Effects of various dietary interventions on the health and performance of Holstein bull calves following an *Eimeria bovis* infection

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Abstract

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treatment difference in the days to consume 100 g of starter with the uninfected CON and Na Percarb group taking the longest to consume 100g of starter and infected CON taking the least amount of time. There was a difference in starter intakes from day 0 to 28, the uninfected CON consumed the least amount of starter during this period when compared to all other treatment groups. Oocyst counts had a treatment x time interaction as well as a treatment x time interaction for weekly fecal scores. There were no treatment differences in any hematological variables.

Keywords: Nutraceutical, coccidiosis

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Chapter 1
Review of Literature

Introduction

Neonatal calves are susceptible to many gastrointestinal diseases during the pre-weaning period (Meale et al. 2017). These diseases are caused by viral, bacterial, and parasitic organisms. One of these parasitic diseases is coccidiosis. Coccidiosis is a disease found in many livestock species and causes hemorrhagic diarrhea and anorexia. In calves, the two most pathogenic species are *Eimeria bovis* and *Eimeria Zuernii* (Hammond et al. 1944). Before the introduction of ionophores in the 1970's, coccidiosis was moderately studied due to the concern of performance loss. It was estimated that in ruminants alone that coccidiosis caused an estimated $15 million loss in production in 1965 and in 1971 an estimated loss of $47 million in calves less than a year old (Fitzgerald 1980). These monetary losses led to a search for a successful anticoccidial drug and ionophores were first introduced into agricultural industries throughout the 1970's. Due to ionophores unique mode of action and production enhancing capabilities they became the preferred coccidiostat for the last 50 years. Recent public concern against antimicrobial use in agriculture has caused ionophores to be questioned due to their extensive use in many livestock industries (Castanon 2007). This has caused current coccidiosis research to focus on finding putative alternatives to ionophores. Hydrogen peroxide producers, sodium percarbonate and glucose oxidase, are considered environmentally friendly and have antimicrobial properties (Heinecke and Buchmann 2009, White et al. 1962). Saponin compounds have also been studied due to antiparasitic effects as well as the effects on the microbiome of poultry and ruminants (Alfaro et al. 2007, Wang et al.

2000). The present hypothesis is that supplementing the milk replacer with these 3 treatments will reduce the fecal oocyst shedding and mitigate the negative performance effects associated with coccidiosis.

Calf Gastrointestinal Tract

The calf's foregut is made up of a four-compartmented stomach which includes the rumen, reticulum, omasum, and abomasum. The first few weeks of life the calf is considered a functional monogastric due to the bypass of liquid feed from the rumen. Suckling causes the closure of the esophageal groove and creates a tube straight to the abomasum. As the calf begins to approach weaning the gut goes through various morphological changes. Ruminal development is influenced by many nutritional factors, including calf starter consumption, increasing volatile fatty acid production, and absorption leading to ruminal papillae development. Once developed, the rumen has large bacterial and protozoan populations whose main function is to degrade plant materials into energy and carbon sources for biosynthesis. As the rumen develops during the pre-weaned and into the post-weaned period, bacterial and protozoan population changes are occurring in the foregut as well as simultaneously in the lower gut. Because of the anatomy of the foregut, feed additives supplemented to pre-weaned calves have the capacity to exert effects in the lower gut, or the small intestine, due to the lack of functionality and microbiome diversity in the rumen during this period (Meale et al. 2017).

The lower gut consists of the small intestine, which includes the duodenum, jejunum, and ileum, and the large intestine composed of the cecum and colon. The first

milk the calf will receive is colostrum, which provides not only essential nutrients but immunity components including immunoglobulins. Immunoglobulins (Ig) are antibodies found in colostrum and are transferred across the lumen of the small intestine by pinocytosis. Pinocytosis is the engulfment of macromolecules, like Ig, into cells. This allows for maternal antibodies to be transferred from the dam to the calf to assist in humoral protection against gastrointestinal disease. This is termed passive transfer of immunity. Between 6 and 24 hours after birth the small intestine of the calf goes through gut closure, where large macromolecules such as Ig will no longer be absorbed intact via transcytosis. If a calf does not receive adequate colostrum, whether in quality and/(or) quantity, this is called failure of passive transfer (FPT). Calves with FPT are considered high risk for disease and suffer the risk of stunted growth parameters. These calves are also not able to protect themselves from pathogens without diminishing their normal growth (Robison et al. 1987). Factors that result in FPT include low quality and volume of colostrum as well as a prolonged duration between birth and the administration of colostrum (Weaver et al. 2000).

The intestines are full of anaerobic bacteria that help digest food and aid in absorption. Intestinal microbial colonization changes dramatically throughout weaning. The major phyla of the intestines are Bacteroides, Proteobacteria, and Firmicutes, and the dominant phyla of the gastrointestinal tract are highly dynamic throughout the first few months of life. The microbiota diversity and abundance of the intestines is important for the development of immunological and physical defense mechanisms. Disease and use of antibiotics could potentially decrease or shift the resident microbiota allowing for

potential colonization of pathogenic bacteria and ultimately cause negative immunological effects (Meale et al. 2010).

Enteric Disease

The gastrointestinal tract is an important system not only for absorption of nutrients and metabolism, but for protective mechanisms against pathogens. The number one cause of morbidity and mortality in the calf population is gastrointestinal disease. Calves go through a very stressful transition during weaning with many digestive tract development changes which in turn raises susceptibility to disease. Pathogens, which include bacteria, viruses, and parasites, cause dehydration, anorexia, and diarrhea in young calves. These symptoms lead to loss of growth efficiency and overall, negatively impacts the farm's income. The severity of disease is impacted by factors such as nutrition, animal density, weather exposure, and FPT. Some of the more common bacterial scours are caused by *Escherichia coli* and *Salmonella spp*. *E. coli* tends to infect calves between 2-5 days of age, whereas *Salmonella* infects calves around 2 weeks of age or older. Viral infections are typically seen in calves between 5-30 days of age. The most common viral enteric diseases are caused by rotavirus and coronavirus. Major parasites that infect neonatal calves are of the protozoan species and include *Cryptosporidium parvum* and coccidia species. *Cryptosporidium* is seen in younger calves while coccidiosis mainly affects calves older than 30 days of age. This is due to the differing life cycles of the parasites. Coccidia is also currently highly controlled through coccidiostats in starter feed, including ionophores such as Monensin (Navarre et al. 2000).

Coccidia

The coccidian species are protozoan parasites that cause coccidiosis in various species of animals. These protozoans are ubiquitous and can live in many different environments. The infection of coccidia is caused by *Eimeria, Isospora, Sarcocytis, Besnoitia,* and *Toxoplasma gondii* species (Fitzgerald 1980). Though coccidia is a significant parasite in the poultry industry, it also has a significant impact on the health of young calves. Clinical coccidiosis is mostly caused by the species *Eimeria bovis* and *Eimeria zuenrii* in calves (Cornelissen et al. 1994).

Life Cycle

The *Eimeria* spp. life cycle consists of asexual and sexual reproduction in three stages. These three stages are called schizogony or merogony, gametogony or gamogony, and sporogony. Throughout the duration of the paper the first and second stages will be referred to as merogony and gamogony, respectively. The oocytes are broken down by the hosts digestive system, mechanically and enzymatically, and the sporozoites penetrate the intestinal epithelial cells to begin the cycle. The asexual stage, merogony, takes place in the intestinal cells of the host. The sporozoite matures into a schizont, which when ruptures releases merozoites. Merozoites are asexually produced and then matured in the infected cell. Once matured, the infected cell ruptures which releases the merozoites to infect other cells. This stage will repeat itself for several generations before moving to the sexual stage. Gamogony, the sexual stage, also takes place in the host's intestinal cells. The merozoites invade other cells and produce either macrogamonts or microgamonts, these then mature to macrogametes and microgametes. A zygote is formed when a

flagellated microgamete fertilizes a macrogamete. A protective cyst wall forms around the zygote and creates an oocyst. The oocyst is resilient and protects against harsh environments. The non-sporulated oocyst enters the intestinal lumen of the host and is ejected through defecation. Sporogony, the transmission stage, takes place outside of the host in moderate and moist environments. After sporulation through meiosis, the oocyst is made up of four sporocysts which each house two sporozoites, the infective units. Depending on the *Eimeria* species the cycle can take 14-21 days to complete (Jolley et al. 2006). The sporulated oocysts can then be transmitted through contaminated environment, feed, and water.

Intestinal Damage & Diagnosis

The symptoms observed in clinical coccidiosis include hemorrhagic enteritis which is caused by lysis of the intestinal cells and it produces dysentery, dehydration, and anorexia. Coccidia causes the most damage in the large intestine of calves. This severely weakens the colon's ability to manage water absorption. The virulence of coccidiosis depends on the species of coccidia, the host's age, the number of oocysts ingested, concurrent infections, and farm management practices (Cornelissen et al. 1994).

A previous study performed on eight Holstein bull calves, 4 to 6 weeks of age, infected with 100,000 oocysts of primarily *E. bovis* showed the histopathological damage inflicted on the intestines. On days 16, 18, 19, 20, 21, 22, 24, and 26 post-infection a calf was killed, and the intestinal damage was assessed grossly and microscopically. When examining the intestines grossly, it was evident that the distal ileum, spiral colon, and the cecum were most affected. On day 16 post-infection, edema was apparent in both the

mucosal and submucosal layers of the cecum and colon. By day 18, that mucosa had thickened and was accompanied by fibrin strands. Day 19 through 21, visible lesions began to form, and the colon became congested and ulcerated. Diphtheritic membranes and fibrin strands became more prominent. Ecchymosis and edema extended from the mucosal layer to the muscle layer. On days 22 through 26, the colon had a granulated appearance with thickened membranes and blood spots. The tissues were then examined microscopically. In the distal small intestine, the first generation schizonts were seen starting day 16 to 18. By day 20 only a few remained and by day 24 they were gone completely. The schizonts were harbored in the cytoplasm of endothelial cells in the central lacteals of the villi. The infected cells exhibited nuclear and cytoplasmic enlargement. Blood vessels and lymphatics were congested and dilated. On days 20 through 23 the villi attained a blunted appearance and cryptal abscesses began to form. In the large intestine the cecum was predominantly infected. Day 16 through 18 showed very high infestation of microgamonts and macrogamonts. The lymphatic glands along with the blood vessels were substantially dilated and the mucosal layers were edematous. Day 19 through 21 exhibited an increase in the number and size of lesions. The cecum and colon both showed necrotizing diphtheritic membranes. The damage to the cecum mucosa was severe. The epithelium was blunted and diminished or completely ulcerated and destroyed. The capillaries were exceptionally thrombosed. The colon was slightly less damaged than the cecum. By day 21 the large intestine, especially the cecum, was demolished. The mucosal appearance was dense and granular with flattened epithelium. Days 22 through 26 showed dramatic lesions, fibrin, erythrocytes, dense tissue, granulocytes, and a profusion of oocytes (Friend and Stockdale 1980).

Host Immune Response

Upon primary infection with *E. bovis* there is a brief proliferation of peripheral blood mononuclear cells (PBMC) during the prepatent period. Starting on day 6 post-infection not only is there proliferation of PBMCs, but the appearance of parasite-specific antigens on host cells can be seen. On day 8 proliferation activity peaks and then rapidly declines thereafter. During this period there is also an increase in antigen-specific IFN-γ which indicates T_H1 associated actions during the early stages of infection. During the patent period the cytokine IL-4 becomes more influential. The T-cell subset $CD4^+$ dominates primary infections; specifically, $CD4^+$ T-cells are first seen in the ileum in late prepatency (day 8) and eventually expand to the colon (day 40) as the infection progresses. In primary infections $CD8^+$ T-cells are only found in the colon, and $\gamma\delta TCR^+$ T-cells are found in both the ileum and colon. When calves are challenged again, also termed a secondary infection with *E. bovis*, both $CD4^+$ and $CD8^+$ T-cells comprise the ileum and colon. There is also a lack of T_H1 activity during this secondary infection which could indicate early elimination of parasites or non-IFN-γ dependent actions. (Taubert et al. 2008, Suhwold et al 2010). Once a host is infected with a specific strain of coccidia the host seems to have protective immunity against future re-infections with the same species.

Management, Prevention, and Treatment

Good management practices play a significant role in keeping shedding counts low. Keeping feed off the ground and housing the animal in a dry, well-ventilated hutch

will help reduce exposure to oocysts. Disinfecting hutches and moving the hutch to new grounds after each calf will help lower contraction from other calves.

Current methods of prevention and treatment of coccidiosis include ionophore antibiotics and other synthetic compounds. Ionophores have been the preventative of choice in the poultry industry since 1972 and it continues to be the most widely adopted strategy. Though ionophores do not have any cases of antibiotic resistance, they are lumped with other antibiotics scrutinized by the public because of their use in agriculture (Noack et al. 2019). This public pressure against the use of ionophores has set in motion the search for an alternative anticoccidial therapy.

Economic Impact

Due to the high pathogenicity of *Eimeria bovis* infection in calves, both clinical and subclinical, the economic loss suffered by the cattle industry is significant. After a severe clinical bout of coccidiosis feed and water consumption may take 6-13 weeks to recover to pre-infection amounts. Though once recovered from the disease and weight gain begins at a normal rate, it will never recover the lost potential for growth of when it was sick. (Fitzgerald 1980). Specifically, in the dairy industry persistently scouring calves have a decreased average daily gain. This will cause a delay in reaching an acceptable weight for reproduction parameters. Due to the animal's delay in lactation, there will be profit loss from feeding cost. Lower body condition scores will also negatively impact milk yields and fat to protein ratio. (Samarütel et al. 2006). A study on *Cryptosporidium muris*, another protozoan parasite, for persistently parasitized dairy cows showed a 13% decrease in overall milk yield (Esteban and Anderson 1995)

Ionophores

An ionophore is a molecule that can bind to ions reversibly. In the early 1970's the ionophorous antibiotic monensic acid, marketed as monensin, was approved for the use as a coccidiostat in the poultry industry. In 1975, it was approved for supplementation to feedlot cattle to increase feed efficiency as well as to control coccidia species. Monensin was not approved for use to increase milk production efficiency until 2004. The selling points of monensin include increased energy metabolism, increased nitrogen metabolism, and decrease of disease due to a shift in ruminal fermentation. Monensin is a by-product of the fermentation of *Streptomyces cinnamonensis* and it is a pentacyclic chemical compound with a molecular formula of $C_{36}H_{62}O_{11}$. This ionophore can adopt a circular shape with the oxygens comprising the middle of the molecule. This configuration along with the polar and non-polar regions allows the molecule to interact with cations to form an ionophore-cation complex. Different ionophores prefer either divalent or monovalent cations. In the case of monensin, monovalent cations are favored. Monensin is considerably small in size which allows it to selectively diffuse across the cell membrane of certain bacteria and protozoan species. The alkyl groups extend across the outer membrane of the molecule allowing the ionophore to solubilize in the lipid bilayer of the microorganism's cell membrane. Ionophores affect gram-positive bacteria due to the lack of a protective outer membrane. The ionophore ultimately impedes the cells sodium-potassium pumps. It persistently pumps hydrogen into the cell which activates sodium ATPase pumps. The constant utilization of these pumps requires increased ATP expenditure. The hydrogen-sodium antiporter pumps hydrogen out of the

cell as sodium enters the cell. The organism is not able to sustain normal metabolic function. With the decrease of gram-positive bacteria this allows the increased growth of gram-negative bacteria. Gram-negative bacteria boost the production of propionate and increases the overall efficiency in the rumen. This aspect also decreases the amount of methane produced during ruminal fermentation. Monensin uses the same mechanism to terminate protozoan species. The high influx of sodium causes water to enter the cell and eventually causes the parasite to burst. It is important to note that ionophores attack the parasite before it infects the intestinal cell, so it is most efficiently used as a prevention method, not a treatment (Chapman et al. 2010). Due to ionophores unique mode of action against parasites the development of resistance is slow. Ionophores are not considered medically important in humans due to the fact that they are not used in human medical practices (Noack et al. 2019). In recent years, the growing concern for the use of antibiotics in livestock has increased, and this includes ionophores. Research has now begun to study alternative therapies to potentially replace ionophores in the livestock industry.

Hydrogen Peroxide

Hydrogen peroxide is a common antimicrobial compound used in household products and medicine. Hydrogen peroxide is generally considered safe and environmentally friendly. It is produced in many biological functions and is regarded as ubiquitous. The lactoperoxidase (LP) system is found in milk products with antimicrobial effects such a preservation and overall milk quality. Lactoperoxidase is naturally secreted in milk, but by itself does not have any antibacterial properties. It is the addition of

hydrogen peroxide through enzyme reactions, like glucose oxidase with glucose, and end-products of reactions, like sodium percarbonate, that activates the LP system. This system is successful in eliminating gram-negative bacteria in the stomach and upper intestine of calves. The lower digestive tract does not benefit from the antibacterial effects due to the lack of O_2 present, causing an inability to form hydrogen peroxide. When feeding milk replacer with a functional LP system it consequently showed a decrease in overall dry matter intake (Reiter et al. 1980). There is a lack of literature on the effects of hydrogen peroxide supplementation on calf digestive health and performance. Musser patented a product that included adding a halide and a hydrogen peroxide source to milk replacer. The trials showed an increase in overall weight gain, an increase of 25% in starter intake compared to the control, and increased feed efficiency of at least 20% (Musser et al. 2016). A second trial was conducted utilizing the patented product. Even without the addition of the halide there was an increase in body weight throughout the weaning period in supplemented calves when compared to the control. Though there is limited literature about hydrogen peroxide inhibiting coccidia species there is evidence that hydrogen peroxide is effective against *Cryptosporidium*, another protozoan parasite impacting calf health. Isolated *cryptosporidium* oocysts were exposed to different concentrations and exposure times of H_2O_2 and assessed for viability and infectivity. The results did show cryptosporidial activity of the disinfectant. (Hermida et al. 2006).

Sodium Percarbonate

Sodium percarbonate (Na Percarb), $2Na_2CO_3\ 3H_2O_2$, is an oxidizing agent used in many household cleaning products. Sodium percarbonate is non-toxic and considered safe for the environment. It is most notable for its source of hydrogen peroxide.

$$2Na_2CO_3 \cdot 3H_2O_2 \rightarrow 3H_2O_2 + 4Na^+ + 2CO_3^{2-}$$

The peroxide is loosely bound and is easily liberated by water. This aspect has made Na Percarb a popular compound to study in aquaculture. It has shown to be effective against many fish ectoparasites, including the common protozoa *Ichthyophthirius multifilis* (Heinecke and Buchmann 2009). The antiparasitic properties are mainly attributed to hydrogen peroxide production. Sodium percarbonate has also been shown to reduce *E. coli* growth when added to composting chicken manure. To the authors knowledge there is no literature on the supplementation of Na Percarb as a feed additive in livestock species.

Glucose Oxidase

Glucose oxidase (GOD) is an enzyme that oxidizes glucose to gluconic acid by using oxygen as an electron donor and concurrently producing hydrogen peroxide.

$$H_2O + O_2 + C_6H_{12}O_6 \xrightarrow{GOD} C_6H_{12}O_7 + H_2O_2$$

Due to this production of hydrogen peroxide, GOD is a natural antimicrobial agent. A great example of this property is honey. Honey is known to have antimicrobial properties and research showed that hydrogen peroxide produced from GOD is the source (White et al. 1962). In one study, GOD inhibited seven common food-borne pathogens, which may also prove the enzyme to be a possible food preservative (Tiina and Sandholm

1989). In another study, GOD was tested with liquid eggs to test the preservative properties. It confirmed that the antimicrobial effects come from the production of hydrogen peroxide (Dobbenie et al. 1994). Glucose oxidase was also listed as the source of hydrogen peroxide in LP system studies. One particular LP study was successful in showing the inhibitory effects this system had on gram-negative bacteria (Bjorck et al. 1975). It is important to note that the decrease in pH due to the coinciding production of gluconic acid may also contribute to the antimicrobial effects. When GOD was fed directly to chickens as a feed additive the enzyme improved many aspects of broiler health, including increased nutrient absorption, increased enzymatic activity of the small intestine and an improved gut barrier when compared to an unsupplemented control. The GOD also effected the gut by promoting growth of beneficial gut bacteria without diminishing the diversity of the gut microbiome. Finally, GOD had increased daily body weight, meat quality, and increased gut digestibility compared to a unsupplemented control (Wu et al. 2018). There were similar beneficial effects when GOD was supplemented to piglets, including a decrease in *Salmonella* fecal shedding rates (Hou et al. 2017, Tang et al. 2016). These studies demonstrate how GOD would not only work as an antimicrobial agent, but also as a possible successful natural feed additive. To the authors knowledge there is no research on the supplementation of GOD to calves.

Saponin

Saponins are chemical compounds extracted from various plant species. The saponins appear to serve a protective mechanism for the plants attributable to its toxicity to some organisms. Many of these plants were traditionally used for medicinal purposes

as well as soaps due to its ability to create a foam. Saponins chemical makeup consist of a sugar moiety linked to an aglycone. The sapogenin, the lipophilic aglycone, gives rise to 3 different classes: triterpene, steroidal, steroidal alkaloid. Due to the saponins amphiphilic attributes it can stabilize emulsions which makes it useful in many industries. Some other notable properties include hemolytic activity, bitter taste, and possible antimicrobial effects. Saponins lyse cells by disrupting the cell membrane. They change the lateral organization of the bilayer and disrupt lipid rafts. The ordering effect of cholesterol is also inhibited. These modifications ultimately kill the cell whether it be by apoptosis, inhibiting the cell cycle, or just permeabilizing the membrane. *Yucca schidigera* extract (YE) and Fenugreek are two plant sources that contain steroidal saponins. Yucca extract impacts rumen fermentation by stimulating starch-digesting bacteria while hindering fibrolytic bacteria. The extract also disrupts gram-positive bacteria more than gram-negative (Wang et al. 2000). The rumen protozoal population is decreased when yucca is supplemented, which means greater nitrogen is possible in the rumen, causing more microbial protein to be absorbed in the intestine. Several studies also show that adding Yucca to ruminants' diets may directly and indirectly affect ammonia production (Wallace et al. 1994; Hristov et al. 1999). These various effects positively impact feed efficiency. Yucca extract has shown possible antiparasitic characteristics in several studies in livestock and poultry. When adding YE to *Eimeria* infected broiler chicken diets, along with a coccidiostat vaccine, an increase in villi length in the duodenum and a greater weight gain and feed conversion rate occurred than in any other treatment group (Alfaro et al. 2007). Another broiler study showed that chickens treated with a mixture of YE and glutamine had no lesions or immature

parasites in the intestines (Galli et al. 2017). A study performed in sheep with alfalfa saponins, oleanane triterpenoids, showed a decrease in the rumen protozoa population by the saponins affinity towards cholesterol and the subsequent lower ruminal pH. The decrease in the ruminal protozoan population was a sustained throughout the four periods of the experiment (Klita et al. 1996). Similarly, a study supplementing alfalfa saponins to sheep found a decrease in the protozoan population in the rumen when compared to a control group (Lu and Jorgenson 1987). Though alfalfa saponins belong to a different class, they also disrupt the cholesterol membranes of microbes like the steroidal class. The improvement of growth parameters along with its possible antiparasitic properties may prove saponin products to be a good substitute for current coccidiostats. Fenugreek, to the authors knowledge, has not been supplemented to livestock species.

Conclusion

Coccidiosis is a gastrointestinal disease caused by a protozoan parasite in various livestock and poultry species. The coccidian species are ubiquitous and hard to eradicate from the environment due to their protective oocysts shell. Clinical symptoms include hemorrhagic diarrhea and anorexia which cause performance loss in both subclinical and clinical disease. Since the 1970's, ionophores have been successful in limiting the negative effects of coccidiosis while also serving as a performance enhancer in livestock and poultry industries. Due to recent public perception on the use of antibiotics in agriculture, ionophores have been publicly scrutinized. Due to the concern of possible restrictions on the use of ionophores, the search for natural coccidiostat feed additives is of interest.

Chapter 2
Effects of various nutritional interventions on the health and performance of Holstein bull calves following an *Eimeria bovis* infection

Abstract

The objective of the current study was to determine the effects of sodium percarbonate, glucose oxidase, and a saponin-based product on the performance, health, and hematological variables of *Eimeria bovis* infected calves. Ninety Holstein bull calves were transported to the Texas Tech Calf Research Center in New Deal, TX, from a commercial calf ranch. Upon arrival calves were randomly assigned to one of five treatments: negative control not infected with oocysts and no treatment in the milk replacer (uninfected CON), a positive control group that was infected with 130,000 oocysts and did not receive treatment in the milk replacer (infected CON), a treatment group infected with 130,000 oocysts and 2g/hd/day of Sodium Percarbonate (Na Percarb) supplemented in milk replacer, a treatment group infected with 130,000 oocysts and 0.18g/hd/day of Glucose Oxidase (GOD) and 4.6g/hd/day of dextrose supplemented in milk replacer, and a treatment group infected with 130,000 oocysts and 2g/hd/day of Yucca/Fenugreek extract (Saponin) supplemented in milk replacer. All infected calves received an oocyst mixture of 90% *Eimeria bovis*, 5% *Eimeria zuernii*, and 5% other *Eimeria spp*. Body weights and blood samples were collected on d 0, 7, 14, 28, 56, and 84. Blood was analyzed for a complete blood count. Fecal samples were collected on d 14, 21, 23, 25, 27, and 35 post-infection through rectal stimulation and analyzed for fecal oocyst counts via the McMaster method. All repeated, continuous data were analyzed by restricted-maximum likelihood ANOVA using the Mixed procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). There was a treatment difference in mortality when the

calves were less than 21 days of age with both of the CON groups having the greatest mortality during this period when compared to all other treatment groups. There was a treatment difference in the days to consume 100 g of starter with the uninfected CON and Na Percarb group taking the longest to consume 100g of starter and infected CON taking the least amount of time. There was a difference in starter intakes from day 0 to 28, the uninfected CON consumed the least amount of starter during this period when compared to all other treatment groups. Oocyst counts had a treatment x time interaction as well as a treatment x time interaction for weekly fecal scores. There were no treatment differences in any hematological variables.

Keywords: Nutraceutical, coccidiosis

Introduction

Coccidiosis is a gastrointestinal disease caused by a protozoan parasite in livestock and poultry. The coccidian species are ubiquitous, and it is common for livestock to have small numbers of coccidia oocysts in their intestines. In calves the two main species that cause clinical disease are *Eimeria bovis* and *Eimeria zuernii* (Hammond et al. 1944). Clinical symptoms include hemorrhagic diarrhea and anorexia which cause performance loss. Subclinical coccidiosis is of concern due to hidden production loss (Fitzgerald 1980). Since the 1970's, ionophores have been utilized in the livestock and poultry industries and are used as a feed additive to improve health and performance (McGuffey et al. 2001). Due to public pressure on the use of antibiotics in agriculture, ionophores have been publicly scrutinized in recent years. Due to the concern of possible restrictions on the use of ionophores, the search for natural coccidiostat feed additives is of interest.

Saponins are a popular natural antimicrobial studied in recent years due to its possible antiparasitic and performance effects (Wang et al. 2000). Two newly studied products, sodium percarbonate (Na Percarb) and glucose oxidase (GOD), are hydrogen peroxide (H_2O_2) sources. It is hypothesized that the H_2O_2 has antimicrobial effects in the gastrointestinal tract. Sodium percarbonate is an environmentally friendly substance mainly studied in aquaculture with antiparasitic effects on ectoparasites found on fish (Heinecke and Buchmann 2009). Evidence of glucose oxidase anti-parasitic properties is scarce, but it was reported to have antimicrobial properties when fed to piglets and chickens (Hou et al. 2017). There is limited knowledge on the H_2O_2 producing products as coccidiostats in neonatal calves.

The objective of the current study was to determine the effects of sodium percarbonate, glucose oxidase, and a saponin-based product on the performance, health, and hematological variables of *E. bovis* infected calves. It was hypothesized that these three treatments would reduce oocyst shedding and mediate the negative performance effects associated with coccidiosis.

Materials and Methods

Study Design

A total of 90 newborn Holstein bull calves were randomly assigned to 1 of 5 treatments (n=18). Treatments included: negative control not infected with oocysts and no treatment in the milk replacer (Uninfected CON), a positive control group that was infected with 130,000 oocysts and did not receive treatment in the milk replacer (Infected CON), a treatment group infected with 130,000 oocysts and 2g/hd/day of sodium percarbonate (Na Percarb) supplemented in milk replacer, a treatment group infected with 130,000 oocysts and 0.18g/hd/day of glucose oxidase (GOD) and 4.6g/hd/day of dextrose supplemented in milk replacer, and a treatment group infected with 130,000 oocysts and 2g/hd/day of Yucca/Fenugreek extract (Saponin) supplemented in milk replacer. All infected calves received an oocyst mixture of 90% *Eimeria bovis*, 5% *Eimeria zuernii*, and 5% other *Eimeria spp* that was added to the morning milk replacer feeding. All calves were fed 454 g of a colostrum supplement (Fortify Complete, MB Nutritional Sciences, Lubbock, TX) reconstituted with 40.5-degree Celsius water to a total volume of 2 L for the first milk feeding. The colostrum supplement provided approximately 50 g of IgG per calf from bovine plasma and whey fractions. After feeding, the calves were given

a preventative 5mL dose of Resflor. Peripheral blood was drawn via jugular venipuncture to determine total serum protein (TSP) concentration as an indirect measurement of passive immunity. Briefly, the blood collection tube was centrifuged at 1200 x g for 5 minutes and a transfer pipette was used to drop one drop of serum onto a hand held refractometer to measure TSP.

Calf Feeding and Care

The current studies protocols and procedures were reviewed and approved by the Texas Tech University Institutional Animal Care and Use Committee. This study was conducted at the Texas Tech Calf Research Center in New Deal, TX from August 13th, 2020, until November 6th, 2020. Ninety day old Holstein bull calves were sourced from a commercial calf ranch in Eastern New Mexico and transported 150 km to the Texas Tech Calf Research Center. Calves originated from 19 different farms before being brought to the commercial calf ranch. Calves were housed outdoors in individual calf hutches with an attached pen (3.81 x 1.24 m SL Calf Hutch, Agri-Plastics, Cortland, NY) and bedded with sand.

Milk replacer and calf starter nutrient analysis are outlined in Table 1. Calves were given 700 g of milk solids split between two feedings per day at 13.2% dry matter of a 22% protein and 20% fat non-medicated milk replacer (Milk Specialties Global, Eden Prairie, MN). A complete non-medicated calf starter pellet was formulated at 20% crude protein (Hi-Pro Feeds, Friona, TX). Calves were offered a colostrum replacer for their first feeding on d 0 and all calves received 25 g of starter upon arrival to the farm. Milk replacer was fed daily beginning on d 1 at 0700h and 1600h, with milk refusals

being recorded after each feeding. Milk tank and bottles were washed immediately after feeding with hot water and chlorine. Calf starter and water were offered *ad libitum* from day 0 to 84 and refusals were recorded daily. Water buckets were washed weekly with chlorine to prevent algae and bacterial growth. Health scores, including fecal, eye, ear, nasal and cough scores, were recorded daily by trained observers from day 0 to 84 after each morning feeding based on the School of Veterinary Medicine at University of Madison-Wisconsin health scoring system. Briefly, a fecal score of 0 correlates to normal feces, a fecal score of 1 is slightly loose feces, a fecal score of 2 is diarrhea, but does not sift through the bedding, and a fecal score of 3 is watery diarrhea. Calves with a fecal score of 3 or refusing equal to or greater than 1,500g as-fed milk replacer per feeding were offered 2 L of oral electrolytes (ELECTROLIFE Renew: MB Nutritional Sciences LLC, Lubbock, TX). Antibiotic administration and electrolyte consumption were recorded accordingly. Calves were stepwise weaned beginning at 49 d of age where they began to receive milk replacer only in the morning, with the daily dose of each treatment being added completely to the morning milk. At 56 d of age calves were fed milk in the morning and then were fully weaned, where milk was no longer offered thereafter. Calves were monitored for immediate treatment carry-over effects on performance or health through day 84.

Weather was recorded daily for the entirety of the study. There was a total of 9 precipitation events which averaged 0.94 cm per event. There was an average high temperature of 27.7℃ and an average low temperature of 12.7℃. The highest temperature over the course of the study was 41.1℃ and the lowest temperature was -5℃.

Data Collection

Milk refusals were recorded daily. Daily water and starter intake were calculated by measuring the difference between the amounts offered from the previous day and refused each morning. Performance measures included weight, height, heart girth, and length. Height is the measurement from the bottom of the hoof to the top of the scapula when the calf is standing straight. Heart girth is the measurement taken around the ribs behind the scapula. Length is the measurement from the scapula to the tail bone when the back is flat. These measurements were taken by a single trained individual by tailor's tape on d 0, 7, 14, 56, and 84.

Health scores were taken daily after each morning milk feeding and included fecal, eye, ear, nasal, and cough scores. Any calf that exhibited a health score of 5 was administered Resflor (Resflor Gold, Merck Animal Health). If the calf had a health score of 4, a rectal temperature was taken. If the calf had a fever of 40℃ or greater, then the health score became a 5 and Resflor was administered. Any calf lying laterally recumbent received Resflor immediately. Persistent disease was treated with Excede (Excede, Zoetis Animal Health) followed by a 3-day monitoring period. Individual treatment records for all calves were collected and included: electrolytes offered, electrolytes refused, and antimicrobial treatments. Post-infection any calf that had severe diarrhea of clinical note, including blood or intestinal sloughing, was recorded as well.

Blood and Fecal Samples Collection and Analysis

Fecal samples were taken from each calf on d 14, 21, 23, 25, 27, and 35 post-infection to quantify coccidia oocyst shedding. The fecal samples were collected directly from digital rectal stimulation and immediately placed into a sterile bag. The samples were then taken directly to the laboratory and assessed following the McMaster protocol by a single trained individual (Zajac & Conboy 2012). Briefly, 2 g of feces per sample were placed into a conical tube with 28 mL of a saturated salt solution. After 5 minutes the solution was strained through a tea strainer into another conical tube and immediately a pipette was used to fill both chambers of the McMaster slide. The slide was then left undisturbed for 5 minutes to allow the oocysts to float to the top of the chamber for viewing. The slide was then placed under the microscope and viewed at 10X objective. The eggs that lay within the grid were counted and the total count obtained from both chambers was multiplied by 50 to give the eggs per gram (EPG).

Blood samples were taken from each calf on d 7, 14, 28, 56, and 84 via jugular venipuncture into a vacutainer tube with EDTA (BD vacutainers, Fisher Scientific, Waltham, MA). Blood samples were collected prior to morning milk feeding. The samples were taken to lab and immediately run for a complete blood count through the ProCyte hematology analyzer (IDEXX ProCyte DX, Westbrook, ME).

Statistical Analysis

All repeated, continuous data were analyzed by restricted-maximum likelihood ANOVA using the Mixed procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). The statistical model included the fixed effects of treatment, time, and treatment x time

interactions. The subject of the repeated statement was calf nested within treatment. Each model was run with all available covariance structures for the within-animal measurement. The appropriate covariance and variance structures for each variable were chosen for each analysis based on the smallest Schwarz Bayesian information criterion. Fecal oocyst counts were log-base 10 transformed prior to statistical analysis; however, the data are reported as the non-transformed LSMEANS. All non-repeated, continuous data were analyzed by restricted-maximum likelihood ANOVA using the Mixed procedure of SAS. The statistical model included the fixed effect of treatment. Covariates tested in all models included initial BW and TSP and remained in the model if significant. Normality of the residuals in the final models were confirmed by evaluating the Shapiro-Wilk statistic and normal probability plots of the residuals using the Univariate procedure of SAS. Pairwise comparisons were made for each significant effect using a Duncan adjustment to control the family-wise error. Count data were analyzed with the Fisher's exact test using the Freq procedure of SAS. Statistical significance was declared at $P \leq 0.05$ and a tendency was considered if $0.10 \leq P > 0.05$. Data are presented as least squares means ± the standard error of the mean.

Results

Mortality

Over the course of the study there was an overall mortality rate of 12.2%, and the age and suspected cause of death are reported in Table 2. A tendency was shown in overall morality ($P = 0.056$) with the CON groups having the greatest mortality loss when compared to all other treatment groups throughout the study. There was a treatment

difference in mortality during the first 2 weeks of life ($P = 0.026$) with both CON groups having the greatest mortality during this period. A total number of eleven calves died during the study. Nine calves died within the first two weeks of the study from suspected sepsis. Eight of the nine calves that died were in the control treatments and one calf was in the saponin treatment. Further, on days 41 and 62, two calves, one control and one GOD treatment respectively, were euthanized due to lameness.

Performance

Total serum protein, body weights, average daily gain (ADG), feed to gain ratio, and body measures are reported in Table 3. There were no differences among treatment groups in either total serum protein ($P = 0.810$) or initial BW ($P = 0.686$). Further, there was no difference in weaned BW ($P = 0.804$) or final BW ($P = 0.602$). There were no treatment x time interactions or treatment differences ($P \geq 0.555$) for ADG. Similarly, there were no treatment x time interactions or treatment differences ($P \geq 0.199$) in the feed to gain ratio. Lastly, there were no treatment x time interactions or treatment differences ($P \geq 0.123$) for any of the body measurement.

Intakes

Milk dry matter (DM) refused, calf starter intake and water intake are reported in Table 4. The amount of milk DM refused ($P = 0.096$) showed a tendency among treatments, whereas the Na Percarb refused less milk solids than the uninfected CON, infected CON, and GOD treatments. There was a treatment difference in the days to

consume 100 g of calf starter ($P = 0.027$). The infected CON group consumed 100 g of starter earlier than the uninfected CON and Na Percarb calves. Calf starter intakes were different on day 0 to 28 ($P = 0.005$), whereas the uninfected CON had decreased intakes compared to all other treatments. There was a tendency for a difference in calf starter intakes from d 0 to 56 ($P = 0.086$). The uninfected CON calves consumed the least amount of calf starter from d 0 to 28, whereas the GOD calves consumed more starter than the sodium percarbonate calves from d 0 to 56. The other time points showed no treatment differences ($P \geq 0.130$). There were no treatment x time interactions or treatment differences ($P \geq 0.244$) in bucket water intakes.

Health

Eimeria bovis oocyst counts, fecal scores, electrolytes offered and consumed, and percentage of antibiotic treatments are reported in Table 5. There was a treatment x time interaction ($P \geq 0.001$) in the oocyst counts shown in Figure 1. There were treatment differences on d 21 and 23 post-inoculation, whereas the Na Percarb and Saponin groups had increased fecal oocyst counts when compared to both the uninfected and infected CON groups. Further, there was a treatment x time interaction ($P \geq 0.001$) in average weekly fecal score which is shown in Figure 2. There were treatment differences at week 1 ($P = 0.045$), 4 ($P = 0.047$), 5 ($P < 0.001$) and 9 ($P = 0.0424$) and a tendency at week 8 ($P = 0.077$). There was a treatment difference in electrolytes offered ($P = 0.021$) and electrolytes consumed ($P = 0.019$), where uninfected CON was offered the least volume of electrolytes throughout the study when compared to all other treatment groups. There

was no treatment difference in the percentage of calves treated with antibiotics (P = 0.309).

Hematology

Hematological variables are shown in Table 6. There was a treatment x time interaction for the percentage of monocytes ($P \geq 0.029$). The treatment differences sliced by time showed a difference at d 84 with the Na Percarb treatment having a greater monocyte percentage than other treatments (data not shown). All other hematological variables showed no treatment x time interactions or treatment differences ($P \geq 0.6$).

Discussion

This study investigated the health and performance effects of Na Percarb, GOD, and Saponin extract following an *E. bovis* infection in Holstein calves. *Eimeria bovis* is one of the leading parasites that causes coccidiosis in cattle. Coccidiosis results in hemorrhagic diarrhea and performance losses that ultimately causes negative economic impact in many livestock and poultry industries (Jolley et al. 2006). Though ionophores have been the coccidiostat of choice the past 50 years, the concern of antimicrobials in agricultural industries has producers searching for an alternative product to serve as a successful coccidiostat as well as a growth additive. Coccidiosis causes high infection, but it is a self-limiting pathogen whose rate of clinical disease is low (Constable 2015).

It is suspected that no calves died directly from the *E. bovis* infection, but two did have lasting performance effects. Eight of the nine calves that died within the first two weeks of life were in the control treatments, with only one calf (Saponin group) from a

treatment. Although the current study was not originally designed to evaluate mortality, these findings may indicate a potential difference in general health between all treatment calves and the control group calves during the first two weeks of life, before the animals were challenged. It is interesting to note the majority of early calfhood mortality seen in the current study was due to suspected sepsis in CON calves, thus it is hypothesized that supplementation of the treatments may have improved enteric disease resistance in calves during the first 2 weeks of life. Enteric disease is one of the leading causes of mortality in neonatal calves. Out of the total mortality of pre-weaned U.S dairy calves, 56.4% is attributable to gastrointestinal disease with *Salmonella* and *E. coli* causing 6.4% mortality in pre-weaned calves. *Salmonella* and *E. coli* are both the main causes of neonatal scours and sepsis for the first two weeks of life. The number of attributable deaths of these bacteria are underreported due to the range of symptoms. Glucose oxidase supplemented to broiler chickens showed improvements in intestinal health by affecting the diversity of the microbiota, with an increase in the phylum Bacteroides that produce propionic acid which can inhibit gram-negative bacteria (Wu et al. 2018; Zhao et al. 2020). Glucose oxidase had antibacterial activity when used as the H_2O_2 source in the LP system in abomasal cannulated calves (Reiter et al. 1980). A study performed in piglets reported that GOD supplemented piglets had decreased in fecal pathogenic bacteria when compared to a control group (Tang et al. 2016). Similarly, another study supplementing GOD to piglets decreased the incidence of piglet diarrhea (Hou et al. 2017). Glucose oxidase also inhibited bacterial growth when preserving food and feed products due to its production of H_2O_2 (Dobbenie 1994, Sandholm et al. 1988). Sodium percarbonate reduced *E. coli* growth when used as a biocide in composting chicken manure (Ryk et al.

2020). Saponins increased starch-digesting microbes while reducing fibrolytic microbes and affects gram-positive bacteria more than gram-negative due to the lack of a cell membrane. (Singer et al. 2007, Wallace et al. 1994, Wang et al. 2000).

On d 7 of the current study calves were infected with 130,000 *E. bovis* oocysts. The symptoms ranged from no clinical signs to fecal shedding of oocysts to sloughing of the mucosal lining of the intestines. Prior coccidia challenges infected calves anywhere from 5,000 oocysts to one million oocysts (Hammond et al. 1944, Niilo 1969, Sink et al. 1992, Suhwold et al. 2010, Taubert et al. 2008). Most of the prior research infected on average between 100,000-200,000 oocysts (Hammond et al. 1944, Quigley et al. 1997). The current study's infection timeline closely reflected a study completed by Hammond et al. (1944), where 2-month-old dairy calves were infected with 125,000 *E. bovis* oocysts. The infected calves' feces were covered with slight hemorrhagic spots by d 18 post-infection and by d 20 the hemorrhagic diarrhea was prominent and included fibrous mucus strands. The hemorrhagic diarrhea had subsided on d 30 post-infection and by d 34 the oocyst shedding had stopped for all calves (Hammond et al. 1944). These results were mimicked in the current study exactly as described, confirming the current challenge model was successful at inducing coccidiosis.

A total of 4 calves, all GOD treatment, started to slough the mucosal lining of the intestine on d 21 post-infection. The intestines were rough, granular, and pale pink to grey in color. The sloughing was accompanied by watery hemorrhagic diarrhea. It is interesting to note that the oocyst counts in these samples were very low, potentially due to a dilution effect from the inflammatory edema response seen in the intestines during a coccidia infection. The treatment GOD is broken down into gluconic acid, that can be

used to lower the pH, and H_2O_2 that can be used for the antibacterial effects. Hydrogen peroxide is also known to cause oxidative stress, which could further perpetuate an inflammatory response, particularly during an infection that takes place in the intestines, although it is unknown if this treatment makes it to the colon of the calf to exert such inflammatory effects. Two of the calves that sloughed their intestines also had reduced feed intake and refused milk replacer during the post-infection period, and subsequently took longer to wean off milk than the other calves due to the consistently lower calf starter intake.

Oocysts counts were sampled and analyzed through the McMaster technique on d 14, 21, 23, 25, 27, and 35 post-infection. These sampling dates are consistent with the life cycle of the parasite as well as the optimum period for quantifying high shedding counts. Oocysts counts for the duration of collection ranged from 0 EPG to 78,000 EPG. Coccidiosis is commonly considered clinical once oocyst shedding reaches or exceeds 5,000 EPG. For the oocyst to form the parasite goes through an asexual and sexual stage in the cells of the intestine, which causes severe damage to the enterocytes. This means the higher the oocyst count, the more severe the disease. In a study with *Cryptosporidium parvum*, another protozoan parasite, the treatment supplemented group had prevented the binding of the parasite to enterocytes, effectively neutralizing the parasite, which reduced oocyst shedding and overall inflammation of the intestines. On the other hand, the control calves showed *C. parvum* attaching to the enterocytes, ultimately causing increased inflammation and subsequent oocyst shedding (Watarai et al. 2008).

Coccidiosis primarily infects calves between three and eight months of age. In the current study calves were infected on the 7th day of life. Though this is quite early for an

infection with 130,000 oocysts, this early infection model is similar to previous studies. In the earliest coccidia infection models, calves were infected at various ages with varying oocysts counts. The youngest calves were infected at d 1 of life with a low dose of 1,000 to 10,000 oocysts. These calves did not show any clinical symptoms throughout the infection due to the low oocyst count (Hammond et al. 1944). Another study infected neonatal calves at d 11 of life with 100,000 or 200,000 oocysts suspended in milk replacer, and contrary to the current study, calves infected with 100,000 oocysts at birth did not inflict the desired diarrheal response, so they raised the oocyst count to 200,000, after which the desired clinical response was observed (Conner et al. 2013). Many past coccidia studies infected calves later in life, ranging between 1 and 12 months of age, which more closely reflects the natural course of infection (Friend & Stockdale 1980, Niilo 1969, Quigley et al. 1997, Sink et al. 1992, Suhwold et al. 2010, Taubert et al. 2008). In the current study, the earlier infection model may have affected the progression of the disease due to the anatomical and microbial changes the calf is going through at this time.

The neonatal calf gastrointestinal tract and corresponding microbiota are going through many changes during the first few months of life. The diversity of the microbiome is impacted through both calfhood nutrition and environment. The intestinal microorganisms and their interplay with enterocytes are heavily involved in the calf's immune system development and maintenance. There is a possibility that infecting neonatal calves while the intestines are developing worsens their ability to respond immunologically to the parasite. The antimicrobial treatments of the current study may have compromised the integrity of the intestines and time

was not allowed for the gastrointestinal tract to adapt to the treatments before being infected with a moderate dose of 130,000 sporulated *E. bovis* oocysts. It is known that the use of antibiotics in young calves can decrease the microbial diversity, which can lead to negative immunological effects (Meale et al. 2017). The infected CON calves may have had the undisrupted barriers to lessen the effects of the parasite, as seen by their decreased oocyst counts and fecal scores when compared to all other infected treatments.

The infection period of the current study did not cause infected calves to decrease feed intake, which is in direct contrast with past coccidia infections. Coccidiosis causes hemorrhagic diarrhea which typically leads to anorexia and stunted growth parameters. Only two calves lasting health and performance deficits, whereas the vast majority did not. In contrast to prior belief, the infected CON calf starter intake throughout the study was not different when compared to the other infected treatment groups due to reasons unknown to the author.

To the authors knowledge there is no information on the effects of Na Percarb supplementation on the health or performance of livestock. Glucose oxidase has improved performance measures such as ADG and feed efficiency in piglets and broiler chickens by improving the intestinal barrier and promoting beneficial bacteria colonization (Tang et al. 2015; Hou et al. 2017; Wu et al. 2018). Saponins have equivocal results on performance measures in livestock and poultry. The current study did not see a difference among the Saponin group and both CON groups when looking at performance and coccidiostat properties which agreed with a few studies (Benchaar et al. 2008, Holtshausen et al. 2009, McMurphy et al. 2014, Sliwinski et al. 2004) and disagreed with others (Alfaro et al. 2007, El-Din et al. 2008, Galli et al. 2018, Singer et al. 2008).

The Na Percarb treatment had the greatest oocyst shedding throughout the infection period, but this did not coincide with a greater fecal score. Sodium percarbonate is broken down into sodium carbonate and H_2O_2 when mixed with water.

$$2Na_2CO_3 \cdot 3H_2O_2 \rightarrow 3H_2O_2 + 4Na^+ + 2CO_3^{2-}$$

The H_2O_2 component is believed to have antimicrobial effects. In a completely closed system, the amount of Na Percarb supplemented in the current study would produce about 125ppm of H_2O_2. Due to the environmentally friendly characteristics of Na Percarb, this product is predominantly studied in aquaculture and environmental impact studies. One major area of the aquatic studies involves the protozoan parasite *Ichthyophthirius multifiliis* which infects wild freshwater fish. Several studies show a negative correlation between the amount of Na Percarb used and the number of parasite eggs. Fish farmers treat fish tanks with 50-100 ppm of Na Percarb to kill these ectoparasites. In a closed system, the amount of treatment we supplemented would produce about 125ppm of H_2O_2. Though *I. multifiliis* is a protozoan parasite like *E. bovis* they infect different systems of the body. *E. bovis* is a bovine intestinal parasite where as *I. multifiliis* infects the epidermis of the fish (Heinecke and Buchmann 2009, Buchmann et al. 2002, Buchmann and Kristensson 2002). Sodium percarbonate is also a popular substance studied in water sanitation to decrease microbial load in water. To the author's knowledge there is no literature discussing supplementation of Na Percarb to livestock or poultry species, therefore it is unknown if this product would even reach the colon of the calf to exert similar antimicrobial or antiparasitic effects.

Glucose oxidase is an enzyme that catalyzes the breakdown of glucose into H_2O_2 and gluconic acid.

$$H_2O + O_2 + C_6H_{12}O_6 \xrightarrow{GOD} C_6H_{12}O_7 + H_2O_2$$

In a completely closed system, the amount of daily GOD supplemented in the current study would produce about 140ppm of H_2O_2. This is based on the assumptions that the dextrose is the rate-limiting step, and that dextrose is the main sugar the enzyme is targeting. To the author's knowledge there is no literature on calves or livestock being treated with GOD for coccidiosis or data that confirms if the treatment has an antimicrobial impact on the gastrointestinal tract or if the H_2O_2 reaches the lower digestive tract. The gluconic acid may lower the pH of the gastrointestinal tract and the H_2O_2 has antimicrobial capabilities. The H_2O_2 is known to cause oxidative stress that can induce inflammation. Coccidiosis can also cause severe inflammation to the lower intestinal tract and with the inflammatory effects of GOD it potentially could have contributed to the sloughing of the intestines. The GOD treatment had the lowest oocyst counts out of the treatment groups, but it was the only group that had intestinal sloughing and severe hemorrhagic diarrhea. It is possible that the severity of the coccidia infection is not properly represented by the greater oocyst count due to dilution of the feces, and ultimately the oocyst counts, from the severe inflammatory effects occurring in the intestines.

The Saponin treatment had a greater oocyst count than the infected CON calves and had the greatest fecal scores on average during weeks 4 and 5 of the study, corresponding with the peak shedding times of the parasite. The yucca and fenugreek saponins used in the current study are classified as steroidal saponins. Steroidal saponins exert antimicrobial effects by interrupting sterol and lipids in the membranes of microbes. The main areas of study of steroidal saponins in cattle involve its effects on rumen

microbes, especially protozoa, and how this effects performance. Some studies did report reduced rumen protozoal numbers (Hristov et al. 1999, Makkar et al. 1998, Wallace et al. 1994) and others that showed negligible effects (Benchaar et al. 2008, Holtshausen et al. 2009). There have also been studies in sheep and chickens supplemented with saponins containing potential anti-protozoal properties. One study observed how saponins affect ruminal digestion in sheep, there was a reduced ruminal protozoal population of 34% and 66% in a low and high dose of saponin treatments, respectively, when the saponins were administered directly into the rumen (Lu and Jorgenson 1987). A similar ovine study showed that the reduced rumen protozoal numbers were sustained and not short-termed throughout the duration of saponin supplementation (Klita et al. 1996). In a natural infection with an avian *Eimeria* species, reduced parasite loads were observed in saponin and glutamine supplemented broiler chickens when compared to a control (Galli et al. 2017). Though neonatal calves are considered functionally monogastric due to the bypass of the rumen it is possible that the saponins affected the microbiota of the intestinal tract. It is also important to note that the current study is not a natural infection model, and that neonatal calves were infected directly with oocysts, so it is possible the infection masked the beneficial effects of the saponins seen in prior studies.

Conclusion

The current study evaluated the effects of Na Percarb, GOD, and a Saponin extract on the performance, health, and hematological variables of *E. bovis* infected calves. There were no differences in performance or hematological variables. No differences in health were determined, but it is important to note that though the GOD

treatment showed the most severe symptoms, it did not correspond with higher oocyst counts probably due to a diarrhetic dilution effect. It is important to note that during the first 2 weeks of life there may be beneficial antibacterial impacts, however this may have increased the risk to the moderate coccidia infection. Due to the neonatal infection model, it is possible that treatment effects were masked by the severity of the challenge in pre-weaned calves. Future research is needed to determine efficacy of these treatments on pathogens, confirmation of where they exert their effects in an *in vivo* model, and ultimately the application of each product for feed additives in milk replacer.

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Table 1. The formulated nutrient content of the milk replacer and calf starter fed to pre-weaned Holstein calves.

Nutrient	Milk replacer[1]	Starter[2]
Dry matter, %	95	95
Crude protein, %	22	22.3
Ether extract, %	20	3.3
Neutral detergent fiber, %	-	23.2
Net energy for lactation, Mcal/kg	4.65	1.69
Calcium, %	1.25	1.08
Phosphorus, %	0.7	0.57
Selenium, ppm	-	0.3
Vitamin A (min), IU/kg	13,500	6,300
Vitamin D3 (min), IU/kg	4,545	2,500
Vitamin E (min), IU/kg	100	50

[1]Milk replacer was non-medicated and was formulated by Milk Specialties Inc. (Milk Specialties Global, Eden Prairie, MN) and included: Dried Whey, Dried Whey Protein Concentrate, Dried Whey Product, Animal and Vegetable Fat (Preserved with BHA and BHT), Lecithin, Dicalcium Phosphate, Calcium Carbonate, Citric Acid (Preservative), L-Lysine Monohydrochloride, DL-Methionine, Vitamin A Supplement, Vitamin D Supplement, Vitamin E Supplement, Ascorbic Acid, Magnesium Oxide, Zinc Sulfate, Ferrous Sulfate, Niacin Supplement, Manganese Sulfate, Calcium Pantothenate, Vitamin B_{12} Supplement, Thiamine Mononitrate, Riboflavin Supplement, Copper Sulfate, Pyridoxine Hydrochloride, Ethylenediamine Dihydroiodide, Folic Acid, Choline Chloride, Cobalt Sulfate, Selenium Yeast, Sodium Silico Aluminate, Mono and Diglycerides of Edible Fats or Oils, Artificial Flavor.
[2]Complete calf starter pellet was made by Hi-Pro Feeds (Hi Pro Feeds, Friona, TX) and included: Grain Products, Processed Grain By-Products, Roughage Products, Plant Protein Products, Molasses Products, Calcium Carbonate, Montmorillonite Clay, Zinc Amino Acid Complex, Manganese Amino Acid Complex, Copper Amino Acid Complex, Cobalt Glucoheptonate, Zinc Sulfate, Vitamin E Supplement, Selenium Yeast, Vitamin A Supplement, Vitamin D Supplement, Manganese Sulfate, Ethylenediamine Dihydroiodide, Salt and Artificial Flavors.

Table 2. The age, treatment, and suspected cause of death of calves during the observation period.

Calf ID	Treatment[1]	Total Serum Protein	Age of Death	Suspected Cause of Death
20	Infected	5.9	1	Dehydration/Heat stress
8	Infected	3.8	1	Sepsis
9	Uninfected	4	3	Sepsis
49	Infected	5.2	4	Sepsis
54	Infected	3.2	6	Sepsis
46	Infected	4.8	7	Bloat
68	Uninfected	3.6	11	Sepsis
19	Saponin	3.9	15	Sepsis
26	Uninfected	4.1	15	Sepsis
64	Uninfected	4	41	Euthanized due to lameness caused by sepsis
31	Glucose Oxidase	4.1	62	Euthanized due to lameness caused by sepsis

[1]During the study, calves were inoculated with 130,000 *E. bovis* oocysts on d 7. Treatments included Uninfected Control which were not inoculated with *E. bovis* and received no treatment (n=14), an Infected Control which were inoculated with *E. bovis* and received no treatment (n=13), a treatment group that were inoculated with *E. bovis* and treated with 2g of Saponin extract a day (n=17), a treatment group inoculated with *E. bovis* and treated with 0.18g/hd/d of Glucose Oxidase (GOD) and 4.6g/hd/d of Dextrose (n=17), and a treatment group inoculated with *E. bovis* and treated with 2g/hd/d of Sodium Percarbonate (Na Percarb) (n=18).

Table 3. The effects of Saponin, GOD, and Na percarb treatments on the performance variables of Eimeria bovis infected dairy calves.

	Treatments[1,2]					Largest	Fixed Effects		
	Uninfected	Infected	Saponin	GOD	Na Percarb	SEM	Trt	Time	Trt*Time
Item	Control	Control					*P* ≤		
Total serum protein	4.86	4.87	4.78	5.13	5.08	0.244	0.810	...	...
Failure of passive transfer, %	72.2	61.1	83.3	55.5	55.5	…	...	...	...
Initial body weight, kg	38.1	37.6	40.1	38.7	38.7	1.317	0.686	…	…
Weaned body weight, kg	58.9	60.8	60.8	62.2	59.3	2.246	0.804	…	…
Final body weight, kg	84.0	89.7	88.2	85.7	83.5	3.397	0.602	…	…
Average daily gain, kg/d[3]	0.52	0.58	0.54	0.57	0.52	0.042	0.802	0.001	0.555
0 d to 28 d[4]	0.25	0.33	0.28	0.30	0.40	0.068	0.453	…	…
0 d to 56 d[4]	0.37	0.41	0.37	0.42	0.42	0.046	0.826	…	…
57 d to 84 d[4]	0.84	0.96	0.91	0.78	0.81	0.071	0.292	…	…
0 d to 84 d[4]	0.53	0.60	0.55	0.54	0.55	0.039	0.755	…	…
Feed to gain, kg[3]	2.8	2.76	3.12	3.03	2.72	0.158	0.199	0.005	0.239
0 d to 28 d[4]	3.53	2.99	3.28	3.27	3.18	0.441	0.936	…	…
0 d to 56 d[4]	2.7	2.7	3.14	2.93	2.69	0.293	0.680	…	…
57 d to 84 d[4]	2.27	2.24	2.52	2.73	2.56	0.251	0.551	…	…
0 d to 84 d[4]	2.44	2.28	2.81	2.94	2.41	0.223	0.107	…	…
Length, cm	69.9	70.3	71.2	71.1	70.7	0.87	0.801	0.001	0.795
Height, cm	86.9	87.2	87.5	87.7	86.3	0.79	0.651	0.001	0.123
Heart girth, cm	88.7	89.5	90.3	90.6	89.4	0.89	0.515	0.001	0.991

[1]During the study, calves were inoculated with 130,000 E. bovis oocysts on day 7. Treatments included a Uninfected Control which were not inoculated with E. bovis and received no treatment (n=14), a Infected Control which were inoculated with E. bovis and received no treatment (n=13), a treatment group that were inoculated with E. bovis and treated with 2g of Saponin extract a day (n=17), a treatment group inoculated with E. bovis and treated with .18g of Glucose Oxidase (GOD) and 4.6g of Dextrose a day (n=17), and a treatment group inoculated with E. bovis and treated with 2g of Sodium Percarbonate (Na Percarb) a day (n=18).
[2]Rows with differing superscripts indicate treatment differences with $P \leq 0.05$.
[3]Repeated measures analysis are the main effects from the repeated analysis reported as least square means and are the weekly average.
[4]Summary statistics were analyzed over the entire period listed.

Table 4. The effects of Saponin, GOD, and Na percarb treatments on the milk DM, starter, and water intakes of Eimeria bovis infected dairy calves.

	Treatments[1,2]					Largest	Fixed Effects		
	Uninfected	Infected	Saponin	GOD	Na Percarb	SEM	Trt	Time	Trt*Time
Item	Control	Control					*P* ≤		
Milk refused, DM kg	0.58[a]	0.62[a]	0.27[ab]	0.55[a]	0.13[b]	0.165	0.096	…	…
Days to consume 100g starter, d	24.6[a]	16.0[b]	19.4[ab]	18.9[ab]	24.7[a]	2.37	0.027	…	…
Starter intakes, kg/d	0.80	0.90	0.85	0.90	0.77	0.068	0.485	0.001	0.771
Starter summary, kg/d[3]									
0 d to 28 d[4]	0.020[a]	0.047[b]	0.043[b]	0.045[b]	0.036[b]	0.006	0.005	…	…
0 d to 56 d[4]	0.278[ab]	0.310[ab]	0.280[ab]	0.337[a]	0.224[b]	0.035	0.086	…	…
57 d to 84 d[4]	2.17	2.22	2.16	2.24	2.02	0.118	0.601	…	…
0 d to 84 d[4]	0.88	0.93	0.89	0.98	0.81	0.056	0.130	…	…
Water intake, L/d[3,5]	4.8	5.0	5.0	5.0	5.0	0.27	0.959	0.001	0.877
0 d to 28 d[4,5]	2.1	2.2	2.3	2.8	2.5	0.25	0.244	…	…
0 d to 56 d[4,5]	2.3	2.6	2.5	2.9	2.6	0.19	0.428	…	…
57 d to 84 d[4,5]	7.1	7.4	7.5	7.5	7.3	0.42	0.940	…	…
0 d to 84 d[4,5]	3.8	4.1	4.1	4.4	4.1	0.24	0.628	…	…

[1]During the study, calves were inoculated with 130,000 E. bovis oocysts on day 7. Treatments included a Uninfected Control which were not inoculated with E. bovis and received no treatment (n=14), a Infected Control which were inoculated with E. bovis and received no treatment (n=13), a treatment group that were inoculated with E. bovis and treated with 2g of Saponin extract a day (n=17), a treatment group inoculated with E. bovis and treated with .18g of Glucose Oxidase (GOD) and 4.6g of Dextrose a day (n=17), and a treatment group inoculated with E. bovis and treated with 2g of Sodium Percarbonate (Na Percarb) a day (n=18). [2]Rows with differing superscripts indicate treatment differences with $P \leq 0.05$.

[3]Repeated measures analysis are the main effects from the repeated analysis reported as least square means.

[4]Summary statistics were analyzed over the entire period listed.

[5]Bucket water analyzed as liters per day.

Table 5. The effects of Saponin, GOD, and Na Percarb treaments on the health variables of Eimeria bovis infected dairy calves.

	Treatments[1,2]					Largest	Fixed Effects		
Item	Uninfected Control	Infected Control	Saponin	GOD	Na Percarb	SEM	Trt	Time	Trt*Time
							$P \leq$		
E. bovis oocyst count, EPG[3]	704[a]	2496[ac]	5321[b]	3884[b]	9630[c]	1054.5	0.001	0.001	0.001
Fecal score[4]	1.13	1.22	1.3	1.21	1.14	0.050	0.0597	0.001	0.001
Electrolytes offered, L	8.1[a]	13.6[b]	16.6[b]	12.15[ab]	12.44[ab]	1.79	0.0215	…	…
Electrolytes consumed, L	8.1[a]	13.0[ab]	16.5[b]	12.0[ab]	12.0[ab]	1.76	0.0198	…	…
Antibiotics, % treated	33.3	16.7	5.6	27.8	22.2	…	0.309	…	…
Mortality , % < 21 days of age	16.7[a]	27.8[a]	5.6[b]	0[b]	0[b]	…	0.026	…	…

[1]During the study, calves were inoculated with 130,000 E. bovis oocysts on day 7. Treatments included a Uninfected Control which were not inoculated with E. bovis and received no treatment (n=14), a Infected Control which were inoculated with E. bovis and received no treatment (n=13), a treatment group that were inoculated with E. bovis and treated with 2g of Saponin extract a day (n=17), a treatment group inoculated with E. bovis and treated with .18g of Glucose Oxidase (GOD) and 4.6g of Dextrose a day (n=17), and a treatment group inoculated with E. bovis and treated with 2g of Sodium Percarbonate (Na Percarb) a day (n=18).
[2]Rows with differing superscripts indicate treatment differences with $P \leq 0.05$.
[3]Shedding determined by the McMaster Technique where coccidia oocysts are counted from a fresh feces sample and recorded as eggs per gram (EPG).
[4]Fecal scores were obtained daily based on the School of Veterinary Medicine at University of Wisconsin Madison's scoring system from a 0 to 3, where 0=normal, 1=semi-formed, pasty, 2=loose but stays on top of bedding, and 3=watery, sifts through bedding. Scores were then averaged per week for analysis.

Table 6. The effects of Saponin, GOD, and Na Percarb treatments on the hematological variables in Eimeria bovis infected dairy calves.

	Treatments[1,2]					Largest	Fixed Effects		
	Uninfected	Infected	Saponin	GOD	Na Percarb	SEM	Trt	Time	Trt*Time
Item	Control	Control					$P \leq$		
Hematocrit , %	28.9	28.3	30.1	29.5	30.2	1.156	0.695	0.001	0.259
Mean corpuscular volume, (fL)	36.7	36.4	37.7	36.9	37.1	0.478	0.380	0.001	0.173
Hemoglobin, (g/dL)	10.4	11.0	11.5	11.2	11.1	0.441	0.514	0.001	0.172
Total leukocyte , (10^6/mL)	9.91	8.82	10.28	10.18	9.17	0.530	0.173	0.025	0.562
Polymorphonuclear leukocytes , (10^6/mL)	4.53	3.61	4.59	4.45	4.04	0.376	0.285	0.001	0.333
Lymphocyte, (10^6/mL)	4.4	4.22	4.5	4.6	4.06	0.266	0.529	0.001	0.813
Monocytes, (10^6/mL)	0.79	0.79	0.97	0.91	0.86	0.086	0.463	0.001	0.136
Eosinophils, (10^6/mL)	0.03	0.03	0.03	0.02	0.03	0.004	0.407	0.001	0.587
Basophils, (10^6/mL)	0.16	0.17	0.18	0.19	0.18	0.042	0.985	0.001	0.294
Polymorphonuclear leukocytes, %	44.1	40.69	44.1	41.61	42.51	1.975	0.647	0.001	0.329
Lymphocytes, %	46.74	48.77	44.78	47.33	45.43	1.830	0.520	0.001	0.656
Monocytes, %	8.0	8.5	8.9	9.1	8.8	0.68	0.822	0.001	0.029
Eosinophils, %	0.03	0.03	0.03	0.02	0.03	0.004	0.407	0.001	0.587
Basophils, %	0.2	0.2	0.2	0.2	0.2	0.04	0.985	0.001	0.294

[1]During the study, calves were inoculated with 130,000 E. bovis oocysts on day 7. Treatments included a Uninfected Control which were not inoculated with E. bovis and received no treatment (n=14), a Infected Control which were inoculated with E. bovis and received no treatment (n=13), a treatment group that were inoculated with E. bovis and treated with 2g of Saponin extract a day (n=17), a treatment group inoculated with E. bovis and treated with .18g of Glucose Oxidase (GOD) and 4.6g of Dextrose a day (n=17), and a treatment group inoculated with E. bovis and treated with 2g of Sodium Percarbonate (Na Percarb) a day (n=18).
[2]Rows with differing superscripts indicate treatment differences with $P \leq 0.05$.

Figure 1

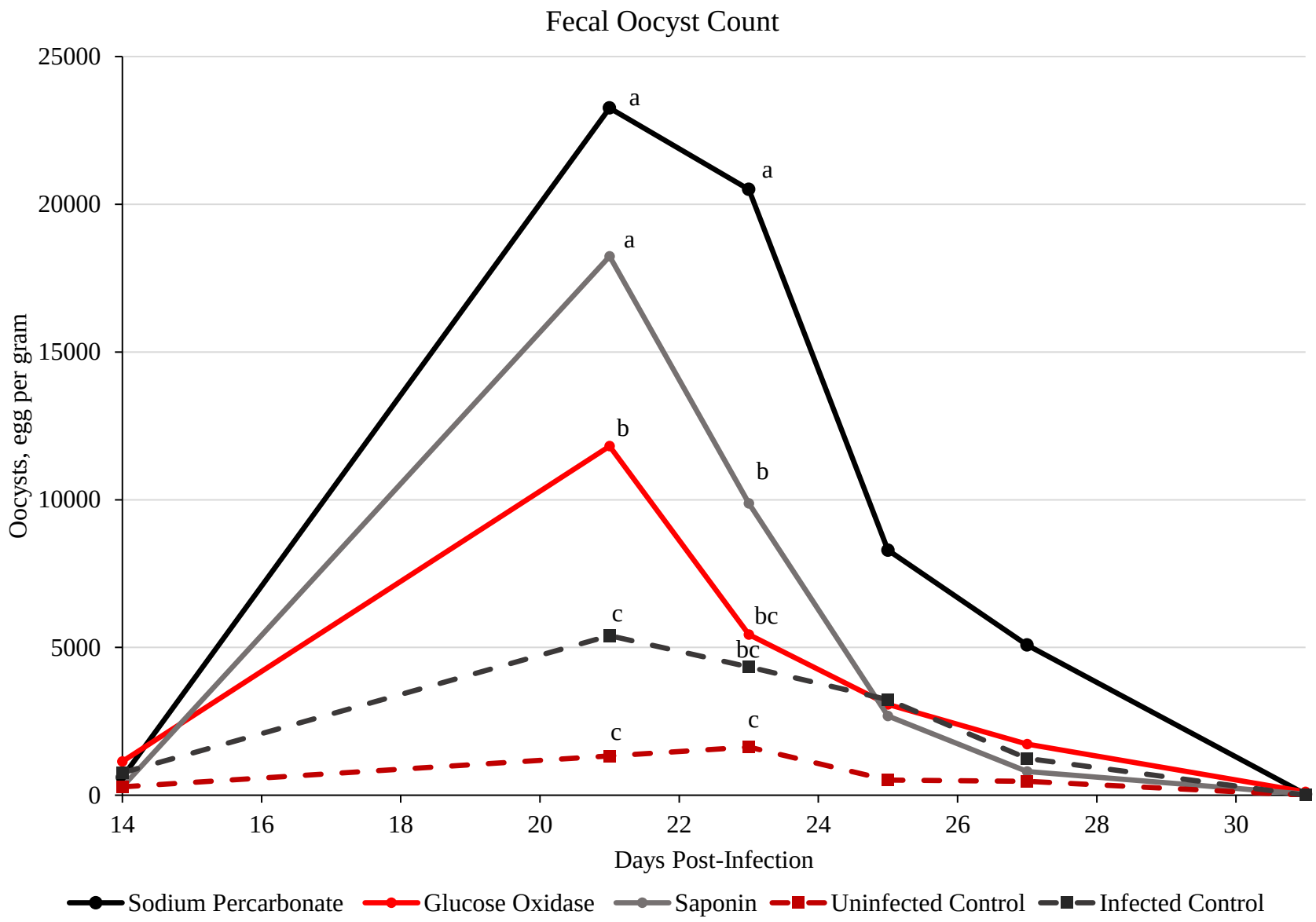
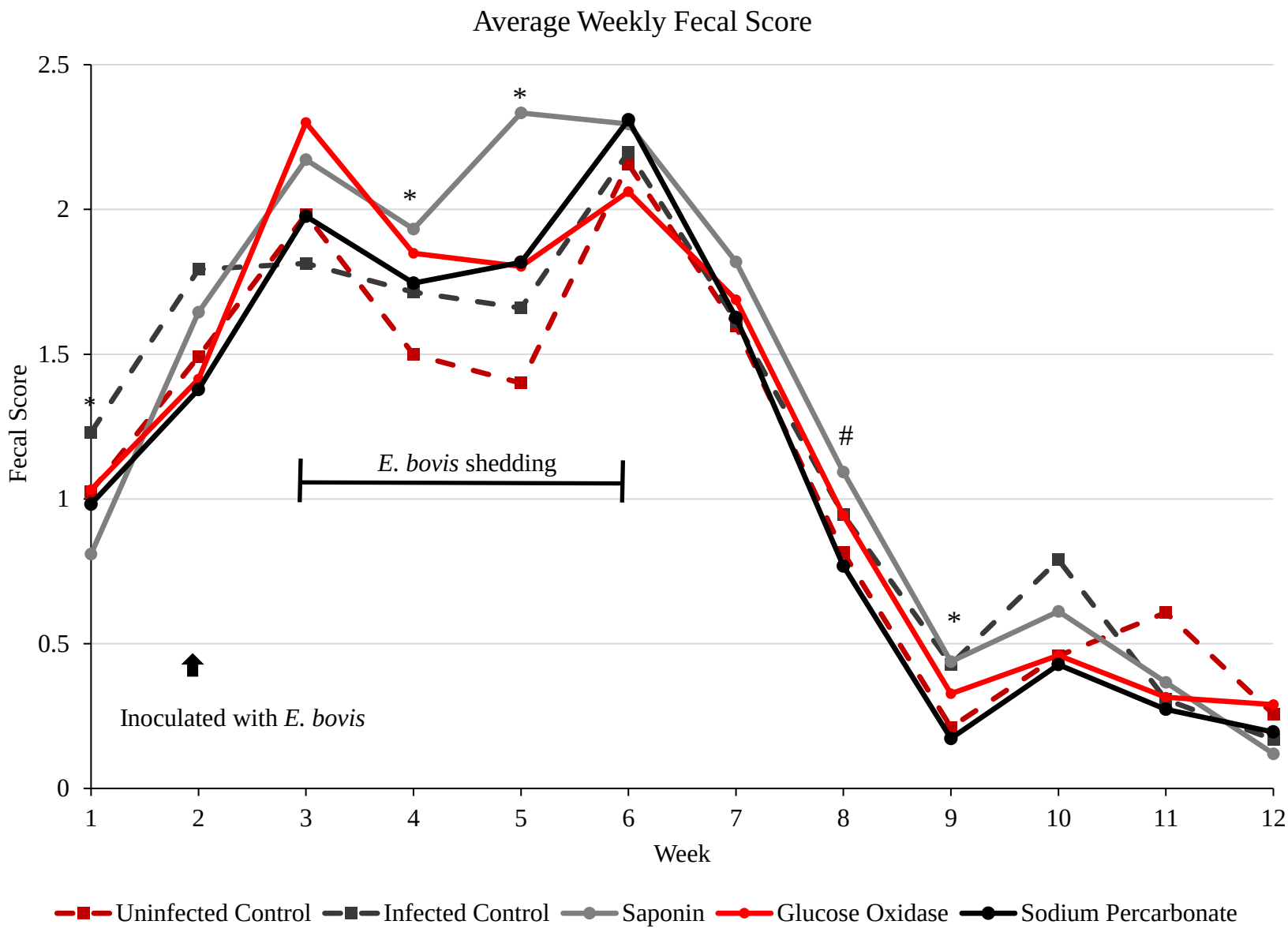

Fecal Oocyst Count
25000
20000
15000
10000
5000
0
Oocysts, egg per gram
14
16
18
20
22
24
26
28
30
Days Post-Infection
a
a
a
b
b
c
bc
bc
c
c
Sodium Percarbonate
Glucose Oxidase
Saponin
Uninfected Control
Infected Control

Figure 2


Average Weekly Fecal Score
Fecal Score
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
10
11
12
Week
*
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E. bovis shedding
Inoculated with E. bovis
Uninfected Control
Infected Control
Saponin
Glucose Oxidase
Sodium Percarbonate

FIGURE LEGENDS

Figure 1. The effects of Saponin, glucose oxidase (GOD), and sodium percarbonate (Na Percarb) on the shedding of oocysts in *Eimeria bovis* infected dairy calves. Differing superscripts indicate treatment differences with $P \leq 0.05$ at the day of post-infection. There was a treatment x time interaction with sliced treatments differences on d 21 and 23 ($P < 0.001$). On d 21, Na Percarb treated calves oocyst shedding was increased when compared to the GOD, uninfected CON, and infected CON groups ($P \leq 0.002$); GOD was increased when compared to both the uninfected and infected CON groups ($P \leq 0.053$); the Saponin treatment group was increased compared to the GOD and both uninfected and infected CON groups ($P \leq 0.0002$). On d 23, Na Percarb treated calves had increased oocyst shedding when compared to GOD, Saponin, and both uninfected and infected CON groups; Saponin treated calves had increased oocyst counts when compared to the uninfected CON calves.

Figure 2. The effects of Saponin, glucose oxidase (GOD), and sodium percarbonate (Na Percarb) on the average weekly fecal score of *Eimeria bovis* infected dairy calves. Treatment differences at each week shown as * $P \leq 0.05$ and tendencies are shown as # $0.05 < P \leq 0.10$. There were treatment differences at week 1 ($P = 0.045$), 4 ($P = 0.047$), 5 ($P < 0.001$) and 9 ($P = 0.0424$) and a tendency at week 8 ($P = 0.077$). During week 1, infected CON calves had increased average weekly fecal scores compared to Na Percarb and Saponin treatments ($P \leq 0.058$); GOD treated calves had increased average weekly fecal scores when compared to the Saponin treated group ($P = 0.086$). During week 4,

uninfected CON calves had decreased average weekly fecal scores when compared to Na Percarb, GOD, and Saponin treated calves ($P < 0.080$). During week 5, Saponin calves had increased average weekly fecal scores when compared to Na Percarb, GOD, and both infected and uninfected CON calves ($P < 0.004$). During week 8, Saponin treated calves had increased average weekly fecal scores when compared to Na Percarb and uninfected CON calves ($P < 0.080$). During week 9, Saponin treated calves had increased average weekly fecal scores when compared to Na Percarb and uninfected CON calves ($P < 0.032$); whereas infected CON calves had increased weekly average fecal scores when compared to the uninfected CON calves ($P < 0.060$).