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Evaluation of Essential Oils (Stay Strong) for Dairy Calves

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EVALUATION OF ESSENTIAL OILS (STAY STRONG) FOR DAIRY CALVES

BY

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The thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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LIST OF ABBREVIATIONS

µL	Microliter
ADG	Average Daily Gain
BL	Body Length
BRSV	Bovine Respiratory Syncytial
BSA	Bovine Serum Albumin
BW	Body Weight
CS	Calf Starter
d	Day
DMI	Dry Matter Intake
ELISA	Enzyme-linked Immunosorbent Assay
EO	Essential oil
FE	Feed efficiency
FOS	Fructo-oligosaccharide
g	Gram
h	Hour
HG	Heart Girth
HH	Hip Height
HW	Hip Width
IgA	Immunoglobulin A
IgG	Immunoglobulin G
kg	kilogram
L	Liter

m	Meter
M	Molar
Mcal	Mega Calories
ME	Metabolizable Energy
mg	milligram
min	Minute
mL	Milliliter
MOS	Mannan-oligosaccharide
MR	Milk Replacer
N	Nitrogen
ng	Nanogram
ºC	Degree Celsius
OVA	Ovalbumin
PBS	Phosphate Buffered Saline
TRT	Treatment
VFA	Volatile Fatty Acid
WH	Wither Height
YCW	Yeast Cell Wall

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ABSTRACT

EVALUATION OF ESSENTIAL OILS (STAY STRONG) FOR DAIRY CALVES

KELLY ANN FROEHLICH

2016

Stay Strong (SS, Ralco Inc., Marshall, MN) is a blend of essential oils (EO) and prebiotic fiber technologies designed to promote immunity and stimulate appetite, helping to diminish health challenges and stresses experienced by newborn calves. Current feeding inclusion rates are unknown to achieve optimal performance in the first 8 weeks of life. The study objectives were to determine the optimal feeding inclusion rate of SS when added to milk replacer (MR) to achieve optimal performance when compared to a yeast cell wall (YCW). One hundred Holstein calves were blocked by birth date and randomly assigned to 1 of 5 treatments. Treatments were: Control (C): a 24:20 MR, EO mixed into 24:20 MR at a rate of 1.25 g/feeding (EO-0.5), 2.5 g/feeding (EO-1.0) or 3.75 g/feeding (EO-1.5), or 24:20 MR with an inclusion of YCW at a rate of 2 g/calf/feeding. Calves were sourced from a commercial dairy farm where they were fed colostrum for the first 2 d and then were transported to South Dakota State University (SDSU). The 24:20 MR was fed via bucket 2 x/d at a rate of 0.28 kg/calf/d for 14 d, increased to 0.43 kg/calf until 35 d and were reduced to 1 x/d at 36 d to facilitate weaning at 42 d. Decoquinate was added to MR at 37.8 g/ton for coccidiosis control. Calves were housed in individual Calf-Tel hutches bedded with straw with ad libitum access to a 20% CP calf starter (CS) and water. It was hypothesized that calves supplemented with the medium dose (EO-1.0) of the commercial EO would be most optimal, and in general calves supplemented with commercial EO would have improved growth, health, and immunity

compared to calves not supplemented. All data was analyzed using PROC MIXED in SAS as a completely random design. Calves fed EO-0.5 demonstrated greater ADG (0.65, 0.71, 0.64, 0.64, and 0.63 kg/d for C, EO-0.5, EO-1.0, EO-1.5, and YCW, respectively) through 56 d compared to calves fed EO-1.0 and YCW, and tended to be greater for C and EO-1.5. Total body weight gains (36.8, 39.9, 35.5, 35.8, and 35.4) were greater for calves fed C and EO-1.5 compared to calves fed EO-1.0 and YCW. Body length gains were greater for calves fed EO-0.5 compared to calves fed other treatments. Hip width gains were similar among treatments. Wither height gains were greater for calves fed EO-0.5 compared to calves fed the remaining treatments. Hip height gains were also increased for calves fed EO-0.5 compared to calves fed C, EO-1.0, and EO-1.5, while being similar for calves fed YCW. These results demonstrate that supplementing EO-0.5 (1.25 g/calf/d) in a 24:20 MR may be optimal to enhance growth rates compared to calves fed a 24:20 MR and a 24:20 MR containing YCW technology or other inclusion rates of EO.

Keywords: Calf, essential oils, yeast cell wall

CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Animal agriculture is under increasing scrutiny as social pressures and new laws have prohibited and restricted the use of antibiotics in animal production. These social changes are severely impacting agriculture on the treatment and prevention of illnesses in livestock. Dairy calves in particular are feeling the impact. Raising calves is both expensive and labor intensive. Successful calf rearing programs are designed to grow fast, healthy calves that are weaned at a young age to be the most economical. This naturally puts a lot of stress on a newborn animal.

In the past, it was not uncommon to supplement milk replacer (MR) fed to calves with antibiotics to combat these stresses and prevent any illnesses that might naturally occur. There have been numerous studies demonstrating the benefits of adding antibiotics in MR including increased gains, feed consumption, and decreased scours, mortality, and protein requirements (Morrill et al., 1976). However, antibiotic supplementation in MR is becoming more difficult as new FDA laws restrict the use. As an antibiotic alternative, many new technologies are being studied, to decrease calf morbidity and mortality, and include nutritional additives such as essential oils (EO) or microbial additives, such as yeast. Many of these technologies show promising benefits that could improve calf performance, health, and immunity.

ESSENTIAL OILS

Essential oils are a diverse group of secondary plant metabolites that contain naturally occurring volatile components that support smell and taste of plants (Calsamiglia, et al. 2007). In nature, EO play an important role in allelopathic

communication between plants, attracting insects for pollination and dispersal of seeds, helping to deter herbivores by reducing their appetite for the plant, and also may act as antibacterials, antivirals, antifungals, insecticides or herbicides (Miguel, 2010). They are defined as a product obtained from a plant or some parts through hydrodistillation, steam distillation, or dry distillation, without heating by a mechanical process (Miguel, 2010). Typically, EO are classified into two specific chemical groups, terpenoids (most common) and phenylpropanoids, which are derived from different metabolic precursors, contributing to more than 15,000 unique components (Miguel, 2010, Calsamiglia, et al. 2007).

Essential oils offer unique properties that could be a huge potential benefit for animal agriculture. One such potential benefit that has a gained a lot of attention, is the antibacterial properties. There is excitement that EO could be a potential mechanism to improve feed efficiency, nutrient utilization, and animal health as an alternative to antibiotics. However, at this point there is still a need for more research to provide information for practical feeding recommendations. Most studies conducted have been conducted using *in vitro* settings with very limited research *in vivo*. Complicating matters more, differences in EO efficacy varies depending on what part of the plant is harvested, time of year, where it was grown, and chemical type (chemical structure) (Calsamiglia, et al. 2007). Inconsistencies of results also arise between research trials due to differences involving diet, dosage rates, and animal management. As will be reported in the following section, this literature summarization of some the important EO properties and studies outlining EO potential benefit for animal agriculture.

Antibacterial Modes

Contrary to their name, EO are not true oils, but are lipids. The hydrophobic nature of EO contributes to its antimicrobial properties. It is through antimicrobial properties that allows the alteration of bacterial growth and bacterial metabolism. A couple modes of action have been identified, and is not likely caused by one specific mode, but several target areas in the bacterial cell (Benchaar et al., 2008, Dorman and Deans, 2000).

Essential oils have a high affinity for the lipid components in the bacterial cell membranes. This affects cell membrane processes and thought to cause interruptions in processes, such as electron transport, ion gradients, protein translocation, phosphorylation, and other enzyme-dependent reactions (Dorman and Deans 2000).

One such reaction involves conformational changes in bacterial cell membrane structure causing loss of membrane stability. It is hypothesized this happens when the EO accumulates in the lipid bilayer of the bacteria cell membrane resulting in fluidification and membrane expansion, causing the membrane to become leaky and decreasing the transmembrane ionic gradient (Griffen, et al. 1999). This loss of membrane stability affects a wide range of microorganisms including gram-positive (GP) and gram-negative (GN) bacteria however, GP bacteria are more sensitive to EO. It is thought (GN) bacteria are less susceptible because the outer cell wall is hydrophilic, thereby preventing lipophilic essential oils from penetrating this barrier (Benchaar et al., 2008, Calsamiglia et al., 2007). This interaction and subsequent loss of membrane stability typically does not result in bacterial cell death, but results in slowed bacterial growth, which causatively also affects the rumen fermentation profile (Griffen, et al. 1999).

Oregano

Origanum vulgare, also known as oregano, is one well known EO that is shown to disrupt bacterial cell membrane ion gradient, inhibiting both GP and GN bacteria (Helander et al., 1998, Dorman and Deans 2000). The active components of oregano are thymol and carvacrol, which chemically are a terpenoid with a phenolic structure. It has been suggested that phenolic compounds, such as thymol and carvacrol, have effective antimicrobial activity due to hydroxyl groups in the phenolic structure (Benchaar et al., 2008). These active components are thought to disrupt the bacterial cell membrane in several different ways. One study demonstrated that the proton motive force was disrupted, and that the H^+ and K^+ ion gradients were dissipated affecting intracellular ATP processes (Benchaar et al., 2011). Another proposed mechanism is similar to that of ionophore antibiotics involving the hydroxyl group acting as a trans-membrane proton carrier (Benchaar et al., 2011).

Essential oils, such as oregano that have active components containing phenolic structures have been shown to be highly effective against specific microorganisms (Dorman and Deans 2000, Benchaar et al., 2008), and has been well documented in many lab based settings. One study conducted by Helander et al. (1998) reported that oregano inhibited GN bacteria, such as *Escherichia coli,* and *Salmonella tryphimurium*. This is further supported by Marino et al. (2001), who reported that oregano not only inhibited GN *Escherichi coli* and *Salmonella tryphimurium,* but also *Proteus mirabilis*, *Pro. Vulgaris, Serratia marcescens, Yersinia enterocolitica, Pse. Fluorescens* and *Pse. Putida*. Oregano was shown to have inhibitory effects against a wide range of bacteria including GP bacteria, such as *Micrococcus sp*., *Sarcina flava, S. aureus, B. licheniformis, B.*

thuringiensis and *L. innocua*. Furthermore, compared to other EO, oregano was the most effective and in high concentrations (800 ppm) caused 100% inactivation of all the tested bacteria (Marino et al., 2001). It has been suggested as well, that certain EO may have the ability to inhibit parasites, such as *Cryptosoridium*, coccidia, and nematodes (Benchaar, et al. 2007).

Bampidis et al. (2006) conducted an *in vivo* study using calves that exhibited *Escherichia coli* scours. Administration of dried oregano leaves in a liquid solution, may be as effective in the treatment of *E. coli* scours, as an oral solution of neomycin sulphate. In this study, calves were treated with either 10 mg of neomycin sulphate per kg of calf weight or 10 mg of oregano per kg of calf body weight per 24 hours with results indicating no significant differences in overall scouring days or calf mortality (Bampidis, et al. 2006). These results should be interpreted with care as administration of oral plant components could have the possibility of controlling a wide range of microorganisms, that could also cause an imbalance in normal gut microorganisms. Potentially allowing pathogens an opportunity to become established in the gut with detrimental results (Dorman and Deans 2000). More research needs to be conducted to elucidate the optimal EO inclusion rates that are effective at controlling various pathogens without inhibiting or consequentially affecting other beneficial gut organisms or processes.

High dosages of EO, such as oregano and its major constituents (carvacrol and thymol), has been shown to inhibit rumen microbial fermentation. In an *in vitro* study conducted by Busquet et al. (2006), high dosage (3,000 mg/L) of oregano oil resulted in decreased rumen total concentration of volatile fatty acids (VFA), ammonia N, and an increase in ruminal pH, and this was true for almost all of the EO evaluated. These results

indicate that EO, such as oregano oil, may be detrimental to rumen microbial fermentation at high dosages (3,000 mg/L), but at marginal (300 mg/L) and low dosages (3 mg/L) can be administered safely without inhibiting rumen fermentation (Busquet, et al. 2006). These results correspond well with other *in vitro* studies Castillejos et al. (2007) reported that a high dose (500 mg/L) of EO including oregano, decreased total VFA concentrations, ammonia N, and increased ruminal pH, and acetate to propionate ratios. Lower dosages (5 and 50 mg/L) had no effect on ammonia N, rumen pH, acetate to propionate ratios, but did increase concentration of VFA (Castillejos, et al. 2007).

Testing the pure components of oregano, such as thymol also yields similar results in terms of rumen fermentation (Calsamiglia et al., 2007). It was suggested that ruminal deamination was inhibited when there was an accumulation of AA and a reduction in ammonia N concentrate in an *in vitro* study incubated with casein in rumen fluid containing thymol (1000 mg/L). Overall, there seems to be a consensus that thymol demonstrates similar results *in vitro* as oregano. At higher doses, thymol can affect overall nutrient digestion, decrease volatile fatty acid production, decrease ammonia N concentrations and that overall microbial metabolism within in the rumen is inhibited. It has been suggested that an optimal dose of thymol ranges approximately between 50 and 500 mg/L (Calsamiglia et al., 2007).

Carvacrol, the other main active component of oregano, has been suggested to either inhibit proteolysis or stimulate peptide lyses. An *in vitro* study using higher doses of carvacrol (300mg/L) decreases acetate-to-propionate ratios, VFA production, and increases pH and butyrate proportion. Lower doses (2.2 mg/L) decreased large peptide concentrations and increased ammonia N concentrations (Calsamiglia et al., 2007).

In both the pure components of oregano and oregano itself, *in vitro* studies demonstrate similar results in that high dosages can be inhibitory to ruminal fermentation. At moderate doses, there can be beneficial effects, however it is hard to quantity what that optimal dose should be. The optimal dosage maybe dependent on type of diet fed to the animal and has been suggested that pH can play a role. The antimicrobial effect of thymol has shown pH dependency, the lower the pH (6.5 versus 5.5) the more efficacious the effect. Furthermore, the percentage of active ingredients in oregano (carvacrol and thymol) are dependent on specific cultivar and processing methods. The chemical structures of thymol and carvacrol are different and these variations in chemical structures can affect results (Calsamiglia et al., 2007).

Essential oils and calves

Very limited EO research exists *in vitro* and even less *in vivo* results are available in dairy calves. Published results among projects are variable but shows many promising benefits, however more research needs to be done on optimal dosage and routes of EO supplementation. Hill et al. (2007) reported improved average daily gains (ADG), calf starter intake (CS) and efficiency in pre-weaned calves fed a commercial blend of essential oils. There were two studies conducted. Study one fed calves either an all MR or a MR with 45% of the crude protein from soy protein with or without the mix of EO. In study two, calves were fed with or without EO in the CS. The MR was formulated to be a 20:20, and the CS was formulated to be 18% CP. All inclusion of EO was fed a 0.05% of either the CS or MR or both. Calves were less than seven days old when enrolled in the trial and were weaned at 42 days of age. At the conclusion of both studies, calves supplemented with the commercial blend of EO had improved body weight gains, CS,

and feed efficiency (FE) compared to calves that were not fed EO. It was also noted that inclusion of the EO in both the CS and MR had additive effects on calf performance in improved body weight gains and feed efficiency (Hill et al., 2007).

Santos et al. (2015), reported no increase in calf performance or health when calves were supplemented with a commercial EO blend. In this study, neonatal calves were fed a 20:15 MR and a 26% crude protein CS and were assigned to one of three treatments: 1) Control with no added EO to MR or CS; 2) MR supplemented with 400 mg/kg of EO; and 3) EO supplemented at 200 mg/kg in both MR and CS. The authors reported no treatment differences in feed intake, body weight, structural gains, ADG, blood metabolites (plasma β-hydroxybutyrate and glucose), fecal scores, and counts of intestinal microorganisms (enteboracteria and lactic acid bacteria). Rumen fluid samples collected from the calves demonstrated no difference in ruminal pH, VFA, acetate to propionate ratio, or counts of protozoa, cellulolytic and amylolytic bacteria. However, ammonia-N concentrations were significantly higher in calves supplemented with EO in MR and CS. It was concluded that addition of EO in the MR, CS, or both did not hinder, but also did not improve the health or performance of dairy calves (Santos et al., 2015).

In a post-weaned study, Vakili et al. (2013) supplemented Holstein calves fed a high concentrate diet with either thyme or cinnamon EO. This study utilized three treatments: 1) control with no EO supplemented to the diet; 2) control diet with 5 grams a day per calf of thyme EO added; and 3) control diet with 5 grams a day per calf of cinnamon EO added. Diets fed were a total mix ration (TMR) composed of 15% alfalfa hay and 85% concentrate and formulated to meet NRC (1996) nutrient requirements. Similar to the study conducted by Santos et al. (2015) in pre-weaned calves Vakili et al.

(2013) reported no effect on calf performance in ADG, FE, DMI, blood metabolites (glucose, urea N, triglyceride, total cholesterol, β-hydroxybutyrate, alanine aminotransferase, aspartate aminotransferase). Rumen fermentation was also similar among treatments for ruminal pH, ammonia nitrogen, and total VFA. There was however, a decrease in molar proportions of calves supplemented with EO compared to control calves in acetate to propionate ratios, and acetate levels. Molar proportions of propionate were increased for calves supplemented with EO compared to control, Butyrate proportions were also significantly increased for calves fed the cinnamon EO over the control calves (Vakili et al., 2013).

PREBIOTICS

Prebiotics are non-digestible food additive that promote the growth and activity of specific gut microbes that beneficially affect the host (Samanta et al., 2012 and Uyeno et al., 2015). Specifically, prebiotics are carbohydrate biomolecules in the form of fructans. There are a variety of biomolecules that can be considered as a prebiotic, which can be of plant origin, or obtained from microbial additives such as yeast. These molecules can be further classified into a variety of subcategories, such as oligosaccharide, polysaccharide, or trisaccharide (Samanta et al., 2012). Some examples of oligosaccharides used in in the livestock industry are Mannan-oligosaccharide (MOS), or Fructooligosaccharides (FOS), galactosyl-lactose is a trisaccharide, and a well-known polysaccharide is inulin.

In ruminants, rumen microbes can ferment prebiotics and utilize it as a source of energy. The non-digestibility of prebiotics ensures that the microbes receive it as an energy source and is used directly by the host. This subsequently can cause an alteration in the microbial population activity and composition which can result in other effects,

such as drop in pH and increased gas production (Papatsiros et al., 2013). Furthermore, these prebiotics when fed have demonstrated increases in N retention, beneficial microflora, weight gain in calves, decreased rumen ammonia nitrogen, and better fecal consistency in calves (Samanta et al., 2012). It has been suggested, that prebiotics can be advantageous to ruminants when exposed to a variety of stresses, such as transportation, weaning, etc. that can result in a variety of adverse health events. Prebiotics promote the growth and activity of healthy gut microflora excluding pathogens that can reduce the adverse health events caused by stress, such as diarrhea, depression of growth and feed intake (Samanta et al., 2012 and Uyeno et al., 2015). It has also been shown that prebiotics can reduce the colonization of gut pathogens such as *Salmonella* and *E. coli* (Papatsiros et al., 2013), as well as, increase concentrations of immunoglobulins (Heinrichs et al., 2009). These characteristics specifically can have beneficial results in neonates in which the gut microflora is adapting and developing under a variety of stresses.

Feeding oligosaccharides to calves has shown similar results as supplementing antibiotics in milk replacer (Quigley et al., 1997 and Donovan et al., 2002). Supplementation of galactosyl-lactose increased body weight gain, feed efficiency and decreased severity of scours when supplemented to calf MR, contributing to increased intestinal health (Quigley et al., 1997). In a commercial prebiotic mix fed to calves tended to increase beneficial bacteria, such as *Lactobacilli* and increased fecal IgA, which suggested an increased intestinal health (Heinrichs et al., 2009). Similarly, MOS, and FOS have shown promising benefits in calf health and performance (Uyeno et al., 2015).

Modes of Action

Prebiotics promotes its benefits through a variety of mechanisms. Consumption of prebiotics results in the production of short chain fatty acids (propionate, acetate, butyrate) and lactic acid which decreases luminal pH. Decreased luminal pH is also thought to play an important role in enhancing mineral absorption, as some minerals, such as calcium has a higher solubility in lower pH and increases the gradient across the epithelium. It is hypothesized that the feeding of prebiotics interacts with the intestinal tight junctions in the epithelium increasing the permeability for mineral absorption. Calcium, magnesium, iron, and zinc have all demonstrated increased absorption with the feeding of prebiotics (Samanta et al., 2012).

Production of butyric acid promotes stimulation of goblet cells and subsequent increased mucous production in the intestines, which plays an important role in immune defense. Butyric along with lactic acid production plays an important role in gut epithelium development, increasing length and width of crypts and subsequent nutrient absorption (Samanta et al., 2012).

Consumption of prebiotics has also been shown to increase beneficial bacteria in the intestine such as *Bidobacteria* and *Lactobacilli*. The production of these bacteria are beneficial due to their ability to produce short chain fatty acids, reduce gut pH, ferment non-digestible carbohydrates, and stimulate immunoglobulin production (Samanta et al., 2012). These beneficial bacteria exclude gut pathogens through competitive exclusion by suppressing growth, reducing toxic fermentation products. This is accomplished by preventing the adhesions of pathogens to mucosa by competing with the sugar receptors (Samanta et al., 2012 and Papatsiros et al., 2013).

Mannan-oligosaccharide (MOS)

Mannan-oligosaccharide is a mannose sugar that is derived from the cell wall fragments of yeast, such as *Saccharmyces cerevisiae.* It is known for its ability to adsorb pathogens with type-1 mannose-specific fimbriae that are found in many GN bacteria, such as *Escherichia coli* and *Salmonella*. This increases competition for binding sites on the intestinal epithelium, decreasing the pathogens ability to colonize the gastrointestinal tract (Heinrichs et al., 2003 and Brady et al., 2015). This allows MOS with the ability to help improve immunity, performance, and health of dairy calves.

There has been varying results published in literature on calves supplemented with MOS. Heinrichs et al. (2003), found that when Holstein calves were supplemented with MOS they performed similarly as calves supplemented with antibiotics. In this study, calves were randomly assigned to one of three treatments: 1) control 20:20 all MR; 20:20 all MR with 400g/440 kg of neomycin + 200 g/440 kg of oxytetracycline; and 3) 20:20 all MR with 4 g calf/ d of Bio-Mos supplemented. It was found that calves supplemented with antibiotics and MOS had a greater probability of having normal feces throughout the study meaning less scours overall compared to control calves. There was no difference in ADG or FE among the three treatments. However, calves supplemented with MOS had significantly higher grain intakes during week six (when calves were weaned) than that of calves supplemented with antibiotics. Greater feed intake did not affect growth performance such as body weights and body parameters (hip width, wither height, heart girth, hip height) were similar among treatments. The authors reported no significant differences in metabolic problems between calves, blood urea N and total

blood protein between treatments. It was concluded that MOS could be as effective as an antibiotic alternative in calf MR (Heinrichs et al., 2003).

Similarly, Terre et al. (2007), found that supplementing a MOS product stimulated an increase in CS intake after weaning, as did Heinrichs et al (2003), but did not improve growth rates. In this study, Holstein calves were enrolled in an enhanced-growth feeding program in a group pen. Calves were assigned to one of two pens; pen one calves were fed MR supplemented with 4 g/d of Bio-Mos, pen two was control calves just fed MR with no supplementation. Calf performance throughout the trial was similar between treatments. Body weight, and ADG, and gain: feed was not significantly different between treatments ($P > 0.05$). Dry matter intake of MR and CS were similar with the exception of MOS supplemented calves having higher CS intake compared to control calves right after weaning. The incident of loose feces or scours between treatments were similar as well as total fecal counts of *Escherichia coli* and *Clostridium perfringens*. Numerically, the probability of observing presence of *Cryptosporidium* in a fecal smear was lower for calves supplemented with MOS compared to control calves in the first two weeks of the study. However, in week three the control calves had numerically lower presence of *Cryptosporidium* than calves supplemented with MOS. Lastly, there was no difference in serum haptoglobin concentrations between treatments. The authors concluded that supplementing calves with MOS did increase CS intake after weaning, but did not affect the growth performance, nor reduced fecal bacterial counts in calves (Terre et al., 2007).

Unlike Heinrich et al (2003) and Terre et al. (2007), Ghosh and Mehla (2012) reported that supplementing cross-bred dairy calves with 4 g/d of Bio-Mos compared to

control calves increased body weight gain. Feed intake overall was increased throughout the study, however the results were not separated by pre- and post-weaning stages so it is difficult to ascertain if similar patterns were observed as reported by Heinrich et al (2003) and Terre et al. (2007) were intake was increased just after weaning. Fecal scores for calves supplemented with MOS was significantly lower compared to control calves and unlike Terre et al. (2007), there was a significant decrease in fecal coliform counts (Ghosh and Mehla, 2012).

It has also been hypothesized that feeding of MOS to dry cows pre-calving or supplementing calves with MOS in colostrum could improve immunity. Franklin et al. (2005) reported that supplementation of periparturient dairy cows with MOS resulted in a tendency for calves to have increased serum protein concentrations (passive transfer) from birth to 24 hours of age. Supplementing the ration with MOS enhanced the cow's immune response to vaccination such as rotavirus, which resulted in the calves receiving better antibodies against rotavirus. It was proposed that MOS supplementation to periparturient cows could enhance immunity transfer to calves leading to better calf immunity when born, which could result in decrease treatment costs (Franklin et al., 2005). Increased serum protein concentrations were not observed when calves were supplemented with 30 g of MOS in the colostrum. Colostrum supplementation with MOS actually resulted in negative effects. Brady et al. (2015) recommended not to supplement MOS in calf colostrum. In that study, MOS supplementation in fresh maternal colostrum led to lower apparent efficiency of IgG absorption and reduced serum IgG concentrations compared to control calves (Brady et al., 2015). These results are similar to Robichaud et al. (2014), who reported that MOS supplementation to colostrum calf replacer resulted in

similar apparent efficiency of absorption of IgG and serum IgG concentrations compared to control. It was also reported that there were no effects on incidences of diarrhea, pneumonia, survival, or ADG in calves supplemented with MOS in colostrum replacer between birth and weaning (Robichaud et al., 2014).

Fructo-oligosaccharides (FOS)

Fructo-oligosaccharide is a soluble fiber that is composed of several β-(1,2) or β-(1,6) linked fructose residues. It is obtained from the partial hydrolysis of inulin, a carbohydrate energy source for plants most often sourced from chicory root (Franck, 2006). Fructo-oligosaccharide is known to stimulate the production of beneficial bacteria in the gut, including *Bifidocteria* and *Lactobacilus,* which can ferment FOS to short chain fatty acids (Quigley et al., 2002, Grand et al., 2013, and Jenkins et al., 1999). Increased production of short chain fatty acids is used as a source of energy, can enhance mucosal structure, and prevent the colonization of pathogens, such as *Clostridium difficile* (Grand et al., 2013 and Donovan et al., 2002).

In rat and human studies, FOS or oligofructose, has shown clear benefits in mineral absorption and bone mineralization. Supplementation of FOS demonstrated that calcium and magnesium absorption is enhanced (Franck et al., 2006). It has also been shown that FOS increases calcium balance, true and apparent intestinal absorption and mineral density in growing rats (Morohashi, 2002).

In calves, studies evaluating FOS supplementation reported that calf performance can be enhanced as a result of the modification of microbial fermentation activity, possibly as an antibiotic replacement, and a useful tool in times of stress (Grand et al., 2013, Quigley et al., 2002, and Donovan et al., 2002). Grand et al. (2013), studied the

effects of different FOS dosages on the growth performance, carcass characteristics and fecal concentrations of short chain fatty acids on Holstein cross veal calves. Calves were fed a MR containing 5% soluble wheat proteins and supplemented with either 0, 3, or 6 g/d of FOS. Body weight, cold carcass weight, DMI, ADG, and feed conversions were similar between treatments. Numerically, calves supplemented with FOS had a reduction in feed conversion and increased carcass weight, and ADG. There was also an observation of an overall increased production of short chain fatty acids with an increased percentage of butyrate and a decrease in acetate production in calves supplemented with FOS compared to control, however not statistically significant. It concluded that supplementation of FOS in MR allowed enhanced growth parameters (Grand et al., 2013).

Grand et al. (2013) reported results similar to Kaufhold et al. (2000) who fed veal calves supplemented with or without FOS. Kaufhold et al. (2000), reported that calves supplemented with 10 g/d of FOS tended to have greater weight gains compared to control calves. Feed intakes among the two treatments were similar. Instead of studying carcass and short chain fatty acids in feces, Kaufhold et al. (2000) was interested in metabolic and endocrine functions of calves supplemented with FOS. It was reported that FOS supplementation decreased post-prandial glucose and increased insulin concentrations in very small amounts when calves were given lactose. It was concluded that FOS had similar effects on the metabolic and endocrine traits as humans with diabetes mellitus and that FOS may aid in coping with insufficient control of glucose metabolism (Kaufhold et al., 2000).

Quigley et al. (2002) studied the effects of feeding spray-dried animal plasma with or without the addition of FOS on the growth and health of calves. This study was conducted into two separate experiments in which Holstein calves were used. Calves were fed MR in study one with 0 to 20% of the crude protein being spray-dried animal plasma and experiment two was with 0 to 16% of the crude protein containing spray-dried animal plasma. Both studies had treatments containing 30 or 60 g of additive that had FOS added that contained either bovine serum or whey protein. In both studies, calves fed milk with FOS had fewer scour days, and improved fecal scours compared to calves fed control. One experiment reported increased body weight gain and subsequently feed efficiency when feeding FOS compared to control fed calves (Quigley et al., 2002).

IMMUNITY

Immunity plays an important role in calf health and is often an overlooked area of research. Immunity is the normal function of the immune system that helps to keep an individual healthy by maintaining homeostasis by recognizing and attacking foreign substances that disrupts balance. The immune system can be classified into two distinct types: innate and acquired/adaptive immune responses. Innate immune system is referred to as the first line of defense and is non-specific in nature. In neonates, the innate immune system is the most important, because it is what the calf is born with. Development of the calf is required to develop an acquired/adaptive immune system. Innate immune system uses mechanisms, such as external defenses, which include the use of physical barriers, like skin, mucous, etc. It can use internal defenses, such as phagocytic cells such as macrophages. Both external and internal defenses are used to keep and clear foreign substances from the body. The innate immune cells can also release signaling proteins,

such as cytokines, that help connect to the adaptive immune response (Mak et al., 2014 and Lippolis 2007).

The adaptive immune system is the second line of defense and is considered specific in nature because it is designed to recognize and remember specific pathogens. Adaptive immune response is triggered when signals are received from the innate immune system (cytokines) when the system is unable to remove or overcome a threat. Adaptive immunity is divided into both passive and active immunity. Active immunity is developed upon contact with an antigen, pathogen, or vaccination which can be further divided into humoral and cell-mediated immunity. Humoral immunity is composed of B-cells that are produced and mature from the bone marrow, that become antibody-producing or memory cells. The cell-mediated immunity is composed of T-cells that mature in the thymus to give rise to T-helper or T-cytotoxic cells. B-cells are specialized to recognize whole antigens, whereas, T-cells recognize fragments of antigens presented on the major histocompatibility complex molecules which are used to enhance immune responses. Overall, active immunity uses humoral and cell-mediated approaches to mature B-cells into making their own antibodies. (Mak et al., 2014 and Lippolis 2007).

Passive immunity is recognized as the transfer of antibodies to a non-immune individual, such as antibodies received by a neonate from its mother. Compared to active immunity, this is a relatively fast immunity development. Passive immunity is vitally important for neonatal calves as they are born agammaglobulinemic (no antibodies). Neonatal calves are unique in that they receive no maternal antibodies (immunoglobulins) via placental transfer. Immunoglobulins are taken up mostly across the gut epithelium by receiving colostrum, making it imperative that quality colostrum is

received when they are born (Pastoret et al., 1998). Passive immunity is an evolutionary response that has developed as antibodies, such as immunoglobulin G (IgG), may take as long as 6-12 months to develop adequate amounts in serum and secretory Igs to be produced by the newborn (Mak et al., 2014).

Immunoglobulins (Ig)

Antibodies or immunoglobulins are glycoproteins members of the immunoglobulin super family. Their basic structure is composed of two light chains and two heavy chains, which come together to form a Y shaped molecule. These molecules have two Fab sections that retains antibodies antigen-binding ability and one Fc region that is considered the tail of the antibody. This Fc region does not bind to antigens, but instead recognizes and binds to Fc receptors on innate cells, and controls antibody effector functions. There are four antibody effector functions which include neutralization, classical complement activation, oponization, and antibody-dependent cell-mediated cytotoxicity (ADCC) (Mak et al., 2014). When an infection or vaccination occurs there is a humoral immunity response in which immunoglobulins are secreted and the foreign entity or pathogen is eliminated or neutralized through the effector functions. There are five different immunoglobulin isotypes- IgG, IgA, IgE, IgD and IgM. Each type has different biological functions and physical properties; typically IgG, IgA, and IgM are found in serum and IgD and IgE are only found in serum in low concentrations (Cervenak and Kacskovics, 2009).

Immunoglobulin G (IgG)

Immunoglobulin G is the most common immunoglobulin isotype found in tissue and blood with a long half-life (Cervenak and Kacskovics, 2009). There are three

recognized subclasses recognized in bovine; IgG_1, IgG_2, and IgG_3 (Pastoret et al., 1998). The IgG class uses a mix of antibody effector functions including opsonization, ADCC, and classical complement activation to eliminate pathogens. Opsonization and ADCC are exerted by the FcγR- bearing cells, such as phagocytes and lytic cells, that has a high affinity to IgG_1 and IgG_3 subclasses (Mak et al., 2014).

Calves are born agammaglobulinemic and must receive maternal antibodies through colostrum. Bovine colostrum is specifically high in IgG (Pastoret et al., 1998), and has a 90% absorption rate (Roy, 1979). Specifically, colostrum is composed mainly of IgG1 subclass, which binds to FcRn (neonatal Fc receptor) antibodies with a high affinity. This FcRn receptor is important in transporting maternal immunity and protects IgG from fast degradation until the calf is able to develop their own antibodies (Cervenak and Kacskovics, 2009).

Ingestion of colostrum is time dependent and it is important for a calf to receive colostrum before gut "closure" and before establishment of gut microflora, otherwise bacteria can be absorbed the same way as maternal IgG (Roy, 1979). It is critical for calves to receive colostrum for adequate IgG absorption within the first 24 hours. Calves are 74 times more likely to die in the first 3 weeks if they do not receive colostrum (Hulbert and Moisa, 2015). Furthermore, calves that do not receive adequate amounts of colostrum with low IgG absorption are said to have a failure of passive transfer. Successful passive transfer is defined as having a serum IgG reading of above 10 mg/mL by 48 hours of age. Generally, higher serum IgG levels are associated with healthier calves that have lower morbidity and mortality rates (Barrington et al., 2001).

Immunoglobulin A (IgA)

Immunoglobulin A is often considered the first line of defense when it comes to immunoglobulins because it binds to pathogens at the mucosal surfaces, which are common sites of pathogen attacks (Mak et al., 2014). It is found in a secretory form referred to as secretory IgA (SIgA), such as tears, saliva, milk, and mucus, and is produced in the gastrointestinal, urogenital, and respiratory tracts. Immunoglobulin A uses neutralization by cross-linking microorganism and macromolecules to prevent pathogens from attaching to the surface of epithelial cells at mucosal surfaces (Corthesy, 2013 and Mak et al., 2014). Low production of IgA is linked to a variety of recurring gastrointestinal, respiratory infections and even allergies/asthmas in humans (Corthesy, 2013).

The serum concentration of IgA is found to be low and is lower in bovine colostrum then IgG (Pastoret et al., 1998), but never less still important. When a calf receives colostrum there is a high concentration of serum IgA but disappears quickly into the intestinal lumen. High concentration of immunoglobulins (300 to 400 g Ig in first 48 hours) from colostrum regardless of isotype are associated with reduced calf mortality and morbidity and is vitally important (Roy et al., 1979). Calves that receive no colostral Igs will remain basically agammaglobulinemic until immune system develops leaving the calf susceptible to infections and even death (Butler, 1969).

CONCLUSION

Milk replacer additives such as EO or prebiotics show many promising benefits to increase calf health and performance with the out use of antibiotics. However, more

research needs to be conducted to elucidate the optimal dosages, and the most efficacious to enhance calf growth, performance, and health. Furthermore, it is important to understand calf immunity and ways to improve immunity; either through feed additives or changing the management of calves to reduce stresses. This literature review was to illuminate the current research conducted to date leading well into the study presented in the next chapter evaluating a commercial blend of EO and prebiotics, mainly oregano, and how performance compares to prebiotics or calves not supplemented with either additive.

CHAPTER 2: EVALUATION OF STAY STRONG FOR DAIRY CALVES

INTRODUCTION

Neonatal calves often experience numerous stresses caused by being exposed to pathogens eliciting various enteric and respiratory problems resulting in pre-weaning deaths. The United States has a 7.8% mortality rate in pre-weaned heifer calves. Of these mortality rates, 56.5% is attributed to scours and digestive problems and another 46.7% is attributed to respiratory problems (USDA-NAHMS, 2007). Supplementing antibiotics in MR has many benefits including reduction in mortality rates, scours and increased feed consumption and gains (Morrill et al., 1977). However, increased FDA regulation and public scrutiny makes the supplementation of antibiotics in MR quite difficult. It is estimated that the treatment of respiratory and enteric problems in calves exceeds $250 million annually (Simmons and Bywater, 1991). This is of huge economic significance to the industry, resulting in investigations for identifying alternatives to antibiotics.

One such alternative is the use of essential oils (EO). Essential oils are a diverse group of secondary plant metabolites and contain naturally occurring volatile components that support the smell and taste of plants (Calsamiglia et al., 2007). In nature, EO play a role in attracting insects, and allelopathic communication in plants. Additionally, EO may also act as antibacterials, antivirals, antifungals, insecticides, and herbicides (Miguel, 2010). Antibacterial is one of the most noted contributing feature of EO in animal agriculture. It is this ability that has the potential to alter bacterial growth, by causing conformational changes to membrane structures affecting cell membrane processes of bacteria (Benchaar et al., 2007, Calsamiglia et al., 2007).

Typically, EO have more of an effect on GP bacteria than GN bacteria due to the cell membrane being less impermeable (Benchaar et al., 2007). However, certain EO such as oregano have been shown to inhibit growth of several GN bacteria, including *E. coli* (Marion et al., 2001). Bampidis et al. (2006), found that administration of an oral oregano solution to calves with E. coli scours may be as effective as neomycin. Further studies supplementing pre-weaned calves with EO have found increases in CS intake, feed efficiencies, and body gains (Hill et al., 2007). As well as increase proportion of beneficial microorganisms in the intestinal flora (Santos et. Al., 2015). As a result EO show promising benefits as a potential feed additive to help reduce neonatal stresses, while improving performance without the use of antibiotics. Thus, the objective of this study was to determine optimal inclusion rate of a commercial EO mix to promote calf growth, and health, when added to the MR of pre-weaned dairy calves compared to calves fed a control or yeast cell wall (YCW) product. It was hypothesized that calves supplemented with the medium dose (EO-1.0) of the commercial EO would be most optimal, and in general calves supplemented with commercial EO would have improved growth, health, and immunity compared to calves not supplemented.

MATERIALS AND METHODS

Calf Feeding and Management

This research project was conducted at the South Dakota State University (**SDSU**) Animal Research Wing (ARW, Brookings, SD) from September 7 to December 8, 2015, all procedures were approved by SDSU Institutional Animal Care and Use Committee before the start of the study. One hundred Holstein calves were sourced from a commercial dairy farm and were housed in Calf-Tel hutches bedded with straw. Calves

were blocked by birth date and randomly assigned to 1 of 5 treatments. Treatments were: Control (C): a 24:20 MR, EO mixed into 24:20 MR at a rate of 1.25 g/feeding (EO-0.5), 2.5 g/feeding (EO-1.0) or 3.75 g/feeding (EO-1.5), or 24:20 MR with an inclusion of YCW at a rate of 2 g/calf/feeding. Essential oil was Stay Strong for dairy calves manufactured by Ralco (Marshall, MN) and the YCW technology (Bio-Mos) was manufactured by Alltech (Nicholasville, KY).

Prior to enrollment in the study, calves were tested for successful passive transfer of colostrum, and bovine viral diarrhea (BVD). Successful passive transfer was determined by serum samples that were collect via jugular puncture and were read for total protein (TP) using a Brix refractometer. Calves with total protein greater than 5.5 g/dl were considered successful. Ear notch samples were submitted to South Dakota Animal Disease Research and Diagnostic Laboratory (**SDSU-ADRDL**) for BVD testing. Upon arrival, calves were castrated using an elastic ring (Animal Health International, Greenly, CO) and vaccinated with Inforce 3 via intranasal (Zoetis; Florham Park, NJ).

Calves were fed colostrum before their arrival at SDSU for the first two d of life. A 24:20 MR was fed at a rate of 0.28 kg/calf at each feeding (0630 and 1730 h) daily for 14 d via bucket, and then increased to a feeding rate of 0.43 kg/calf at 2x/d until 35 d. Feedings were reduced to 1x/d at 36 d to facilitate weaning at 42 d. Decoquinate was added to MR at 37.8 g/ton for coccidiosis control. A 20% CP pelleted calf starter (CS) and water was offered ad libitum throughout the study. All MR and CS were sourced from Hubbard Feeds Inc. (Mankato, MN). Intakes and refusals of CS were recorded daily in the morning, and any MR refusal were also recorded. Both CS and MR samples were

collected every two weeks and stored frozen (-20ºC) before being composited by month and sent to Analab (Fulton, IL) for analysis.

Ovalbumin Vaccination

Calves were subjected to an ovalbumin (OVA) challenge, and were vaccinated in the beginning of week three (21 d) and then given a booster on week six (42 d). Calves were given 4 mL subcutaneously of an OVA solution in four, 1 mL injections to the side of the neck to minimize abscesses. The OVA solution contained 4 mg of crystallized OVA (Sigma Chemical, St. Louis, MO) suspended in a phosphate buffered saline (PBS). The concentration of the OVA-PBS solution was 2 mg of OVA/ml of PBS. The OVA-PBS solution was filtered sterilize through a 0.45 micron filter, and was then diluted with 1:1 (ml:ml) Freund's incomplete adjuvant (ThermoFisher Scientific, Waltham, MA). The OVA-PBS-Freunds solution was emulsified using a 22 gauge micro-emulsifying needle and glass mixing syringes for several minutes. The OVA vaccine was transferred to plastic 5 mL syringes with 18 gauge needles for administration to calves. The vaccine was made weekly and stored in refrigerator for up to four days before administration.

Nasal Secretion Collection and Analysis

Nasal secretion samples were collected from calves twice throughout the study. Secretion samples were collected when the calves arrived (0 d) before vaccination of Inforce 3 via intranasal (Zoetis; Florham Park, NJ), and again on week three (21 d). A 50 x 55 mm foam plug (VWR, Radnor, PA) was cut into quarters and one quarter was inserted into the nasal cavity of the calf and was allowed to sit for 5 to 7 minutes or until the foam was saturated. The foam was then pulled out and inserted into a 10 mL plastic

syringe that was used to squeeze the nasal secretions out into a 1 mL microcentrifuge tube that was then stored frozen (-20ºC) until analysis.

Nasal secretions were analyzed for specific IgA titers to Inforce 3 against bovine respiratory syncytial virus (BRSV) using an enzyme-linked immunosorbent assay (ELISA) modified from Woolums et al. (2013). Samples were prepared using a 96-round well plate (Immulon 1B, VWR, Radnor, PA). Every other column of the plate was coated by pipetting 100 µL/well of a 1:10 dilution of UV inactivated BHV-1 Cooper strain. Stock solution of BHV-1 Cooper strain had a titer of 1 X 10^7 $TCID_{50}$/ml and was inactivated by placing in a hood with UV for 20 minutes which was stored at -80ºC until ready to use for coating. Prior to coating the UV inactivated BHV-1 Cooper strain was diluted 1:10 with a coating buffer which was distilled water containing 0.9% sodium carbonate (Sigma Chemical, St. Louis, MO) solution adjusted to a pH of 9.7. The non-coated (no BHV-1 Cooper strain) columns served as a control to account for non-specific binding. All stock solutions were made fresh the day samples were analyzed. Plates were allowed to incubated at 4ºC for at least 12 h (overnight) before analysis, to allow adherence of antigen to each coated well. Following overnight incubation plates were emptied and washed once with phosphate buffered saline tween (washing buffer) and 200 µL of blocking buffer solution was added to each well and allowed to incubate for 1h at room temperature (~22ºC). Phosphate buffered saline tween (PBST) was prepared as PBS with 0.05% of polyoxeyethylene sorbitan monolaurate (Tween 20, Sigma Chemical, St. Louis, MO). The blocking solution was prepared with phosphate buffer solution (PBS) that contained 0.5% bovine serum albumin (BSA, Sigma Chemical, St. Louis, MO). Following incubation of the blocking buffer, plates were emptied and washed 3

times with PBST and blotted dry. Nasal secretion samples were thawed and diluted in a two-fold serial dilatation with PBST that contained 0.01% bovine serum albumin (BSA, Sigma Chemical, St. Louis, MO). Dilution of nasal secretion samples from initial concentration was 1/4 for both week 0 (d 0) and week 3 (21 d). One hundred µL of the diluted samples were added to the wells in duplicate and incubated for 1 h at room temperature (~22ºC). Following incubation plates were emptied and washed 4 times with PBST wash buffer and blotted dry. A second antibody, rabbit anti-bovine IgA (Bethyl Laboratories Inc., Montgomery, TX) was diluted to a concentration of 1:2500 with PBST containing 0.01% BSA and 100 µL was pipetted to each well and incubated for 1 h at room temperature (~22ºC). Following incubation plates were emptied and washed 4 times with PBST wash buffer and blotted dry. A tetramethylbenzidine substrate solution (TMB, ThermoFisher Scientific, Waltham, MA) was added at 100 µL per well and incubated in the dark at room temperature (~22ºC) for 20 minutes for color development. The reaction was stopped by adding 100 µL of a TMB stop solution to each well. Plates were then read at 405 nm optical wavelength using a 96-well plate reader (Biotek, ELx808, Winooski, VT).

Body, Fecal and Health Measurements

Body weights were taken weekly using a digital scale (Digi-Star, Fort Atkinson, WI) after morning feeding. Body measurements including wither height (**WH**), hip height (**HH**), hip width (**HW**), heart girth (**HG**) and body length (**BL**) were taken bi-weekly at the time body weights were taken.

Fecal samples were collected three times for each calf throughout the trial and were taken when they first arrived (0 d) and then the beginning of week two (8 d) and

week five (35 d). Samples were taken fresh to Rural Technologies Inc. (Brookings, SD) for analysis of *Escherichia coli, Salmonella*, *Clostridium*, and *Cryptosporidium*. Samples were analyzed using a streak plate and given semi quantitative descriptions based on growth of the bacteria into different quadrants of the plate. *Escherichia coli* descriptions were established as 1) none detected, 2) Rare amount, 1 phenotype, 3) rare amount, 2 phenotypes, 4) small amount, 1 phenotype, 5) small amount, 2 phenotypes, 6) moderate amount, 1 phenotype, 7) moderate amount, 2 phenotypes, 8) moderate amount, 3 phenotypes, 9) large amount, 1 phenotype, 10) large amount, 2 phenotypes, 11) large amount, 3 phenotypes. *Salmonella*, *Clostridium*, and *Cryptosporidium* descriptions: 1) none detected, 2) rare amount present, 3) small amount present, 4) few present, 5) moderate amount present, 6) large amount present, 7) many present.

Health scores included ear, eye, nasal, and fecal scores and were recorded daily before evening feeding. Health scores were according to the University of Wisconsin calf health scoring chart (McGuirk, 2013), and were based on a 0-3 scale. Fecal scores were established as 0) normal, 1) semi-formed, pasty, 2) loose, but stays on top of bedding, 3) watery, sifts through bedding. Ear scores: 0) normal, 1) ear flick or head shake, 2) slight unilateral droop, 3) head tilt or bilateral droop. Eye scores: 0) normal, 1) small amount of ocular discharge, 2) moderate amount of bilateral discharge, 3) heavy ocular discharge. Nasal scores: 0) normal serous discharge, 1) small amount of unilateral cloudy discharge, 2) bilateral, cloudy or excessive mucus discharge, 3) copious bilateral mucopurulent discharge. All health incidents, and treatments were recorded for the length of the study.

Blood Sampling and Analysis

Blood samples were collected from the jugular vein on all calves weekly after morning feeding. Blood samples were collected using a 10 ml Vacutainer serum separation tube with a 18 gauge needle (Animal Health International, Greenly, CO). Samples were allowed to clot, and serum was harvested by centrifugation at 2,000 x *g* for 20 min and stored frozen in 5 ml polystyrene tubes for later analysis.

VFA- Serum samples from week 5 were analyzed for VFA concentrations using a GC (model 6850, Agilent, Santa Clara, CA). Samples were prepared according to the procedures of Oba and Allen (2003) and 1 µL of sample was injected at a split ratio of 2:1 at the injection port (280ºC). Volatile fatty acids were separated on a capillary column (30 m x 0.32 mm x DB 0.25; Agilent, Santa Clara, CA) with a flow of 45 mL/min of He using 2-ethylbutyrate as an internal standard. Initial oven temperature was 90ºC for 5 min and then was increased by 10ºC/min to 150ºC, where it was held for 5 min. The oven was brought up to 170ºC for purging at the end of the run. The flame ionization detector was maintained at 300ºC.

ELISA Ovalbumin Assay- Serum samples from week 3, 4, 5, 6, 7, and 8 were analyzed for determination of IgG titers to vaccination of calves with ovalbumin using a modified protocol from Rivera et al. (2002). Samples were prepared using a 96-round well plate (Immulon 1B, VWR, Radnor, PA). Every other column of the plate was coated by pipetting 100 µL of a solution containing 0.005 mg of ovalbumin/mL of PBS or 500 ng of ovalbumin per well. The non-coated (no ovalbumin) columns served as a control to account for non-specific binding. All stock solutions were made fresh the day samples were analyzed. Plates were allowed to incubated at 4ºC for at least 12 h (overnight) before analysis, to allow adherence of ovalbumin antigen to each coated well. Following

overnight incubation plates were emptied and 200 µL of a blocking buffer solution was added to each well and allowed to incubate for 1 h at room temperature (~22ºC). The blocking solution contained PBST with 2% Casein (Sigma Chemical, St. Louis, MO). PBST was prepared as PBS with 0.05% of polyoxeyethylene sorbitan monolaurate (Tween 20, Sigma Chemical, St. Louis, MO). Following incubation of the blocking buffer, plates were emptied and washed 3 times with PBST and blotted dry. Serum samples were thawed and diluted in a two-fold serial dilution with PBST that contained 0.1% bovine serum albumin (BSA, Sigma Chemical, St. Louis, MO). Dilution of serum samples were as followed week 3 (21 d) and 4 (28 d) were diluted 1/4 from initial concentration, week 5 (35 d) diluted 1/16, week 6 (42 d) diluted 1/64, week 7 (49 d) and 8 (56 d) diluted 1/512 from initial concentration. One hundred µL of the diluted samples were added to the wells in duplicate and incubated for 1 h at room temperature (~22ºC). Following incubation plates were emptied and washed 3 times with PBST wash buffer and blotted dry. A second antibody, alkaline phosphatase anti-bovine IgG (Sigma Chemical, St. Louis, MO) was diluted to a concentration of 1:5000 in PBST containing 0.1% BSA and 100 µL was pipetted to each well and incubated for 1 h at room temperature (~22ºC). Following incubation plates were emptied and washed 3 times with PBST wash buffer and blotted dry. A substrate solution was added at 200 µL per well and incubated in the dark at room temperature (~22ºC) for 13 min. Substrate solution was made fresh prior to addition to wells and was made by using SIGMAFAST p-nitrophenyl phosphate tablets (Sigma Chemical, St. Louis, MO) added to diethanolamine substrate buffer (ThermoFisher, Waltham, MA) that was diluted from 5x concentration to 1x with distilled water. The reaction was stopped by adding 50 µL of a 2 M NaOH solution to

each well. Plates were then read at 405 nm optical wavelength using a 96-well plate reader (Biotek, ELx808, Winooski, VT).

Statistical Analysis

All data were subjected to least squares ANOVA for a randomized complete block design using the PROC MIXED procedure of SAS (version 9.3, SAS Institute Inc., Cary, NC). Calves were blocked by birth date. Treatment, week, and treatment x week were considered to be fixed effects, with calf within treatment as random effect. Least squares means were separated by PDIFF. Body weights, and measurements were adjusted by the covariate to account for difference in initial measurements. IgA and IgG titers were determined by subtracting control wells (non-coated) from corresponding optical density reading for the value of the sample well (coated) to account for non-specific binding. Fecal pathogen descriptions were grouped into 1) none detected, 2) rare amount, 3) small amount, 4) moderate amount, 5) large amount for *Escherichia coli* and 1) none detected, 2) rare/small present, 3) few/moderate present, 4) large/many present for *Salmonella*, *Clostridium*, and *Cryptosporidium.* Significance was declared at $P < 0.05$ and trends at $0.05 < P < 0.10$. Dry matter intake, fecal, nasal, eye, an ear scores were averaged by week. Means for the remaining variables were calculated from the data gathered during the collection time for each period.

RESULTS AND DISCUSSION

Feed Analysis

Nutrient composition and DM of MR and CS are provided in Table 1. The 20:24 MR slightly exceeded the for CP formulation specification. However, the fat was slightly

below the formulation specification (19.5% compared to 20%) but still is adequate for neonatal calves. The CS met or exceed formulation requirements for nutrients.

Immunological Performances

Immunoglobulin A titers were similar ($P > 0.10$) among all treatments (Table 2 and Figure 1). However, numerically calves fed EO-0.5 had higher IgA titers compared to calves fed the other treatments, and calves fed EO-1.0 and EO-1.5 had the lowest titers.

Immunoglobulin G titers were similar between treatments for week 3, 4, 5, and 6 (Table 2 and Figure 2). It was not until the booster ovalbumin vaccine was given during week 6 that numerical differences could be observed. Statistically, calves fed EO-1.0 and EO-1.5 had the lowest titers compared to calves fed C, EO-0.5 and YCW for weeks 7 and 8. Similarly to IgA results calves fed EO-0.5 had the highest IgG titer values.

Calves fed the EO-0.5 had the greatest immunological response. Too much of an immunological response can be detrimental to a growing calf because of the energy expenditure needed. However, in this case calves fed EO-0.5 had improved growth rates compared to other treatments (Table 5) as well as an improved immunological response, indicating that the EO-0.5 improved the immunity of the calves. Calves fed EO-1.0 and EO-1.5 had the lowest titer results compared to C, YCW, and EO-0.5 indicating that the immune system was possibly overwhelmed and was shut down as a protective response. As an overactive immune system response could lead to self-damage from inflammation, a properly working immune system can be thought of as a teeter totter. An immunological response is great when a challenge arrives but too much of a response will result in self-inflicted damages (Mak et al., 2014).

Health Performances and Fecal Pathogens

Total serum blood protein averaged 6.5 ± 0.15 g/dl (Table 3). The mean serum protein levels were not different between treatments and were above 5.5 g/dl indicating successful passive transfer of immunity.

Health scores were averaged weekly for the 56 d study (Table 3). Overall health scores were acceptable for all calves across all treatments. Fecal scores (Table 3 and Figure 3) on weeks one, five, seven, and eight were similar ($P > 0.05$) for calves fed all treatments. Fecal scores were higher on week two for calves fed C compared to calves fed EO-1.0, and were significantly greater for calves fed C compared to calves fed EO-0.5 and YCW on week three. During week four, calves fed EO-1.5 had significantly ($P < 0.05$) higher fecal scores compared to calves fed C and EO-0.5. Again on week six fecal scores were significantly ($P > 0.05$) greater for calves fed EO-1.5 compared to calves fed EO-1.0. There were some significant differences in nasal, ear, and eye scores ($P < 0.05$) among some treatments, but the differences were small and calves were healthy overall.

Fecal pathogens were analyzed for samples that were collected on 0, 8, and 35 d (Table 4.) Analysis of fecal pathogens indicated no detection of s*almonella* throughout the study with no treatments differences in *clostridium* and *cryptosporidium* when the calves first arrived for the trial (0 d). Calves fed the control treatment had higher ($P < 0.05$) *cryptosporidium* levels on d 8 compared to calves fed the other treatments, but by d 35 no difference were observed between treatments. *Clostridium* was significantly ($P < 0.05$) increased for calves fed EO-0.5 compared to calves fed control on 8 d. Numerically, by 35 d calves fed EO had lower *clostridium* levels than calves fed YCW and C and EO-1.0 fed calves were significantly ($P < 0.05$) lower compared to calves fed

YCW and C. *Escherichia coli* was significantly higher for YCW calves than EO-1.5 fed calves on 0 d of the trial but by 8 and 35 d no significant differences were detected.

Body Growth and Measurements

Initial, final, and total gains of body weight and body measurements are shown in Table 5. The mean initial body weight (BW) of all the calves was 38.9 ± 1.5 kg. Calves fed EO-1.0 and EO-1.5 had significantly ($P < 0.05$) different initial body weights at the start of the trial, with no significant ($P > 0.10$) differences among the other treatments. All data were covariate adjusted to minimize pretreatment differences. Total BW gain and final BW were increased for calves fed EO-0.5 compared to calves fed EO-1.0, YCW, and C. Calves fed EO-0.5 demonstrated greater ($P < 0.05$) ADG through 56 d compared to calves fed EO-1.0 and YCW, while tending ($P < 0.10$) to be greater for calves fed C and EO-1.5. These growth measurements are industry acceptable with calves doubling their starting body weight by eight weeks. These growth measurements are also exceptional compared to a similar study by Santos et al., (2015) who fed lower amounts of EO but similar amounts of MR. In that study, Santos et al., (2015) reported an ADG of .38 kg for calves supplemented with a blend of EO (0.4 g/kg).

Calves fed EO-1.0 had significantly ($P < 0.05$) greater initial BL and HG compared to calves fed C, but similar to calves assigned to the other treatments. Calves fed EO-1.0 had significantly ($P < 0.05$) greater initial WH compared to calves fed YCW, but similar to calves assigned to the other treatments. Initial HH and HW measurements were similar for calves assigned to all treatments. Numerically, by the end of the study, calves fed EO-0.5 had the greatest body measurement gains. Calves fed EO-0.5 were significantly ($P < 0.02$) greater than calves fed EO-1.5 in BL gains. Gains in HW were

greater for calves fed EO-0.5 compared to calves fed C, but were similar among the remaining treatments. Gains of WH through 56 d was greater for calves fed EO-0.5 compared to calves C, EO-1.0, EO-1.5, while being similar for calves fed YCW. Gains in HH were greater for calves fed EO-0.5 compared to calves fed C, EO-1.0, EO-1.5 and YCW. Gains in HG calves fed C were significantly increased compared to calves fed YCW, however, calves fed EO-0.5 had the highest numerical HG gains while calves fed EO-1.5 had the lowest numerical gains. The reasons for these values not showing up significantly different is attributed to the standard errors of the samples being less than that of the other treatments. This data demonstrates that feeding EO-0.5 (1.25 g/calf/d) to a 24:20 MR will enhance growth rates compared to calves fed a modified accelerated 24:20 MR and a 24:20 MR containing YCW technology.

Volatile Fatty Acids

Serum blood samples from week five were analyzed for volatile fatty acids (**VFA**) as an indirect measurement of rumen development (Table 6). Quigley et. al., (1990) found that differences in plasma VFA were greatest between five and eight weeks of age in calves that were weaned early (28 d) and late weaned calves (56 d). Week five was the start of weaning and the blood VFA concentrations were similar between all treatments. Blood concentrations of isobutyrate and valerate were below detection limits. There was no significant difference ($P > 0.10$) in propionate and butyrate concentrations between treatments. These results are similar to data reported by Santos et al., (2015) who found no significant differences in propionate, butyrate, and acetate in calves supplemented with and without EO. Calves fed YCW were significantly lower in acetate concentration compared to calves fed C, EO-0.5, EO-1.0, and EO-1.5. Calves fed YCW were

significantly lower in isovalerate concentration compared to calves fed EO-1.0 and EO-1.5, but were similar in concentration to C and EO-0.5 calves. The VFAs are known to initiate epithelial and papillary growth within the rumen and butyrate is considered the most effective followed by propionate and acetate (Sakate and Tamate, 1979). Based on this knowledge it can be concluded that the treatments in this study neither inhibited nor enhanced ruminal development.

Dry matter intake, feed efficiency

Calf starter intake on a dry matter basis had no significant ($P > 0.10$) differences among treatments during the initial four weeks (Figure 5 and Table 7). The start of weaning (week five) calves fed EO-0.5 had significantly ($P < 0.05$) greater CS consumption than calves fed YCW, which continued through week 7 and 8. During week six, calves fed EO-1.0 had significantly greater CS intake compared to calves fed YCW, while calves fed EO-0.5 had greater CS intake compared to calves fed C and YCW. Total dry matter intake (MR plus CS) yielded similar results as CS DMI (Table 7). There were no significant ($P > 0.10$) differences in gain per feed (feed efficiency) among the different treatments (Table 7). Similar studies supplementing calves with EO have reported varying results. In a study conducted by Santos et al., (2015) found that calves supplemented with a blend of EO were similar in CS intake compared to non-supplemented calves. However, these results are in contrast to data reported by Hill et al., (2007) that demonstrated a commercial blend of EO actually increased CS intake and efficiency.

CONCLUSIONS

In this study the hypothesize proved partly true. Dairy calves supplemented with a commercial EO performed well compared to non-supplemented calves. However, dairy calves fed EO-0.5 demonstrated the optimal response compared to the hypothesized EO-1.0. Body weight, measurements, and ADG gains were numerically higher for EO-0.5 then calves fed C, YCW, and higher doses of essential oils. Similarly, calves fed EO-0.5 had the highest numerical IgA and IgG titers corresponding well with growth performance data indicating healthier calves. Calves fed EO-0.5 compared to C calves had significantly higher HG, WH, HH, WH, and BW gains. Furthermore, blood VFA concentrations were similar among treatments, indicating that the EO neither inhibited nor enhanced ruminal development. Health scores among treatments were acceptable and calves supplemented with EO were neither healthier or sicker compared to calves fed the other treatments. In some weeks, calves supplemented with EO numerically had improved fecal scores compared to calves fed C and YCW.

Overall, calves benefit with supplementation of a commercial EO. Questions still remain and future direction would be to see if a different dosage of this commercial EO could improve immunological, and growth of calves even more either through a lower dose or possibly a slightly higher (but lower than EO-1.0) dose. Other potential options would be to see if there is any benefit of feeding EO in the solid feed (pellets) of calves for an extended period of time that would extend past weaning.

Table 1. Milk replacer and calf starter nutrient analysis

Item[1]	**Milk Replacer**	**Calf Starter**
DM, %	95.6	87.3
Crude Protein, %	26.0	23.5
ADF, %	0.93	12.0
Fat, %	19.5	3.3
Starch, %	0.93	21.5
Ash, %	9.87	7.5
Calcium, %	0.94	0.85
Phosphorus, %	0.72	0.61
Magnesium, %	0.14	0.38
Mcal, kcal/kg	2730.9	2097.3

[1]Nutrient analysis conducted by Analabs (Fulton, IL). Values are reported as % DM.

Table 2. Immunological titers

	Treatments[1]					
Item	**C**	**EO-0.5**	**EO-1.0**	**EO-1.5**	**YCW**	**SEM**
No. calves	20	20	20	19	20	
IgA						
0 d	8.2	7.7	2.8	4.6	5.3	4.1
21 d	17.3	26.5	17.1	19.9	17.9	4.6
IgG						
21 d	10.1	7.5	7.3	10.2	5.7	597.0
28 d	30.8	10.3	19.9	7.3	9.4	597.0
35 d	178.1	141.3	166.7	78.9	142.6	613.3
42 d	411.2	441.0	405.4	270.9	405.9	597.0
49 d	4536.4^{ac}	5058.5^{ac}	2602.6^{bc}	1956.8^{b}	4037.0^{ac}	597.0
56 d	4224.7^{ab}	5310.0^{ab}	2339.6^{c}	2192.1^{c}	3684.2^{b}	597.0

[1]Control (**C**): 24:20; **EO-0.5**: 24:20 MR with 1.25 g Stay Strong added; **EO-1.0**: 24:20 MR with 2.5 g Stay Strong added; **EO-1.5**: 24:20 MR with 3.75 g Stay Strong added; **YCW**: 24:20 MR with 2 g Bio-Moss added. All MR were fed at 0.28 kg 2x/d for 14d, then 0.43 kg 2x/ d from 15 d to 35 d, MR then fed 1x/d from 36 d to weaning at 42 d.

Table 3. Total serum proteins and health scores

	Treatments[1]					
Item	**C**	**EO-0.5**	**EO-1.0**	**EO-1.5**	**YCW**	**SEM**
No. calves	20	20	20	19	20	
Serum Protein	6.40	6.50	6.56	6.43	6.62	0.21
Fecal Scores[2]						
1 to 7 d	1.09	1.19	1.22	1.27	1.18	0.08
8 to 14 d	2.01^{a}	1.83^{ab}	1.75^{b}	1.84^{ab}	1.93^{ab}	0.08
15 to 21 d	1.28^{a}	0.97^{bc}	1.05^{abc}	1.13^{abc}	1.08^{bc}	0.08
22 to 28 d	0.33^{b}	0.38^{b}	0.45^{ab}	0.58^{a}	0.40^{ab}	0.08
29 to 35 d	0.21	0.20	0.23	0.24	0.24	0.08
36 to 42 d	0.19^{ab}	0.15^{ab}	0.05^{b}	0.28^{a}	0.09^{ab}	0.08
43 to 49 d	0.10	0.07	0.06	0.005	0.09	0.08
50 to 56 d	0.05	0.03	0.03	0.03	0.02	0.08
Nasal Scores[3]						
1 to 7 d	0.05	0.06	0.06	0.07	0.05	0.02
8 to 14 d	0.01^{b}	0.01^{b}	0.05^{b}	0.14^{a}	0.02^{b}	0.02
15 to 21 d	0	0.01	0.03	0.05	0.03	0.02
22 to 28 d	0^{b}	0.07^{a}	0.07^{a}	0.05^{ab}	0.06^{ab}	0.02
29 to 35 d	0.02	0.06	0.03	0.01	0.03	0.02
36 to 42 d	0	0.03	0.02	0.04	0.01	0.02
43 to 49 d	0.07	0.01	0.02	0.06	0.04	0.02
50 to 56 d	0.01^{b}	0.02^{b}	0.03^{ab}	0.08^{a}	0.01^{b}	0.02
Eye Scores[4]						
1 to 7 d	0.03	0.04	0.08	0.10	0.04	0.05
8 to 14 d	0.06	0.06	0.06	0.07	0.10	0.05
15 to 21 d	0.26	0.18	0.27	0.29	0.30	0.05
22 to 28 d	0.31^{a}	0.15^{b}	0.21^{ab}	0.22^{ab}	0.24^{ab}	0.05
29 to 35 d	0.17^{b}	0.19^{b}	0.35^{a}	0.23^{ab}	0.14^{ab}	0.05
36 to 42 d	0.18^{ab}	0.18^{ab}	0.27^{a}	0.19^{ab}	0.12^{b}	0.05
43 to 49 d	0.17	0.20	0.17	0.18	0.27	0.05
50 to 56 d	0.25	0.23	0.27	0.27	0.24	0.05
Ear Scores[5]						
1 to 7 d	0.42^{bc}	0.42^{bc}	0.53^{abc}	0.59^{ac}	0.61^{a}	0.06
8 to 14 d	0.75	0.63	0.59	0.61	0.65	0.06
15 to 21 d	0.65	0.63	0.65	0.71	0.58	0.06
22 to 28 d	0.68^{a}	0.67^{a}	0.61^{ab}	0.44^{b}	0.61^{ab}	0.06
29 to 35 d	0.67^{a}	0.43^{b}	0.62^{a}	0.46^{b}	0.53^{ab}	0.06
36 to 42 d	0.47	0.42	0.43	0.51	0.58	0.06
43 to 49 d	0.41	0.39	0.47	0.42	0.37	0.06
50 to 56 d	0.37	0.31	0.28	0.24	0.41	0.06

[1]Control (**C**): 24:20; **EO-0.5**: 24:20 MR with 1.25 g Stay Strong added; **EO-1.0**: 24:20 MR with 2.5 g Stay Strong added; **EO-1.5**: 24:20 MR with 3.75 g Stay Strong added; **YCW**: 24:20 MR with 2 g Bio-Moss added. All MR were fed at 0.28 kg 2x/d for 14d, then 0.43 kg 2x/ d from 15 d to 35 d, MR then fed 1x/d from 36 d to weaning at 42 d.

[2] Fecal score = 0 to 3; 0 = normal, ≥ 2 = scours

[3]Nasal score = 0 to 3; 0 = normal, 3 = excessive mucopurulent discharge

[4]Eye score = 0 to 3; 0 = normal, 3 = heavy ocular discharge

[5]Ear score = 0 to 3; 0 = normal, 3 = droopy

Table 4. Fecal pathogens

	Treatments[1]					
Item	**C**	**EO-0.5**	**EO-1.0**	**EO-1.5**	**YCW**	**SEM**
No. calves	20	20	20	19	20	---
***Escherichia coli*[2]**						
0 d	4.3^{ab}	4.2^{ab}	4.3^{ab}	4.0^{b}	4.5^{a}	0.3
8 d	3.8	3.9	3.8	3.8	4.1	0.5
35 d	3.8	3.1	3.2	3.1	3.1	0.4
***Salmonella*[3]**						
0 d	ND	ND	ND	ND	ND	---
8 d	ND	ND	ND	ND	ND	---
35 d	ND	ND	ND	ND	ND	---
***Clostridium*[4]**						
0 d	1.4	1.6	1.7	1.5	1.6	0.3
8 d	1.0^{b}	1.4^{a}	1.4^{ab}	1.2^{a}	1.3^{ab}	0.2
35 d	1.3^{a}	1.2^{ab}	1.1^{b}	1.2^{ab}	1.4^{a}	0.2
***Cryptosporidium*[5]**						
0 d	1.0	1.1	1.0	1.0	1.0	0.0
8 d	2.2^{a}	1.8^{ab}	1.5^{ab}	1.5^{ab}	1.6^{b}	0.4
35 d	1.0	1.1	1.1	1.0	1.1	0.1

[1]Control (**C**): 24:20; **EO-0.5**: 24:20 MR with 1.25 g Stay Strong added; **EO-1.0**: 24:20 MR with 2.5 g Stay Strong added; **EO-1.5**: 24:20 MR with 3.75 g Stay Strong added; **YCW**: 24:20 MR with 2 g Bio-Moss added. All MR were fed at 0.28 kg 2x/d for 14d, then 0.43 kg 2x/ d from 15 d to 35 d, MR then fed 1x/d from 36 d to weaning at 42 d.
[2]*Escherichia coli* = 1 to 5; 1 = absent, 5 = large amount present
[3]*Salmonella* = 1 to 4; 1 = absent, 4 = large amount present
[4]*Clostridium* = 1 to 4; 1 = absent, 4 = large amount present
[5]*Cryptosporidium* = 1 to 4; 1 = absent, 4 = large amount present

Table 5. Body weight, measurements, ADG

	Treatments[1]					
Item	**C**	**EO-0.5**	**EO-1.0**	**EO-1.5**	**YCW**	**SEM**
No. calves	20	20	20	19	20	
BW, kg						
Initial	38.1^{ab}	39.2^{ab}	41.1^{a}	37.0^{b}	38.9^{ab}	3.4
Final	75.7^{b}	79.2^{a}	72.1^{b}	74.7^{ab}	74.4^{b}	6.4
Gain	36.8^{b}	39.9^{a}	35.5^{b}	35.8^{ab}	35.4^{b}	6.4
ADG, kg	0.65^{ab}	0.71^{a}	0.64^{b}	0.64^{ab}	0.63^{b}	0.08
BL, cm						
Initial	62.52^{b}	63.99^{ab}	64.49^{a}	63.47^{ab}	62.77^{ab}	1.09
Final	76.62^{ab}	78.16^{a}	76.58^{ab}	75.63^{b}	76.67^{ab}	1.06
Gain	13.11^{ab}	14.66^{a}	13.08^{ab}	12.12^{b}	13.17^{ab}	1.06
HG, cm						
Initial	77.10^{b}	79.48^{ab}	80.26^{a}	77.74^{ab}	79.0^{ab}	1.20
Final	98.12^{b}	98.45^{ab}	96.52^{bc}	96.42^{bc}	96.57^{c}	0.80
Gain	19.30^{b}	19.68^{ab}	17.70^{bc}	17.60^{bc}	17.75^{c}	0.80
WH, cm						
Initial	75.43^{ab}	76.08^{ab}	77.09^{a}	74.96^{ab}	74.89^{b}	1.03
Final	85.56^{b}	87.13^{a}	85.58^{b}	85.18^{b}	86.15^{ab}	0.54
Gain	9.78^{b}	11.34^{a}	9.80^{b}	9.40^{b}	10.36^{ab}	0.54
HH, cm						
Initial	79.87	80.21	81.98	79.75	79.73	1.02
Final	90.58^{b}	92.58^{a}	90.0^{b}	90.20^{b}	90.97^{b}	0.73
Gain	10.13^{b}	12.13^{a}	9.56^{b}	9.77^{b}	10.52^{b}	0.73
HW, cm						
Initial	46.29	47.31	47.47	46.71	46.92	1.57
Final	69.18^{b}	71.76^{a}	69.62^{ab}	69.38^{ab}	69.47^{ab}	1.52
Gain	22.18^{b}	24.76^{a}	22.62^{ab}	22.38^{ab}	22.47^{ab}	1.52

[1]Control (**C**): 24:20; **EO-0.5**: 24:20 MR with 1.25 g Stay Strong added; **EO-1.0**: 24:20 MR with 2.5 g Stay Strong added; **EO-1.5**: 24:20 MR with 3.75 g Stay Strong added; **YCW**: 24:20 MR with 2 g Bio-Moss added. All MR were fed at 0.28 kg 2x/d for 14d, then 0.43 kg 2x/ d from 15 d to 35 d, MR then fed 1x/d from 36 d to weaning at 42 d.

Table 6. Volatile Fatty Acids

	Treatments[1]					
Item	**C**	**EO-0.5**	**EO-1.0**	**EO-1.5**	**YCW**	**SEM**
Acetate, mM	0.64[a]	0.61[a]	0.60[a]	0.61[a]	0.48[b]	0.06
Propionate, mM	0.05	0.04	0.07	0.06	0.03	0.01
Isobutyrate, mM	ND	ND	ND	ND	ND	
Butyrate, mM	0.06	0.07	0.07	0.06	0.06	0.01
Isovalerate, mM	0.002[ab]	0.004[ab]	0.006[a]	0.007[a]	0.001[b]	0.002
Valerate, mM	ND	ND	ND	ND	ND	

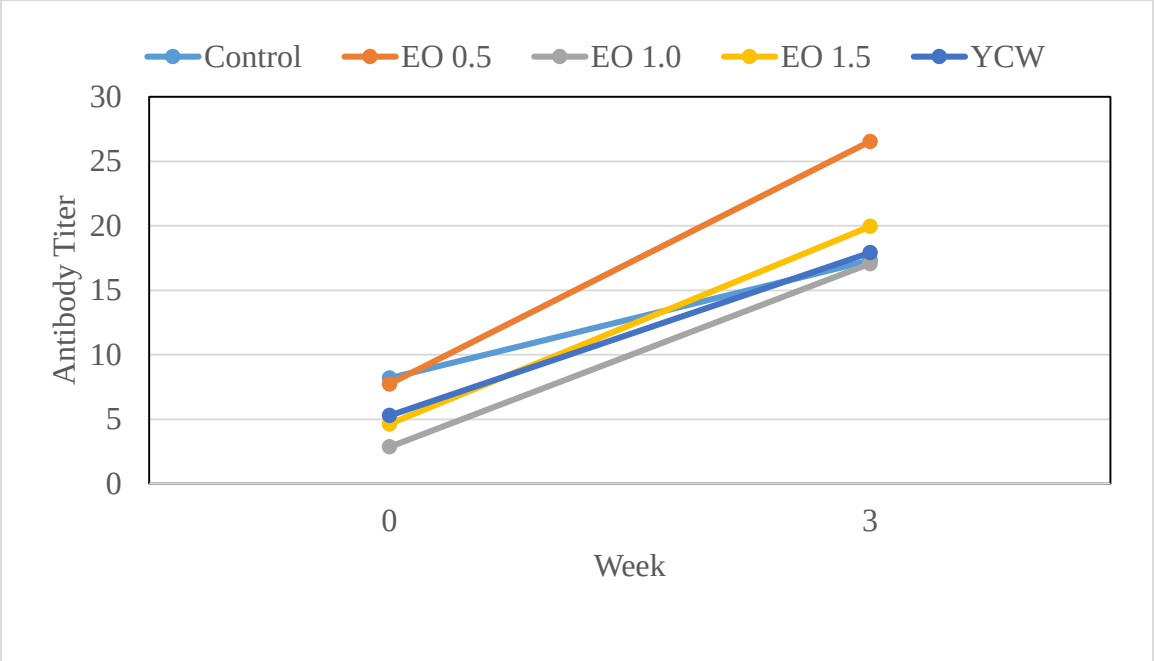
[1] Control (**C**): 24:20; **EO-0.5**: 24:20 MR with 1.25 g Stay Strong added; **EO-1.0**: 24:20 MR with 2.5 g Stay Strong added; **EO-1.5**: 24:20 MR with 3.75 g Stay Strong added; **YCW**: 24:20 MR with 2 g Bio-Moss added. All MR were fed at 0.28 kg 2x/d for 14d, then 0.43 kg 2x/ d from 15 d to 35 d, MR then fed 1x/d from 36 d to weaning at 42 d.

Table 7. CS DMI, Total DMI, Gain per Feed

	Treatments[1]					
Item	**C**	**EO-0.5**	**EO-1.0**	**EO-1.5**	**YCW**	**SEM**
No. calves	20	20	20	19	20	
CS DMI, kg/d						
1 to 7 d	0.02	0.01	0.01	0.02	0.01	0.08
8 to 14 d	0.05	0.05	0.05	0.05	0.02	0.08
15 to 21 d	0.13	0.18	0.16	0.18	0.12	0.08
22 to 28 d	0.29	0.33	0.28	0.33	0.27	0.08
29 to 35 d	0.46^{ab}	0.58^{a}	0.54^{ab}	0.52^{ab}	0.41^{b}	0.08
36 to 42 d	0.97^{bc}	1.17^{ac}	1.07^{ac}	1.04^{abc}	0.90^{b}	0.08
43 to 49 d	1.84^{ab}	1.90^{a}	1.86^{ab}	1.87^{ab}	1.73^{b}	0.08
50 to 56 d	2.25^{ab}	2.31^{a}	2.17^{ab}	2.19^{ab}	2.14^{b}	0.08
Total DMI, kg/d						
1 to 7 d	0.55	0.55	0.55	0.55	0.55	0.08
8 to 14 d	0.58	0.59	0.58	0.59	0.56	0.08
15 to 21 d	0.95	1.00	0.98	1.00	0.94	0.08
22 to 28 d	1.11	1.15	1.10	1.16	1.09	0.08
29 to 35 d	1.27^{ab}	1.40^{a}	1.36^{ab}	1.34^{ab}	1.23^{b}	0.08
36 to 42 d	1.38^{bc}	1.58^{a}	1.48^{ab}	1.45^{abc}	1.31^{c}	0.08
43 to 49 d	1.85^{ab}	1.90^{a}	1.86^{ab}	1.87^{ab}	1.73^{b}	0.08
50 to 56 d	2.25^{ab}	2.31^{a}	2.17^{ab}	2.19^{ab}	2.14^{b}	0.08
Gain per Feed						
1 to 7 d	0.90	0.94	0.91	0.92	0.88	0.11
8 to 14 d	0.19	0.30	0.09	0.33	0.12	0.11
15 to 21 d	0.76	0.81	0.70	0.72	0.73	0.11
22 to 28 d	0.56	0.63	0.64	0.61	0.70	0.11
29 to 35 d	0.70	0.63	0.64	0.57	0.55	0.11
36 to 42 d	0.34	0.50	0.40	0.38	0.39	0.11
43 to 49 d	0.42	0.38	0.44	0.39	0.41	0.11
50 to 56 d	0.49	0.47	0.44	0.41	0.53	0.11

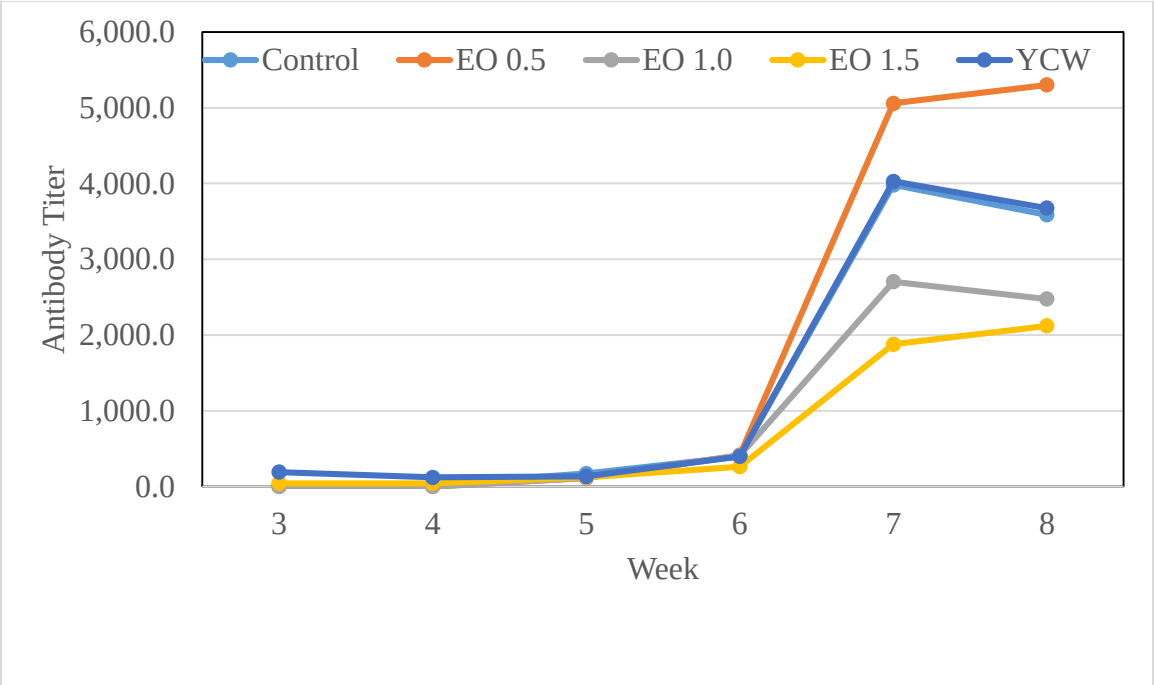
[1]Control (**C**): 24:20; **EO-0.5**: 24:20 MR with 1.25 g Stay Strong added; **EO-1.0**: 24:20 MR with 2.5 g Stay Strong added; **EO-1.5**: 24:20 MR with 3.75 g Stay Strong added; **YCW**: 24:20 MR with 2 g Bio-Moss added. All MR were fed at 0.28 kg 2x/d for 14d, then 0.43 kg 2x/ d from 15 d to 35 d, MR then fed 1x/d from 36 d to weaning at 42 d.

Figure 1. IgA titers



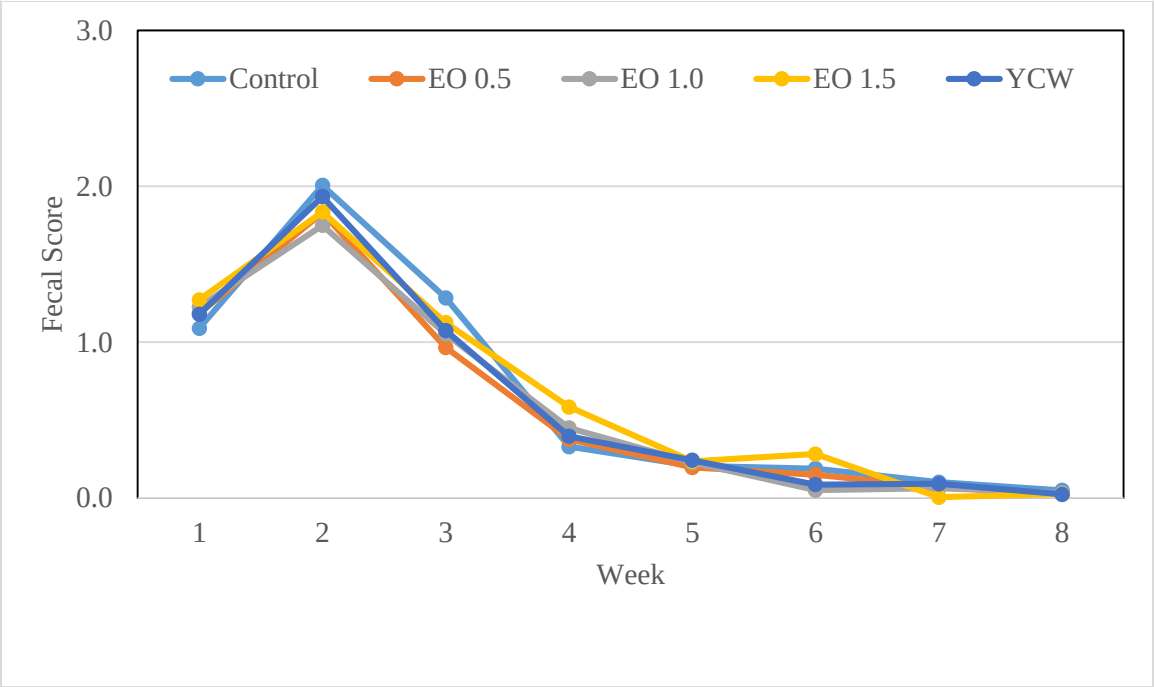
Control (**C**): 24:20; **EO-0.5**: 24:20 MR with 1.25 g Stay Strong added; **EO-1.0**: 24:20 MR with 2.5 g Stay Strong added; **EO-1.5**: 24:20 MR with 3.75 g Stay Strong added; **YCW**: 24:20 MR with 2 g Bio-Moss added. All MR were fed at 0.28 kg 2x/d for 14d, then 0.43 kg 2x/ d from 15 d to 35 d, MR then fed 1x/d from 36 d to weaning at 42 d.

Figure 2. IgG titers



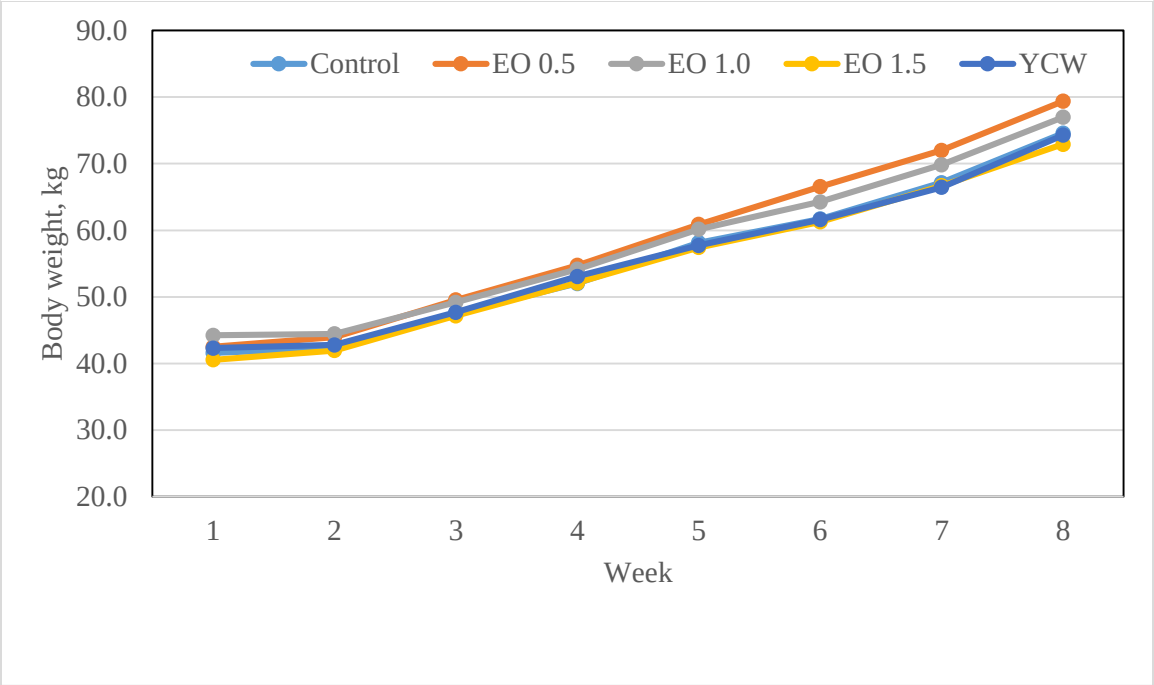
Control (**C**): 24:20; **EO-0.5**: 24:20 MR with 1.25 g Stay Strong added; **EO-1.0**: 24:20 MR with 2.5 g Stay Strong added; **EO-1.5**: 24:20 MR with 3.75 g Stay Strong added; **YCW**: 24:20 MR with 2 g Bio-Moss added. All MR were fed at 0.28 kg 2x/d for 14d, then 0.43 kg 2x/ d from 15 d to 35 d, MR then fed 1x/d from 36 d to weaning at 42 d.

Figure 3. Fecal scores



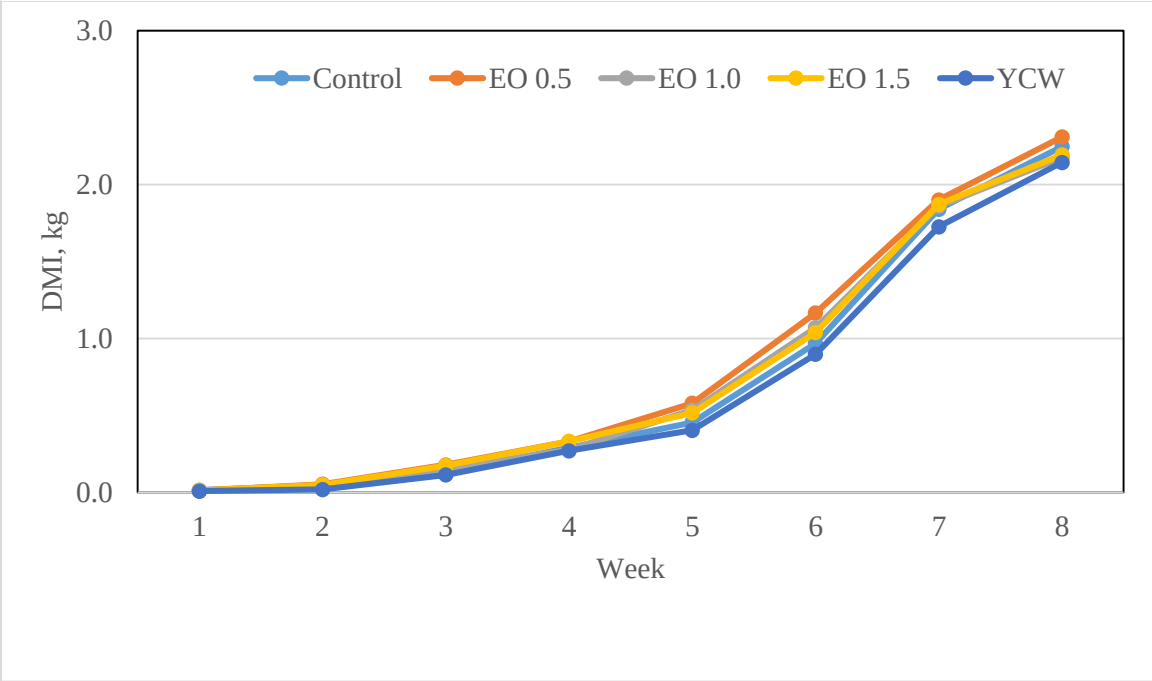
Control (**C**): 24:20; **EO-0.5**: 24:20 MR with 1.25 g Stay Strong added; **EO-1.0**: 24:20 MR with 2.5 g Stay Strong added; **EO-1.5**: 24:20 MR with 3.75 g Stay Strong added; **YCW**: 24:20 MR with 2 g Bio-Moss added. All MR were fed at 0.28 kg 2x/d for 14d, then 0.43 kg 2x/ d from 15 d to 35 d, MR then fed 1x/d from 36 d to weaning at 42 d.

Figure 4. Body weight



Control (**C**): 24:20; **EO-0.5**: 24:20 MR with 1.25 g Stay Strong added; **EO-1.0**: 24:20 MR with 2.5 g Stay Strong added; **EO-1.5**: 24:20 MR with 3.75 g Stay Strong added; **YCW**: 24:20 MR with 2 g Bio-Moss added. All MR were fed at 0.28 kg 2x/d for 14d, then 0.43 kg 2x/ d from 15 d to 35 d, MR then fed 1x/d from 36 d to weaning at 42 d.

Figure 5. Calf starter intake



Control (**C**): 24:20; **EO-0.5**: 24:20 MR with 1.25 g Stay Strong added; **EO-1.0**: 24:20 MR with 2.5 g Stay Strong added; **EO-1.5**: 24:20 MR with 3.75 g Stay Strong added; **YCW**: 24:20 MR with 2 g Bio-Moss added. All MR were fed at 0.28 kg 2x/d for 14d, then 0.43 kg 2x/ d from 15 d to 35 d, MR then fed 1x/d from 36 d to weaning at 42 d.

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