Improving efficiency in beef production systems using composite parasitism diagnostics and rumen microbial modulation in beef cattle

by

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Abstract

Improving efficiency in beef production systems requires integrating sound animal health management with optimal rumen function. A variety of diagnostic and management approaches have been developed to identify opportunities for targeted interventions that improve both animal health and rumen performance. Using these approaches, a series of experiments evaluated diagnostics and nutritional strategies influencing cattle health, rumen microbial activity, and greenhouse gas emissions in beef production systems. In experiment 1, a mob composite fecal sampling protocol was evaluated as an alternative to traditional individual fecal egg count diagnostics for estimating parasite loads in commercial feedlot cattle. Fecal samples (n = 1,060) were collected from cattle in commercial feedlots (n = 12) and sale barns (n = 1), with pen sizes ranging from 64 to 225 head. Individual fecal egg count values were determined using 20 individual samples per pen (IDV). Mob composite samples were created by combining fecal material from 10 fresh fecal pats to create two composite samples representing 20 fecal pats within each pen (MOB). Lin's concordance correlation coefficient compared mean fecal egg counts between sampling methods. Mob composite and individual sampling methods demonstrated a 90.2% agreement in mean fecal egg counts (95% CI: 84.3% to 93.9%). Results indicate that the two 10-sample mob composite protocol provides an effective and cost-efficient method for evaluating parasite loads in commercial feedlot cattle. Experiment 2 evaluated rumen microbial populations, greenhouse gas emissions, and growth performance of commercial beef steers fed a high forage diet supplemented with or without a commercial essential oil blend, Agolin©(Agolin Ruminant, Alltech, Nicholasville, KY). Steers (n = 36; initial BW = 317 ± 34 kg) were assigned to either a control diet (CON) or a diet supplemented with Agolin (AGO) during a 70-d trial. Methane and carbon dioxide emission were measured using Greenfeed

Automated Head Chamber Systems (AHCS; C-Lock Inc., Rapid City, SD). Steers supplemented with Agolin produced 4% less methane (240 g/d) compared with CON steers (250 g/d; $P = 0.03$). Carbon dioxide emissions did not differ between treatments ($P = 0.15$). Average daily gain and dry matter intake did not differ between treatments ($P \geq 0.42$). Shifts in rumen protozoal populations away from Holotrich populations towards Entodiniomorph populations indicated altered rumen fermentation dynamics for steers supplemented with Agolin. Results from these experiments demonstrate that efficiency of beef cattle systems can be influenced by improvements to practical diagnostic approaches. Additionally, dietary interventions, such as Agolin supplementation, can alter rumen fermentation dynamics to further enhance production efficiency. Improved parasite diagnostics and targeted rumen microbial modulation support each enhance beef cattle production efficiency. These results emphasize the importance of continued research to expand diagnostic tools and product solutions that support sustained improvements in beef production efficiency.

Keywords

methane, protozoa, essential oil, beef cattle, gastrointestinal nematodes

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Dedication

This thesis is dedicated to the memory of…

Dr. Grant Crawford – His endless knowledge, guidance, encouragement, and passion for parasitology and continuing education has left a lasting impact on my academic and professional journey and has inspired me to continue to pursue the path I am on. Thank you for teaching me that worms aren't so bad after all (even though it took some convincing).

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Chapter 1 - Literature Review

Parasitism Diagnostics Methods

1 - Introduction

Controlling internal parasites while limiting development of anthelmintic resistance is a challenge faced by animal health scientists and cattle producers worldwide. In the United States, the most reported gastrointestinal parasites of cattle include: *Ostertagia*, *Nematodirus*, *Trichuris*, *Oesophagostomom*, *Trichostrongylus*, *Strongyloides*, *Dictyocaulus*, *Bunostomum*, *Cooperia*, and *Heamonchus* (Zajac et al., 2021). Parasite species composition varies among herds depending on geographic location, climate, and management practices (Hildreth et al., 2007; Navarre, 2020). Gastrointestinal nematode infection is associated with suppressed immune function, reduced feed efficiency, decreased weight gain and overall production losses, making diagnostics of herd parasite burden increasingly important for producers (Rashid et al., 2019; Zajac et al., 2021).

Fecal egg counts for parasite identification remain a standard diagnostic tool used to guide targeted anthelmintic treatment and mitigate nematode drug resistance. Many fecal egg counting techniques used today are based on methods developed in the early twentieth century and have undergone limited methodological change (Zajac et al., 2021). As concern regarding anthelmintic resistance continues to increase, emphasis has shifted toward improving diagnostic efficiency and expanding numbers of animals evaluated within a herd to better characterize parasite burden and treatment efficacy. Assessment of resistance to specific anthelmintic classes commonly relies on fecal egg count reduction tests (FECRT), which compare fecal egg counts before and after treatment to quantify drug efficacy. Numerous studies have employed fecal egg count reduction test methodologies to evaluate resistance of gastrointestinal nematodes in cattle

following administration of individual anthelmintics or combination therapies (George et al., 2017; Rinaldi et al., 2019; Ward et al., 1997).

Several laboratory techniques are available for determining fecal egg counts, including McMaster, Wisconsin, and FLOTAC methods. Comparative evaluations of techniques demonstrate that accuracy, precision, and limits of detection vary among methods, resulting un differences in reported fecal egg counts depending on analytical approach used (Levecke et al., 2012). Findings highlight the importance of using a consistent fecal egg counting methodology throughout duration of fecal egg count reduction test to minimize analytical variation and ensure reliable interpretation of treatment efficacy.

1.1 – Historical Overview/Landscape of Parasites in Beef Cattle

1.1a - Gastrointestinal Parasite Loads in Cattle

Life Cycle – General Overview

Gastrointestinal parasite infection risk for pasture-based cattle generally peaks during warmer months, when environmental conditions favor larval development and transmission. Elevated temperature accelerates larval development, resulting in shorter life cycles and increased potential for pasture contamination. Gastrointestinal parasite infection risk for pasture-based cattle generally increases during warmer periods, when environmental conditions favor larval development and pasture contamination (Craig, 2018). In contrast, cooler temperature slow larval development and reduce their activity, but larvae may survive for extended periods under favorable moisture conditions, allowing infective stages to persist on pasture for longer durations (Craig, 2018).

Gastrointestinal nematodes undergo multiple developmental stages between egg deposition and reinfection of host. The life cycle begins when adult female worms residing in

abomasum of host animals or fecal pats, eggs hatch and larvae develop through successive stages, molting from first stage (L1) to infective third-stage larvae (L3) under suitable environmental conditions. Infective L3 larvae migrate from fecal pats onto surrounding herbage, where they are ingested by grazing cattle (Zajac et al., 2021). Larval migration from fecal pats is strongly influenced by moisture and rainfall events. Successful transmission occurs under conditions of moderate temperature and adequate moisture, whereas extreme heat and dry conditions limit larval survival and reduce infection pressure (Stromberg, 1997).

Prevalence of Species

Multiple studies conducted across North America identify *Cooperia* and *Ostertagia* as the predominant gastrointestinal genera affecting cattle, with additional strongyle species contributing to regional or age-specific variation in parasite communities.

A large-scale molecular epidemiology study evaluated 1,772 fecal samples collected from weaned beef calves of 99 operations, reporting infection in 85.6% of samples. Genus-level identification using PCR revealed high-prevalence of *Cooperia spp.* (91%), *Ostertagia spp.* (79%), *Haemonchus spp.* (53%), *Oesophagostomum spp.* (38%), *Nematodirus spp.* (17%), *Trichuris spp.* (7%), and *Trichostrongylus spp.* (3%). The study also demonstrated regional variation in fecal egg counts, with higher mean egg counts observed in southern states (58.1 eggs per gram (EPG)) compared with central (30.3 EPG) and western (11.3 EPG) regions (Stromberg et al., 2015).

Another study evaluating 180 breeding-age dairy heifers from Saskatchewan farms reported a strongylid prevalence of 67.2% in June, increasing to 88.3% in August. Species-level analysis indicated *Cooperia oncophora* and *Ostertagia ostertagi* dominated nematode

populations, comprising approximately 85% of total infections while *Nematodirus* and *Trichuris* were detected in 16-24% of samples (Scott et al., 2019).

Earlier epidemiological surveys further supported these patterns, identifying *Ostertagia, Cooperia, Haemonchus, Trichostrongylus, Oesophagostomum*, and *Nematodirus* as core gastrointestinal nematode genera present in U.S. cattle herds with relative abundance varying by climatic zone. *Ostertagia ostertagi* and *Cooperia oncophora* predominate in northern and Midwestern regions, while *Haemonchis placei* and *Cooperia punctata* are more common in southern and subtropical areas. Parasite burdens are consistently highest in first-season grazers and stocker cattle (Yazwinski & Tucker, 2006). In contrast, mature cows often exhibit lower fecal egg counts, although their greater manure output can contribute to pasture contamination and influence the environmental distribution of infective larvae (Yazwinski & Tucker, 2006).

Collectively, studies confirm that gastrointestinal nematode prevalence in North American cattle populations frequently exceeds 80% infection, with *Cooperia oncophora* and *Ostertagia Ostertagi* being the primary nematodes identified. Although *Haemonchus spp.* occur at lower overall prevalence, they remain seasonally and regionally important, particularly in warmer southern climates.

Regional Differences

Regional differences in gastrointestinal nematode prevalence, species composition, and transmission dynamics are evident among U.S. cattle populations and are driven primarily by climatic gradients and management practices. Studies conducted across the Great Plains and southern regions demonstrate that temperature, precipitation, and grazing season length substantially influence parasite diversity and infection pressure on cow-calf and stocker systems.

In northern U.S regions and northern Great Plains, parasite transmission is constrained by prolonged freezing winters. Fecal egg and oocyst counts in cow-calf herds in Minnesota, North Dakota, and South Dakota decrease along an east-west precipitation gradient, with significantly lower nematode burdens in drier, western sites (Hildreth et al., 2007). Cooler and drier northern environments further limit gastrointestinal nematode intensity by shortening grazing periods to approximately 140-180 days and disrupting life cycle through winter-associated larval mortality. *Ostertagia ostertagi* and *Cooperia oncophora* predominate, while *Haemonchus placei* and *Cooperia punctata*, species more common in warmer climates, remain comparatively rare (Hildreth & McKenzie, 2020). Persistence of *O. ostertagi* and *C. oncophora* in northern systems is supported by their capacity to overwinter as hypobiotic larvae within hosts or as infective third-stage larvae (L3) in soil (Zajac et al., 2021).

In contrast, southern U.S. cattle systems experience near year-round parasite transmission due to higher ambient temperatures and humidity. Across the Gulf Coast and south-central regions, *Haemonchus spp.*, *Cooperia spp.*, and *Ostertagia ostertagi* are dominant gastrointestinal nematodes of veterinary importance. Warm temperatures and frequent rainfall promote rapid larval development and extend survival on pasture, with infection pressure often peaking during late summer and early fall (Navarre, 2020).

Overall, north-south climactic gradients produce a continuum of parasite ecology in U.S. cattle systems. Northern herds are typically characterized by subclinical *Ostertagia* and *Cooperia* infections and are limited by cold-induced mortality, whereas southern herds face persistent mixed-species infections dominated by *Haemonchus* and *Cooperia*. Regional distinctions support contrasting parasite control strategies. Northern systems often emphasize strategic treatment timed to overwintering phases and preservation of refugia, while southern systems

require integrated pasture management and refugia-based approaches to mitigate continuous reinfection and escalating anthelmintic resistance.

Part 1.1b – Repercussions of Parasites in Animal

Gastrointestinal Nematode Infection Impacts on Cattle Performance

Gastrointestinal nematode infections negatively affect growth efficiency and overall productivity in cattle, with magnitude of performance loss closely related to infection intensity and duration of exposure. A meta-analysis of 26 studies by Shephard et al. (2022) demonstrated a clear dose-dependent relationship between strongyle burden and growth rate in young cattle. Across diverse geographies and production systems, cattle with heavier infection rates achieved approximately 11% lower of growth performance than that observed in lightly infected or uninfected counterparts. Moreover, each ten-fold increase in fecal egg count was associated with an additional 13% reduction in average daily gain, quantitatively illustrating biological cost of parasitism on growth performance (Shephard et al., 2022). Findings provide strong quantitative support for the established association between gastrointestinal nematode infection and reduced feed efficiency, reinforcing need for parasite management in both grazing and confined systems.

Immune Responses to Gastrointestinal Nematode Infections

Gastrointestinal nematode infections cause a complex immune response in cattle that influences both host physiology and productivity efficiency. Bovine immune response to nematode infections is predominantly type-2 (Th2)-mediated and characterized by increased expression of cytokines including IL-4, IL-5, IL-9, and IL-13. Cytokines coordinate activation of mast cells, eosinophils, and goblet cells, leading to increased mucus secretion, enhanced epithelial turnover, and altered abomasal motility. While effector mechanisms contribute to

larval expulsion, they also disrupt digestive processes and reduce nutrient absorption efficiency (Hendawy, 2018).

The most economically significant nematode in cattle, *Ostertagia ostertagi*, causes localized damage within abomasal glands as larvae penetrate and develop. Larval invasion results in loss of parietal cells, leading to elevated abomasal pH, impaired pepsinogen activation, and compromised protein digestion. Infected cattle commonly exhibit increased circulating concentrations of pepsinogen and gastrin, which serve as biomarkers of abomasal mucosal damage and correlate with parasite burden and disease severity (Fox, 1997). Chronic infection produces an immune-mediated hypochlorhydria that further disturbs feed intake and nutrient utilization, directly linking immune pathology to performance losses observed in infected cattle (Yazwinski & Tucker, 2006).

Prolonged immune activation also imposes a metabolic cost that affects animal performance even when parasite burdens are subclinical. Gasbarre (2014) reported herds under chronic gastrointestinal nematode challenge exhibit persistent inflammatory signaling and diversion of nutrients toward immune function rather than growth or milk production (Gasbarre, 2014). Reallocation contributes to reductions in feed efficiency and average daily gain demonstrating that immune-associated costs, rather than parasite load alone, can depress productivity in absence of overt clinical disease (Gasbarre, 2014).

Variation in competence of host immunity competence further influences infection outcomes. Immunogenic studies document differences among cattle in Th2 polarization, mucosal immune responses, and expression of defense-related genes, resulting in phenotypes characterized by either resistance (lower parasite burden) or resilience (maintained productivity despite infection). Biological variation provides a foundation for selective breeding and

management strategies aimed at enhancing natural immunity while reducing reliance on anthelmintic dependence (Sweeney et al., 2016).

Collectively, gastrointestinal nematode infections stimulate a protective Th2 immune response that limits parasite establishment but imposes physiological and metabolic costs on hosts. Interactions between immune activation, tissue pathology, and nutrient partitioning underlies declines in growth and milk production, reinforcing need for integrated control strategies that balance suppression of infection with preservation of immune function.

Economic Losses Due to Gastrointestinal Nematodes

Gastrointestinal nematode infections impose substantial economic losses on U.S. cattle industry by decreasing productivity, increasing treatment costs, and decreasing feed-efficiency. Total annual losses attributed to parasitism across beef and dairy operations are estimated to exceed $2 billion, with majority of losses resulting from subclinical infections that impair animal performance without causing visible clinical disease (Gasbarre, 2014; Stromberg et al., 2015). Even modest reductions in average daily gain (approximately 0.05-0.10 kg/d) associated with subclinical gastrointestinal nematode infections can translate to losses of $25-$60 per head over a grazing period, which, when extrapolated across U.S. cattle populations, contribute to industry-wide losses exceeding $1 billion annually (Gasbarre, 2014).

Anthelmintic intervention can partially mitigate losses. Sustained parasite control has been associated with improvements in carcass marbling and quality grade, increasing proportion of animals grading Choice or higher and enhancing carcass value. Economic analysis show that effective parasite management programs can return about $2.50 to $3.50 per dollar invested, depending on pasture contamination, infection pressure, and market conditions (Strydom et al., 2023).

In dairy industry, gastrointestinal nematode infections contribute to persistent reduction in milk yield, reproductive efficiency, and herd longevity. Chronic parasite exposure, even at low infection intensity, diverts nutrients towards immune and inflammatory processes rather than milk synthesis (Gasbarre, 2014). Reported reductions in milk yield of approximately 0.5 to 1.0 kg per cow per day corresponded to losses of roughly $150 to $190 per lactation, based on milk prices ranging from $0.50 to $0.62 per kg with an average milk yield reduction of 0.5 to 1.0 kg (Gasbarre, 2014; USDA Agricultural Marketing Services (AMS), 2025). Additional economic impacts have risen from extended calving intervals from 10 to 20 days which reduced overall production efficiency associated with chronic infection (Gasbarre, 2014).

Increasing anthelmintic resistance among *Cooperia* and *Haemonchus* populations further amplifies losses, as ineffective treatments allow continued parasite exposure and residual performance suppression. Under such conditions, unresolved parasitism may result in losses exceeding $60 per head per grazing season (Gasbarre, 2014). Implementation of integrated parasite management programs that combine targeted selective treatment, refugia preservation, and genetic selection for resilience can reduce costs by up to 40 percent, improving both herd profitability and sustainability (Strydom et al., 2023).

Collectively, gastrointestinal nematode infections represent a major economic constraint on profitability in U.S. beef and dairy cattle systems. While beef operations incur large aggregate losses through reduced growth efficiency and carcass value, dairy systems experience sustained financial pressure from decreased milk production and reproductive inefficiency. Economic consequences reinforce the importance of parasite control strategies that limit production losses, preserve anthelmintic efficacy, and support long-term profitability.

1.2 – Gastrointestinal Nematode Anthelmintic Treatments

Field-based evaluations further demonstrate production benefits associated with anthelmintic control. Andersen et al. (2019) compared extended-release eprinomectin (EPR) with a conventional injectable doramectin (DOR) treatment in 13 commercial cow-calf herds in seven U.S. herds. Treatment with eprinomectin significantly reduced fecal egg counts ($P < 0.05$) but did not affect pre-weaning cow body weight, body condition score, or pregnancy outcomes. Similarly, calf average daily gain and weaning weight did not differ between treatments during grazing phases. However, differences were observed later in production. Feedlot-finished calves originating from eprinomectin-treated cows exhibited greater marbling scores, improved carcass quality grades, and a greater percentage grading Choice or higher ($P < 0.05$). Results indicate that early-season parasite control can enhance carcass deposition efficiency, even in absence of immediate growth changes during grazing period (Andresen et al., 2019).

Similar findings have been reported in grazing dairy systems. Dudley and Smith (2020) evaluated extended-release eprinomectin administration in pastured dairy heifers over a 123-day grazing period. Treated heifers achieved an average daily gain of 0.85 kg d^{-1} compared with 0.64 kg d^{-1} in controls ($P < 0.01$), with corresponding reductions in strongyle fecal egg counts throughout grazing season. Predominant nematode genera identified included *Haemonchus placei*, *Cooperia spp.* and *Ostertagia spp.*, parasites commonly linked to subclinical production losses in temperate grazing environments. Although milk yield was not assessed due to pre-lactation status of heifers, results demonstrate a direct and measurable performance response to parasite control in grazing dairy systems (Dudley & Smith, 2020).

Anthelmintics remain primary tools used to control gastrointestinal nematode infections and other parasites diseases in livestock. Compounds are classified according to their chemical

structure and mechanisms of action, with each class exhibiting distinct pharmacological properties, parasite targets, and host-specie approvals. In cattle and other ruminants, major anthelmintic classes include benzimidazoles, macrocyclic lactones, imidazothiazoles and tetrahydropyrimidines, amino-acetonitrile derivatives, and salicylanilides and substituted phenols. Newer classes such as amino-acetonitrile derivatives are currently approved for use in small ruminants, but not cattle in the United States.

Benzimidazoles (BZs)

Benzimidazoles, including fenbendazole, albendazole, and oxfendazole, exert their anthelmintic activity by selectively binding to β-tubulin subunits of nematodes, thereby inhibiting microtubule polymerization. Disruption of microtubule formation interferes with glucose uptake and essential intracellular transport processes, ultimately leading to energy depletion and death of the nematode (Lacey, 1990). Benzimidazoles exhibit high efficacy against immature and adult nematodes living in abomasum and small intestine, including *Ostertagia ostertagi, Cooperia oncophora, Trichostrongylus axei, and Haemonchus placei* (Gasbarre, 2014). Additionally, benzimidazoles are active against bovine lungworm *Dictyocaulus viviparus* and display partial flukicidal and ovicidal activity, primarily with albendazole at elevated doses against adult Fasciola spp., depending on compound and dose (Martin, 1997).

Beyond cattle, benzimidazoles are widely used across livestock species. They are commonly administered in sheep and goats for control of *Haemonchus contortus, Teladorsagia circumcincta*, and *Trichostrongylus colubriformis*; in horses for and small strongyles (*Strongylus* spp. and *Cyathostome*); in swine for *Ascaris suum* and *Trichuris suis*; and in poultry for *Ascaridia galli* and *Heterakis gallinarum* (Zajac et al., 2021). Broad host applications and

parasite target spectrum of benzimidazoles has contributed to their extensive and prolonged use in veterinary medicine.

Resistance to benzimidazoles is becoming widespread and is primarily associated with single-nucleotide polymorphisms in β-tubulin gene at codons F200Y, E198A, and F167Y. Mutations reduce drug binding affinity and stabilize microtubules, thereby conferring resistance (Demeler et al., 2013). In cattle, benzimidazole resistance has been documented most frequently in *Cooperia* and *Haemonchus* species on U.S. beef operations, contributing to reduced treatment efficacy and increased reliance on alternative anthelmintic classes (Gasbarre, 2014).

Macrocyclic Lactones (MLs)

Macrocyclic lactones are among the most widely used anthelmintic in both beef and dairy cattle production including ivermectin, doramectin, eprinomectin, and moxidectin. Macrocyclic Lactones exert their anthelmintic activity by acting as agonists at glutamate-gated chloride (GluCl) channels located in nematode nerve and muscle cell membranes. Activation of channels induces chloride ion influx, resulting in membrane hyperpolarization, flaccid paralysis, and ultimately parasite expulsion and death (Shoop et al., 1995). Macrocyclic lactones also potentiate γ-aminobutyric acid (GABA)-mediated neurotransmission, further prolonging neuromuscular paralysis. When neuromuscular tissue function is inhibited, parasites lose their ability to maintain attachment or orientation in the gastrointestinal environment and become susceptible to displacement by normal gut motility and expulsion from the host (Shoop et al, 1995; Martin, 1997).

High lipophilicity of macrocyclic lactones contributes to prolonged tissue persistence and extended residual activity, which can increase selection pressure when parasites are exposed to declining drug concentrations over time. However, this same property increases selective

pressure for development of resistance. Macrocyclic lactones exhibit a broad spectrum of activity against gastrointestinal and pulmonary nematodes, including *Ostertagia*, *Cooperia*, *Trichostrongylus*, *Haemonchus*, *Nematodirus*, and *Dictyocaulus viviparus*, as well as ectoparasites including lice, mites and grubs. In dairy cattle, eprinomectin, a semi-synthetic avermectin, is approved for use due to its low milk partitioning, which minimizes drug residues in milk (Gasbarre, 2014; Lespine et al., 2005; Shoop et al., 1995).

Beyond cattle, macrocyclic lactones are widely used across livestock species. They are effective against *Haemonchus* and *Teladorsagia* in sheep and goats; *Strongylus* spp. and *Parascaris* spp. in horses; *Ascaris suum* and *Strongyloides ransomi* in swine; and numerous nematode and arthropod parasites in companion animals (Zajac et al., 2021; Shoop et al., 1995).

Resistance to macrocyclic lactones is increasingly documented in North American cattle populations, particularly in *Cooperia* and *Haemonchus*. Proposed resistance mechanisms include altered expression or function of GluCl channel subunits, increased activity of P-glycoprotein efflux transporters, and reduced cuticular permeability which limits drug uptake (Blackhall et al., 1998; Gasbarre, 2014; Lespine et al., 2005).

Imidazothiazoles/Tetrahydropyrimidines (LEV/PYR)

Imidazothiazoles and tetrahydropyrimidines, including levamisole, pyrantel, and morantel, act as agonists at nicotinic acetylcholine receptor (nAChR) located at nematode neuromuscular junctions. Activation of receptors induces sustained depolarization, resulting in spastic paralysis and rapid expulsion of susceptible parasites (Aceves et al., 1970). Compounds inimidazothiazole and tetrahydropyrimidine class act quickly and are primarily effective against gastrointestinal nematodes such as *Cooperia*, *Trichostrongylus*, *Haemonchus*, and *Dictyocaulus*

viviparus, but exhibit limited to no activity against inhibited or hypobiotic *Ostertagia* larvae (Zajac et al., 2021).

Imidazothiazoles and tetrahydropyrimidines are approved for use across multiple host species. In cattle, this class is commonly used as an alternative treatment option in herds where resistance to macrocyclic lactones is present. In small ruminants, imidazothiazoles and tetrahydropyrimidines compounds are effective against *Haemonchus* and *Teladorsagia* species. In swine, this class is used to control *Ascaris suum* and *Oesophagostomum* spp. In horses, it remains widely used for control of *Strongylus* spp. and *Parascaris* spp. In companion animals, it is a primary treatment for common ascarid and hookworm infections, including *Toxocara* and *Ancylostoma* spp (Zajac et al., 2021).

Amino-Acetonitrile Derivatives

Amino-acetonitrile derivatives represent a newer class of anthelmintics, with monepantel as the primary compound currently in use. Monepantel exerts its anthelmintic activity by selectively activating nematode-specific ACR-23 receptor, a member of the DEG-3/DES-2 family of ligand-gated ion channels that is absent in vertebrates. Activation of receptor induces sustained depolarization of nematode neuromuscular cells, resulting in paralysis and parasite death (Kaminsky et al., 2008).

Monepantel demonstrates high efficacy against several economically important gastrointestinal nematodes, including *Haemonchus contortus*, *Teladorsagia circumcincta*, and *Trichostrongylus colubriformis*, and retains activity against isolates resistant to macrocyclic lactones and benzimidazoles. Despite its mode of action and effectiveness against multidrug-resistant nematodes, amino-acetonitrile derivatives are currently approved for use in sheep and goats but have not been approved for use in cattle in the United States (Zajac et al., 2021).

Salicylanilides and Substituted Phenols

Salicylanilides and substituted phenols, represented primarily by closantel and oxyclozanide, exert their anthelmintic activity by disrupting mitochondrial oxidative phosphorylation. Salicylanilides and substituted phenols act as protonophoric uncouplers, collapsing the proton gradient across mitochondrial membranes, depleting adenosine triphosphate (ATP), and leading to energy failure and parasite death (Martin, 1997).

Salicylanilides and substituted phenols class is most effective against blood-feeding helminths, particularly *Haemonchus placei* and *Bunostomum phlebotomum*, and demonstrates activity against trematodes such as *Fasciola hepatica*. In feedlot and pastured beef cattle, use of salicylanilides is generally restricted to production systems where liver fluke and *Haemonchus* overlap. In small ruminants, compounds are commonly used for control of *Haemonchus contortus*. Salicylanilides and substituted phenols are not routinely used in horses, swine, or companion animals due to limited efficacy and narrow parasite spectra (Zajac et al., 2021).

1.3 – Diagnostics for Gastrointestinal Infection Detection

Importance of Gastrointestinal Nematode Infection Diagnostics

Accurate diagnostic testing is fundamental to effective treatment and long-term management of gastrointestinal nematodes in livestock production systems. Among range of available diagnostic tools, fecal egg counts (FECs) remain the most widely used method for estimating parasitic infection intensity and monitoring herd or flock level risk (Nielsen, 2021). Quantification of nematode EPG of feces provides an indirect measure of adult parasite populations and serves as a practical indicator of pasture contamination pressure, treatment efficacy, and overall health of animals. Although fecal egg counts do not measure worm burden

directly, they offer information on transmission dynamics and production risk when interpreted within appropriate biological and management context.

In grazing systems, routine fecal egg count surveillance supports evidence-based parasite control by allowing producers to identify high-shedding animals, create deworming schedules, and limit unnecessary anthelmintic use. Practices are important in maintaining animal performance while avoiding losses associated with parasites (Lejeune et al., 2023).

Beyond individual animal assessment, fecal egg counts are applied at herd or flock level to allow for group-based management decisions. Composite sampling methods, which has been increasingly adopted in cattle and small ruminants, allows for a cost-effective way to determine infection intensity of a group of animals (George et al., 2017). By utilizing FEC data in strategic parasitic control programs, producers can implement targeted selective treatment by only treating animals whose burden levels are greater than predefined thresholds. This allows for producers to maintain a refugia population of susceptible parasites and reduce selection pressure for resistance (Rinaldi et al., 2019).

Diagnostics also play an important role in evaluating anthelmintic efficiency and detecting resistance. Fecal egg count reduction test (FECRT) is industry standard method for field-based efficacy assessment by comparing pre-treatment and post-treatment egg counts to determine percentage of reduction in egg shedding (Kaplan et al., 2023). Reductions of less than 95% with a confidence interval less than 95% indicate potential resistance (Coles et al., 1992). Routine application of FECRT allows for early identification of reduced drug efficacy and supports decisions regarding drug rotation and parasite control strategies. Accurate interpretation requires consideration of host immunity, fecal consistency, sampling intervals, and parasite species composition, all of which can influence egg shedding dynamics (Zajac et al., 2021). As a

result, following standardized protocols and appropriate statistical analyses is essential for reliable conclusions.

While traditional copromicroscopic techniques are labor-intensive, continued methodological refinement has improved their precision and applicability. Comparative evaluations of McMaster, Mini-FLOTAC, and Wisconsin techniques have demonstrated important differences in sensitivity and repeatability, particularly under conditions of low egg output (Levecke et al., 2012). Improvements in molecular diagnostics, such as quantitative PCR and digital droplet PCR, allow for increased specificity, but higher cost and technician requirements limit their ability to be performed in the field (Reslova et al., 2021). Fecal egg count-based diagnostics remain the most practical and accessible option for parasite monitoring in most production settings, particularly where financial and logistical resources are constrained (Nielsen, 2021; Zajac et al., 2021).

Techniques for Diagnosis of Gastrointestinal Nematodes

There are many methods available to diagnose gastrointestinal nematode infections in livestock including copromicroscopic, immunologic, and molecular. Each method varies in sensitivity, precision, labor requirements, and suitability for research or field application (Levecke et al., 2012). The following section details major fecal egg count methods while noting advantages and limitations.

Fecal Flotation

Fecal flotations techniques are the most widely used diagnostic approach for detection and quantification of gastrointestinal nematode infections in livestock. Methods are based on differences in specific gravity, whereby parasite eggs and oocytes have a lower specific gravity than flotation solution allowing them to rise to the surface for microscopic evaluation (Zajac et

al., 2021). While floatation techniques primarily detect nematode eggs rather than protozoal oocytes in routine parasite monitoring, simplicity and adaptability have made them central to both field diagnostics and laboratory-based surveillance (Zajac et al., 2021; Nielson, 2021). Various floatation methodologies exist, including McMaster, Cornell-Wisconsin, and FLOTAC systems, each differing in sensitivity, labor requirements, and applicability.

McMaster

The McMaster technique is a quantitative fecal flotation method most used for estimating EPG of feces. Its popularity comes from its cost-effective, fast, and ease of replication, allowing it to be a good method for routine herd and flock level monitoring and on-farm applications (Nielsen, 2021). Standard McMaster protocols typically have a detection limit of approximately 50 EPG, which limits sensitivity in animals with low-level infections or following anthelmintic treatment (Zajac et al., 2021; Levecke et al., 2012). As a result, it is less appropriate for confirming light parasite burdens or evaluating treatment efficacy in low-shedding populations. Sensitivity and accuracy are frequently improved through protocol modifications, such as increasing fecal sample volume or altering dilution ratios, however, changes may compromise standardization if not controlled (Levecke et al., 2012; Nielsen, 2012).

Cornell-Wisconsin

Cornell-Wisconsin method is a centrifuge-based flotation technique designed to increased diagnostic sensitivity through use of larger fecal volumes and egg conservation prior to microscopic examination. Centrifugation improves egg recovery by overcoming debris interference and flotation inefficiencies commonly encountered in passive methods. Increased sensitivity makes Cornell-Wisconsin method particularly useful for detecting light infections, validating post-treatment egg suppression, and confirming parasite presence in low-prevalence

settings (Nielsen, 2021; Zajac et al., 2021). Despite advantages, Cornell-Wisconsin method is more labor intensive and time-consuming than McMaster, requiring centrifugation equipment and additional processing steps. Limitations reduce practicality for routine field use but support its role as a confirmatory or research diagnostic tool (Levecke et al., 2012).

FLOTAC/Mini-FLOTAC

The FLOTAC system, and field adapted Mini-FLOTAC, represents a refinement of flotation-based diagnostics aimed at improving sensitivity, precision, and repeatability. Systems use standardized chambers and calibrated flotation volumes to reduce operator variability and achieve detection limits as low as 5 EPG (Ghafar et al., 2021). High analytical sensitivity and repeatability make FLOTAC-based methods well suited for research applications, including anthelmintic efficacy trials and resistance monitoring. However, widespread adoption is constrained by higher costs associated with specialized equipment, the need for precise solution calibration, and increased technical training requirements (Nielsen, 2021). As a result, FLOTAC systems are most used in controlled laboratory or research settings rather than routine field diagnostics.

Direct Smear

Direct smear method is a rapid, quantitative technique used to detect and identify protozoa and other cell structures that fail to rise or are disformed due to flotation solution. A very small fecal sample is mixed with saline and smeared on microscope slide for reading under a microscope (Zajac et al., 2021). Due to samples being examined without concentration or flotation, parasite morphology is better preserved, allowing preliminary identification.

Despite speed and minimal equipment requirements, the direct smear method has low diagnostic sensitivity. Small sample volume and lack of concentration result in a high probability

of false-negative findings, particularly in cases of low parasite burden. Additionally, uneven distribution of eggs or protozoal stages within the smear further limit's reliability. As a result, direct smears are not considered suitable for routine surveillance or quantitative assessment of gastrointestinal nematode infections, instead they are used as a complementary screening tool for rapid detection of protozoa or abnormal cellular components, with confirmatory testing performed using more sensitive copromicroscopic techniques (Zajac et al., 2021).

Fecal Sedimentation

Fecal sedimentation is a diagnostic method used to detect parasite eggs with a high specific gravity that do not float in standard flotation solutions (flukes, acanthocephalans, and certain other helminths whose density exceeds that of commonly used flotation media) (Zajac et al., 2021). Sedimentation provides a necessary alternative to flotation-based diagnostics, because eggs remain in suspension or sink rather than rise.

In fecal sedimentation method, fecal samples are mixed with water and allowed to stand so heavier particles, including parasite eggs, settle to the bottom of containers. Supernatant is carefully decanted, and sediment is resuspended and washer through multiple sedimentation steps to progressively concentrate eggs in final sediment fraction. Recovered sediment is subsequently examined microscopically for parasite identification. While the procedure is relatively simple and requires minimal specialized equipment, it is inherently less quantitative than flotation-based methods and is not designed to estimate EPG of feces.

Diagnostic sensitivity is influenced by several procedural factors, including number of washings performed, volume of feces processed, and care taken during decanting to avoid loss of sediment. Inadequate washing or improper technique can reduce egg recovery and lead to false-negative results. Despite limitations, fecal sedimentation remains the method of choice for

detecting heavy eggs, particularly in cases where trematode infections are suspected and serves as an essential complement to flotation techniques in comprehensive parasite diagnostic programs (Zajac et al., 2021).

Baermann Test

Baermann test is a specialized diagnostic technique used for detection of larval stages of parasites, particularly lungworms and certain strongyloid nematodes. Unlike flotation and sedimentation methods, which are designed to recover parasite eggs, Baermann technique targets motile larvae, making it especially valuable for diagnosing active larval infections that may not be detected through standard fecal egg count methods (Zajac et al., 2021).

In Baermann test procedures, fresh fecal material is wrapped in gauze or cheesecloth and suspended in a water filled funnel or Baermann apparatus. Over time, live larvae actively migrate out of fecal material and move downward through water column, accumulating in stem of funnel where they are collected and examined microscopically. Reliance on larval motility necessitates use of fresh, non-refrigerated samples and adequate incubation time, typically several hours to overnight to allow sufficient larval migration.

While Baermann test offers high specificity for detecting larval stages, its sensitivity is influenced by sample freshness, larval viability, and incubation conditions. Baermann test also relatively time intensive and requires laboratory equipment, limiting its practicality for routine field diagnostics. It remains diagnostic method of choice for suspected lungworm infections and for parasites that shed larvae rather than eggs, and it plays a critical role in comprehensive parasitological diagnostic workflows (Zajac et al., 2021).

Immunologic Diagnostic Techniques

Immunologic diagnostic techniques complement traditional copromicroscopic methods by detecting parasite-derived antigens or host-produced antibodies rather than relying on visualization of eggs or larvae. Assays offer improved analytical sensitivity and species specificity and can detect infections during prepatent or low-burden stages that may not be identifiable through microcopy alone (Zajac et al., 2021; Nielsen, 2021). As a result, immunologic diagnostics are particularly valuable for early infection detection, herd level surveillance, and evaluation of treatment efficacy (Charlier et al., 2023; Mazeri et al., 2016).

Antigen ELISA: Enzyme linked immunosorbent assays

Antigen-capture enzyme-linked immunosorbent assays (ELISAs) detect parasite specific proteins released during active infection, providing direct evidence of ongoing parasitism. Assays normally use monoclonal antibodies bound to a microplate to find soluble antigens in feces, serum, or milk. Coproantigen ELISAs, such as described for *Fasciola hepatica,* can identify infections before eggs appear in feces (Kajugu et al., 2015).

Antigen ELISAs are well suited for assessing treatment efficacy and monitoring parasite activity at herd or flock level as antigen presence reflects active infection rather than historical exposure (Charlier et al., 2023; Mazeri et al., 2016). However, assay performance may be influenced by antigen clearance rates following treatment and variability in antigen producing among parasite species, making careful interpretation within context of sampling timing and management practices important (Zajac et al., 2021; Nielsen, 2021).

Lateral flow immunochromatographic assay

Lateral flow immunochromatographic assays (LFIAs), commonly referred to as rapid tests, use antibody-coated membranes to visually detect parasite antigens within minutes. Lateral flow immunochromatographic assays are portable, inexpensive, and require minimal laboratory

infrastructure, making them a viable option for point of care and field-based diagnostics (Aboelsoued & Abdel Megeed, 2022). Commercial lateral flow kits are widely available for protozoan parasites such as *Cryptosporidium* and *Giardia* in both ruminants and companion animals (Campos-Ruiz et al., 2023).

Despite their practicality and rapid turnaround, LFIAs generally exhibit lower sensitivity compared with laboratory-based ELISA platforms and produce qualitative rather than quantitative results. As a result, they have increased suitability for screening purposed or rapid confirmation of suspected infections, with positive or ambiguous results often requiring follow-up testing using more sensitive laboratory methods (Aboelsoued & Abdel Megeed, 2022; Campos-Ruiz et al., 2023).

Antibody ELISA: Enzyme linked immunosorbent assays

Antigen enzyme-linked immunosorbent assays (ELISAs) measure host immune responses against specific parasite antigens and are used to assess previous or current exposure rather than active infection. Assays typically screen serum or milk samples for parasite-specific immunoglobulins, most commonly IgG and, less frequently, IgM (Zajac et al., 2021; Nielsen, 2021). Antibody ELISAs are particularly well suited for herd level surveillance and epidemiological monitoring of gastrointestinal and hepatic parasites in cattle, including *Ostertagia ostergagi, Cooperia oncophora,* and *Fasciola hepatica*, as antibody production persists beyond parasite clearance (Charlier et al., 2023; Mazeri et al., 2016)

Milk and serum antibody ELISAs targeting *O. ostertagi* have been widely applied in dairy production systems to evaluate pasture exposure, estimate infection pressure, and predict parasite-associated production losses at herd level (Charlier et al., 2023; Sanchez et al., 2004). Similarly, antibody ELISAs for *F. hepatica* demonstrate high diagnostic sensitivity and

specificity in naturally infected cattle, supporting their use in epidemiological surveillance and assessment of control program effectiveness (Mazeri et al., 2016). While antibody ELISA tests cannot distinguish between current and past infections, their relatively low cost, scalability, and compatibility milk and serum sampling methods make them valuable in modern parasite monitoring and decision support frameworks (Charlier et al., 2023; Nielsen, 2021).

IFA: immunofluorescent assay

Immunofluorescent assay (IFAs) are serological techniques used to detect host antibodies directed against specific parasite antigens through visualization of antigen-antibody complexes using fluorescently labeled antibodies (Zajac et al., 2021; World Organization for Animal Health (WOAH), 2023). In indirect immunofluorescence assays, parasite antigens, often whole organisms, larvae, or antigen-coated cells, are fixed onto microscope slides and incubated with serum samples. If parasite-specific antibodies are present, they bind to antigens and are detected using fluorophore-conjugated secondary antibody, producing a characteristic fluorescence pattern under fluorescence microscopy (WOAH, 2023).

Immunofluorescent assays are commonly used to assess host exposure and humoral immune responses rather than active infection and have been widely applied in veterinary parasitology for detection of antibodies against protozoan and helminth parasites across livestock species (WOAH, 2023). In cattle, immunofluorescent assays have been used in seroprevalence studies and immune-epidemiological studies, specifically when direct parasite detection is impractical or when antibody dynamics provide insight into immune status and exposure history (Zajac et al., 2021; WOAH, 2023).

Immunofluorescent assays offer a high analytical sensitivity and allow semi-quantitative interpretation of antibody titers through serial dilution, making it useful for comparative studies

and longitudinal monitoring of immune responses following infection or treatment (Zajac et al., 2021). Although, interpretation may be influenced by observer subjectivity and potential cross reactivity among related parasite species. Despite limitations, IFAs remain an important reference method due to their strong specificity, visual confirmation of antigen-antibody binding, and long-standing validation in parasitological research. Similar to antibody ELISAs, IFAs do not distinguish between current and past infections, but continue to play a critical role in research-based surveillance and immune characterization of parasitic diseases in cattle and other livestock (World Organisation for Animal Health, 2023).

Molecular: PCR (qPCR)

Polymerase chain reaction (PCR)-based assays are molecular diagnostic techniques used to detect parasite specific DNA, providing highly sensitive and species-specific identification that is independent of host immune response and egg shedding dynamics. Conventional PCR assays are designed to confirm presence or absence of target DNA, while quantitative PCR (qPCR) monitors DNA amplification in real time allowing for estimation of relative parasite DNA abundance within a sample. Assays typically target conserved or species-specific genomic regions, most commonly ribosomal DNA sequences such as internal transcribed spacer (ITS-1 and ITS-2) regions, which enable reliable discrimination among closely related gastrointestinal nematode species (Zarlenga et al., 2001).

PCR-based diagnostics are particularly effective for detecting low intensity infections and mixed-species parasite communities that may be underestimated or misclassified using microscopy based fecal egg count techniques. By bypassing limitations associated with egg morphology and shedding variability, molecular assays provide improved taxonomic resolution

and facilitate more accurate characterization of parasite populations within individual animals and herds (Avramenko et al., 2015; Reslova et al., 2021).

In cattle parasitology, PCR and qPCR approaches have been increasingly applied for detection and differentiation of economically important gastrointestinal nematodes such as *Ostertagia ostertagi*, *Cooperia oncophora*, *Haemonchus placei*, and *Trichostrongylus* spp. directly from fecal samples. Molecular assays demonstrate improved species resolution compared with traditional coprological methods and have been shown to correlate with fecal egg counts while offering additional insight into parasite community comparison (Avramenko et al., 2015; Reslova et al., 2021). Such information is particularly valuable in epidemiological investigations and in evaluating impact of management strategies on parasite species dynamics.

Although PCR based methods detect parasite DNA rather than viable organisms, meaning residual DNA may be present in feces following anthelmintic treatment. As a result, molecular results must be interpreted cautiously when assessing treatment efficacy or active infection status. In addition, high costs, specialized laboratory infrastructure, and technical expertise requirements limit routine field application. High specificity, ability to multiplex multiple targets at the same time, and utility in epidemiological and resistance-related studies make PCR and qPCR valuable tools in modern parasite surveillance and research in cattle production systems (Avramenko et al., 2015; Reslova et al., 2021).

Method Comparisons

Traditional fecal-based methods differ primarily in whether parasite stages are concentrated prior to examination and which parasite life stages they are designed to recover. Direct fecal smear method is a basic diagnostic coprological technique and involves microscopic examination of a small quantity of fresh feces without a concentration step. As a result,

diagnostic sensitivity is low, particularly for low-intensity or intermittently shedding infections, limiting its utility for surveillance, quantitative assessment, or treatment-efficacy studies (Zajac et al., 2021). As a result, direct smears are restricted to rapid screening in cases of suspected heavy infection rather than routine parasite monitoring.

Fecal floatation methods improve diagnostic sensitivity by concentrating parasite eggs using solutions of high specific gravity. Quantitative flotation methods vary in analytical sensitivity and precision, which can significantly influence interpretation of egg counts and fecal egg count reduction tests (FECRT). McMaster technique is widely used because it is rapid, inexpensive, and provides quantitative EPG estimates. Limitations show impacts on fecal egg count reduction test interpretation when post-treatment egg counts approach methods threshold, potentially leading to misclassification of anthelmintic efficacy (Coles et al., 1992). Cornell-Wisconsin method increases egg recovery through centrifugation and larger sample volumes, improving detection of trichostrongylid eggs under low intensity infection conditions, although it is more labor and equipment intensive (Dryden et al., 2005). FLOTAC and Mini-FLOTAC methods were developed to further increase sensitivity and repeatability, with multiple comparative studies demonstrating that Mini-FLOTAC provides improved agreement, lower detection limits, and greater precision than McMaster, especially in cases where egg counts are low or when FECRT outcomes are being evaluated (Barda et al., 2013; Rinaldi et al., 2019).

Certain parasite stages are poorly detected by flotation, making alternative coprological approaches needed. Fecal sedimentation relies on gravitational settling rather than buoyancy and is therefore better suited for detecting dense eggs, such as *Fasciola hepatica*. Large-scale, studies in cattle demonstrate sedimentation supports herd-level fasciolosis surveillance, especially when composite sampling is used. Diagnostic sensitivity remains constrained by intermittent egg

shedding and inability to detect prepatent infections (Mazeri et al., 2016; Rapsch et al., 2006). The Baermann technique is specifically designed to recover live, motile larvae and is widely used for diagnosis of lungworm infections, such as *Dictyocaulus viviparus*. Field based studies demonstrate that larval recovery using Baermann method is closely associated with patent infection status and is influenced by infection dynamics, larval viability, sample freshness, and timing of fecal collection relative to larval shedding (May et al., 2018).

In comparison to coprological approaches, immunologic diagnostic techniques detect either parasite derived antigens or host immune responses. Antigen detection assays, including coproantigen ELISAs, identify parasite molecules released during active infection and can improve detection when egg shedding is low or inconsistent. In cattle, coproantigen ELISAs for *Fasciola hepatica* have demonstrated higher sensitivity than coproscopy in naturally infected animals and show declining signal following successful treatment, supporting their application in control program evaluation (Brockwell et al., 2013; Mazeri et al., 2016). Lateral flow immunochromatographic assays (LFIA) provide rapid, point-of-care testing and offer logistical advantages for field screening; however, diagnostic performance is variable and results are qualitative and require confirmation by laboratory-based methods (Posthuma-Trumpie et al., 2009).

Antibody detection methods, including antibody ELISAs and immunofluorescent assays (IFA/IFAT), measure host humoral immune responses and therefore reflect exposure rather than current parasite burden. Antibody ELISAs are well suited for high-throughput herd surveillance and have been widely applied in cattle, including bulk-talk milk ELISAs for *Ostertagia ostertagi*, which correlate with pasture exposure and parasite associated production losses in dairy herds (Charlier et al., 2023; Sanchez et al., 2004). Immunofluorescent assays are more

labor-intensive and observer-dependent, but provide visual confirmation of antigen-antibody binding, and are commonly used as a reference or comparative methods in serological studies (Lira-Amaya et al., 2021). As with antibody ELISAs, IFAs cannot distinguish between active and past infections due to antibody persistence following parasite clearance.

Molecular diagnostics using PCR and quantitative PCR (qPCR) enable species-level identification of gastrointestinal nematodes directly from cattle fecal samples and allow resolution of mixed infections that cannot be differentiated morphologically. Amplicon-based sequencing and multiplex qPCR approaches have demonstrated improved sensitivity and strong concordance with fecal egg counts while providing substantially greater taxonomic resolution and insight into parasite community composition (Avramenko et al., 2015; Reslova et al., 2021). Although PCR-based methods detect parasite DNA rather than viable parasites and may reflect residual DNA following treatment, their sensitivity, multiplexing capacity, and utility in epidemiological and resistance-related studies make them increasingly important components of modern parasite surveillance frameworks.

1.4 – Anthelmintic Resistance and Efficacy against Gastrointestinal Nematodes

Anthelmintic Resistance in Gastrointestinal Nematodes

Anthelmintic resistance in gastrointestinal nematodes of cattle is an increasing concern for sustainable parasite control. Resistance compromises efficacy of the limited anthelmintic classes available and allows persistent infections that negatively affect animal health and productivity. Reviews focused on cattle production systems emphasize that resistance is no longer restricted to isolated herds but has been recorded across multiple regions, with macrocyclic lactones showing the most widespread reductions in efficacy. *Cooperia* spp. are frequently identified as early and consistent indicators of resistance development in cattle

populations, often preceding detectable resistance in *Ostertagia ostertagi* and other abomasal nematodes (Gasbarre, 2014; Kaplan & Vidyashankar, 2012). Due to macrocyclic lactones being heavily relied on for endo- and ectoparasite control, reduced efficacy has substantial implications for parasite management strategies in beef and dairy systems.

Resistance develops through selection of heritable traits that allow parasites to survive drug exposure, with management practices playing a central role in accelerating the process. Frequent whole-herd treatments, repeated use of the same anthelmintic class, underdosing, and limited refugia all increase selection pressure for resistant genotypes. The concept of refugia, maintaining a population of parasites unexposed to treatment, is now widely recognized as a critical factor in slowing evolution of resistance by diluting resistant alleles in subsequent generations (Kaplan & Vidyashankar, 2012; van Wyk, 2001). Field-based studies and modeling approaches support targeted selective treatment strategies as effective tools for preserving refugia while maintaining animal health, particularly in systems where blanket deworming has historically been routine (Kaplan & Vidyashankar, 2012).

Detection and monitoring of anthelmintic resistance rely on both phenotypic and genotypic diagnostic approaches. Fecal egg count reduction test (FECRT) remains primary field-based method for resistance assessment because it directly measures treatment impact on parasite egg output; however, its diagnostic sensitivity is influenced by study design, baseline egg counts, and analytical performance of fecal egg counting method used. Molecular tools increasingly complement FECRT by providing species-level resolution and enabling detection of resistance-associated alleles and shifts in parasite community composition following treatment. Large scale amplicon sequencing, such as nemabiome analysis, reveals that resistance may be concentrated

within specific taxa in mixed infections, a pattern that is often undetected when relying solely on total egg counts (Avramenko et al., 2015; Queiroz et al., 2020).

Anthelmintic Drug Efficacy

Anthelmintic drug efficacy in cattle has historically been high across major classes, including benzimidazoles, imidazothizoles/tetrahydropyrimidines, and macrocyclic lactones. However, large variation in performance has been documented under field conditions. Early controlled studies demonstrated that benzimidazoles and levamisole-based products were highly effective against adult and developing stages of abomasal and intestinal nematodes, particularly *Ostertagia ostertagi* and *Cooperia oncophora*, when administered at recommended dosages and under optimal management conditions (McKellar & Jackson, 2004). Introduction of macrocyclic lactones, such as ivermectin and moxidectin, represented a major advancement in parasite control due to their broad spectrum of activity, prolonged persistence, and ease of administration, leading to widespread adoption in both beef and dairy systems (Kaplan & Vidyashankar, 2012).

While having initial success, various field studies have demonstrated that anthelmintic efficacy, particularly for macrocyclic lactones, is often substantially lower than expected. Studies conducted in North America and Europe consistently report reduced fecal egg counts reductions following macrocyclic lactone treatment, most often associated with *Cooperia* spp., while efficacy against *O. ostertagi* often remains comparatively higher but variable (Edmonds et al., 2010; Gasbarre, 2014). Findings highlight species-dependent nature of drug efficacy and highlight how mixed infections can obscure treatment failure when efficacy is assessed only using fecal egg counts. Reduced efficacy does not always indicate complete resistance but may reflect shifts in parasite community composition toward less susceptible species following treatment (Kaplan & Vidyashankar, 2012).

Assessment of anthelmintic efficacy in cattle commonly relies on fecal egg count reduction tests. It is important that efficacy estimates be interpreted in context of study design, parasite biology, and drug pharmacokinetics, particularly for macrocyclic lactones that suppress egg output without complete parasite elimination (Coles et al., 1992; Kaplan & Vidyashankar, 2012). As a result, efficacy estimates can vary across studies when similar treatments are applied. Interpretation is further complicated for macrocyclic lactones, which may suppress egg output for extended periods without fully eliminating adult parasites, particularly when post-treatment sampling occurs shortly after administration (Kaplan & Vidyashankar, 2012)

Drug formulation and route of administration also influence observed efficacy. A previous study indicates extended-release formulations of macrocyclic lactones prolong suppression of fecal egg shedding and reduce reinfection pressure by maintaining persistent antiparasitic activity following treatment (Rehbein et al., 2013). However, prolonged exposure to sub-therapeutic drug concentrations associated with extended-release formulations may increase selection pressure for resistance, particularly in systems with limited refugia (Kaplan & Vidyashankar, 2012). Injectable formulations generally achieve more consistent systemic drug concentrations than pour-on products, which are more susceptible to variability due to application technique and environmental conditions (McKellar & Jackson, 2004).

Literature indicates anthelmintic efficacy in cattle can no longer be assumed based only on drug class or label claims. Field-based evaluations demonstrate that efficacy varies across parasite species, production systems, and management practices, reinforcing need for routine efficacy monitoring and informed treatment decisions. Integrating drug efficacy assessment with ongoing parasite surveillance allows producers and veterinarians to optimize anthelmintic use,

preserve effectiveness of existing anthelmintic drugs, and mitigate long-term impacts of declining drug performance (Gasbarre, 2014; Kaplan & Vidyashankar, 2012).

1 - Conclusions

Effective control of gastrointestinal nematodes in cattle requires a shift from treatment centered models toward management strategies that integrate diagnostic information into decision making. Evidence presented demonstrates reliance on a single diagnostic approach or an assumption of uniform drug performance is insufficient for contemporary cattle production systems characterized by heterogeneous parasite populations and variable treatment responses.

Sustained parasite control depends on alignment of diagnostic outputs with management objectives, ensuring that intervention decisions are based on meaningful biological signals rather than generalized thresholds. Incorporating diagnostic insight into treatment selection enables more deliberate use of anthelmintics, reduces unnecessary exposure, and supports long-term preservation of drug-efficacy.

Moving forward, parasite control frameworks must prioritize adaptability, emphasizing ongoing assessment and refinement rather than static protocols. Embedding diagnostic interpretation within herd health planning allows producers and veterinarians to respond to changing parasite pressures while maintaining productivity and minimizing risk of further resistance development.

Efficacy and Sustainability in Beef Production

2 - Introduction

Animal scientists and cattle producers across the world are facing an increasing pressure to improve production efficiency while also reducing environmental impacts of beef systems (Gerber, 2013). As demand for animal protein rises, producers are challenged with maximizing

growth performance and feed utilization without compromising animal health or long-term sustainability (Alexandratos, 2012). Inefficient nutrient utilization not only limits animal productivity, but also contributes to increased resource use and greenhouse gas emissions, specifically in forage-based beef production systems (Wu, 2018).

Enteric methane production represents a major contributor to greenhouse gas emissions from ruminant livestock and represents both an environmental concern as it represents a significant source of agricultural emissions, ultimately contributing to climate change. Enteric methane production also presents a loss of gross dietary energy otherwise used for animal growth and production (Johnson & Johnson, 1995). In growing cattle, energy losses negatively impact average daily gain and feed efficiency while contributing to agricultural greenhouse gas emissions (Wu, 2018). As a result, increasing attention has been directed toward nutritional strategies capable of improving rumen fermentation efficiency while mitigating methane output.

Feed-based interventions have increased in popularity as tools for improving sustainability, as opposed to genetic, management, or infrastructure-based strategies, without requiring major changes to infrastructure or management. Among feed-based strategies, plant-derived feed additives included in cattle diets, such as essential oils, have gained interest due to their potential to modify rumen microbial populations and fermentation pathways. As a result, numerous studies have effects of evaluated impacts on methane emissions; however, responses have been variable and are often influenced by diet composition, dosage, and microbial adaptation. Limited research has simultaneously evaluated impacts of essential oil supplementation on rumen microbial ecology, methane emissions, and animal performance. Therefore, a comprehensive evaluation of feed additives that integrates efficacy and

sustainability outcomes is necessary to support their application in modern beef production systems (Hristov et al., 2013).

2.1 – Rumen Fermentation and Methane Production

Anaerobic Fermentation/Biochemical Pathways of Methane Synthesis

Anaerobic rumen fermentation is a microbially driven process in which bacteria, protozoa fungi, and archaea degrade plant structural and non-structural carbohydrates into volatile fatty acids, which supply most of the metabolizable energy to the host animal. Primary volatile fatty acids produced, acetate, propionate and butyrate, are generated alongside carbon dioxide and methane. Proportions of fermentation products are not fixed, but vary according to forage quality, dietary composition, and use of feed additives, reflecting shifts in microbial activity and metabolic routing (Reece & Rowe, 2017).

While volatile fatty acid production is often emphasized because of its importance to host energy supply, fermentation simultaneously generates reduced electron carriers whose re-oxidation is essential for continued microbial metabolism. Sustained fermentation relies on the ability of the rumen microbial community to regulate reducing equivalents, therefore preventing hydrogen accumulation (Janssen, 2010).

Following hydrolysis of polymers such as cellulose, hemicellulose, and starch into monomers, microbial glycolysis and associated fermentative pathways generate reduced cofactors, like NADH, and release reducing equivalents in the form of molecular hydrogen (H_2), along with CO_2 and organic acids. Overall direction and efficiency of fermentation are constrained by thermodynamics as the rumen operates under anaerobic conditions. Continued fermentation depends on maintaining low partial pressure of hydrogen, permitting re-oxidation of reduced electron carriers and sustains microbial ATP production. Accumulation of hydrogen

causes slowing of fermentation and compromised microbial growth (Janssen, 2010). Consequently, the distribution of volatile fatty acids (acetate, propionate, and butyrate) reflects how rumen ecosystems partition carbon and reducing equivalents, whereas methane is produced independently as a sink for excess hydrogen via methanogenesis (Mackie et al., 2024).

Methane synthesis is as an important portion of ruminal electron partitioning rather than a passive byproduct of fermentation. In typical rumen conditions, hydrogenotrophic methanogenesis predominates, whereas methanogenic archaea reduce carbon dioxide (CO_2) using Dihydrogen (H_2) as the primary electron donor ($CO_2 + 4H_2 = CH_4 + 2H_2O$). The hydrogenotrophic methanogenesis pathway removes H_2 from the system, supporting upstream fermentative microbes, particularly acetate- and butyrate-producing bacteria that generate H_2 as a metabolic byproduct (Beauchemin et al., 2020; Janssen, 2010). In comparison, propionate formation functions as an alternative hydrogen sink, consuming reducing equivalents during its synthesis. As a result, dietary or microbial conditions that promote propionate production can reduce hydrogen availability for methanogenesis, even when total fermentation activity remains unchanged (Janssen, 2010; Mackie et al., 2024). With this context, these relationships explain why methane output is often described not simply as a microbial "byproduct", but as a redox-balancing endpoint tightly coupled to volatile fatty acid profiles and hydrogen flux.

At the biochemical level, methanogenesis proceeds through a conserved series of one-carbon transfer reaction unique to archaea, using specialized cofactors such as methanofuran, tetrahydromethanopterin, coenzyme F420, coenzyme B, and coenzyme M. Cofactors facilitate stepwise reduction of CO_2-derived intermediates to methane. Terminal reaction, reduction of methyl-coenzyme M to methane, is catalyzed by methyl-coenzyme M reductase, and enzyme widely regarded as a biochemical hallmark of methanogenesis and a primary target for methane

mitigation strategies (Beauchemin et al., 2020; Khairunisa et al., 2023). Although CO_2/H_2 reduction generally dominates in a mature rumen, methanogens may also utilize methylated substrates such as methanol or methylamines through methylotrophic methanogenesis. Contribution of alternative pathways can vary with substrate availability, diet composition, and microbial community structure (Khairunisa et al., 2023; Pitta et al., 2022).

Hydrogen production and utilization are distributed across a complex microbial consortium rather than being controlled by methanogens alone. Fermentative bacteria and certain rumen protozoa generate H_2 through hydrogenase enzymes during carbohydrate metabolism. Multiple hydrogen-consuming processes compete with methanogenesis, including propionate formation and other reductive pathways whose relevance depends on diet and microbiome composition (Mackie et al., 2024; Pitta et al., 2022). Recent syntheses emphasize that methane yield (g CH_4 per dry matter intake) is strongly influenced by microbial network structure, not just abundance of methanogens, and that interventions which suppress methanogens must also account for where reducing equivalents are redirected to avoid accumulation of H_2 or shifts that impair fermentation efficiency (Beauchemin et al., 2020; Pitta et al., 2022). Thus, methane synthesis is most appropriately framed as an emergent property of anaerobic fermentation kinetics, thermodynamic constraints on electron flow, and ecological balance among hydrogen-producing and hydrogen-utilizing microbes in the rumen (Janssen, 2010; Mackie et al., 2024).

Relationship Between Fermentation End Products and Energy Availability to Host Animals

Microbial fermentation in the rumen converts dietary substrates into end-products that are used for energy by the ruminant host. During ruminal fermentation, microbes generate volatile fatty acids, gases, microbial biomass, and heat. Balance among outputs determine how efficiently dietary energy is recovered and partitioned toward maintenance and productive functions such as

growth and lactation (Dehority, 2023). Consequently, shifts in fermentation pathways influence not only total quality of energy retained by host animals, but also metabolic forms in which energy is delivered to tissues, thereby affecting feed efficiency, nutrient partitioning, and growth performance (Wu, 2018).

Relative production of volatile fatty acids varies with substrate type, rumen physiochemical conditions, and microbial community structure, meaning diet-driven fermentation shifts can alter both magnitude and energetic quality of absorbed end products. Experimental work evaluating dietary energy density repeatedly shows manipulating fermentation profiles are accompanied by measurable differences in volatile fatty acid concentrations, feed efficiency, and metabolic signaling, supporting the idea that volatile fatty acids function act as regulators of physiology as well as fuels (Ahmad et al., 2020; Wang et al., 2021). The described relationships are most evident when comparing forage versus concentrate based feeding systems, where fermentation end products and efficiency of energy capture can diverge substantially.

Acetate is generally the predominant volatile fatty acid in the rumen and is strongly associated with fiber fermentation. From an energy-availability standpoint, acetate production is closely linked to metabolic hydrogen generation, in turn influencing downstream competition among hydrogen sinks such as methanogenesis and alternative reduced products. When rumen conditions promote acetate-dominant fermentation, a greater proportion of reducing equivalents redirect toward methane formation, representing an energetic loss to the host and decreasing net energetic efficiency (Ungerfeld, 2020). Acetate remains essential for host metabolism as it serves as a major oxidative fuel for peripheral tissues and provides a primary carbon source for lipogenesis. Accordingly, acetate-rich fermentation can support adipose tissue accretion and, in

lactating animals, milk fat synthesis, even if it does not always maximize efficiency of body weight gain.

Propionate plays a uniquely important role as it directly links ruminal fermentation to host glucose supply. In ruminants, systemic glucose availability depends greatly on hepatic gluconeogenesis rather than intestinal glucose absorption, and propionate is a primary gluconeogenic precursor. Propionate-favoring fermentation patterns improves fractions of dietary energy retained by animals by supporting glucose-dependent functions and reducing hydrogen availability for methane formation (Ungerfeld, 2020). Thus, propionate increases energy availability through both metabolic supply and improved fermentation efficiency.

Butyrate contributes less to circulating energy supply than acetate or propionate on a molar basis, yet it exerts a disproportionate influence on rumen capacity to capture and transfer fermentation energy to hosts. Butyrate plays a central role in rumen epithelial development and metabolism, as epithelial tissues preferentially oxidize butyrate and convert it to ketone bodies such as B-hydroxybutyrate (Steele et al., 2011; Zhuang et al., 2024). Butyrate metabolites contribute to host energy metabolism and reflect specialized metabolic function of the rumen wall. Increases in fermentable substrate supply can stimulate epithelial proliferation, transporter expression, and absorptive surface area, thereby enhancing volatile fatty acid uptake efficiency (Ahmad et al., 2020; Zhang et al., 2025). Butyrate functions not only as an energy source, but also as a driver of structural and functional infrastructure governing how effectively fermentation energy becomes metabolizable energy.

Energy losses during fermentation represent a major constraint on net energy recovery, and methane formation being a most prominent loss. Methane production reflects diversion of carbons and reducing equivalents away from host use and has been consistently described as a

meaningful fraction of gross dietary energy loss, with magnitude dependent on diet composition and fermentation pattern (Carmichael et al., 2024; Ungerfeld, 2020). Mechanistic links explain why interventions that increase propionate production are frequently characterized energetically favorable.

Diet composition and rumen conditions ultimately determine which fermentation end products predominate and how efficiently they are captured by host animals. High-forage systems generally favor acetate production and greater rumination, while high-concentrate systems tend to increase propionate and decrease ruminal pH. While shifts can enhance energetic efficiency, prolonged acidotic conditions may impair epithelial function and compromise absorptive capacity (Wang et al., 2021; Zhang et al., 2025). Therefore, relationships between fermentation end products and energy availability cannot be reduced to simply "more propionate is better". Rather, optimal energy availability emerges from a balance among fermentation intensity, minimization of avoidable energy losses, and maintenance of a rumen epithelium environment capable of efficiently absorbing and metabolizing fermentation

Proportion of Gross Energy Intake Lost as Methane in Growing Cattle

Enteric methane is an unavoidable by-product of ruminal fermentation and represents a direct loss of dietary energy from ruminant systems. In ruminant nutrition, methane energy loss is commonly expressed as Ym, defined as the proportion of gross energy intake (GEI) lost as methane. While methane formation functions as a hydrogen sink, it also represents a loss of carbon and reducing equivalents from the host, reducing energy available for productive use. As a result, proportions of gross energy intake lost as methane is a meaningful indicator of energetic efficiency in cattle production systems, where maximizing energy retention is essential for supporting frame growth and lean tissue increase (Gavrilova et al., 2019).

Across ruminants, methane energy losses are typically reported to range from approximately 2 to 12% of gross energy intake, reflecting strong influences of diet composition, intake level, and rumen fermentation characteristics on hydrogen balance (Johnson & Johnson, 1995). Variation within this range is attributed to differences in fermentable substrate type, dry matter intake, rumen passage rate, and the partitioning of metabolic hydrogen among competing sinks which collectively drive differences in Ym across diets and production systems.

From a cattle-feeding perspective, Y_m is consistently lower for high-concentrate, feedlot style growing programs than for forage-based growing systems. United States inventory documentation reports recommended Y_m values of approximately 3% (±1%) for feedlot cattle and approximately 6.5% (±1%) for other well-fed cattle consuming temperate-climate forage diets (Gavrilova et al., 2019, NASEM, 2016). Differences reflect underlying fermentation patterns, as structural carbohydrate fermentation typically favors acetate production and greater hydrogen release, providing substrate for methanogenesis, as discussed previously. In contrast, higher-starch diets promote propionate formation, reduce hydrogen availability, and consequently lower fractions of feed energy lost as methane (Ungerfeld, 2020). Collectively, relationships indicate that reductions in Y_m correspond, indirectly, to support growth, reinforcing relevance of methane mitigation strategies for production efficiency in cattle (Ungerfeld, 2020).

Implications for Feed Efficiency

Variation in feed efficiency among growing cattle reflects differences in how effectively animals capture and utilize metabolizable energy derived from ruminal fermentation. Even when gross energy intake is similar, cattle can differ in efficiency due to differences in fermentation pathways, hydrogen partitioning, and host utilization of absorbed metabolites (Van Soest, 1994). As a result, feed efficiency is interpreted as an integrated outcome of microbial energy

conversion and host-level energy retention rather than intake alone (Ungerfeld, 2020; Van Soest, 1994).

Feed efficiency is also influenced by intake dynamics and rumen passage rate, which interact with fermentation to determine how much dietary energy is lost relative to energy retained. Higher intake levels and faster passage rates can shorten rumen retention time, reduce extent of fermentative energy dissipation while increasing the flow of digestible nutrients to lower digestive tract. Consequently, efficiency advantages observed in some growing systems reflect not only differences in fermentation stoichiometry, but also differences in how rapidly and effectively fermentation products are captured and used by host animals (Gavrilova et al., 2019).

Beyond microbial fermentation, host factors such as rumen epithelial function further contribute to variation in feed efficiency. Differences in absorptive surface area, epithelial metabolism, and transport efficiency can lead to meaningful differences in energy utilization even when fermentation outputs are similar. Experimental studies examining dietary energy supply and rumen development demonstrate that improved epithelial function enhances volatile fatty acid uptake and utilization, thereby increasing efficiency independent of intake (Ahmad et al., 2020; Steele et al., 2011).

2.2 – Rumen Protozoa as Modulators and Fermentation and Methane Production

Diversity of Rumen Protozoa

Rumen protozoa are anaerobic, unicellular eukaryotes that represent a functionally diverse component of rumen microbial ecosystems. Although protozoa typically comprise a smaller portion of total microbial biomass than bacteria, protozoa can account for a substantial fraction of total microbial volume due to their relatively large cell size. Through their size,

motility, and feeding behavior, protozoa exert disproportionate effects on rumen fermentation dynamics and microbial interactions. Protozoal populations are broadly classified into two major functional groups: Entodiniomorphs and Holotrichs. Classification reflects differences in morphology, substrate preference, feeding behavior, and metabolic activity rather than simply taxonomic lineage alone (Dehority, 2023).

Entodiniomorph protozoa are the dominant group in most rumen environments and are strongly associated with digestion of particulate feed material. Entodiniomorphs actively ingest starch granules, plant cell wall fragments, and are associated bacteria, making them a key contributor to turnover of solid-associated microbial populations (Dehority, 2023; Coleman, 1986). Through selective predation and substrate uptake, Entodiniomorphs influence both rates and extents of fermentation of particulate substrates, particularly under forage-based or mixed diets where solid feed fractions are abundant (Dehority, 2023). In contrast, Holotrich protozoa preferentially utilize soluble carbohydrates such as sugars and respond rapidly to increases in soluble substrate availability following feeding (Dehority, 2023; Newbold et al., 2015). Holotrich activity is associated with short-term increases in fermentation rate and hydrogen production, with potential downstream implications for methane formation as they rapidly ferment substrates (Ungerfeld, 2020).

Protozoa differ from bacteria in their growth kinetics and metabolic strategies. Protozoal generation times are generally slower than bacteria, and many protozoa store fermentable substrates intracellularly as reserve polysaccharides. Storage capacity enables protozoa to sequester rapidly fermentable substrates during periods of high availability and metabolize them over extended periods, contributing to temporal smoothing of fermentation activity (Dehority,

2023). Through described mechanism, protozoa can buffer short-term fluctuations in substrate supply and fermentation rate.

Functional diversity of protozoa therefore contributes to both stability and variability in fermentation processes (Dehority, 2023). Differences in protozoal community composition across diets and feeding systems influence substrate utilization patterns, microbial predation dynamics, hydrogen production, and downstream fermentation outcomes (Ungerfeld, 2020). Consequently, protozoa function as a collection of functionally distinct organisms whose abundance and activity shape rumen microbial ecology and fermentation behavior (Dehority, 2023; Ungerfeld, 2020)

Protozoal Regulation of Rumen Microbial Populations

A defining functional role of rumen protozoa is their regulation of bacterial populations through predation. Protozoa actively engulf large numbers of rumen bacteria, consuming microbial biomass and thereby influencing bacterial abundance, turnover rates, and community structure. Predatory activity has direct consequences for microbial protein synthesis and flow, as bacterial cells consumed by protozoa are retained within the reticulo-rumen rather than passing to the abomasum and, later, the small intestines for host absorption (Dehority, 2023; Jouany, 1996). As a result, presence of protozoa is commonly associated with reduced microbial protein outflow to hosts, despite continued microbial growth within the rumen.

Protozoal predation also exerts selective pressure on bacterial populations. Protozoa preferentially graze on rapidly growing and metabolically active bacterial species, which can limit dominance by specific taxa and prevent unchecked bacterial proliferation (Coleman, 1986). Through top-down regulation, protozoa promote a more balanced microbial community and can contribute to stabilization of fermentation patterns, particularly under conditions of high

substrate availability that favor rapid bacterial growth (Dehority, 2023). Protozoa function as ecological regulators within rumen microbial ecosystems, rather than simply contributing to fermentation, by influencing microbial population dynamics, substrate turnover, and fermentation pathways.

Protozoal regulation of bacterial populations via predation has important implications for nitrogen metabolism. As protozoa digest engulfed bacteria, microbial protein is partially deaminated and recycled within the reticulo-rumen, resulting in increased ammonia production. Elevated ruminal ammonia concentrations can reduce nitrogen-use efficiency when microbial uptake is not synchronized with energy availability, leading to greater nitrogen absorption across rumen walls and subsequent excretion as urea (Jouany, 1996; Newbold et al., 2015). However, intra-ruminal nitrogen recycling also contributes to nitrogen availability for microbial growth, particularly under conditions of limited dietary protein (Leng & Nolan, 1984). Therefore, protozoal activity both stabilizes microbial populations and modulates nitrogen cycling, with context-dependent effects on the efficiency of microbial protein supply to the host (Dehority, 2023; Newbold et al., 2015).

Protozoal Contributions to Methanogenesis

Protozoa contribute indirectly to ruminal methane production through their metabolic activity and interactions with methanogenic archaea. Many rumen protozoa produce molecular hydrogen as a by-product of carbohydrate fermentation, creating localized microenvironments enriched in hydrogen. Hydrogen serves as a key substrate for methanogens, which reduce carbon dioxide to methane as part of their energy metabolism. Protozoa influence methanogenesis by modulating both the availability and spatial distribution of reducing equivalents (Dehority, 2023).

In addition to hydrogen production, protozoa interact closely with methanogenic archaea through physical and ecological associations. Numerous studies have demonstrated that methanogens frequently attach to or reside in close proximity to protozoal cells, forming functionally linked microenvironments that facilitate efficient interspecies hydrogen transfer (Newbold et al., 2015). Protozoa-associated methanogens often differ in abundance and activity relative to free-living methanogens, indicating that protozoa create specialized niches that enhance methanogenic efficiency.

Evidence from defaunation studies, studies that intentionally cause the removal or absence of rumen protozoa, consistently demonstrates contribution of protozoa to methane production. Removal or suppression of protozoa from rumen has been shown to reduce methane emissions across a range of diets, supporting role of protozoa in methanogenic activity (Dehority, 2023; Newbold et al., 2015). Reductions in methane following defaunation are generally attributed to decreased hydrogen availability, disruption of protozoa-associated methanogen populations, and altered microbial community structure (Newbold et al., 2015; Hook et al., 2010; Ungerfeld, 2020).

Diet composition strongly influences protozoal populations and, in turn, methane production by shaping rumen physiochemical conditions, substrate availability, and microbial interactions. High-forage diets typically promote greater protozoal populations because structural carbohydrate fermentation supports a rumen environment with higher pH and longer retention times, conditions that favor protozoal survival and activity. Under conditions that promote protozoal activity, protozoa contribute substantially to hydrogen production through carbohydrate fermentation and provide favorable niches for protozoa-associated methanogens, resulting in elevated methane emissions (Dehority, 2023; Newbold et al., 2015).

In comparison, high-concentrate diets typically reduce protozoal abundance due to decreased ruminal pH, faster passage rates, and shifts toward readily fermentable starch substrates. Acidic conditions directly inhibit protozoal growth and survival, while reduced availability of particulate substrates limits their feeding activity. Suppression of protozoa under concentrate-rich feeding systems is consistently associated with lower hydrogen availability and reduced abundance of protozoa-associated methanogens, contributing to lower methane production relative to forage-based systems (Hristov et al., 2013; Newbold et al., 2015). Consequently, diet-induced changes in protozoal populations provide a partial explanation for variation in methane emissions across feeding systems and reinforce roles of protozoa as key intermediaries linking dietary composition to methane production

Overall, protozoa influence methane production through their roles in hydrogen generation, microbial interactions, and regulation of rumen community structure. As described by Dehority (2023), protozoa function as key ecological regulators whose presence and activity can subsequently affect methane output. Understanding protozoal contributions to methanogenesis is therefore essential for interpreting variation in methane emission and for evaluating nutritional strategies aimed at mitigating methane production while maintaining rumen function and fermentation efficiency.

Protozoal Community Structure and Methane Output

Differences in methane output among ruminants are strongly associated with whether animals are faunated or defaunated, as well as with variation in protozoal population size and community composition. Naturally, beef cattle typically harbor protozoal population on order of 10^4-10^6 cells per milliliter of rumen fluid, whereas defaunated animals lack protozoa entirely. Across a wide range of studies, defaunation consistently results in lower methane emissions

relative to faunated controls, demonstrating that presence of protozoa substantially alters rumen microbial environment in ways that favor methanogenesis (Newbold et al., 2015).

Beyond binary distinction between faunated and defaunated states, methane output also varies with protozoal population density and species dominance. Systems characterized by high total protozoal abundance are generally associated with greater methane production than systems with lower protozoal populations, even when animals consume similar diets. Experimental comparisons indicate that reducing protozoal populations, without complete defaunation, results in intermediate decreases in methane emissions. This reflects a dose-dependent relationship in which decreased protozoal abundance reduces hydrogen production and associated methanogenic activity, thereby lowering methane output (Newbold et al., 2015).

Variation in rumen microbial community composition contributes to differences in methane production. Entodiniomorph protozoa often dominate protozoal communities numerically, particularly in forage-based and mixed feeding systems. Dominance of Entodiniomorphs may support sustained methanogenic activity over time, due to their persistence throughout feeding cycles and ability to maintain relatively stable population sizes. In comparison, Holotrich protozoa typically fluctuate more markedly in abundance in response to feeding events, which may lead to greater temporal variability in methane production rather than consistently elevated emissions (Dehority, 2023).

Observations indicate protozoal community structure influences methane output along a continuum, ranging from complete absence of protozoa in defaunated systems to high-abundance, Entodiniomorph-dominated communities in faunated animals. Understanding variation in protozoal population density and functional group dominance is critical for interpreting differences in methane emissions both within and among cattle populations, and for

evaluating nutritional or microbial intervention that partially suppress protozoa without eliminating them entirely.

2.3 – Essential Oils as Rumen Modifiers and Methane Mitigation Strategies

Classification of Essential Oils Used in Ruminant Nutrition

Essential oils (EOs) are complex mixtures of volatile, lipophilic secondary plant metabolites that have received increasing attention as natural rumen modifiers. In ruminant nutrition, essential oils are not classified by plant species but rather by their dominant chemical constituents, as chemical structure largely determines biological activity, antimicrobial spectrum, and stability under ruminal conditions (Burt, 2004; Calsamiglia et al., 2007). Major functional classes include terpenoids, phenylpropanoids, and related aldehydes and alcohols, each of which differ in antimicrobial potency, target specificity, and persistence in rumen conditions (Benchaar et al., 2008; Calsamiglia et al., 2007).

Terpenoids, including compounds such as thymol, carvacrol, and menthol, are among most extensively studied essential oil components in ruminant systems due to their strong antimicrobial properties. Many terpenoids are phenolic in nature and exert their effects primarily through disruption of microbial cell membranes, leading to increased membrane permeability and leakage of cellular contents (Griffin et al., 1999). Compounds tend to exhibit stronger activity against gram-positive bacteria, which partially explains their influence on rumen fermentation patterns and hydrogen balance (Calsamiglia et al., 2007; Patra & Saxena, 2010). Phenylpropanoids, including eugenol and cinnamaldehyde, are structurally distinct and often produce more selective effects on microbial metabolism, including inhibition of specific enzymatic pathways, rather than broad suppression of microbial growth.

Aldehydes and alcohols, including compounds such as citral and linalool, are less frequently evaluated as isolated additives but are commonly included in essential oil blends. They typically display moderate antimicrobial activity and are often used to complement stronger phenolic compounds, contributing to a broader functional spectrum while reducing risk of excessive inhibition of fiber-degrading microbes (Patra & Saxena, 2010). Their inclusion in blends reflects practical need to balance antimicrobial efficacy with rumen stability, as excess inhibition of rumen microbes can negatively impact fiber digestion and overall performance (Calsamiglia et al., 2007; Patra & Saxena, 2010).

From a nutritional standpoint, distinctions among essential oil chemical classes are important because chemical structure influences solubility, membrane affinity, volatility, and resistance to ruminal degradation (Calsaminglia et al., 2007; Burt, 2004). Phenolic compounds generally exhibit strongest antimicrobial effects but may negatively affect fiber digestion or total fermentation when included at excessive concentrations. In contrast, non-phenolic essential oil compounds often exert more subtle modulatory effects on microbial metabolism and may be better tolerated within rumen ecosystem, particularly when used in combination or at low inclusion rates (Calsamiglia et al., 2007).

In practical feeding systems, essential oils are rarely supplied as single purified compounds. Instead, most commercial products contain blends of multiple essential oil constituents designed to broaden antimicrobial spectra, stabilize fermentation responses, and reduce variability across animals and diets (Benchaar et al., 2008; Calsamiglia et al., 2007). However, wide variation in chemical composition, inclusion rate, and delivery method among essential oil products complicates direct comparison of biological responses across studies and contributes to inconsistent outcomes reported in literature (Calsamiglia et al., 2007).

Plant-Derived Bioactive Compounds and Their Antimicrobial Properties

Biological activity of essential oils in the rumen is primarily attributed to their antimicrobial properties (Griffin et al., 1999). Many essential oil compounds exhibit bacteriostatic or bactericidal effects through interaction with microbial cell membranes and intracellular targets (Burt, 2004). Due to essential oils being lipophilic, they readily associate with lipid bilayers, disrupting membrane integrity and altering permeability, which can interfere with ion gradients, nutrient transport, and enzyme function (Benchaar et al., 2008). Effects can suppress microbial growth or metabolic activity without necessarily eliminating entire microbial populations, allowing fermentation to continue while being functionally altered (Calsamiglia et al., 2007; Patra & Saxena, 2010).

Sensitivity to essential oils varies substantially among rumen microbial groups. Gram-positive bacteria are generally more susceptible than gram-negative bacteria due to fundamental differences in cell wall structure. Outer membrane of gram-negative bacteria acts as a permeability barrier to hydrophobic compounds, reducing essential oil penetration and conferring greater resistance (Burt, 2004). Selectivity is nutritionally relevant because many gram-positive rumen bacteria are involved in acetate production and hydrogen generation, metabolic processes closely linked to methanogenesis (Calsamiglia et al., 2007). As a result, preferential inhibition of gram-positive populations can alter hydrogen availability and downstream fermentation end products.

Protozoa and methanogenic archaea also display differential sensitivity to essential oils. Protozoa appear particularly sensitive to essential oil compounds, with reductions in protozoal abundance frequently reported at moderate inclusion rates. However, protozoal responses are inconsistent across studies and depend on compound type, dose and microbial adaptation to

essential oil supplementation (Patra & Saxena, 2010). However, methanogenic archaea are generally less sensitive to direct essential oil inhibition, suggesting that methane reductions often arise indirectly through essential oil mediated effects on hydrogen-producing bacteria and protozoa rather than through direct suppression of methanogens (Calsamiglia et al., 2007; Patra & Saxena, 2010; Ungerfeld, 2020).

Importantly, essential oils do not function as broad-spectrum antimicrobials in the rumen in ways disinfectants or antibiotics do. At low inclusion rates, essential oil compounds may selectively inhibit specific microbial groups or metabolic pathways, leading to shifts in fermentation end products without compromising overall fermentative capacity. Such selective modulation can result in altered volatile fatty acid profiles, reduced hydrogen availability, or changes in microbial interactions while maintaining fermentation activity (Calsamiglia et al., 2007; Patra & Saxena, 2009; Patra & Yu, 2012). Dose dependent, selective antimicrobial action underpins potential of essential oils as rumen modifiers rather than as agents of microbial suppression.

Antimicrobial properties of essential oils therefore provide mechanistic basis for their effects on rumen fermentation and methane production. Understanding patterns of microbial sensitivity, adaptation, and selectivity is critical for interpretating essential oil responses reported in literature and for designing supplementation strategies that modulate fermentation in targeted ways without impairing rumen function (Patra & Saxena, 2009; Patra & Yu, 2012).

Proposed Mechanisms of Action of Essential Oils in the Rumen

Disruption of Microbial Cell Membranes

One primary mechanism by which essential oils influence rumen microorganisms is disruption of microbial cell membrane structure and function. Due to their hydrophobic nature,

many essential oil compounds partition into lipid bilayers, where they alter membrane fluidity and permeability. Interactions disrupt membrane integrity, leading to leakage of ions and intracellular metabolites, dissipation of proton motive force, and disruption of transmembrane gradients required for ATP synthesis and nutrient transport. Together, the effects impair cellular energy metabolism and reduce microbial growth and activity (Benchaar et al., 2008).

Extent of membrane disruption is strongly dose dependent. At relatively low inclusion rates, essential oils may partially impair membrane function, resulting in reduced growth rates, altered enzyme activity, and decreased metabolic efficiency without causing cell death. At higher concentrations, membrane integrity is sufficiently compromised to inhibit microbial survival. Under typical rumen conditions, sublethal effects are more commonly observed, leading to shifts in microbial activity, competitive balance, and fermentation pathways rather than complete suppression of fermentation (Benchaar et al., 2008; Calsamiglia et al., 2007).

Membrane-based mode of action also explains selective antimicrobial effects commonly observed with essential oil supplementation. Microorganisms with membrane compositions that are more susceptible to lipid disruption, such as differences in fatty acid composition, membrane thickness, or absence of protective outer membranes, are disproportionately affected. As a result, essential oils can exert selective pressure that alters microbial community structure and fermentation end-product profiles (Calsamiglia et al., 2007; Patra & Saxena, 2009). Selectivity is particularly relevant for hydrogen-producing bacteria and rumen protozoa, which have been reported to exhibit greater sensitivity to certain essential oil compounds than other rumen microorganisms, thus indirectly influencing hydrogen availability and methanogenesis (Patra & Saxena, 2009; Patra & Yu, 2012).

Importantly, because membrane disruption is a relatively nonspecific mechanism, excessive inclusion of essential oils can negatively affect overall rumen function. High essential oil concentrations have been associated with reductions in fiber digestion and total fermentation activity, reflecting broad inhibition of fibrolytic and fermentative microbial populations (Benchaar et al., 2008; Calsamiglia et al., 2007). As a result, practical applications of essential oils in ruminant diets depends on identifying inclusion rates that modulate microbial activity and fermentation pathways without inducing extensive membrane damage or compromising rumen efficiency (Patra & Saxena, 2009; Patra & Yu, 2012).

Alteration of Enzymatic Activity and Metabolic Pathways

In addition to membrane-mediated effects, essential oils can influence rumen fermentation by altering microbial enzymatic activity and intracellular metabolic pathways. Certain essential oil compounds inhibit or modulate activity of enzymes involved in carbohydrate and nitrogen metabolism, thereby modifying substrate utilization and fermentation end-product formation (Patra & Saxena, 2009). Effects may occur without substantial reductions in microbial population size, indicating essential oils can alter microbial function independently of broad antimicrobial suppression.

Inhibition of specific enzymatic processes can lead to measurable changes in ruminal metabolite profiles. For example, suppression of deaminative enzymes reduces amino acid deamination and ammonia production, improving nitrogen retention within microbial biomass. Similarly, inhibition or modulation of key carbohydrate-fermenting enzymes can alter flux through central fermentative pathways, resulting in shifts in volatile fatty acid profiles. Enzymatic effects can redirect metabolic fluxes within rumen, influencing hydrogen production and utilization while maintaining overall fermentative activity (Patra & Yu, 2012).

Mechanisms of action related to essential oil supplementation are relevant for methane mitigation, as changes in microbial metabolic pathways can reduce hydrogen availability for methanogenesis. By favoring fermentation pathways that consume reducing equivalents, such as propionate formation, essential oils may indirectly suppress methane production while maintaining total fermentation and energy supply to hosts (Calsamiglia et al., 2007; Patra & Saxena, 2009). Multiple studies have reported essential oil supplementation shifts volatile fatty acid profiles toward increased propionate and decreased acetate proportions, thereby decreasing pool of metabolic hydrogen available to methanogenic archaea (Benchaar et al., 2008; Patra & Yu, 2012).

Ability of essential oils to modulate enzymatic activity and redirect metabolic pathways distinguishes them from ionophores and other traditional antimicrobial feed additives. Rather than exerting broad inhibitory effects on microbial growth, essential oils often act at sublethal concentrations to fine-tune fermentation pathways and alter end-product profiles (Calsamiglia et al., 2007; Patra & Saxena, 2009). Targeted modulation of ruminal metabolism has contributed to growing interest in essential oils as rumen modifiers for improving fermentation efficiency and mitigating methane emissions without compromising rumen function (Benchaar et al., 2008; Patra & Yu, 2012).

2.4 – Agolin©(Alltech, Nicholasville, KY) as an Essential Oil-Based Feed Additive in Cattle Production

Agolin is an essential oil blend developed to modify rumen fermentation with the goal of reducing enteric methane emissions while maintaining animal performance. Formulation consists of chemically defined plant-derived compounds, including coriander seed oil, eugenol, geranyl acetate, and geraniol, delivered at low inclusion rates (Belanche et al., 2020). Unlike studies

evaluating single essential oil compounds, Agolin research focuses on functional outcomes of blended product, recognizing that synergistic interactions among compounds may contribute to its biological activity and consistency of response.

Evidence from dairy production systems demonstrate Agolin supplementation can reduce methane emissions without negatively affecting feed intake or rumen fermentation. A meta-analysis of 23 in-vivo studies in dairy cows reported that long-term supplementation (≥ 4 weeks) with Agolin decreased methane production expressed on a daily basis, per unity of dry matter intake, and per unit of fat and protein corrected milk, while simultaneously improved milk yield and feed efficiency (Belanche et al., 2020). Reductions in methane occurred without consistent changes in volatile fatty acid profiles, suggesting that Agolin acts through subtle modulation of microbial metabolism rather than broad suppression of rumen fermentation activity.

Studies conducted in beef and dairy-beef cattle further support methane-mitigating potential of Agolin across production systems. Miller at al. (2023) evaluated Agolin supplementation in dairy-beef steers using respiration chambers and observed significant reductions in methane yield (g CH4/kg DMI) at both 46 and 116 days of supplementation, although total daily methane production did not differ between treatments (Miller et al., 2023). Findings indicate Agolin primarily improves methane efficiency relative to intake, highlighting importance of expressing methane responses in relation to feed consumption when evaluating mitigation strategies.

Recent work has expanded Agolin delivery strategies beyond feed-based inclusion, demonstrating applicability in extensive production systems. Batley et al. (2024) evaluated Agolin delivered by drinking water to beef cattle and reported reductions in methane production under both in-vitro and in-vivo conditions, with no negative effects on feed intake, water intake,

live-weight gain, or rumen fermentation parameters (Batley et al., 2024). Ability to deliver Agolin through drinking water presents a practical mitigation strategy for pasture-based systems where controlled feed delivery is challenging.

Beyond methane mitigation, Agolin has been evaluated for effects on emissions and nitrogen utilization. Carrazco at al. (2020) reported reductions in methane intensity and ammonia emissions in dairy cows supplemented with Agolin, with no changes in dry matter intake, milk yield, milk composition, or feed efficiency (Carrazco et al., 2020). Results suggest that Agolin does not compromise productivity and may contribute to improved nitrogen-use efficiency, further supporting its role as a fermentation modifier rather than a growth-limiting antimicrobial.

Together, available information indicates Agolin consistently reduces methane emissions or methane intensity across dairy, dairy-beef, and beef production systems while maintaining animal performance. Responses appear to be influenced by diet composition, production system, duration of supplementation, and delivery method, with greater consistency observed following adequate adaptation periods. While precise microbial mechanisms remain incompletely defined, current data support Agolin as a viable essential oil-based strategy for methane mitigation that aligns environmental benefits with productive efficiency.

2.5 – Effects of Essential Oil Supplementation on Cattle Performance

Essential Oil Influence on Average Daily Gain, Dry Matter Intake and Feed Efficiency

Performance responses to essential oil supplementation in cattle are most evaluated using average daily gain, dry matter intake, and feed efficiency as primary outcome measures. Across beef and dairy production systems, essential oil supplementation at recommended inclusion rates generally results in neutral effects on dry matter intake, indicating essential oils do not adversely affect palatability or voluntary feed intake (Benchaar et al., 2008; Calsamiglia et al., 2007).

Consistency in intake response is critical for interpreting subsequent effects on growth performance and energetic efficiency, as it suggests observed performance responses are not driven by changes in feed consumption.

Responses in average daily gain to essential oil supplementation are more variable than intake responses and appear to depend ono production stage, diet composition, and duration of supplementation. In growing and finishing cattle, essential oil supplementation most frequently results in no measurable change in average daily gain, with occasional modest improvements reported under dietary or management conditions (Benchaar et al., 2008). Findings suggest essential oils do not function as growth promoters but may influence how efficiently consumed nutrients are utilized.

In contrast, feed efficiently responses are more consistently affected by essential oil supplementation than average daily gain. Several studies report modest improvements in gain-to-feed ratio or production efficiency metrics in absence of changes in dry matter intake or average daily gain (Belanche et al., 2020; Miller et al., 2023). Responses suggest improved energetic efficiency, likely reflecting altered rumen fermentation patterns and nutrient partitioning rather than increased nutrient intake. Findings are consistent with proposed role of essential oils as fermentation modifiers that enhance utilization efficiency rather than as intake stimulants or anabolic agents.

Collectively, available performance data indicates implication of essential oil supplementation can occur without compromising feed intake or growth across a range of cattle production systems. Potential for modest improvements in feed efficiency under certain conditions highlights compatibility of essential oils with production goals and provides a

foundation for evaluating their mechanistic, environmental, and sustainability implications in ruminant systems.

Potential Mechanisms Linking Microbial Modulation to Performance Outcomes

Performance responses observed with essential oil supplementation are supported by changes in rumen microbial metabolism rather than alterations in feed intake or digesta passage rate. Essential oils modulate microbial activity through selective effects on enzymatic systems and metabolic pathways, leading to improvements in nutrient utilization efficiency without substantial reductions in total fermentation or digestibility (Calsamiglia et al., 2007; Patra & Saxena, 2009). These mechanisms allow fermentation to be maintained while metabolic efficiency is improved.

One proposed mechanism linking essential oil supplementation to improved efficiency is enhanced conversation for fermented substrates into host-available energy through modulation of microbial metabolic flux. Essential oils selectively influence microbial groups and enzyme systems, reducing energetically wasteful pathways while favoring production of metabolites that are more efficiently utilized by host, such as propionate and microbial biomass (Benchaar et al., 2008; Calsamiglia et al., 2007). Improvements in metabolic efficiency increase proportion of digestible energy captured for growth or production without increasing feed intake, which is consistent with reports of unchanged dry matter intake accompanied by modest improvements in feed efficiency in essential oil supplemented cattle (Belanche et al., 2020; Benchaar et al., 2008).

Protozoa represent an important microbial target through which essential oils may influence performance outcomes. Several essential oil compounds have been shown to reduce protozoal abundance or activity, either directly through antimicrobial effects or indirectly through changes in rumen environments (Patra & Saxena, 2009; Patra & Yu, 2012). Due to

protozoa contributing to bacterial predation and microbial turnover, reductions in protozoal populations may decrease intraruminal recycling of microbial nitrogen, thereby increasing flow to the small intestine. While this can improve nitrogen utilization efficiency, the net benefit is context-dependent and may be offset by negative effects on fiber degradation or rumen stability.

In addition to changes on protozoal abundance, essential oils may alter protozoal metabolic activity, with implications for hydrogen production and fermentation efficiency. Protozoa are significant contributors to ruminal hydrogen generation during carbohydrate fermentation, and suppression of protozoal activity can reduce hydrogen availability for competing metabolic sinks (Patra & Yu, 2012). By limiting protozoa-associated hydrogen production, essential oil supplementation may indirectly favor more energetically efficient fermentation pathways, thereby improving net energy utilization by the host.

Essential oils may also influence nitrogen metabolism through combined effects on protozoa and bacterial enzymatic activity. Reduced protozoal predation on bacteria, together with inhibition of amino acid deamination enzymes, can improve synchronization between energy and nitrogen availability in the rumen and enhance efficiency of microbial protein synthesis (Calsamiglia et al., 2007; Patra & Saxena, 2009). Changes support productive performance by improving nutrient capture and utilization rather than by increasing intake.

Importantly, mechanisms emphasize gains in efficiency rather than direct stimulation of performance. Essential oils act by refining microbial metabolism and protozoal activity at sublethal concentrations rather than promoting growth through increased feed intake or accelerated passage rate (Benchaar et al., 2008; Calsamiglia et al., 2007). Mode of action explains why performance responses to essential oil supplementation are often modest and

context dependent, underscoring roles of protozoa as key intermediaries linking microbial modulation to animal performance outcomes (Patra & Yu, 2012).

Importance of Maintaining/Improving Performance While Targeting Methane Reduction

From a production and sustainability perspective, methane mitigation strategies must be evaluated in context of animal performance. Enteric methane represents a direct loss of dietary energy to the animal, however, mitigation approaches that impair dry matter intake, average daily gain, or feed efficiency are not viable in commercial cattle systems (Beauchemin et al., 2020; Patra & Yu, 2012). As a result, maintaining performance while targeting methane reduction is a central criterion for evaluating essential oil-based interventions.

Evidence from studies evaluating essential oils and commercial blends such as Agolin indicate methane reductions are generally achieved without detrimental effects on animal performance. Meta-analytical and long-term in-vivo studies report reductions in methane yield or methane intensity with no adverse effects on dry matter intake, average daily gain, or feed efficiency in both dairy and beef systems (Batley et al., 2024; Belanche et al., 2020; Miller et al., 2023). Separation of environmental benefit from performance loss is particularly important, as it suggests that methane mitigation is achieved without compromising productivity.

In some cases, reduced methane intensity coincides with modest improvements in feed efficiency, suggesting lower gaseous energy losses may translate to greater net energy retention by the animal (Belanche et al., 2020). However, such responses are not universal and appear to depend on diet composition, duration of supplementation, and baseline rumen fermentation characteristics (Miller et al., 2023; Patra & Yu, 2012). As a result, performance maintenance, rather than performance enhancement, is be viewed as the primary benchmark for evaluating essential oil-based methane mitigation strategies.

For growing cattle, where dietary energy is prioritized for lean tissue growth and frame growth, strategies that reduce methane while maintaining performance are especially valuable. Essential-oil based interventions, including blended products such as Agolin, have demonstrated capacity to reduce methane emission or methane intensity without compromising growth performance or feed efficiency across a range of production systems (Batley et al., 2024; Belanche et al., 2020). Compatibility with production goals supports integration of essential oils into sustainable cattle production systems that aim to balance environmental mitigation with efficient performance outcomes.

2 - Conclusions

Enteric methane production represents both an environmental liability and a measurable loss of dietary energy in beef systems, making mitigation strategies relevant to efficient and sustainability goals. Literature reviewed here supports methane formation as an outcome of ruminal redox balance, hydrogen flux, and microbial network structure, with volatile fatty acid profiles serving as functional indicators of electron partitioning. Fermentation patterns that increase propionate production and limit hydrogen availability to methanogens consistently align with reduced methane yield and improved energetic retention, although optimization requires maintaining rumen stability and epithelial capacity for volatile fatty acid absorption (Ungerfeld, 2020; Hristov et al., 2013).

Rumen protozoa become a key intermediate linking diet composition to methane output and nitrogen-use efficiency. Protozoal predation on bacteria increases intraruminal nitrogen recycling and ammonia production, reducing microbial protein flow to small intestine, while protozoal hydrogen production and close associations with methanogenic archaea promote methanogenesis. Defaunation studies consistently demonstrate reduced methane emissions and

improved non-ammonia nitrogen supply to the intestine, yet responses in animal performance remain diet and environmentally dependent, reflecting tradeoffs between reduced methanogenesis and potential declines in ruminal fiber digestion under select feeding conditions. Findings reinforce value of approaches modulating protozoal abundance or activity without disrupting overall fermentation function.

Essential oils provide a biologically plausible and logistically feasible route or targeted rumen modification due to selective antimicrobial and metabolic effects. At appropriate inclusion rates, essential oil compounds can shift fermentation end products, alter nitrogen metabolism, and suppress protozoal activity with minimal effects on intake or growth, although responses vary with dose, diet, and adaptation. Evidence specific to Agolin indicates reductions in methane yield or methane intensity across dairy, dairy-beef and beef systems while maintaining performance, including delivery through drinking water in grazing contexts. Collectively, findings indicate essential oil-based strategies offer a practical pathway to lower methane emissions while preserving productivity, with greatest potential when supplementation is paired with diets and management conditions that support stable fermentation and efficient nutrient capture. Continued work integrating microbial ecology, hydrogen partitioning, and performance outcomes across forage-based beef systems will strengthen application guidelines and clarify conditions under which methane mitigation translates into consistent efficiency gains.

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Chapter 2 - Field Validation of a Mob Composite Sampling Protocol to Estimate Herd Parasitism in Cattle

Introduction

Gastrointestinal nematode infections impact many cattle herds in the United States with severity often varying by region. Gastrointestinal infections were found to be of greatest concern and have most of their negative impacts on animals fresh off pasture. Negative impacts of nematode infections include poor growth (Craig, 2018) and suppressed immune function (Verocai et al., 2020) which can translate to significant economic loss (Craig, 2018; Verocai et al., 2020) when cattle are placed in feedlots. Parasite infections in cattle may vary based upon location and climate. Warm, humid environments and regions with extended grazing seasons increase the survival and transmission of infective larvae on pasture increasing infection risk.

Even with basic anthelmintics, gastrointestinal nematode infections remain a great concern for cattle producers. Myers and Kieth (1993) completed a study analyzing over 5,500 cattle herds among all regions in the United States. Results revealed a 90% nematode prevalence across all classes of cattle, with comparable results in cattle herds outside of the United States (Myers & Keith, 1993).

Industry discussion estimates less than 1% of cattle on feed are surveyed for nematodes (Grant Crawford, personal communication, January 15, 2025). With a limited percentage of US cattle herds being tested, it is difficult for veterinarians, field representatives, and parasitology professionals to deeply understand the presence of anthelmintic resistance of gastrointestinal nematodes in U.S. beef cattle. Additional methodology development is needed to expand surveillance capacity and increase the number of animals surveyed.

The gold standard method to quantify gastrointestinal nematode infections is to perform a necropsy and count adult worms and larvae present in the abomasum and intestines (Preston et al, 2014). However, since necropsies are invasive and require a deceased animal, it is not a feasible method for cattle producers to survey groups of cattle within the live animal production chain. Alternatively, common field practice to determine parasite burden in live animals is to perform fecal egg counts. Fecal egg counts are completed to quantify and identify various gastrointestinal parasites including *Ostertagia, Nematodirus, Trichuris, Oesophagostomum, Haemonchus, Cooperia, Bunostomum, Strongyles*, mite and protozoa (such as Coccidia) EPG of feces (Craig, 2018).

To complete the diagnostic test, a fresh fecal sample is collected from an animal. Methods such as FLOTAC, McMaster or Wisconsin are commonly used to process and analyze fecal samples. FLOTAC is a high-precision quantitative method that uses a specialized chamber and is best used for epidemiology studies. While FLOTAC method is highly sensitive with the sensitivity of ~1 EPG, it is a less common method as it requires specialized equipment. McMaster is a quantitative method for estimating EPG with a sensitivity of ~50 EPG. While the sensitivity is low and results may vary by operator, the McMaster method is cost effective, time efficient and requires minimal equipment. The Cornell-Wisconsin test is a highly sensitive (~1-5 eggs per gram (EPG)) floatation method using a centrifuge. It is most reliable for low-burden infections with advantages of clear slides and increased sensitivity. However, the Cornell-Wisconsin method tends to be more time consuming and requires additional investment in lab equipment (Levecke et al., 2012).

Strategic timing of fecal egg counts before and after drug administration can support field efficacy for anthelmintics. Fecal egg count reduction tests (FECRT) are the preferred assay for

detection of herd level resistance of gastrointestinal nematodes of ruminants to anthelmintic drugs (Coles, G.C. et al., 1992; G.C. Coles et al., 2006). Fecal egg count reduction tests are typically used to assess anthelmintic efficacy after field application and identify potential gastrointestinal nematode resistance to anthelmintic drugs. Fecal egg count reduction tests involve sampling ~15-20 animals within a cattle herd (Coles et al., 1992). Animals are sampled at day 0 to provide a baseline parasitism infection level prior to being treated with anthelmintics. Resampling occurs again 10-14 days after anthelmintic application to determine effectiveness of the drug used, as well as determine any resistance within nematodes causing the infection. The difference in eggs counted prior to and after drug administration represents the reduction of parasites due to treatment of choice. A reduction in FEC less than 95% reveals potential anthelmintic resistance (Coles et al., 1992).

Methods are used to test for infections at the individual animal level, for companion animals (e.g., cats and dogs) or to test groups of animals. When testing groups of animals, like herds or lots of cattle, the number of tests needed are decreased to a minimum number of animals that represent the group. George et al. (2017), Rinaldi et al. (2019), and Ward et al. (1997) each completed studies comparing individual sampling to various methods of composite sampling. Each trial found high correlations between individual animal sampling and composite sampling determining composite testing is a suitable alternative to individual testing methods.

Based on knowledge of current uses of fecal egg count tests to support understanding of herd parasitism, anthelmintic efficacy and nematode resistance, the opportunity to enhance sampling methodology was recognized. It was hypothesized that a traditional fecal collection method or modified mob sampling method could be suitable for fecal egg count numerical evaluation. It was also hypothesized that a modified mob sampling approach would increase

efficiency of fecal egg count reduction test for detection of herd-level parasitism in cattle by reducing costs and time required for analysis. The trial was performed with an objective of evaluating the efficacy of a proposed modified mob composite sampling approach against existing industry fecal sampling protocols to identify levels of herd parasitism in beef cattle.

Materials And Methods

Experimental Procedures

Calf-fed and yearling cattle were selected from regions known to have high prevalence of internal parasites. Seven individuals collected fecal pat samples (n = 1,060) from selected lots of cattle placed in feedyard (n = 12) and housed at sale barn (n = 1) locations (Figure 2.1). Samples came from 53 pens of cattle ranging from 64 to 225 head per pen. After collection, the samples were brought to one of the two following locations for analysis: 1) Merck Animal Health Diagnostic Laboratory, Lawrence, KS; 2) Kansas State University, Olathe, KS. No IACUC sought because no animal handling occurred outside of owner protocols.

To account for inconsistent concentration of gastrointestinal parasite egg throughout the fecal pats due to motility of the large intestine and cecum, when collecting fecal pat samples, 15 ml were collected from three different locations on each fecal pat. Each sample was placed in a resealable plastic bag which combined the three 15 mL spot samples into a 45 ml sample that represented one fecal pat within one pen. This process was repeated 20 times per pen, resulting in 20 individual samples per pen. Samples were transported and stored at 35 degrees Fahrenheit prior to processing to prevent egg development and hatching. Once samples arrived at designated lab, they were processed by pen to test two methods: 1) Industry Standard Sampling; 2) Modified Mob Sampling.

Industry Standard Sample Processing

Industry-standard sample processing (IDV) started with the 20 fecal pat samples collected per pen. Fecal pat samples were individually homogenized inside the resealable plastic bag they were collected in by generously massaging the bag. After homogenizing each fecal pat sample, three grams from each fecal pat sample was weighed using a 2-place balance into individual smooth sided cups. Smooth sided cups were used to facilitate uniform homogenization and minimize retention of fecal matter on cup surfaces, thereby improving parasite egg recovery and reducing variability in quantitative fecal egg counts. Each three-gram sample was prepared for counting via modified Wisconsin procedure with the process repeated 20 times per pen.

Modified Mob Sample Processing

Modified mob sample processing (MOB) started with the 20 fecal pat samples collected per pen. Fecal pat samples were individually homogenized inside the resealable plastic bag they were collected in by generously massaging the bag. The 20 samples were split into 2 groups of 10 while noting which samples were assigned to each group.

For each group of 10, after individual sample homogenization, 3 grams from each sample were weighed, into a smooth sided cup resulting in 2 smooth sided cups with 30 grams of sample each. Composite samples were thoroughly homogenized in smooth-sided cups using a tongue depressor to ensure uniform distribution of parasite eggs.

Homogenization of the two 30-gram samples was completed by generously stirring samples in smooth sided cups with a tongue depressor. Liberal homogenization of samples allows for increased accuracy of fecal egg count analysis at the pen-level. After homogenizing the two 30-gram samples per pen, 3 grams were weighed out into a smooth sided cup so that 2 smooth sided cups containing 3 grams each represented each pen. These samples were then evaluated for fecal egg count using the Modified Wisconsin procedure.

Modified Wisconsin Sample Preparation

Samples for both industry standard method and modified mob method were evaluated using the modified-Wisconsin procedure by D. H. Bliss (2024b). Sheather's solution was prepared by dissolving 1.28 grams of sugar per mL of warm water until all sugar is dissolved. Sheather's solution (15ml) was added to each smooth sided cup containing 3 grams of sample. For each pen, there were 20 smooth sided cups; 2 representing industry standard method and 2 representing the modified mob method. Using a tongue depressor, fecal sample was mixed into Sheather's solution creating a consistent slurry mixture. Slurry was filtered through tea strainer into a clean cup. Filtered slurry was decanted into 15-mL falcon tube and centrifuged at 1000 rpm for 5 minutes. After centrifuging, a small amount of Sheather's solution was added to tubes to produce a reverse meniscus. A coverslip was placed on reverse meniscus and left to sit for 30 minutes to allow for gastrointestinal nematode eggs to rise. The coverslip was then placed on a microscope slide and analyzed for fecal egg counts per 3 grams of fecal matter under a microscope.

Statistical Analysis

Fecal egg counts (FEC) were expressed as eggs per 3 g (EP3G). For the industry standard method (IDV), mean was calculated as the arithmetic mean of 20 individual samples collected per pen. For the modified mob composite method (MOB), mean FEC was calculated as the arithmetic mean of two composite samples representing the same population.

Agreement between IDV and MOB sampling methods was evaluated using Lin's Concordance Correlation Coefficient (Lin's CCC) and 95% confidence intervals. Lin's CCC was used to assess precision and accuracy between the two methods and was calculated using epiR package in R (version 4.4.1; R Foundation for Statistical Computing, Vienna, Austria).

A Bland-Altman analysis was conducted to further evaluate the agreement by estimating the mean difference (bias) and limits of agreement between methods. Due to the positive nature of fecal egg counts, values were $\log_{10}$-transformed prior to analysis. Bland-Altman Analysis was performed using BlandAltmanLeh package in R.

Results And Discussion

Fecal egg counts of the sample population ranged from 3 to 390 eggs per 3 grams (EP3G) of feces (equivalent to 1 to 130 EPG), with an overall mean of 90 EP3G (approximately 30 EPG). All results were summarized at the pen level. For the modified mob approach, pen-level values represent the mean of two independent 10-sample composite subsamples derived from the same 20 fecal pat samples collected per pen. Composite size being reduced to 10 samples per composite rather than a single composite of 20 samples, as recommended for fecal egg count testing, reduced dilution effects associated with larger pooled samples while maintaining adequate representation of within-pen variability. While composite fecal egg counts reliably estimate herd-level infection, prior studies suggest that excessive pooling can reduce sensitivity as low fecal egg counts due to dilution (George et al., 2017; Rinaldi et al., 2019; Ward et al., 1997). Dividing samples into two independent 10-sample composites therefore preserve sensitivity while providing pen-level replication and allowing assessment of within-pen variability.

The distribution found was consistent with published reports for growing cattle on pasture in the Southeastern United States, where group mean fecal egg counts commonly fall within the tens to low hundreds of EPG, reflecting light to moderate gastrointestinal nematode burdens (Gasbarre et al., 1996; Hernandez et al., 2022). Infection intensity was classified as light load (1-10 eggs per 3 grams), moderate (11-50 eggs per 3 grams), and heavy (>50 eggs per 3

grams) load categories to facilitate comparison between Industry Standard and Modified Mob sampling methods (Bliss, 2024a). These categories were defined analytically rather than biologically and were used solely to assess agreement between sampling approaches. Minor numeric differences in the infection category assignment were observed between the industry-standard method (IDV) and modified mob (MOB) methods in classification of infection load; however, both techniques produced similar overall distributions across infection categories, suggesting comparable performance across the observed range of parasite burdens (Table 1.1).

Agreement between IDV and MOB sampling methods was high, as demonstrated by a Lin's Concordance Correlation Coefficient (CCC) of 0.902 with a (95% CI: 0.843 to 0.939) (Figure 1.1). Results confirm mob composite sampling provides a comparable estimate of herd-level parasitism to the industry standard sampling method.

Figure 1.3 presents results from Bland-Altman analysis of log-transformed fecal egg counts. This analysis further supports the identified agreement between the sampling methods as the mean difference between IDV and MOB sampling was -6 EP3G. This can be interpreted to indicate that the MOB sampling produced slightly lower counts than the industry-standard method. The limits of agreement ranged from -61 to 49 EP3G. Bland-Altman analysis indicated a small directional bias associated with mob composite sampling relative to individual sampling. Observed bias represents a predictable consequence of fecal pooling and likely reflects dilution of high-shedding individuals within composite samples. Minimal bias did not affect interpretation of herd-level parasitism across evaluated infected intensities.

Implementation of a two-sample mob composite method to replace the industry standard 20-sample method substantially reduced numbers of samples requiring microscopic evaluation. Reduction from 20 individual fecal pat samples per pen to two composite samples per pen

represents a reduction in laboratory workload of approximately 90% while maintaining diagnostic precision. Findings demonstrate that the modified MOB protocol offers a practical and cost-efficient alternative for assessing gastrointestinal nematode burden in commercial feedlot settings without compromising analytical reliability.

Strong agreement observed between the IDV and MOB methods supports the methodological decision to use two 10-sample composites rather than a single 20-sample composite. Use of smaller composite groups reduced dilution effects associated with larger pooled samples while maintaining adequate representation of within-pen variability. Maintaining variability remains particularly important in cattle populations, where fecal egg counts frequently remain low and egg shedding exhibits an aggregated distribution.

Results align with and extend previous work completed by George et al. (2017), which demonstrated strong agreement between individual and composite fecal egg count methods in cattle.

Conclusions

Trial results demonstrate that modified mob composite method provides a field test with confident results that has an increase in efficiency compared to traditional industry-standard methodology for estimating herd-level gastrointestinal parasitism in cattle. Findings confirm that herd-level infection status can be characterized using composite sampling reducing laboratory analysis workload by 90%.

Adoption of a MOB method has important implications for parasite surveillance in commercial cattle systems. Simplification of sampling logistics and reduction of analytical burden increases the practicality of routine fecal egg count monitoring at pen level. In turn,

improved feasibility of herd level surveillance may support broader implementation of evidence-based parasite management programs in feedlot and backgrounding operations.

The MOB method, which used two independent 10-sample composite subsamples per pen, preserved analytical sensitivity while maintaining adequate representation of within-pen variability. The small directional bias associated with mob composite sampling reflects a predictable consequence of pooling and does not compromise the application of method for herd-level parasitism assessment.

Expanded use of scalable sampling approaches, like MOB method, has potential to improve early detection of gastrointestinal nematode challenges and enhance monitoring of anthelmintic performance across diverse production settings. Use of MOB methodology has the capability of enhancing surveillance efforts and support economic performance and sustainability of cattle production systems as gastrointestinal nematodes continue to challenge productivity and anthelmintic efficacy.

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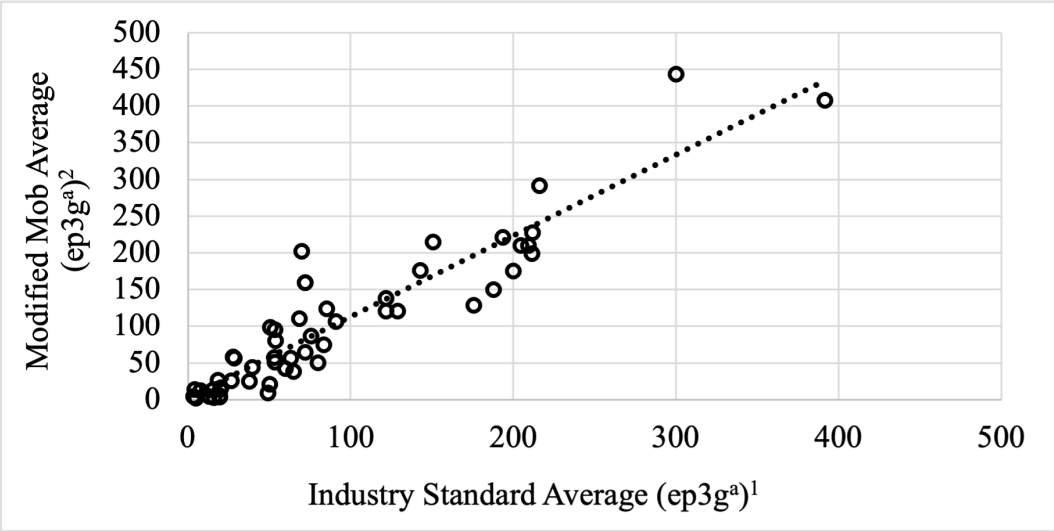
Figures

Figure 2.1. Correlation between Industry Standard Average and Modified Mob Average Fecal Egg Counts



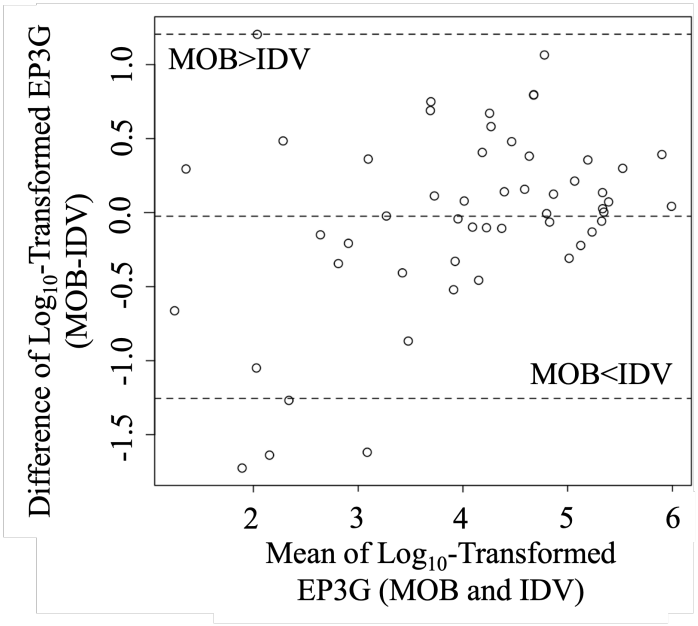
[1]Industry standard (IDV) is the average of 20 samples fecal egg count values

[2]Modified mob (MOB) is the average of 2 mob samples fecal egg count values that represent the original 20 samples

[a]EP3G: eggs per 3 grams

Figure 2.2. Field locations of fecal sample collections for the validation experiment

Figure 2.3. Bland-Altman plot comparing modified mob (MOB) and industry standard (IDV) methods for log-transformed fecal egg counts



Tables

Table 2.1. Fecal pat sample[1] fecal egg count[2] pen average results categorized by load class

Load Classes[3]	IDV	MOB
Light Load	4	7
Medium Load	15	12
Heavy Load	34	34

[1]One sample represents a pen

[2]Fecal egg counts include stomach worms (Haemonchus (barberpole worm), Trichostrongylus (bankrupt worm), Ostertagia (brown stomach worm)), Nematodirus, Cooperia (small intestinal worm), Bunostomum (hookworm), Strongyloides (threadworm), Trichuris (whipworm), Oesophagostomum (nodular worm)

[3]Load classes defined by (Bliss, 2024a)

Chapter 3 - Field Performance and Enteric Gas Emissions in Growing Beef Steers Supplemented with Agolin

Introduction

Enteric methane production represents a loss of dietary energy in ruminant livestock and contributes significantly to agricultural greenhouse gas emissions (Johnson & Johnson, 1995). Methanogenesis within the rumen is driven by microbial hydrogen turnover that occurs during fermentation (Ungerfeld, 2020). Rumen microbial populations play a very important role in determining hydrogen utilization pathways, thereby influencing the amount of methane produced (Hristov et al., 2013). Rumen protozoa are important in this process as they produce hydrogen and have close associations with methanogenic archaea, which together support methane formation. Among protozoal groups, Holotrich protozoa (Dasytrich and Isotrich) are associated with hydrogen production, and reductions in their populations may decrease hydrogen available to methanogens and potentially reduce methane emissions (Hook et al., 2010). As a result, strategies that target rumen microbial pathways involved in hydrogen metabolism may redirect energy use within the rumen and influence overall animal performance (Hristov et al., 2013).

A range of methane (CH_4) mitigation approaches, including feed supplements, phytocompounds, and genetic selection strategies, have been explored to reduce CH_4 production in cattle (Bačėninaitė et al., 2022). However, practical adoption of CH_4 mitigation strategies depend on several factors, including consistency of response, palatability, impacts on intake and performance, animal health considerations, and overall economic feasibility (Pandya & Varun, 2024). While some mitigation strategies may be effective under controlled conditions, their

application in commercial production systems requires CH_4 reductions to occur without compromising growth performance or feed efficiency.

Agolin (Alltech, Nicholasville, KY) is an essential oil-based feed additive composed of coriander oil, eugenol, geraniol, and geranyl acetate that has demonstrated antimicrobial activity against a range of rumen microorganisms (Benchaar & Hassanat, 2025). Previous work suggests that Agolin may improve feed efficiency and palatability, while reducing grams of CH_4 per day and grams of CH_4 per dry matter intake (Belanche et al., 2020). These improvements are suggested to be due to the redirection of hydrogen away from methanogenesis toward alternative fermentation pathways, such as propionate formation, without disrupting overall rumen function (Brambila & Noricumbo-Saenz, 2022). Based on previous work, Agolin is suggested an attractive CH_4 mitigation strategy for growing cattle systems, while maintaining animal performance and efficiency for system profitability.

Despite growing interest in essential oil-based feed additives, limited information is available regarding how Agolin influences rumen microbial ecology under practical feeding conditions, particularly in growing beef cattle. As previously mentioned, rumen protozoa play an important role in hydrogen turnover and are closely associated with CH_4production, with Holotrich protozoa such as Dasytrich and Isotrich commonly linked to increased CH_4 output (Dai et al., 2022). These protozoa contribute to CH_4 formation by producing hydrogen during fermentation and by forming close associations with methanogenic archaea that utilize this hydrogen to produce CH_4. As a result, protozoal populations within the rumen can influence the availability of hydrogen for methanogenesis and ultimately affect overall CH_4 emissions.

Therefore, the objective of this was to evaluate effects of Agolin supplementation on dry matter intake, growth performance, enteric CH_4 emissions, emission intensity, and rumen

protozoa population in growing steers. It was hypothesized that Agolin supplementation would reduce CH_4 emissions without negatively affecting intake or performance and reductions would be associated with shifts in rumen protozoa community composition, particularly reductions in protozoa groups associated with increased CH_4 production.

Materials And Methods

Facilities and Diet

Over 70 days, 38 crossbred steers (317 ± 34 kg) were fed and maintained in one pen at the Kansas State University Intake Unit in Manhattan, KS from February 2025 to May 2025. Steers were cared for according to the KSU Stocker Unit wellness management protocol and Kansas State University Institutional Animal Care and Use Committee (IACUC; protocol #5112). Steers were stratified by weight during allocation to treatment (n = 18 and 18, respectively).

Prior to trial start, steers were acclimated to two GreenFeed systems (AHCS; C-Lock Inc., Rapid City, SD) and Insentec feed bunks (Insentec B.V., Marknesse, Netherlands) for 52 days. Intake data was collected on all steers through Insentec feed bunks and emission data was collected on all steers using two AHCS units located within the pen.

To simulate supplementation of forage fed animals, the experimental diet was divided with cattle allowed ad libitum access to a forage blend of wheatlage and alfalfa (Tables 3.1 and 3.2) with separate limited access to a distillers-based supplement containing the treatment (Table 3.3). The forage blend consisted of wheatlage (72.05% DM inclusion), alfalfa hay (27.12% DM inclusion), and a commercially available grazing cattle supplement (0.82% inclusion; Hubbard Stockmaster® Wheat Pasture Mineral (Hubbard Feeds, Mankato, MN)). The forage blend was provided to the animals twice daily to ensure consistent availability of the forage, similar to a

grazing system. A distillers grains-based supplement was provided once daily in separate smaller Insentec modules that were set to limit daily individual animal intake of the supplement to 0.41 kg DM per head per day. Based on the treatment allocation steers were assigned to supplement containing Insentec modules, which contained:

- **AGO (Agolin treatment):** 0.41 kg DM per head per day of dry distillers grains containing Agolin, providing approximately 725.8 mg/hd/d
- **CON (Control treatment):** 0.41 kg DM per head per day of dry distillers grains

Forage samples (wheatlage and alfalfa hay) were collected weekly and compiled into composites representing two weeks for nutrient analysis. Mineral samples were collected once a month and analyzed individually. One sample from the control supplement and two samples from the Agolin supplement were collected and submitted to verify product nutrient composition. Feed and supplement samples were sent to a commercial laboratory (Servitec Inc., Dodge City, KS) and were evaluated for dry matter, organic matter, crude protein, neutral digestible fiber, acid digestible fiber, total digestible nutrients, net energy (maintenance), and net energy (gain). Values for results of analysis were averaged by ingredient (Table 3.2 and Table 3.3).

Cattle Care

All cattle were equipped with electronic identification (EID) to ensure compatibility with Insentec and AHCS equipment used for intake and enteric emissions measurement, respectively. Individual body weights were collected every 14 d using a calibrated chute scale. Average daily gain was calculated over the five 14 d timepoints of the trial for each steer.

Protozoal Enumeration

At trial start, five treatment steers and five control steers had rumen fluid collected via their esophagus to be evaluated for protozoa population. Due to challenges with sampling equipment on d 0, collections on first day of trial were stopped. With the suggested 28 d adaptation period, d 14 of trial was selected for resampling. Since the selected sampling day was still prior to the point of rumen adaptation, there was limited concern of product effects on protozoal populations for this initial baseline sample collection. Statistical analysis was used to ensure the two sampling groups did not differ in protozoal populations at this baseline timepoint. Esophageal collection of rumen fluid was repeated at trial end (day 70) using the same animals to compare protozoal numbers before and after supplementation adaptation. Rumen fluid was collected using protocol described by Lage et al. (De Assis Lage et al., 2020). After placing a speculum and esophageal tube, approximately 200 mL of rumen fluid was collected and discarded due to potential saliva contamination without removing the esophageal tube. After discarding initial collection, 500 additional mL of rumen fluid is collected and filtered through two layers of Grade 90 cheesecloth.

After filtering, samples were then processed and evaluated using the process published by Dehority (Dehority, 1984). In brief, to prevent inability to pipette larger protozoa, 10-mL pipette tips were cut off. A 1:2 dilution of 37% formaldehyde was produced to create 50% formalin solution using distilled water. Filtered rumen contents (10 mL) were pipetted into a 20 x 150 mm culture tube to which formalin (10 mL) was added to. Formalized sample (1 mL) was removed from the original culture tube into a 16 x 150 mm culture tube for staining. Brilliant green dye was created by combining 2.0 grams of Brilliant green and 2.0 mL of glacial acetic acid. Distilled H_2O was added to brilliant green and glacial acetic acid to dilute to 100 mL. Brilliant Green Dye (2 drops) was added to 1 mL of formalized sample and allowed to stand overnight.

After staining, 9 mL of glycerol were added. Using a wide-orifice pipette, 2 individual drops of dyed sample were added to a microscope slide (2 coverslips per slide). Samples were analyzed in duplicate by steer using a compound microscope. Numerical count results of Entodiniomorph, Isotrich, and Dasytrich were recorded and averaged by steer.

Statistical Analysis

The 70-d trial was divided into five 14-d periods, with d 1-28 defined as the adaptation phase. GreenFeed data were cleaned to include visits with a minimum duration of 3 minutes and minimum airflow rate of 26 L/s. Individual animal greenhouse gas emission values were estimated using a least-squares means approach adjusted for hour of day to account for diurnal variation (Beck et al., 2024).

Performance metrics (ADG, DMI, forage blend intake, supplement blend intake, total intake), gross energy intake, emissions variables (CH_4 and carbon dioxide (CO_2) production (g/d), methane yield (g CH_4/kg DMI), methane intensity (g CH_4/kg ADG) and methane energy loss(CH_4/GEI, MJ) and protozoal populations were analyzed using R (version 4.4.1; R Foundation for Statistical Computing, Vienna, Austria) with linear mixed effects models (lme4 package). Treatment was included as a fixed effect and initial body weight as a covariate. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. Two steers were removed from the data set prior to analysis due to being identified as an outlier (Cook's distance model) and mortality resulting from health complications not associated with the trial.

Dry matter intake (DMI) was evaluated by full trial (d 0-70) and post-adaptation period (d 29-70). Intake variables included forage blend (wheatlage, alfalfa and mineral), treatment (CON or AGO), and total dry matter intake (forage blend plus treatment; Table 3.4). Intake data were recorded daily on as-fed basis and converted to DMI using ingredient-specific DM

concentrations. Values were averaged across days to obtain mean DMI per animal. Individual animal gross energy (GE) intake was calculated from DMI using average ingredient gross energy values (cal/g) and averaged across the trial period.

Methane emissions were expressed as daily production (g/d), methane yield (g CH_4 /kg DMI), emissions intensity (g CH_4 /kg ADG), and energy lost as methane was calculated relative to gross energy intake. Protozoa populations (Dasytrich, Isotrich and Entodiniomorph) were analyzed as both absolute counts and proportions at baseline and d 70 (Table 3.5).

Results And Discussion

Data were analyzed to represent both entire trial duration (d 0 to d 70) and the post-adaptation to product (d 29 to d 70).

Emissions and Growth Performance

Table 3.4 summarizes performance and greenhouse gas emissions over both the full 70-day trial and d 29-70 for CON and AGO treatment groups.

Over the full trial period, there were no differences observed in ADG between treatment groups ($P = 0.38$). Following adaptation, average daily gain (ADG) differed numerically between treatments, but no statistical difference was observed. Control steers averaged 1.28 kg/d ADG and AGO steers averaging 1.35 kg/d ADG ($P = 0.42$; Table 3.4). These findings are consistent with previous research evaluating Agolin supplementation in beef cattle, where ADG was not significantly altered despite changes in methane emissions (Batley et al., 2024). Similarly, no differences in growth performance have been reported in grazing beef systems, although numerical improvements in ADG have been observed in animals receiving Agolin (Diller et al., 2025).

Over full trial period, steers supplemented with Agolin did not differ (P = 0.19) in daily CH_4 emissions compared to CON steers (223 vs. 228 g/d, respectfully). Similarly, no differences (P = 0.53) were observed in CO_2 emissions between cattle fed AGO and CON (7,372 vs 7,408 g/d, respectfully). The lack of treatment effect over the full trial period likely reflects the inclusion of the 28-d adaptation period, during which the rumen microbial populations may not have fully responded to the essential oil supplementation. Previous work has demonstrated that an adaptation period greater than 4 weeks is often required for consistent responses to Agolin supplementation due to microbial adaptation within the rumen (Belanche et al., 2020).

To better evaluate treatment effects following ruminal adaptation, data from d 29 to 70 were analyzed separately (Table 3.4). During the post-adaptation period, AGO steers exhibited reduced (P = 0.03) daily CH_4 emissions compared with CON steers (240 vs. 250 g/d), while CO_2 emissions remained similar (P = 0.15). These results suggest that Agolin selectively influenced methanogenic pathways rather than overall fermentation activity, as CO_2 production is more broadly associated with total fermentation. Similar reductions in methane production without negative impacts on performance have been reported previously (Batley et al., 2024). However, reductions in methane production are not always accompanied by changes in methane yield or intensity, highlighting variability in response depending on diet, delivery method and duration of supplementation (Batley et al., 2024).

Emissions intensity (g CH4/kg ADG) was numerically lower in AGO steers (37.3 vs 41.2 g/kg ADG; P = 0.09; Table 3.4) reflecting the combined effects of reduced methane output and maintained or slightly improved growth. Similar reductions in methane intensity or yield without detrimental impacts on performance have been reported in dairy-beef systems supplemented with Agolin (Miller et al., 2023).

Collectively, these findings indicate that Agolin supplementation reduced methane emissions without negatively impacting animal performance. From a biological perspective, this suggests that energy previously lost as methane may have been partially redirected toward productive pathways, such as propionate production, which serves as a glucogenic energy source in ruminants.

Dry Matter and General Energy Intake

From day 29 to 70, forage blend dry matter intake did not differ between treatments, with control steers consuming 6.89 kg and Agolin-treated steers consuming 6.99 kg ($P = 0.81$). Supplement dry matter intake in CON and AGO groups were similar, with 0.41 kg/d consumed by CON steers and 0.42 kg/d consumed by AGO steers ($P = 0.11$). No differences ($P = 0.44$) were observed for total dry matter intake with control steers consuming 7.57 kg/d compared with AGO steers who consumed 7.21 kg/d (Table 3.4). Results indicate that Agolin did not alter forage, supplement, or total dry matter intake during the post-adaptation period. These findings are consistent with previous studies evaluating essential oil-based feed additives, which report no effect on DMI despite changes in rumen fermentation (Benchaar et al., 2008; Calsamiglia et al., 2007). Agolin specific studies have demonstrated no differences in performance between treated and control animals while reducing methane emissions (Hristov et al., 2013; Batley, 2024), and a meta-analysis reported no effect of Agolin on intake across studies evaluating longer supplementation periods (Belanche et al., 2020).

The absence of intake differences indicates that reductions in methane emissions were not driven by decreased feed consumption. Instead, Agolin supplementation appears to have altered energy partitioning, as reflected by Ym. Methane represents a direct loss of gross energy intake

in ruminants, and reductions in Ym indicate improved energetic efficiency (Johnson & Johnson, 1995).

Methane per Dry Matter Intake

CH_4 production per unit of dry matter intake did not differ between CON and AGO groups in the post adaptation period (34.7 vs 33.5 g CH_4/kg DMI; $P = 0.63$; Table 3.4). Similarly, the methane conversion factor (Ym) between CON and AGO treatment groups did not differ in the post adaptation period (8.0% vs 7.95%; $P = 0.83$; Table 3.4). These Ym values fall within the expected biological range for beef cattle (approximately 6-10%) of gross energy intake; Johnson & Johnson, 1995), indicating overall fermentation efficiency was not substantially altered.

The lack of difference in methane yield and Ym suggests that reductions in total methane production observed in AGO steers were not proportional to intake. Previous work has reported that Agolin supplementation can reduce daily methane production by approximately 8-16% without altering methane yield or intensity (Batley et al., 2024; Belanche et al., 2020).

Collectively, these findings indicate that the magnitude of microbial shift induced by Agolin was sufficient to reduce total methane production, but not large enough to significantly alter methane production relative to unit of intake. This is consistent with the proposed mechanism of action of essential oils, which can modulate methanogenic activity without fundamentally changing overall fermentation efficiency.

Protozoa

At trial initiation, protozoa community composition did not differ between treatments. Entodiniomorphs were the dominant group in both treatments (99.3% vs. 98.5%; $P = 0.34$; Table

3.5), while Dasytrich (0.5% vs 1.1%; $P = 0.39$) and Isotrich (0.2% vs 0.4%; $P = 0.53$) represented small proportions of the total protozoa population (Andersen et al., 2023).

At trial completion following 70 days of product exposure, protozoa community composition differed between treatments. Holotrich populations were divided into Dasytrich and Isotrich. Dasytrich populations tended ($P = 0.08$) to be decreased in AGO steers (1.8%) compared to CON (3.7%). 1.0% Isotrich populations followed a similar pattern with a trend ($P = 0.06$) to be decreased in AGO steers (1.0%) compared to CON steers (2.8%). In contrast Entodiniomorphs were increased ($P = 0.03$) for AGO steers (97.2%) compared to CON steers (93.5%; Table 3.5).

The shift in protozoa population composition provides a mechanistic explanation for the observed reduction in methane emissions. Holotrich protozoa (Dasytrich and Isotrich) are known to rapidly ferment soluble carbohydrates and produce hydrogen as a byproduct (Dai et al., 2022). Methanogenic archaea utilize this hydrogen to produce methane, forming a symbiotic relationship with protozoa (Newbold et al., 2015). Therefore, reductions in Holotrich populations likely decrease hydrogen availability for methanogenesis.

In contrast, the relative increase in Entodiniomorphs may indicate a shift toward microbial protozoal populations with altered hydrogen metabolism and associations with methanogenic archaea (Ungerfeld, 2020; Newbold et al., 2015). This supports the hypothesis that Agolin selectively alters rumen microbial ecology rather than broadly suppressing fermentation.

The observed delay in methane reduction until after the adaptation period supports this interpretation. Microbial population shifts, particularly in Holotrich protozoa, require time to stabilize, which aligns with the emergence of treatment effects after d 28 (Belanche et al., 2020; Calsaminglia et al., 2007).

Conclusions

Agolin supplementation reduced enteric CH_4 emission by approximately 4% (g/d) without negatively affecting dry matter intake or growth performance. This reduction may be associated with shifts in rumen microbial ecology, particularly decreased Holotrich protozoa such as Dasytrich and Isotrich, which contribute to ruminal hydrogen production and support methanogenesis through symbiotic associations with methanogenic archaea. Reduced abundance of these protozoa may decrease hydrogen availability to methanogens and subsequently limit CH_4 formation (Hook et al., 2010). Reductions in CH_4 emissions may allow a greater proportion of energy to be retained for productive purposes such as growth as methane production represents a loss of gross dietary energy (Moss et al., 2000). Agolin supplemented steers exhibited a trend for improvement in emission intensity, producing 9.4% fewer grams of CH_4 per kilogram of average daily gain. Collectively, results suggest that Agolin supplementation may represent a viable nutritional strategy to improve enteric methane efficiency while maintaining animal performance in growing cattle.

Implications

Future research is needed to further evaluate the effects of Agolin supplementation on beef cattle performance under varying production conditions to determine whether reductions in CH_4 emissions can result in measurable improvements in cattle performance. Future studies should also work to quantify changes in emission composition and ruminal fermentation characteristics among supplemented animals to better understand the biological mechanisms driving CH_4 mitigation. Determining the optimal supplementation dosage will allow producers to maximize CH_4 reduction while maintaining or improving animal performance. Together, this work may help to refine nutritional strategies while supporting productive efficiency.

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Tables

Table 3.1. Ingredient Inclusion rates of forage blend (DM basis)

Feedstuff	Inclusion Rate
Wheatlage	72.05%
Alfalfa	27.12%
Mineral[1]	0.82%

[1]Composition of Hubbard Stockmaster Wheat Pasture Mineral (Hubbard Feeds, Mankato, MN): 97.77% DM; 16.8% Ca; 2.155% P; 3.86% Mg; 0.315% K; 0.755% S; 5.93% Na; 3375 mg/kg Zn; 2625 mg/kg Fe; 2725 mg/kg Mn; 2085 mg/kg Cu

Table 3.2. Nutrient composition of individual forage ingredients and combined forage blend

Nutrient, % DM	Combined Forage Blend[1]	Wheatlage	Alfalfa Hay
Dry Matter, % As Fed	58.3	46.2	89.1
Organic Matter	93.0	94.7	91.3
Crude Protein	13.0	10.5	20.2
Neutral Detergent Fiber	47.6	49.8	43.3
Acid Detergent Fiber	33.7	34.1	33.8
Total Digestible Nutrients	63.2	64.2	62.6
Net Energy for Maintenance (mcal/kg)	0.7	0.7	0.6
Net Energy for Gain (mcal/kg)	0.4	0.4	0.4

[1]Forage Blend: 72.05% Alfalfa; 27.12% Wheatlage; 0.82% Hubbard Stockmaster Wheat Pasture Mineral (Hubbard Feeds, Mankato, MN)

Table 3.3. Nutrient composition of control and Agolin distillers-based supplements

Nutrient, % DM	Control	Agolin
Dry Matter, % As Fed	87.6	86.7
Organic Matter	85.9	86.4
Crude Protein	32.5	31.0
Neutral Detergent Fiber	12.3	11.2
Acid Detergent Fiber	30.8	30.6
Total Digestible Nutrients	79.4	80.3
Net Energy for Maintenance (mcal/kg)	0.9	0.9
Net Energy for Gain (mcal/kg)	0.6	0.6

Table 3.4. Growth performance and greenhouse gas emissions of steers supplemented with Agolin during full trial (d 0-70) and periods 3-5 (d 29-70)

	Full Trial[2]				Periods 3-5[2]			
Item[1]	Control	Agolin	SEM	*P*-Value	Control	Agolin	SEM	*P*-Value
CH_4, g/d	228	223	3.43	0.19	250	240	3.76	**0.03**
CO_2, g/d	7408	7372	92.6	0.53	7825	7679	94.9	0.15
ADG, kg	1.37	1.43	0.04	0.38	1.28	1.35	0.05	0.42
EI, g CH_4/kg ADG	34.7	32.5	0.99	0.12	41.2	37.3	2.8	**0.09**
Forage DMI, kg	6.8	6.8	0.26	0.94	6.89	6.99	0.30	0.81
Treatment DMI, kg	0.41	0.41	0.005	0.39	0.41	0.42	0.004	0.11
Total DMI, kg	7.21	7.21	0.26	0.95	7.57	7.21	0.32	0.44
CH_4/DMI, g/kg	31.5	31.3	1.64	0.93	34.7	33.5	1.78	0.63
CH_4/GEI, MJ	7.84%	7.77%	0.28%	0.79	8.00%	7.95%	0.33%	0.83

[1]CH_4, methane; CO_2, carbon dioxide; g/d, grams per day; ADG, average daily gain; lb, pound; EI, emission intensity; Forage: wheatlage and alfalfa; Treatment: control or Agolin; Total: Wheatlage, alfalfa, mineral and treatment; g, gram; kg, kilogram; GEI, gross energy intake; MJ, megajoules.

Table 3.5. Rumen protozoa enumeration in a subset of cattle (n = 10) at the start[1] and end[2] of trial

	Start[1]				End[2]			
Item	Control	Agolin	SEM	*P*-Value	Control	Agolin	SEM	*P*-Value
Total Count	435	764	213	0.20	292	487	131	0.31
Dasytrich	1.4	3.5	1.0	0.17	6.7	6	1.7	0.78
Isotrich	0.5	1.3	0.5	0.27	5.9	4.9	1.4	0.70
Entodiniomorph	433	760	173.5	0.20	280	476	130	0.30
Dasytrich, % total protozoa	0.5%	1.1%	0.38%	0.39	3.7%	1.8%	0.72%	**0.08**
Isotrich, % total protozoa	0.2%	0.4%	0.19%	0.53	2.8%	1.0%	0.63%	**0.06**
Entodiniomorph, % total protozoa	99.3%	98.5%	0.54%	0.34	93.5%	97.2%	1.08%	**0.03**

[1]Protozoa counts prior to product exposure at start of trial

[2]Protozoa counts after 70 days of product exposure at conclusion of trial