses in mesenteric artery from steers, not exposed to ergot alkaloids, with a $-\log^{EC50}$ value of 6.03 ± 0.4 M. Whereas, the shape of mesenteric vein contractile response curves were in contrast to the observations of Egert et al. (15), which did not drop to negative values after reaching a maximum. Further, the maximum contractile response of mesenteric vein to ERT in the current study was also lower (10% vs. 45% of KCl maximum). The blood vessels used in the previous study (15) did not undergo an *in vitro* pre-myograph incubation and utilized blood vessels from steers compared to heifers in the current study.

Substantial evidence has shown that many ergot alkaloids are vasoconstrictive in multiple types of vessels in various animal models (7, 8). Among these vasoactive ergot alkaloids, ERT and ergovaline were indicated as more potent vasoconstrictors with lower EC50 values (relative to other alkaloids) in bovine saphenous vein (45), ruminal vasculature (13), and mesenteric vasculature (15). The current observation of the contractile response induced by ERT was consistent with the previous findings in terms of vasoactivity for this alkaloid. Even though not completely defined yet, numerous studies have been conducted to investigate the mechanism of ergot alkaloid-caused vasoconstriction. The structural similarities of the ergoline ring system and several biogenic amines [i.e., (nor)epinephrine, serotonin, and dopamine] allows ergot alkaloids to interact with corresponding biogenic amine receptors as ligands (30, 46). Substantial evidence has shown that ergot alkaloids interact with dopamine-2 receptors (47, 48), α_1-adrenergic receptors (49, 50), α_2-adrenergic receptors (51), and 5-HT_{2A} receptors (14, 16, 17). The binding with these G protein-coupled receptors activates the subunit of the heterotrimeric G protein and then triggers various secondary messaging systems and corresponding cytoplasmic signaling transductions. The vasoconstrictive response induced by ERT shown in the current study (**Figure 2**) could be explained by these agonistic mechanisms.

The pre-myograph incubation with EXT altered the contractile capacity of the mesenteric artery and vein. Using a bovine lateral saphenous vein model, Klotz et al. (16) found that 2,5-dimethoxy-4-iodoamphetamine (DOI), a 5-HT_{2A} receptor agonist, induced vessel contractile intensities were 35% lower in high endophyte-infested tall fescue than in low-endophyte-infested tall fescue, whereas 5-carboxytryptamine (5-HT_7 receptor agonist) produced greater (37%) contractile intensities in high endophyte-infected tall fescue. Taken together, this indicated that chronic exposure to ergot alkaloids through grazing endophyte-infested tall fescue altered the vasoconstriction via serotonergic receptors. In a subsequent study, Klotz et al. (17) reported a suppression of the contractile response to ergovaline and 5-HT in steers grazing Kentucky-31 tall fescue infected with wild-type endophyte. Further, Egert et al. (15) demonstrated a similar reduced contractile response to ERT in mesenteric vasculature of steers treated with endophyte-infected tall fescue seed. Unsurprisingly, the antagonistic effects of ergot alkaloids to certain 5-HT receptors have been shown previously (49, 50, 52). Collectively, it is possible that the EXT pretreatment in the present experiment reduced the vasoconstriction to ERT via altering the biogenic amine receptor activities.

Many *in vitro* bioassays have demonstrated that, in various vessel types and in numerous species, the binding of ergovaline and receptor was irreversible, or the dissociation from the receptor was very slow (14, 45, 50, 53). Likewise, an apparent bioaccumulation

was reported in bovine lateral saphenous veins after repetitive exposures of ergovaline *in vitro* (54). Additionally, studies have indicated that ergot alkaloid-induced constriction is prolonged and wash-resistant in human superficial temporal artery (55) and coronary artery (56). These findings may explain the high contractile response to the initial ERT addition (5×10^{-9} M) in mesenteric arteries that were pretreated with EXT (1×10^{-6} M ergovaline) in the current study. Furthermore, using an inositol phosphate accumulation assay, Unett et al. (57) reported that as a 5-HT_{2B} agonist, ERT maintained similar potencies after 2 or 4-h extensive washout and this was due to internalization or sequestration of the active ERT-bound receptor. It has been demonstrated that ERT and ergovaline are equally potent vasoconstrictors inducing similar contractile responses (45). Thus, these findings support the observations in the current study that the elevated contractile response to very low concentrations of ERT pretreated with EXT was numerically similar to 1×10^{-6} M ERT-induced contractile response of mesenteric artery without EXT incubation. This can be attributed to prior exposure to ergovaline (in EXT) leading up to the myograph portion of the experiment, and possible carry over of the vascular response to ergovaline to the dose response to ERT.

In the present study, prior exposure to *F* and *B* failed to consistently alter the mesenteric vasculature contraction induced by ERT. As discussed earlier in this section, numerous studies have shown the vasodilative effect of isoflavones and their metabolites in different vessel types. Pretreatment with *F* (3×10^{-5} and 5×10^{-5} M) antagonized contractile responses of rat thoracic aortas to norepinephrine in a non-competitive manner (38). Sun et al. (37) hypothesized that pre-incubation with *F* (3×10^{-5} and 1×10^{-4} M) depressed the contraction of rat mesenteric artery to phenylephrine and 5-HT, and similarly, depression was not observed when *F* at 1×10^{-5} M. The 1×10^{-6} M *F* and *B* concentrations in the present study were possibly not high enough to elicit sufficient vasorelaxation to offset the ERT-induced vasoconstriction. Other evidence of the chronic antihypertensive effect of *F* has been reported based on male spontaneously hypertensive rats (SHR) (58). They found that the vasoconstriction of mesenteric artery segments induced by phenylephrine or 5-HT was reduced in *F* (50 mg/kg per day) orally administrated to SHR. The expression of α_1-adrenoceptors and 5-$HT_{2A/1B}$ receptors in mesenteric artery of *F* treated SHR decreased (58).

Even though there might be some species differences between bovine and SHR, the 2-h pre-myograph incubation with *F* or *B* in the present study may not be long enough to permit expression or induction of NOS by the tissues that were used. However, sodium- and ATP-dependent K+ transport mechanisms, like those described by Wu et al. (29), are very rapid. Further, *F* and *B* are metabolized to daidzein and genistein in the rumen (59). As many metabolic studies have found, daidzein is further metabolized by rumen and intestinal bacteria to equol, which has a higher estrogenic potency than its isoflavone precursor (22, 23, 59). Equol undergoes renal clearance by clover-fed ruminants (60, 61), which indicates that equol, rather than formononetin, is present in the blood. It is possible that equol and other bacterial metabolites of isoflavones could have greater effects on bovine blood vessels. Thus, the different routes of administration of isoflavone treatments (*in vivo* vs. *in vitro*) may account some of the inconsistencies, and the idea that bacterial or animal metabolism might influence vasorelaxation is consistent with the aforementioned results by Sun et al. (58).

With EXT in the pretreatment media, *F* and *B* increased the contractile response of mesenteric artery at several ERT concentrations. This has provided the first evidence that the isoflavones, *F* and *B*, have potential to alleviate the vasoconstrictive effect of ergot alkaloids. However, based on the current knowledge from ergot alkaloids and isoflavones, there is little to mechanistically explain this phenomenon, so more investigations on their interactions are needed.

CONCLUSION

In conclusion, this study indicated that a pre-myograph incubation with *F*, *B* at 1×10^{-6} M and their combination did not affect the contractile response to ERT in mesenteric vasculature. The pre-myograph incubation of mesenteric vasculature with EXT (equivalent to 1×10^{-6} M ergovaline) reduced the vasoactivity of ERT, and there were some indications that *F* and *B* may alleviate this reduction. At higher concentrations, *F* and *B* may alleviate this reduction in vasoactivity caused by prior exposure to ergot alkaloids. Future studies with higher isoflavone dosages or longer exposure may be helpful to further investigate the ergot alkaloids and isoflavones interaction. Additionally, this study was confined to the mesenteric veins and arteries. It is plausible that effects could be elicited in other vessels with different expression levels of 5-HT, NOS, or other factors. *In vivo* research, which takes isoflavone metabolism into account, is necessary to better understand the interaction from a practical perspective.

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Potential of plant essential oils and their components in animal agriculture – *in vitro* studies on antibacterial mode of action

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The antimicrobial activity of essential oils and their components has been recognized for several years. Essential oils are produced as secondary metabolites by many plants and can be distilled from all different portions of plants. The recent emergence of bacteria resistant to multiple antibiotics has spurred research into the use of essential oils as alternatives. Recent research has demonstrated that many of these essential oils have beneficial effects for livestock, including reduction of foodborne pathogens in these animals. Numerous studies have been made into the mode of action of essential oils, and the resulting elucidation of bacterial cell targets has contributed to new perspectives on countering antimicrobial resistance and pathogenicity of these bacteria. In this review, an overview of the current knowledge about the antibacterial mode of action of essential oils and their constituents is provided.

Keywords: essential oils, foodborne pathogens, mode of action, quorum sensing, antibacterial

Introduction

Plant materials including flowers, roots, bark, leaves, seeds, peel, fruits, and wood can be used to extract aromatic and volatile liquids known as essential oils (EOs) (1–3). These EOs have a long history of use for medical purposes, in perfumes and cosmetics, and as herbs and spices for foods. EOs are considered to be secondary metabolites in plants; secondary metabolites are organic compounds that are not directly involved in the normal growth, development, or reproduction of the plant (4). These secondary metabolites are often involved in plant defense and thus may possess antimicrobial properties (4, 5). The first experiment to determine the bactericidal properties of EOs is said to have been carried out by de la Croix in 1881 (6). In more recent years, many EOs, or their components, have been shown to possess broad-range antibacterial properties (7).

Increased resistance to infectious diseases, including parasitic infections such as coccidiosis, has been noted when plant phytonutrients were fed to animals. For instance, Lee et al. (8) found that feeding plum powder to laying hens increased their immune response as well as conferring immunity to coccidiosis. Lillehoj et al. (9) determined the effects of feeding capsicum oleoresin or cinnamaldehyde on the global gene expression profiles of broilers. Capsicum oleoresin induced gene changes in genes associated with metabolism and immunity, whereas cinnamaldehyde affected genes related to antigen presentation, humoral immune response, and inflammatory disease. Feeding of these compounds also protected the birds against infection with live coccidiosis parasites. Mathlouthi et al. (10) found that oregano or rosemary EOs had different antimicrobial effects *in vitro* against pathogenic and non-pathogenic bacteria but had the same growth promoting effects as avilamycin

when added to broiler diets. Authors speculated that the *in vivo* growth promotion effects were due to ecological changes in the bacterial gut flora rather than antibacterial effects against a single bacterial genus and species. Betancourt et al. (11) confirmed a shift in gut flora in the foregut but not ceca and colon in broilers fed oregano EOs during a 42-day grow out period.

Alali et al. (12) tested a mixture of carvacrol, thymol, eucalyptol, and lemon for the ability to prevent colonization and shedding in broilers intentionally fed *Salmonella* Heidelberg. They determined that feeding 0.05% (v/v) of the EO mixture significantly reduced the colonization of the crops of challenged birds as well as lowering feed conversion and improving weight gain in the birds. However, cecal colonization and shedding were not significantly decreased. Cerisuelo et al. (13) fed an EO mixture composed of cinnamaldehyde and thymol to broilers, either with or without butyric acid. They determined that the EO blend reduced cecal numbers of *Salmonella*, especially when combined with butyric acid, as compared to control feed. Ricke et al. (14) provide an overview of the anti-*Salmonella* effects of EOs in agriculture.

Benchaar et al. (15) investigated the effects of EOs *in vitro* rumen microbial fermentation. They determined that only the phenolic compounds, carvacrol, thymol, and eugenol affected ruminal fermentation, relative to the control, increasing pH and butyrate and decreasing propionate, indicating antibacterial activity which was not nutritionally beneficial. Callaway et al. (16) studied the *in vitro* effects of orange peel and orange pulp, both sources of EOs, against *Escherichia coli* O157:H7 and *Salmonella typhimurium* in rumen fluid. Growth of both pathogens was reduced by addition of 0.002 g/ml of orange pulp or orange peel. Callaway et al. (17) were able to demonstrate that the orange peel products when fed to experimentally inoculated sheep reduced *S. typhimurium* populations in the gut, with a significant reduction reached in the ceca.

The antimicrobial properties of EOs are a recent focus for agricultural applications because of a desire on the part of many consumers to reduce the use of "hazardous or unnatural chemicals" in their food (18–20). Although there are many studies on the antimicrobial activities of EOs, few take the next step and determine the mode of action of these compounds. However, application of EOs as antibacterial substances for food animals or as food preservatives requires detailed knowledge about their properties, including the mode of action. The purpose of this review is to provide an overview of current knowledge about the antimicrobial mode of action of EOs and their constituents.

Effects on Cell Wall and Membrane

Antimicrobial activity of EOs is strongly linked to their hydrophobicity (21–28). The cell walls of Gram-positive bacteria are made up predominantly of peptidoglycan linked with other molecules such as proteins or teichoic acid (29). On the other hand, Gram-negative bacteria possess an outer membrane of containing hydrophilic lipopolysaccharides (LPS), which creates a barrier toward hydrophobic compounds such as those found in EOs (30, 31). Gram-negative bacteria are thus considered to be less susceptible to the effects of EOs than Gram-positive bacteria (32). However, the hydrophobic constituents of EOs are able to gain access to the periplasm of Gram-negative bacteria through the porin proteins of their outer membrane (24), through which they can slowly travel (33) See **Table 1** for an overview of the bacterial targets of select EOs and their constituents.

TABLE 1 | Target of antibacterial action of some essential oils.

Target	Essential oil or component	Reference
Cell wall or membrane	Carvacrol	Helander et al. (24), Ultee et al. (28, 34), Fitzgerald et al. (37), Xu et al. (48)
	Cinnamon	Bouhdid et al. (40)
	Cinnamaldehyde	Gill and Holley (57)
	Cymene	Ultee et al. (28)
	Eugenol	Walsh et al. (36)
	Farnesol	Inoue et al. (38)
	Nerolidol	Inoue et al. (38)
	Oregano	Bouhdid et al. (43), De Souza et al. (53)
	Plaunotol	Inoue et al. (38)
	Rosemary	De Souza et al. (53)
	Tea tree oil	Cox et al. (26)
	Thymol	Helander et al. (24), Walsh et al. (36), Xu et al. (48)
	Vanillin	Fitzgerald et al. (37)
Respiration	Cinnamon	Bouhdid et al. (40)
	Tea tree oil	Cox et al. (26)
	Vanillin	Fitzgerald et al. (37)
Quorum sensing	Clove	Khan et al. (64)
	Geranium	Szabo et al. (65)
	Lavender	Szabo et al. (65)
	Oregano	Alvarez et al. (66)
	Rose	Szabo et al. (65)
	Rosemary	Szabo et al. (65)

Potassium Leakage from Cells

The leakage of potassium into the extracellular space is considered an indicator for an increase in membrane permeability and ultimate loss of viability for the cell. This particular result was observed in multiple studies (22, 26, 34–40). Carvacrol is a fraction of many EOs and is the major component of oregano EO, which consists of 60–74% carvacrol and thyme EO which consists of 45% carvacrol (41, 42). Ultee et al. (34) examined carvacrol for its antimicrobial properties against *Bacillus cereus* and determined that, at a concentration of 0.15 ml/l, carvacrol caused an immediate decrease in intracellular potassium and an increase in the extracellular potassium. Fitzgerald et al. (37) studied the effects of carvacrol and vanillin on *E. coli*, *Lactobacillus plantarum*, and *Listeria innocua*. At concentrations of 7.6 and 0.496 ml/l of vanillin and carvacrol, respectively, they saw that intracellular potassium levels decreased and the extracellular potassium levels increased in all organisms. Both thymol and eugenol were able to increase extracellular potassium at concentrations of 0.50 ml/l in *E. coli* and *Staphylococcus aureus* (36). Tea tree oil at 2.50 ml/l caused the release of 100% of the total cellular potassium in *E. coli* within 30 min, but only approximately 20% was released by *S. aureus* in the same time (26), which is in contrast to the reported greater susceptibility of Gram-positives (32) and illustrates the great diversity in efficacy of EOs. Bouhdid et al. (43) demonstrated

that oregano EO caused potassium leakage in both *Pseudomonas aeruginosa* and *S. aureus*. Oil-treated cells were examined by transmission electron microscopy (TEM), which revealed mesosome-like structures, coagulated cytoplasmic material, and intracellular material outside the cell; these effects were more noticeable in *P. aeruginosa* compared with *S. aureus* (43). Bouhdid et al. (40) determined the effects of EO of *Cinnamomum verum* on *P. aeruginosa* and *S. aureus* and discovered that a concentration of 1.25 ml/l was able to increase extracellular potassium levels in both bacteria. Inoue et al. (38) tested the terpene alcohols farnesol, nerolidol, and plaunotol for their antimicrobial effects on *S. aureus*. All three compounds increased the extracellular potassium levels when applied to *S. aureus* at a concentration of 0.020 ml/l. Togashi et al. (39) found that when geraniol was added to farnesol in a ratio of 0.010:0.005 ml/l, the ability of farnesol to cause potassium leakage was enhanced, while geranylgeraniol inhibited the potassium leakage activity of farnesol. Bajpai et al. (44) used scanning electron micrographs of *B. cereus* and *E. coli* to demonstrate that cells treated with *Ginkgo biloba* EO (250 and 500 μg/ml, respectively) had disruption of cell membranes and swelling of the cells which led to leakage of potassium from the cells. The studies discussed in this section indicate that these EOs and components act in a similar fashion on the membrane to cause the bacterial cell to lose the ability to regulate potassium transfer across the membrane, leading to an outpouring of potassium from the cell and a subsequent loss of viability.

Leakage of Other Cell Components

The release of other cellular components has also been an object of study for those seeking to determine microbial action of EOs. Carboxyfluorescein diacetate is commonly used as a probe for viable cells (45, 46). Carboxyfluorescein diacetate was used to stain live cells of *L. innocua* which were then exposed to the EOs of *Cymbopogon citratus*, *Ocimum gratissimum*, or *Thymus vulgaris* (47). Loss of fluorescence was taken to be indicative of leakage of carboxyfluorescein from within the cell and thus a measure of the disruption of the cell membrane. EOs of *C. citratus* (7.80 ml/l), *O. gratissimum* (5.0 ml/l), and *T. vulgaris* (4.30 ml/l) were adjusted to a concentration of 1.04×10^4 arbitrary units (AUs) for treatment; AUs were determined by the formula AU ml^{-1} = mean diameter (mm) of the minimal inhibition zone × dilution factor × 50. All EOs were shown to cause a decrease in fluorescence as compared to an unexposed control (47). Xu et al. (48) also examined the leakage of carboxyfluorescein by *E. coli* after exposure to components of thyme EO, carvacrol and thymol, at different concentrations (0.10 and 0.20 ml/l). They found that both carvacrol and thymol caused an increase in carboxyfluorescein released by *E. coli* cells. Trombetta et al. (32) assessed the release of carboxyfluorescein from large unilamellar vesicles (LUVs) that were exposed to (+) menthol, thymol, and linalyl acetate at several concentrations (0.10, 0.25, and 0.50 ml/l). They concluded that (+) menthol and thymol were both effective in causing the release of carboxyfluorescein from the LUVs, but linalyl acetate was only slightly effective. Helander et al. (24) researched the release of fatty acids from *E. coli* cells treated with thymol, carvacrol, (+)-carvone, or *trans*-cinnamaldehyde. Thymol and carvacrol were shown to release a significant amount of fatty acids into the supernatant, while (+)-carvone and *trans*-cinnamaldehyde were ineffective at releasing fatty acids. The results produced by Helander et al. (24) demonstrated that thymol and carvacrol actually break down the cell membrane. Proteins absorb light at 280 nm; therefore, release of 280 nm materials was considered indicative of macromolecular leakage by the cell; *E. coli* and *S. aureus* exposed to tea tree oil were found to leak 280 nm absorbing material, but at levels lower than potassium leakage (35). Nucleic acids, on the other hand, absorb light at 260 nm, and the release of 260 nm material from bacterial cells would be indicative of macromolecular leakage by the cell. Ifesan et al. (49); Carson et al. (50), and Oussalah et al. (51) all examined the release of 260 nm absorbing material. Strains of *S. aureus* exposed to a crude extract of a perennial herb, *Eleutherine americana*, had a peak release of 260 nm absorbing materials after 8 h of exposure to 2.50–10.0 ml/l of the extract (49). After 22 h of exposure, the absorbance decreased from the levels seen after 8 h, most likely due to denaturation of the materials, which caused them to become unreactive to the light at 260 nm (49). A significant amount of 260 nm material was released by *S. aureus* cells after an hour of exposure to tea tree oil or its components (50). Oussalah et al. (52) examined Spanish oregano, Chinese cinnamon, and savory EOs against *E. coli* O157:H7 and *Listeria monocytogenes*. They found that at 0.25 ml/l each EO was able to cause an increase in the release of 260 nm absorbing material. A study investigating the effects of the EOs of oregano and rosemary on *Pseudomonas fluorescens* found that cell material was released into the growth medium immediately after contact with either EO singly or in combination (53). Electron microscopy of exposed cells revealed alteration in the cell wall structure, rupture of the plasma membrane, shrinking of the cells, condensation of the cytoplasmic content, and leakage of the intracellular material with a 2 h contact time (53). Confocal scanning laser microscopy revealed increased cell membrane permeability, resulting in cell death after exposure times of only 15 min (53). These studies indicate that EOs and components are able to cause macromolecular permeability in a variety of bacteria.

Uptake of Substances

The cell membrane helps cells regulate what enters and exits the cell. When ions are no longer being regulated, it indicates that at the very least small pores have formed in the cell membrane. When larger molecules, such as propidium iodide (PI) or *N*-phenyl-L-napthylamine (NPN), enter the cell without regulation it indicates much larger pores have formed in the membrane and a much higher probability of cell death. Helander et al. (24) examined the uptake of NPN by *E. coli* and *S. typhimurium* after exposure to thymol, carvacrol, (+)-carvone, and *trans*-cinnamaldehyde. Thymol and carvacrol significantly increased the uptake of NPN by both bacteria, while (+)-carvone and *trans*-cinnamaldehyde did not allow uptake. These findings demonstrate that (+)-carvone and *trans*-cinnamaldehyde are unable to form pores large enough for NPN, unlike thymol and carvacrol. *Trans*-cinnamaldehyde has a similar minimal inhibitory concentration (MIC) to carvacrol and thymol, according to Helander's study, but it does not function as a large pore former like carvacrol or thymol. Therefore, there must be another mode of action for *trans*-cinnamaldehyde. Fisher and Phillips (54) also investigated NPN uptake by *Enterococcus*

faecium and *Enterococcus faecalis* after exposure to a citrus oil blend. They found that the oil blend was able to increase the NPN uptake over twofold, indicating that the blend is able to cause large pore formation in the bacteria. They also examined the cells using TEM and reported morphological changes in the cells including a loss of distinction of the membrane after being in contact with either the EO or its vapor. Cells that had been in direct contact with the EO appeared to have vacuoles which the authors speculated contained the EO within them. Nearly 100% of *E. coli* cells exhibited increased PI uptake after exposure to tea tree oil, while only around 10% of *S. aureus* cells expressed increased PI uptake. (35). Cox's results indicate that there is a possible difference in the effects of tea tree oil on Gram-negative and Gram-positive bacteria. Similarly, Bouhdid et al. (40) determined that cinnamon EO treatment made *P. aeruginosa* much more susceptible, in terms of PI uptake, than *S. aureus*. Fitzgerald et al. (37) exposed *E. coli*, *L. plantarum*, and *L. innocua* to vanillin and measured subsequent PI uptake; *E. coli* exhibited membrane damage after 15 and 60 min of exposure, but appeared to recover overnight, as the percentage of membrane damage decreased from that seen after 60 min. However, *L. plantarum* exhibited slight damage after 15 and 60 min, but rather than recover overnight as *E. coli* did, damage increased (37). *L. innocua* showed little susceptibility to vanillin. Fitzgerald's results indicate that vanillin does not have a Gram-negative or Gram-positive bias, such as observed with tea tree oil or cinnamon EO, although further studies with tea tree oil and cinnamon EO need to be carried out to determine if they are more effective at large pore formation in Gram-negative bacteria. Based on these studies, large pore formation appears to be a mode of action employed by various EOs against a range of bacteria.

Effects on Membrane Potential

Membrane potential is used by the cell to perform actions necessary for life, such as synthesis of enzymes, nucleic acids, polysaccharides, and other cell components, for cell maintenance and repair of damage, for motility, and for numerous other processes (55), and decrease in this membrane potential is indicative of damage to the cell membrane. Bouhdid et al. (40) used bis-oxonol dye to stain depolarized *P. aeruginosa* and *S. aureus* cells. They found that cinnamon EO at MIC levels was able to decrease the membrane potential of *P. aeruginosa*, but not *S. aureus*. Using 3,3′-dipropylthiacarbocyanide iodide ($DiSC_35$) to monitor membrane potential, Veldhuizen et al. (56) exposed *S. aureus* cells to carvacrol, *o*-cresol, or 2-amino-*p*-cymene at MIC concentrations which were all found to decrease the membrane potential of the cells within seconds of contact. Ultee et al. (28) also employed $DiSC_35$ to look at the disruption of membrane potential of *B. cereus* by carvacrol and cymene. Carvacrol was seen to immediately decrease the membrane potential at concentrations above 0.037 ml/l, while cymene was able to decrease the membrane potential to a lesser extent at a maximum concentration of 0.067 ml/l. Cymene lacks the hydroxyl group that is present on carvacrol, indicating that the hydroxyl group plays an important role in the effect of carvacrol on the membrane potential. Fisher and Phillips (54) also monitored membrane potential with $DiSC_35$ in *E. faecalis* and *E. faecium* after exposure to a 1:1 blend (20.0 ml/l each) of orange and bergamot EOs. They found that the blend was able to dissipate the membrane potential within seconds of contact with the cells. These studies show that when an EO affects the membrane potential of a cell it is an almost immediate reaction. Loss of membrane potential is adverse to cell survival, but could be a consequence of membrane disruption in the case of carvacrol.

Effects on ATP: Leakage from Cells and Usage by Cells

Another target for research on the mode of action of EOs has been to examine the levels of ATP both inside and outside bacterial cells. Ultee et al. (28) determined that cymene, a plant aromatic compound found in cumin and thyme, was unable to cause ATP levels to rise in the extracellular environment or decrease in the intracellular environment. However, carvacrol was able to eliminate the internal ATP of *B. cereus* cells within 20 min of contact at a concentration of 0.150 ml/l (34). Fisher and Phillips (54) utilized an EO blend consisting of a 1:1 mixture of sweet orange and bergamot to treat *E. faecalis* and *E. faecium* and determined that the blend was able to completely dissipate internal ATP in within 10 min of contact. Oussalah et al. (51) examined the effects of Spanish oregano, Chinese cinnamon, and savory EO on the ATP of *E. coli* O157:H7 and *L. monocytogenes*. Savory EO was able to increase extracellular ATP levels in both bacteria, while Spanish oregano was only able to increase extracellular ATP in *E. coli* O157:H7 and Chinese cinnamon was not able to increase extracellular ATP in either organism. Helander et al. (24) looked at the effects of carvacrol, thymol, (+)-carvone, and *trans*-cinnamaldehyde on the ATP of *E. coli* cells. Carvacrol and thymol were both found to decrease intracellular ATP over a 20-min period, and slightly increase external ATP, but (+)-carvone and *trans*-cinnamaldehyde did not affect the ATP of the cells, internally or externally. Fitzgerald et al. (37) looked at the effect of vanillin and carvacrol on ATP of *E. coli*, *L. plantarum*, and *L. innocua*. Vanillin was found to be ineffective at reducing intracellular ATP or increasing extracellular ATP in any bacteria. Carvacrol was very effective at decreasing all bacteria's internal ATP, while only increasing external ATP in *E. coli* and *L. innocua*. The increase in extracellular ATP concentration in the presence of some EOs is of course due to intracellular ATP release, most likely as a consequence of envelope damage induced by the oils.

Some EOs and components, such as cinnamaldehyde, directly target the production of ATP in the cell. Gill and Holley (57) investigated ATP generation in *Lactobacillus sakei* and *L. monocytogenes.* Both bacteria were treated with eugenol, and *L. monocytogenes* was additionally treated with cinnamaldehyde. Eugenol was found to inhibit ATP production but was unable to decrease intracellular ATP. Cinnamaldehyde was found to both inhibit production of ATP and decrease intracellular ATP. These results were determined by adding the EOs to the bacteria before or after the addition of 0.25% glucose to energize the cell. Gill and Holley (57) propose that the mode of action for eugenol relies on inhibiting the cell from using glucose due to its effectiveness when added prior to glucose, but not when added after glucose. Cinnamaldehyde reduced ATP levels when added either prior to or after glucose, which led Gill and Holley (57) to hypothesize that there may be more than one plausible mode of action. Eugenol, carvacrol, and cinnamaldehyde have been found to inhibit the membrane-bound ATPase activity of some bacteria. There are several enzymes with

ATPase activity that are associated with bacterial membranes, including one that is involved in ATP generation and cellular pH regulation (58). Gill and Holley (59) hypothesized that ATPase inhibition plays a significant role in reducing the growth rate of pathogens at sublethal concentrations of the EO.

Effects on pH Gradient

The ability to maintain a pH gradient is necessary for cell survival and thus studying the internal pH or the pH gradient helps to determine if a cell has been severely damaged. Oussalah et al. (51) and Lambert et al. (22) both examined the internal pH of bacteria exposed to EOs. Oussalah et al. (51) found that Spanish oregano, Chinese cinnamon, and Savory EOs were effective at decreasing the internal pH of *E. coli* O157:H7 and *L. monocytogenes*. Lambert et al. (22) determined that oregano EO increased the rate of change in internal pH for *S. aureus* compared with an untreated control, indicating a lack of ability by the cell to maintain the pH gradient. Sikkema et al. (58) tested liposomes that had been reconstituted with beef heart mitochondrial cytochrome c oxidase, in order to give them a proton motive force, with various cyclic hydrocarbons (benzene, toluene, ethylbenzene, *o*-xylene, and others) and found that all of them dissipated the pH gradient at concentrations related to their membrane partition coefficient. Fisher and Phillips (54) employed their 1:1 blend of orange and bergamot EOs (20.0 ml/l of each) to determine that the blend was able to completely eliminate the pH gradients of *E. faecium* and *E. faecalis*. Fitzgerald et al. (37) treated *L. plantarum* with vanillin and determined that 15.2 ml/l of vanillin was able to dissipate the pH gradient. Ultee et al. (28, 34) ascertained that 0.15 ml/l carvacrol completely dissipated the pH gradient across the cytoplasmic membrane of *B. cereus*, while 0.268 ml/l of cymene was ineffective. These studies indicate that many oils act to disrupt the membrane in such a way as to make the cell unable to maintain a pH gradient which is essential for generation of a proton motive force, and thus life.

Effect on Respiration

Respiration is an integral part of aerobic metabolism with the potential to be a target for antimicrobial EOs, and several researchers have examined the effects of EOs on respiration to help elicit their mode of action. Reduction of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) is an indication of respiratory enzyme activity; Bouhdid et al. (40) determined that in the presence of cinnamon EO <6% of *P. aeruginosa* cells were able to reduce CTC after a 30-min exposure to the MIC of the oil (1.25 ml/l). However, *S. aureus* was found to be more resistant as approximately 10% of the cells were able to reduce CTC after 60 min of exposure to the MIC (1.25 ml/l) and 1.5× MIC (1.87 ml/l) of the oil. Cox et al. (26) examined the oxygen consumption of *E. coli* and *S. aureus* in the presence of tea tree oil at different concentrations; 5.0 ml/l of tea tree oil reduced the oxygen consumption of *E. coli* by 100% and 10.0 ml/l reduced the oxygen consumption of *S. aureus* by 60%. Fitzgerald et al. (37) determined oxygen consumption in *L. innocua* and *E. coli* when exposed to various concentrations of vanillin (0–6.09 ml/l) and discovered that oxygen consumption was reduced by 19 and 52% in *E. coli* and *L. innocua*, respectively, when exposed to 6.09 ml/l of vanillin. These results indicate that some EOs disrupt the ability of bacterial cells to perform oxidation reduction reactions and could be a potential mode of action for the oil.

Effect on Quorum Sensing

Cell-to-cell communication among bacteria [quorum sensing (QS)] is accomplished using small signaling molecules produced by the bacteria and is utilized by the bacteria to evaluate their external environment and their internal physiological status (60). The signaling molecules are in general known as autoinducers; the Gram-negative bacteria use acyl homoserine lactones, whereas the Gram-positive bacteria use modified oligopeptides as signaling molecules (61). QS is involved in biofilm production, motility, swarming, stress resistance, and virulence (61). Researchers have begun to target QS as a means to reduce antimicrobial resistance and food spoilage (62). Additionally, QS is thought to allow pathogens to minimize host immune responses by delaying the production of virulence factors until a large enough bacterial population is present to overwhelm host defense mechanisms and establish infection (63).

Khan et al. (64) screened 21 EOs for anti-QS activity using biosensor strains, *Chromobacterium violaceum* CV12472 and CVO26. They determined that clove oil demonstrated the most anti-QS activity on both wild and mutant strains followed in activity by cinnamon, lavender, and peppermint oils. The effect of clove oil on pigment production, which is QS controlled, was found to be concentration dependent. At sub-MICs of clove oil, there was a 78.4% reduction of pigment production compared with the control and up to a 78% reduction in swarming motility over the control (64). Szabo et al. (65) performed similar studies with a wider range of EOs and established that rose, geranium, lavender, and rosemary oils were the most potent QS inhibitors of the compounds they tested. Eucalyptus and citrus oils moderately reduced pigment production by CV026, while chamomile, orange, and juniper oils were ineffective. Alvarez et al. (66) researched the use of oregano EO in edible films and the effect on QS as well as the antibacterial activity against several pathogens. Pectin films incorporated with 36.10 and 25.90 ml/l of oregano EO inhibited growth of *E. coli* O157:H7, *Salmonella* Cholerasuis, *S. aureus*, and *L. monocytogenes*. All concentrations of oregano EO (0.015, 0.0312, 0.0625, and 0.125 ml/l) and pectin-EO films (15.70, 25.90, and 36.10 ml/l) showed a significant anti-QS activity. They also determined that the pectin-EO films reduced total coliforms, yeast, and molds on shrimp and cucumber slices stored at 4°C for 15 days. Thus, EOs could be used to explicitly target QS in a novel antimicrobial strategy.

Effect of Compound Structure on Antimicrobial Activity

Many researchers have examined the structures of EOs themselves to seek to determine what allows these compounds to affect the various cellular activities of bacteria. Sikkema et al. (58) examined how biological membranes react to cyclic hydrocarbons and determined that the hydrocarbons accumulate in the interior of the membrane causing it to swell. The amount to which a particular hydrocarbon can accumulate in the membrane was found to

be directly related to its membrane partition coefficient. Sikkema et al. (58) proposed that the accumulation of these compounds in the membrane result in changes to the membrane structure and function, thus leading to inhibition or cell death.

Carvacrol is an EO that has been shown to have high antibacterial activity and therefore it has been the focus or benchmark for many studies, some of which have studied what it is about the structure of carvacrol that makes it antimicrobial. Carvacrol is a cyclic hydrocarbon which, according to Sikkema et al. (58), allows it to accumulate in the membrane. It also has a hydroxyl group on the ring, which Ultee et al. (28) have proposed gives carvacrol its activity. Ultee et al. (28) also studied thymol, menthol, carvacrol methyl ether, and cymene in addition to carvacrol. These compounds possess similar structures to carvacrol that only differ in one or two ways. Thymol has the same chemical formula as carvacrol, but the hydroxyl group is in the meta position rather than the ortho position. Menthol lacks the benzene ring and instead has a cyclic hexane ring. Carvacrol methyl ether has the hydroxyl group replaced with a methyl ether group. Finally, cymene completely lacks a hydroxyl group. Ultee et al. (28) noted that cymene and carvacrol methyl ether lacked any antibacterial activity. Menthol showed slight inhibitory activity at a concentration of 0.320 ml/l for up to 5 h, but after 24 h, the bacteria had rebounded to levels seen with only a 0.016 ml/l concentration of menthol, which showed no inhibition of the bacteria. Thymol showed a very similar pattern of activity to that of carvacrol, with concentrations above 0.113 ml/l completely inhibitory to the bacteria (28).

Veldhuizen et al. (56) achieved similar results when they examined *o*-cresol, 3-isoporpylphenol, 2-amino-*p*-cymene, *p*-cymene, and 3,4-dimethylcumene in comparison to carvacrol. They found that *p*-cymene and 3,4-methylcumene exhibited no antibacterial activity. In 3,4-methylcumene, there is a methyl group in the place that the hydroxyl group of carvacrol occupies, indicating that the hydroxyl group is an important component of the antibacterial activity. The compound 3-isopropylphenol, which lacks the methyl group on the ring, was found to have an MIC approximately 1.5 times that of carvacrol, while *o*-cresol, which lacks the isopropyl group of carvacrol, was found to have an MIC approximately two times that of carvacrol. The MIC of these two carvacrol-related compounds indicates that the R groups do play a small role in the antibacterial activity of carvacrol, though not as much as the hydroxyl group. The compound 2-amino-*p*-cymene has an amino group in the same position as the hydroxyl group of carvacrol, but the rest of the structure remains the same. The MICs of 2-amino-*p*-cymene were approximately 0.450–0.60 ml/l higher than that of carvacrol and had the highest MIC of all antibacterially active compounds tested. Since 2-amino-*p*-cymene shows antibacterial activity, it seems that the hydroxyl group is not essential for antimicrobial activity, although it appears to enhance activity considerably.

Ben Arfa et al. (67) also examined how the structure of carvacrol affects its antibacterial activity. They compared carvacrol with carvacrol methyl ether, carvacrol acetate, eugenol, and menthol. Carvacrol methyl ether and carvacrol acetate were not found to display any antibacterial activity; both of these compounds have substitutions at the hydroxyl group of carvacrol respective of their names, indicating that these groups cannot convey the same activity as the hydroxyl group of carvacrol. Eugenol, which is another aromatic compound like carvacrol, has a hydroxyl group and an ether group which are para and ortho, respectively, to a three-carbon chain that ends in a double bond. Eugenol was found to exhibit a lower antibacterial activity than carvacrol, but was not inactive like carvacrol acetate and carvacrol methyl ether. Ben Arfa et al. (67) only determined MICs for their compounds, so it is difficult to say if the antibacterial action of eugenol is the same as carvacrol, although it can be said that not all antibacterial EO compounds must have a structure similar to that of carvacrol in order to possess activity. Similar to Ultee et al. (28), Ben Arfa et al. (67) found menthol to possess very little antibacterial activity, which supports the theory that the benzene ring is important for antibacterial activity, but only for those compounds that possess a hydroxyl group or an amino group, as was the case with 2-amino-*p*-cymene in the study by Veldhuizen et al. (56).

Gill and Holley (57) studied the two aromatic compounds eugenol and *trans*-cinnamaldehyde. Both compounds were found to exhibit antibacterial activity, although eugenol had a lower MIC. *Trans*-cinnamaldehyde, which contains an aromatic ring with a three-carbon aldehyde chain containing a double bond at the second position, was able to decrease internal ATP of energized cells and inhibit ATP increases of non-energized cells of *L. monocytogenes*. Eugenol, which was described earlier, was able to inhibit ATP increases of non-energized cells, but was unable to reduce ATP levels of energized cells. Their results indicate that *trans*-cinnamaldehyde could be acting via membrane disruption without the need of the carvacrol hydroxyl group. It is difficult to compare carvacrol and *trans*-cinnamaldehyde since they were not used in the same study, but if one extrapolates the results from Gill and Holley (57) and Ben Arfa et al. (67), one can make the assumption that *trans*-cinnamaldehyde has lower antibacterial activity than carvacrol. This is due to the fact that *trans*-cinnamaldehyde was found to have lower antibacterial activity than eugenol and that eugenol was found to have lower antibacterial activity than carvacrol. Therefore, *trans*-cinnamaldehyde probably causes membrane disruption to a lesser extent than carvacrol due to its lack of a hydroxyl group. Bouhdid et al. (40) examined cinnamon EO, which is mainly composed of *trans*-cinnamaldehyde, and found that the EO caused leakage of potassium ions from *P. aeruginosa* and *S. aureus*, which furthers the idea that *trans*-cinnamaldehyde is a membrane disruptor. Helander et al. (24), however, found that *trans*-cinnamaldehyde was unable to induce ATP leakage. This contradictory result is most likely due to the concentration difference used in the two studies; Helander et al. (24) used 0.264 ml/l, whereas Gill and Holley (57) used 5.29 ml/l.

Conclusion

Several plant-derived EOs are effective as natural alternatives to synthetic food additives, particularly as antimicrobial agents. One of the main actions of EOs is to affect the permeability of cell membranes leading to loss of cellular components or influx of other substances into the cell. New studies on the effects of EOs on the QS systems of foodborne and other pathogens offer an opportunity for the development of natural food antimicrobials that could not only be bactericidal but could also lessen pathogenic effects.

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The effects of steroid implant and dietary soybean hulls on estrogenic activity of sera of steers grazing toxic endophyte-infected tall fescue pasture

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Soybean hulls (SBHs) have been fed to cattle pasturing on endophyte-infected tall fescue in attempts to increase rate of gain. Literature reports indicated some symptoms associated with fescue toxicosis were ameliorated by the use of steroidal implants containing estradiol (E2) and progesterone [implantation (IMP)], feeding SBHs, or the combination of the two. While the mechanism for amelioration was unclear, the SBHs were postulated as acting as a diluent of the toxic factors of the fescue. Alternatively, estradiol and phytoestrogens of SBHs might be acting through relaxation of the persistent vasoconstriction found in animals ingesting ergot alkaloids of endophyte-infected fescue. If so, estrogenic activity of serum of steers receiving SBHs, IMP, or a combination of the two should be elevated. Using the cellular proliferation assay of estrogenicity (E-Screen), estradiol equivalents (E_2Eqs) were determined on both SBHs and the serum of steers from a previously reported study. Range of SBHs was 5.0–8.5 ng Eqs g^{-1} DM (mean 6.5, $n = 4$ from different commercial sources of SBHs). At the rate fed, theoretically calculated blood E_2Eq could be physiologically relevant (~80 pg mL^{-1}, based on 2.3 kg SBHs d^{-1}, 300 kg steer, 5.7% blood volume, and 10% absorption). Serum E_2Eqs did increase in steers ($P \leq 0.05$) with steroidal implants or fed SBHs by 56 and 151% over control, respectively, and treatments were additive (211% increase). Serum prolactin was also greatest for the SBH + IMP group (188 ng mL^{-1}, $P < 0.05$), concentrations comparable to values reported for steers grazing endophyte-*free* fescue. Prolactin in the SBH group was higher than IMP or control groups (146 versus 76 and 60 ng mL^{-1}, respectively). Still unknown is if additional E_2Eqs from dietary phytoestrogens or exogenous sources of estradiol can further reduce symptoms of fescue toxicosis. The E-Screen assay was an effective tool in monitoring serum for estrogenic effects of dietary supplementation with SBHs or estrogenic implants.

Keywords: phytoestrogens, isoflavones, fescue toxicosis, prolactin, estrogenic activity

Introduction

Ergot alkaloids produced by a fungal endophyte [*Epichloë coenophiala* (Morgan-Jones & W. Gams) C.W. Bacon & Schardl] that infects tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh] have a strong affinity for selected subclasses of α-adrenergic and serotonin receptors in the vasculature (1, 2). Ergot alkaloids consumed by grazing animals cause persistent constriction of blood flow to peripheral tissues, thereby disabling the animal's ability to regulate core body temperature, resulting in a condition referred to as fescue toxicosis. Various feeding regimens have been evaluated to mitigate the effects of fescue toxicosis on the rate of gain of steers (3–7). Soybean hulls (SBHs) are a high-fiber feed, the feeding of which has been effective in providing a higher plane of nutrition, resulting in a higher rate of gain, when fed to steers grazing toxic tall fescue pasture (6, 7). Feeding SBHs at a daily rate of 2.3 kg day^{-1} $steer^{-1}$ partially reversed the decreased serum prolactin concentrations and decreased the frequency of rough hair coats [Carter et al. (7)], both symptoms of fescue toxicosis (8). An estradiol–progesterone implant with no SBH treatment also reduced prevalence of rough hair, and a combination of the two treatments resulted in an additive effect on mitigating toxicosis. While the authors conjectured that SBHs diluted ergot alkaloids in steer diets, the phytoestrogens in SBHs could have been a contributing factor in the mitigation through positive effects on specific vasculature and overall circulation.

Though phytoestrogens (a subset known as isoflavones) have been linked to reproductive issues in cattle and sheep (9, 10), dietary isoflavones have also been demonstrated to benefit human health and well-being (11, 12). Various effects on the vasculature have been reported. For instance, isoflavones have been identified as an alternative to 17β-estradiol in ameliorating hot flashes in post-menopausal women, through relaxation of the vasculature by binding the estrogen receptors in the vascular endothelium, stimulating nitric oxide synthase (NOS), resulting in nitric oxide-mediated vasodilation (12–14). There also is evidence that genistein, considered the most estrogenic phytoestrogen in soybeans, exerts a vasodilatory effect as a β-adrenergic receptor agonist (15). Based on these findings, isoflavones may have the potential to induce relaxation of alkaloid-induced vasoconstriction, and perhaps mitigate other signs of toxicosis as described by Carter et al. (7) (rough hair coat, low prolactin concentrations, and poor thriftiness/performance).

Depending on species, variety, season and drying time, the amount of isoflavones found in legumes, such as soybeans, alfalfa, and clover, can vary (11, 16–18). While the estrogenic activity of soybean meal (SBM) (45% crude protein) was assessed using the *in vitro* cellular proliferation assay (E-Screen) and found to average 113 ng g^{-1} as fed (19), the estrogenic activity of SBHs has not been evaluated. Therefore, SBH samples collected from various suppliers, and serum samples collected from steers of the Carter et al. (7) study were analyzed by E-Screen to assess the estrogenicity of SBHs and determine if there is an association between serum prolactin and serum estrogenic activity.

Materials and Methods

Soybean Meal and Hulls

Samples of SBHs fed by Carter et al. (7) were no longer available for determination of estrogenic activity, therefore four samples from unrelated lots of SBHs were obtained from three feed suppliers representing three different years of harvest (**Table 1**, Woodford Feed Co., Versailles, KY, USA; Hallway Feed Co., Lexington, KY, USA, and North Dakota State University Feed Mill, Fargo, ND, USA). Sample size ranged from 120 to 750 g (as fed). Sample dry matter was determined in quintuplicate (dried at 100°C). Extractions were done as described for isoflavinoid analysis (20), with the exception of an additional hexane extraction step. Three subsamples of feed (1 g as fed) were extracted using sonication (47 kHz) and 25 mL of 60% acetonitrile at 37°C for 1 h, shaking samples every 10 min to resuspend feed and enhance sample/solvent contact. Particulates were removed by centrifugation (900 × *g*, 10 min). Supernatants were filtered through solvent-washed glass wool to remove remaining fines and volumes recorded. Acetonitrile was volatilized under N_2 at 37°C, and hexane (1:1, v:v) was added, vortexed for 1 min, then held at −20°C for ≥1 h. Phase separation was achieved by centrifugation as described above. The upper hexane layer was discarded, and the aqueous layer was subsequently diluted to ~90 mL with nanopure H_2O (npH_2O) after removing any pelleted material. Phytoestrogens were then concentrated by Oasis HLB solid phase extraction as described [Waters, Milford, MA, USA (21)], and the eluent taken to dryness and stored at −20°C for later analysis. On the day of assay, samples were resuspended in 400 μL of 5% dimethyl sulfoxide in npH_2O. Resuspended SBH samples were diluted (1:10,000–1:25,000) in cell culture medium described below to obtain cellular responses in the linear range of the E-Screen assay (~1×10^{-12} to 1×10^{-11} M).

Animal Treatment, Serum Collection

Estradiol equivalents were assayed on serum collected from steers used in a 2-year grazing experiment, evaluating the effect of combinations of steroid implantation (IMP) and SBH feeding on mitigation of fescue toxicosis (7). The experimental protocol was reviewed and accepted by Institution's Animal Care and Use Committee at the University of Kentucky (00996A2006). Treatments consisted of IMP (Synovex-S 200, 200 mg progesterone and 20 mg estradiol, Fort Dodge Animal Health, Fort Dodge,

TABLE 1 | Estrogenic equivalents of commercial soybean hull feedstuff by E-Screen assay.

Site of purchase/origin	Year of harvest	E_2Eqs[a] ng E_2 Eqs g^{-1} DM	COV[b]
KY/na[c]	2011	8.5 ± 0.7	8%
ND/na	2013	5.0 ± 0.7	14%
KY/IN	2014	7.2 ± 0.3	5%
KY/IN	2014	5.3 ± 0.4	8%
	Mean	6.5 ± 1.6	25%

[a]*Mean ± SD of three replicate extractions, each dilution was tested on 4–6 wells of cells. Values do not reflect activity assayed in hexane phase, as ≤3% of SPE values.*
[b]*Coefficient of variation.*
[c]*Not available.*

IA, USA), SBHs (daily SBH feeding at 2.3 kg steer^{-1} day^{-1}), SBH + IMP (combined feeding of SBHs and IMP), and Control (pasture-only). Steers were assigned to 12 3.0-ha pastures of toxic endophyte-infected tall fescue in a split-plot design. Main- and sub-plot treatments were ±SBH and ±IMP. Blood was collected from the jugular vein on 14 June and 5 July in 2007, and 24 June and 24 July in 2008. Blood was centrifuged for 15 min at 10,000 × *g* to obtain serum, which was stored frozen (−20°C) for prolactin analysis. Detailed descriptions of experimental design, sample preparation, and analyses (including prolactin) were provided by Carter et al. (7). Estrogenic activity (estradiol equivalents, E_2Eqs) was analyzed in serum from a subset of steers for which serum samples were available for all collection dates to provide an estimate of the bioavailability of isoflavones once they are absorbed into the animal's circulation.

Serum Extraction and E-Screen

Prior to E-Screen testing, serum samples required extraction to eliminate factors toxic to the MCF-7 cells as previously described (22). Sterile aliquots (typically 1 mL) of serum were diluted with acetonitrile (ACN, 1:2 v/v) in silanized glass conical centrifuge tubes. Serum weights were recorded, samples vortexed for 1 min, and centrifuged at 800 × *g* for 10 min (room temperature). The serum/ACN was removed from the pellet and transferred into a silanized glass vial, and residual liquid and pellet were weighed. The extracted serum was taken to dryness, and stored at −20°C until E-Screen analysis. Sample weights were used to calculate and adjust final estrogenic activity values for sample loss during extraction. Extracted serum samples were reconstituted in np$H_2$0 at 1/5th the original serum volume. Samples were further diluted in cell culture medium (1:10 to up to 1:25 of the original serum) to obtain a proliferative response in the linear range of the E_2 standard curve. The limit of quantitation was ~0.2 pg mL^{-1} of E_2Eq in the original serum.

The MCF-7 BOS, estrogen-dependent cell line (derived from a human mammary epithelial carcinoma, provided by Drs. Ana Soto and Carlos Sonnenschein, Tufts University, Boston, MA, USA) was used to determine estrogenicity relative to 17β-estradiol as described by Shappell (21). Briefly, resuspended extracts were diluted in cell culture media. Twenty-four hours post-plating, steroid-containing medium (5% FBS) was removed and replaced with steroid-free medium (minus phenol red and containing 10% charcoal dextran-stripped FBS) containing diluted sample extracts of samples or 17β-E_2 (1×10^{-13} to 1×10^{-9} M). After 5 days of incubation, cells were fixed with trichloroacetic acid, stained for protein with sulforhodamine B (Sigma Chemicals, St. Louis, MO, USA), solubilized in buffer, and absorbance measured (490 nm). Estradiol equivalents were determined based on a regression analysis of the 17β-E_2 curve from the same experiment. Assay assessment, including range finding, was performed on serum samples from four steers on endophyte-infected tall fescue pasture, but not on trial. All extracts were tested over a wide range of dilutions, and those resulting in absorbance readings in the linear range of the E_2 standard curve were used for interpolation of the data. Evidence of toxicity was determined by evaluating cellular proliferation in the presence of the sample extract spiked with 17β-E_2 versus 17β-E_2 alone. The estrogen-dependence of cellular proliferation was confirmed by co-incubation with the E_2-receptor antagonist ICI 182,780 [Tocris, Ellisville, MO, USA (23)].

Statistical Analysis

Estradiol equivalents and prolactin concentrations were statistically analyzed using mixed models of SAS (24), with year, treatment, and blood collection month (June versus July) as fixed effects, and animal as a random effect. Mean separations were performed on least square means using the Tukey–Kramer adjustment of multiple comparisons of least square means. Estradiol equivalents data were transformed using the log function to stabilize the variances and analyzed as repeated measures using the AR(1) covariance structure.

Results

Soybean Hull Estrogenic Activity

Quantitation of E_2Eqs from extracts of SBHs yielded reproducible data. Coefficient of variation on triplicate extractions averaged 8.8%, with only one COV over 10% (14.2%). The hexane extractable phase contained ≤3% of the estrogenic activity from the SPE extraction. Estradiol equivalents of the SBH samples harvested in three different years and representing at least two different regions averaged 6.5 ± 1.65 ng g^{-1} dry matter or 5.8 ng g^{-1} wet weight (**Table 1**, overall COV 25%). As expected, the dry matter content of the processed SBHs were essentially identical (89 ± 1%). The estrogenic activity of SBH samples was more variable, though the range of values within a year was similar to the range across years.

Serum E_2Eqs

Range finding samples from the four steers on endophyte-infected tall fescue pasture, but not on trial yielded values similar to those from the control treatment group (4.9 ± 0.92 versus 6.1 ± 1.0 pg mL^{-1} E_2Eqs, respectively). Of the experimental treatments, all had mean serum estrogenicity greater ($P < 0.05$) than control steers (**Figure 1**). The smallest increase in E_2Eqs over control was found when feeding SBHs alone (9.6 ± 1.3 pg mL^{-1}), with the serum from IMP steers ranking next (15.3 ± 1.7 pg mL^{-1}). The highest mean serum E_2Eqs was of steers receiving the combined IMP and SBH treatment (19.0 ± 2.5 pg mL^{-1} and similar to IMP alone ($P = 0.44$). Serum E_2Eqs of IMP-SBH steers was greater ($P < 0.001$) than the SBH steers, indicating an additive effect of the two treatments.

Serum Prolactin

Unlike serum estrogenic activity, serum prolactin concentrations of IMP steers were not different from control concentrations (76.3 ± 7.6 IMP, versus 60.1 ± 12.9 ng mL^{-1} control, $P = 0.38$). SBH treatment alone resulted in elevated prolactin concentrations (145.5 ± 13.9 ng mL^{-1}, $P < 0.05$) with even greater prolactin concentrations when SBH and IMP treatments were combined (187.9 ± 17.3 ng mL^{-1}, $P < 0.05$) (**Figure 2**). There was a weak, but positive correlation between serum E_2Eqs and serum prolactin concentrations ($r = 0.29$; $P = 0.027$; $n = 59$).

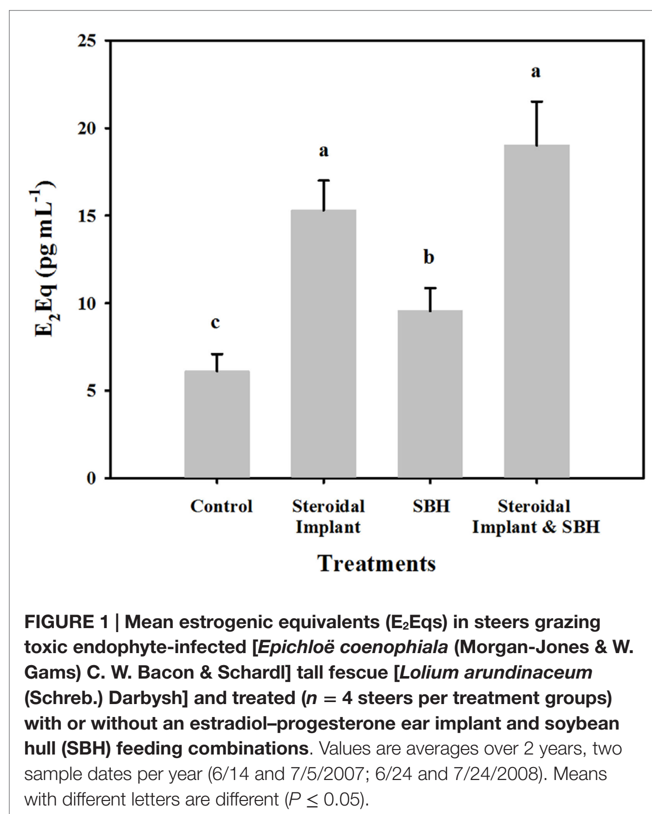

FIGURE 1 | Mean estrogenic equivalents (E_2Eqs) in steers grazing toxic endophyte-infected [*Epichloë coenophiala* (Morgan-Jones & W. Gams) C. W. Bacon & Schardl] tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh] and treated (n = 4 steers per treatment groups) with or without an estradiol–progesterone ear implant and soybean hull (SBH) feeding combinations. Values are averages over 2 years, two sample dates per year (6/14 and 7/5/2007; 6/24 and 7/24/2008). Means with different letters are different ($P \leq 0.05$).

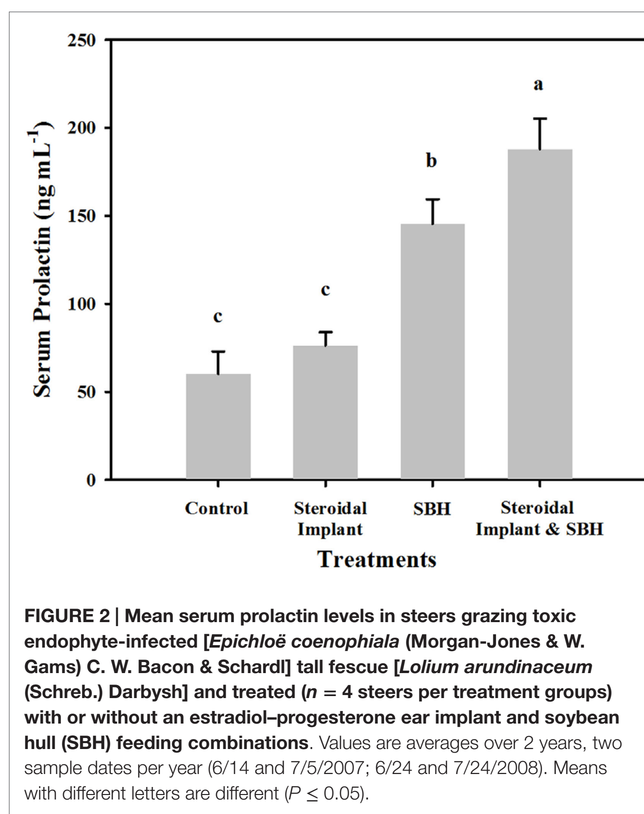

FIGURE 2 | Mean serum prolactin levels in steers grazing toxic endophyte-infected [*Epichloë coenophiala* (Morgan-Jones & W. Gams) C. W. Bacon & Schardl] tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh] and treated (n = 4 steers per treatment groups) with or without an estradiol–progesterone ear implant and soybean hull (SBH) feeding combinations. Values are averages over 2 years, two sample dates per year (6/14 and 7/5/2007; 6/24 and 7/24/2008). Means with different letters are different ($P \leq 0.05$).

Discussion

Estrogenic Activity of SBHs

While soybeans and SBM have been evaluated for phytoestrogens and estrogenic activity, SBHs as an alternative feedstuff had not. The hulls are a co-product of milling meal that is derived from the fibrous, inert, soybean seed coat and are greater than 86% cellulose, hemicellulose, and lignin (25), with the remainder being primarily protein and minerals. Variability of E_2Eqs (5.0–8.5 ng g^{-1}) reported herein for SBHs from different suppliers was less than that reported for soybean *meal* using the yeast-based human α-estrogen receptor *in vitro* assay [YES (26)]. Meal from lots manufactured 1 month apart varied threefold in E_2Eqs by YES assay, while SBHs analyzed here varied by <2-fold. For context, based on data from our laboratory, the estrogenic activity of SBHs could be expected to range from 4 to 20% of SBM. These estimates are based on E-Screen analysis of SBM in our laboratory yielding 127 E_2Eqs ng g^{-1} dry matter (19), and are similar to analysis by YES assay [29–100 E_2Eqs]. The measured quantities of estrogenic activity of SBH are in line with the phytoestrogen content of hulls, reported to be 11–15% of whole soybeans or 15% of SBM from chemical analysis (27).

Physiological Context for Estrogenic Activity

A theoretical assessment of the potential for a physiological effect of SBH feeding of steers follows:

2,070 g DM SBHs per day (2,300 g as fed × 89% dry matter)
2,070 g SBHs DM × 6.5 E_2Eqs ng g^{-1} DM SBHs =
13.5 μg E_2Eqs per day per steer

For a 300 kg steer, blood volume estimates = 5.7% of body weight (28)

300 kg × 5.7% = 17.1 L or 17,100 mL blood volume
Of 13.5 μg E_2Eqs fed, if 10% are absorbed and bioavailable, then 1.35 μg E_2Eqs/17,100 mL blood = 79 pg mL^{-1} E_2Eqs in blood.

Obviously, this estimate has inherent assumptions requiring caution, if not skepticism. First, is the assumption of bolus delivery of phytoestrogens, second, no metabolism of phytoestrogens, either by steer or by their microflora, reducing the estrogenicity of the feedstuff. Though ingested phytoestrogens are found circulating in the serum of cattle (29), estimates of bioavailability were not reported. The peak blood concentrations (relative to time of consumption) resembled those in a study of women consuming phytoestrogen-rich diets (30). In women, the estimates of bioavailability for two phytoestrogens (genistein and daidzein) were 18–27% based on urinary recovery after 48 h. Similarly, oral bioavailability of genistein and daidzein in rats were 18 and 23%, respectively (31). Using a value of 10% bioavailability in the calculation above, though less than these reports, could still result in large overestimates due to ruminal metabolism (32). The estimate above also ignores any receptor-level competition between naturally circulating estrogens and the phytoestrogens. For context, the circulating concentration of estradiol for cows in estrus peak at ~10 pg mL^{-1} (28). Based on this calculation, it appears the dosage of estrogenic activity from SBHs, may be capable of eliciting an estrogenic response.

Supporting the potential for circulating E_2Eqs of steers consuming SBHs to be capable of exerting a physiological impact are the findings in rats receiving phytoestrogens. After 3 days

of oral administration of ~75 mg kg body weight^{-1} day^{-1} of the phytoestrogens, genistein and daidzein (2:1 ratio), to 17 day old rats, an increase in wet uterine weight was observed (33). SBHs (as fed) were reported to contain 111 mg kg^{-1} of genistein and 200 mg kg^{-1} of daidzein (27). Using these values, the steers in our study received an estimated 250 and 460 mg kg body weight^{-1} day^{-1} of genistein and daidzein, respectively, substantially more than the above threshold for a uterine dose response in rats.

Serum E_2Eqs and Prolactin Response to Treatment

Historically little attention has been paid to the estrogenicity of various livestock feeds in comparison to estrogenicity of human foods. Exceptions include red clover and the associated infertility in sheep (34) or the effects of the estrogenic mycotoxin zearalenone-contaminated feed on heifers (35) and ewes (36). Soybeans have been used for livestock and poultry feed in the United States since the 1930s (37), and SBM is referred to as "the most important protein source used to feed farm animals" by the Feedipedia web site sponsored by the Institute for Agricultural Research, French Agricultural Center for International Development, French Association for Animal Production and the Food and Agriculture Organization of the United Nations (38). In contrast, their web site which provides many pages of information on SBM, mentions SBM's estrogenic properties in only one line – "soybean meal contains 1 g kg^{-1} of genistein, which has estrogenic properties." In contrast, a recent review in the International Journal of Endocrinology reports on the effect of phytoestrogens and specifically SBM on reproductive hormones in cows (29). SBHs, which contain only 5–20% of the estimated E_2Eqs of commonly fed SBM, nearly doubled the serum E_2Eq concentration of steer serum relative to control. These findings, at least conceptually, support the theoretical estimates of estrogenic activity made above, and are further supported by empirical findings of increased estrogenic activity of serum from SBH + IMP steers.

Estrogenic activity of serum from implanted steers was almost threefold that of control steers. These findings are in agreement with the plasma E_2 concentrations measured in steers with Synovex-S implants (20 mg estradiol). In two separate trials, Rumsey and Beaudry (39) found mean E_2 concentrations to be 4.4 and 34 times the non-implanted steers concentration, 60 days post-implantation ($n = 10$ steer per treatment per trial, using radioimmunoassay), with mean E_2 concentrations declining to near control concentrations by 106–120 days. Estrogenic activities in serum samples from steers in the current study are a mean of those collected at about 60 and 90 days post-implantation, and therefore could be expected to be lower than at 60 days.

While the findings of enhanced estrogenic activity with SBH feeding is novel, perhaps more unexpected is the trend for additivity of treatments (SBH + IMP) on E_2Eqs. The utility of the E-Screen assay can be fully realized here, because measurement of blood estrogens alone, either by immunoassay or by chemical analysis, would have failed to accurately reflect the full potential for systemic estrogenic activity. There have been some indications of additivity of *in vivo* responses to estrogenic chemicals in the literature, though often evidence of additivity occurs over a very limited range of concentrations [fish: (40, 41); frogs: (42); rats: (33)]. Lack of additive responses are most likely a reflection of the complexity of nature including differential rates of absorption, metabolism, receptor affinity, biofeedback, etc. The E-Screen assay has the unique potential to evaluate the effect of estrogenic modulators mid-experiment for an indication of an *overall* change in estrogenic activity of the animal's serum, not a specific hormonal response. Results from such monitoring could lead investigators to end experiments earlier than anticipated, or extend them.

One of the few measurable parameters indicative of fescue toxicosis is a decreased serum prolactin concentration (8). The control steers grazing endophyte-infected fescue had serum prolactin concentrations similar to bulls fed endophyte-infected fescue seed [~30 ng mL^{-1} (43)], indicating toxicity. While implants caused no significant increase in serum prolactin, SBHs did elevate prolactin, and the combination of SBH + IMP resulted in prolactin concentrations similar to those of bulls fed endophyte-free fescue seed [~250 ng mL^{-1} (43)]. In other words, the ~3-fold decrease in prolactin concentrations measured with consumption of endophyte-infected fescue, was reversed in SBH + IMP steers. The lack of elevation in serum prolactin in the IMP steers is puzzling, but could reflect suppression by progesterone contained in the implants. The literature has no reports of progesterone effects on prolactin in steers. Implants of estradiol alone are not widely used, but it would be of interest to compare serum prolactin in steers implanted with E2 versus E2 plus progesterone. These results appear to indicate that phytoestrogens of SBHs could be acting as surrogates for estradiol, overcoming any potential suppression by progesterone in the implant.

While the actions of prolactin on angiogenesis and the vasculature are complicated [review in Ref. (44)] and its causative role in fescue toxicosis is unclear, E_2 binding to E_2-α receptors in the plasma membrane of pituitary cells increased prolactin release (45). Estradiol also increased angiogenesis in the pituitary (46), another possible connection for an additive effect of the treatments, with phytoestrogens from SBHs. While elevated serum E_2 from implants might be relatively quickly metabolized by endogenous enzymes, the clearance rates of phytoestrogen might be slower, resulting in a more sustained physiological response.

Though Carter et al. (7) suggested that SBHs were acting merely through a passive role, diluting the ergot alkaloids, and thereby mitigating their adverse effects, we propose a more active role for SBHs. Both steers with implants or fed SBHs had a reduced percentage of rough hair coat (7). If a decrease in hair coat shedding (another indicator of fescue toxicosis) is a result of decreased blood flow to the hair follicle caused by the ergot alkaloids, then perhaps E_2 or phytoestrogens/isoflavones can ameliorate the effect by increasing follicular blood flow. Though several reports have found a lack of specific arterial responsiveness to isoflavones [brachial artery (12, 13)], a more global vascular effect was seen, including reduced peak flow velocity and lower peripheral flow resistance. Nestel et al. (47) noted that soy isoflavones increased vascular blood flow in menopausal and perimenopausal women. In addition, using biopsies of subcutaneous fat from men, the phytoestrogen genistein increased relaxation of small arteries, as determined by myograph (14). It is well accepted that arteries often have individual or tissue-specific

responses to chemical stimuli, and that global measures, such as blood pressure and velocity, are not necessarily indicated by the responses of individual arteries (48). Though specific direct evidence may be hard to find, isoflavones of SBHs could be ameliorating coat effects via interaction with the vasculature nourishing the hair coat.

Mechanistically, vasorelaxation can be caused by nitric oxide (NO), which is generated by binding and activation of estrogenic receptors by E_2 or isoflavones (15). While NO was not measured in the current study, Schreihofer et al. (49) measured increased vasorelaxation in ovariectomized rats fed high-soy diets or treated with E_2. The increase was associated with a decrease in caveolin-1 activity, an enzyme that inhibits NO-induced vasorelaxation. Similar mechanisms could be expected to occur with SBH feeding of steers.

There is a tendency to focus on "the most estrogenic compounds" in SBHs and consider them the "players," although it is well known that isoflavones undergo degradation by bacteria in the gut/rumen (32, 50). And while some catabolites or conjugates of phytoestrogens are less active, some are more active, depending on the tissue or assay type. For instance, equol, the ruminal catabolite of daidzein (found in SBHs) has greater vasodilatory effects on the basilar artery of rats than its precursor daidzein (51), though they are equipotent on the carotid artery. Equol has been found not only in ruminal digesta of animals fed legumes, but bioavailability was confirmed by its presence in the kidneys and blood (9, 52). Assay and tissue specificity was also a factor in assignment of a "relative potency factor," as genistein evaluated by E-Screen (breast tissue origin) was ~6 times as potent as daidzein in eliciting MCF-7 cell proliferation (53), with daidzein and equol ~ equivalent, yet the two had different potencies based on E_2-receptor affinities and transcriptional efficiencies (54).

Conclusion

The estradiol–progesterone implant and SBH treatments enhanced E_2Eqs in sera of steers, with an additive effect when combined. It cannot be concluded that the increases in estrogenicity directly caused the mitigation of fescue toxicosis reported by Carter et al. (7) for these steers. But based on research with human subjects, exogenous sources of estradiol and isoflavones may relieve the ergot alkaloid-induced vasoconstriction that persists in livestock grazing toxic endophyte-infected tall fescue. Still unknown is the threshold at which estradiol and or isoflavones can alleviate or mitigate toxicosis. The E-Screen assay was shown to be an effective tool in monitoring serum for estrogenic effects of dietary supplementation with isoflavones or estrogenic implants, though threshold concentrations of estrogenic activity that result in vasculature responses have yet to be established.

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