DETERMINATION OF THE MECHANISM(S) BY WHICH DIRECT-FED MICROBIALS CONTROL *ESCHERICHIA COLI* O157:H7 IN CATTLE

By

LACEY MICHELE GUILLEN

Bachelor of Science in Food Technology
Texas Tech University
Lubbock, Texas
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Master of Science in Food Science
Oklahoma State University
Stillwater, Oklahoma
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Dissertation Approved:

Dr. Stanley Gilliland

Dissertation Adviser

Dr. Clint Krehbiel

Dr. William McGlynn

Dr. Barbara Stoecker

Dr. A. Gordon Emslie

Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Escherichia coli O157:H7 was first implicated in outbreaks of disease linked to undercooked ground beef in the early 1980's (*49*). Cattle are a recognized reservoir of strains of enterohemmorhagic *E. coli* O157:H7 (*17*). The infectious dose of enterohemmorhagic *E. coli* O157:H7 has been estimated to be as low as <100 cells. Enterohemmoraghic *E. coli* O157:H7 infection can lead to hemolytic uremic syndrome which can be distinguished by hemolytic anemia, thrombocytopenia, and possible acute renal failure which can be fatal *(32).* *Escherichia coli* O157:H7 has been shown to have long-term survival in both manure and water (*62, 63*). This can lead to contamination of both water supplies and crops. Because of the risk of food or water borne illness it is important to find methods to reduce the carriage of *E. coli* O157:H7 by cattle.

Probiotic bacteria have been shown to promote intestinal health and immune function (*30*). Enhancement of the immune system is one proposed mechanism by which probiotics may confer benefits to the host.
Direct-fed microbials are also known as probiotics. Both terms refer to a live and naturally occurring bacterial supplement (*27*). The following discussion will use the terms "probiotics" and "direct-fed microbial" interchangeably. Brashears et al. (*9*) reported a decrease in fecal shedding of *E. coli* O157:H7 for cattle fed *Lactobacillus*-based direct-fed microbials. Younts-Dahl et al. (*67*) investigated three different doses of *Lactobacillus acidophilus* NP51 on reductions of *E. coli*

O157 in cattle. The highest dose of NP51 resulted in the greatest decrease of *E. coli* O157 in the cattle. An experiment by Peterson et al. (*41*) also confirmed the previous results by reporting a decrease in *E. coli* O157:H7 for cattle fed *L. acidophilus* NP51.

The objective of this experiment was to look into the mechanism by which direct-fed microbials decrease the prevalence of *E. coli* O157:H7 in live cattle. The steers in this experiment were inoculated with the pathogen. One group of steers was fed direct-fed microbials while the other group was not. Uncovering the mechanism for the decrease in pathogens in cattle fed direct-fed microbials may lead to the isolation of even more effective cultures.

CHAPTER II

REVIEW OF LITERATURE

Characteristics of *Escherichia coli* O157:H7: Pathogenicity, Prevalence, and Other Important Considerations

Escherichia coli O157:H7 has received a lot of attention because of its connection with food and water borne illnesses. The pathogen was first identified as a food borne pathogen in 1982 after an outbreak from contaminated hamburgers (*49*). A brief history of the knowledge of *E. coli* O157:H7 will be included and information on its prevalence in cattle as well as food, water, and soil.

Escherichia coli O157:H7 Emergence and Disease Characteristics

Escherichia coli O157:H7 is thought to have emerged from a combination of events. Virulent bacteria may have genes for toxin production, adhesins, and invasins that reside in certain regions of a bacterial chromosome called pathogenicity islands (*29*). Shiga-like toxins of *E. coli* O157:H7 are bacteriophage encoded (*40*, *58*). New pathogens may be created from the horizontal gene transmission by plasmids or phages (*29*). Some research indicates that *E. coli* O157:H7 was derived from an enteropathogenic *E. coli* (EPEC) ancestral strain that expressed the enzyme β-glucuronidase and had the ability to ferment sorbitol (*24*). *Escherichia coli* O157:H7 contains but does not

express the *uidA* gene for the inducible enzyme β-glucuronidase (*23*). This ancestral EPEC strain gave rise to a strain with O55 somatic and H7 flagellar antigens (*24*). The *rfb* region generally contains 8 to 14 genes and encodes for the enzymes to produce the O side chains that characterize serogroup specificity (*48, 59*). The O side chains or somatic antigens are part of the bacterial lipopolysaccharide (*47, 66*). The O antigens initiate antibody responses where the antibody-antigen complex activates the complement system to produce membrane attack complex or phagocytosis (*47*). Shiga-toxin 2 is thought to have been acquired by transduction by a toxin-converting bacteriophage. A *uidA* +92 mutation occurred and the somatic antigen, O55, became O157. This strain was a precursor to today's *E. coli* O157:H7 but it still retained expression of β-glucuronidase and sorbitol fermentation. Further mutations led to the loss of sorbitol fermentation and the acquisition of Shiga-toxin 1. The loss of β-glucuronidase expression leads us to the current pathogenic strain (*24*).

The disease caused by *E. coli* O157:H7 can be characterized by hemorrhagic colitis which causes bloody diarrhea, non-bloody diarrhea, and hemolytic uremic syndrome. Strains of *E. coli* that cause these diarrheal diseases are referred to as enterohemorrhagic. In the United States the most important enterohemorrhagic serotype currently is O157:H7. Other serotypes such as O26 and O111 cause more problems than O157:H7 in other countries (*32*).

Strains of enterohemorrhagic *E. coli* express intimin. Intimin is required for attachment to the host cell and the formation of attachment/effacing lesions in

cattle (*18, 19*) and humans (*43*). Shiga toxin or verocytotoxin is a key virulence factor for enterohemorrhagic *E. coli* (*28, 32*, *60*). These strains are referred to as Shiga toxin-producing *E. coli* if they produce at least one type of Shiga toxin (*28*). It was discovered that some strains of enterohemorrhagic *E. coli* may produce Shiga-like toxins in 1982. Extracts of toxins produced by different strains of *E. coli* were tested for cytotoxicity in HeLa cells. The extracts were able to be neutralized with Shiga toxin antiserum (*39*). The toxin was further characterized and compared to Shiga toxin in a follow up experiment. The two toxins were found to have many similarities but they differed in molecular weight. These results suggested to the authors that a family of Shiga-like toxins exists (*40*).

There are two subgroups of Shiga toxins which are referred to as Shiga toxin 1 and Shiga toxin 2 (*32*). In order for disease to be caused the Shiga toxin-producing *E. coli* must be ingested. It must survive the harsh, acidic environment of the stomach and the upper gastrointestinal tract so that it may colonize the lower gastrointestinal tract (*60*). Renal inflammation occurs when Shiga toxin travels from the colon to the kidney and induces cytokine and chemokine production. The damage caused by this can lead to hemolytic uremic syndrome. Shiga toxin can also cause damage in the colon which can result in bloody diarrhea and hemorrhagic colitis (*32*).

Pre-harvest Prevalence of *Escherichia coli* O157:H7 in Cattle

Cattle are recognized as the main reservoir for *E. coli* O157:H7 (*7, 14*). Many studies have been conducted to determine the prevalence of *E. coli*

O157:H7 in cattle. Five feedlots in the Midwestern United States were sampled for *E. coli* O157:H7. The pathogen was isolated from the feces of 719 (23%) of the 3,162 cattle tested. At least one animal from each of the 29 pens was positive. Smith et al. (*53*) reported that the prevalence of cattle shedding *E. coli* O157:H7 was not significantly different among feedlots but there was a significant variation within feedlots.

In similar commercial feedlot studies the prevalence of *E. coli* O157:H7 observed was different. LeJeune et al. (*34*) observed that 13.3% of the fecal samples tested were positive. Every pen had at least one positive sample. The largest prevalence survey to date, which included cattle from the 13 states with the most feedlot cattle, was conducted by the USDA's Animal and Plant Health Inspection Service. The percentage of positive fecal samples was 1.8% and 63% of the feedlots tested had at least one positive sample (*3*).

The differences observed in the prevalence data in each experiment can be explained by several factors. First of all the methods for detecting the pathogen were not standardized. Tests conducted by different researchers may vary in their sensitivity to detect the pathogen (*4*). Secondly, the age of the animal may influence infection. Wells et al. (*65*) conducted an experiment of *E. coli* O157:H7 in dairy cattle and they recovered the pathogen more often in young animals than in adults. Cray and Moon (*15*) observed that among experimentally infected animals, calves shed *E. coli* O157:H7 in greater numbers and for a longer time period than adult animals. A third reason for prevalence differences may be the season in which the samples are taken. Elder et al. (*21*)

observed that the peak times for *E. coli* O157:H7 shedding was during summer and early fall.

Post-harvest Prevalence of *Escherichia coli* O157:H7 in Cattle

Elder et al. (*21*) observed what appeared to be a correlation between the fecal prevalence of *E. coli* O157:H7 and carcass contamination at slaughter. Out of 29 lots of cattle tested there were 11 lots (38%) that had hides positive for the pathogen. The prevalence of positive hides within lots ranged from 0% to 89%. There was at least one positive carcass in 27 out of 30 lots (90%). The percentages of positive carcasses varied depending on the processing step. Out of 30 lots the following positives occurred at three different processing steps: 26 positives (87%) at pre-evisceration, 17 positives (57%) at post-evisceration, and 5 positives (17%) at post-processing. Of the 17 positive carcasses at post-evisceration, 16 of those also were positive at the pre-evisceration step.

When monitoring 3 Midwestern fed-beef processing plants, Barkocy-Gallagher et al. (*5*) reported that 60.6% of hide samples and 26.7% of carcass samples were positive for *E. coli* O157:H7 prior to a pre-evisceration wash. The positive carcasses dropped to 1.2% after the washing step. The researchers also observed that while the fecal prevalence of the pathogen peaked in the summer, the hide prevalence remained high from spring through the fall.

The variation seen in post-harvest prevalence appears to be caused by the same inconsistencies as the pre-harvest prevalence data. Detection methods used were not standardized and therefore likely varied in sensitivity to

detect the pathogen. A possible seasonal variability as previously mentioned will certainly affect the post-harvest prevalence as well. Both pre-harvest and post-harvest prevalence studies differ as a result of a lack of knowledge of the shedding patterns of the pathogen by cattle (*34*). More research is needed to expand the knowledge of the behavior of this pathogen in the live animal as well as its survival through the slaughter process.

Prevalence of *Escherichia coli* O157:H7 in Crops, Fresh Produce, and Contributing Factors

In recent years foodborne illnesses from fresh produce reported to the Centers for Disease Control and Prevention (CDC) have been increasing (*52*). Outbreaks of illness caused by *E. coli* O157:H7 were first reported in produce in 1991 and they accounted for 21% of the 183 foodborne illness outbreaks (*46*). Consumption of fresh fruits and vegetables has increased in the United States so the potential for foodborne illnesses from these sources also has increased.

Because cattle and other animals are reservoirs for pathogens it is possible that crops may become contaminated with *E. coli* O157:H7 through the use of manure as a fertilizer (*31*). Contamination can also occur through irrigation of plants with untreated water or sewage (*8*, *55*). Feces from infected cattle may also contaminate fields of crops through surface water runoff from nearby cattle operations (*2*).

The most recent outbreak of *E. coli* O157:H7 in fresh produce was from the consumption of fresh spinach. The illness was reported in 199 people in 26

states between August 19 and September 5, 2006. The people suffered from enteritis caused by a Shiga toxin producing strain of *E. coli* O157:H7. The fresh spinach and other spinach products came from a company in California. The exact cause of the contamination is still not known but the same strain of the pathogen was observed in cattle manure from cattle ranches adjacent to the crop fields. Since 1993 this was at least the 26th outbreak caused by *E. coli* O157:H7 in leafy green vegetables (*36*).

One of the latest theories is that some crops may become tainted through the internalization of *E. coli* O157:H7 from contaminated fertilizer, irrigation water, or run-off water. *Escherichia coli* O157:H7 has been shown to have long-term survival in both manure and water (*62, 63*). Several studies have investigated whether the pathogen may enter the plant through the root system. There are many factors at work but research demonstrates that *E. coli* can enter plants via the root system (*31, 54*, *64*).

Possible Solutions to Decrease the Prevalence of *Escherichia coli* O157:H7 in Cattle

There are many possible interventions to decrease *E. coli* O157:H7 in the live animal. Implementing one or more of these strategies will hopefully reduce the levels of the pathogen in beef products. Reduction in contamination of other products such as fresh produce may also result from successful pre-harvest intervention strategies.

Vaccination Against *Escherichia coli* O157:H7

Vaccines against the pathogen are currently being developed and studied. In an experiment conducted by Potter et al. (*44*) a vaccination with type III secreted proteins derived from *E. coli* O157:H7 was tested on feedlot cattle. Type III secreted proteins are thought to be involved in the colonization of *E. coli* O157:H7 in ruminants. Prior to vaccination the treatment and control groups did not differ significantly in the prevalence of shedding *E. coli* O157:H7. Post-treatment there was significantly less shedding of the pathogen in the vaccinated cattle pens when compared to the unvaccinated control group pens (*44*).

Peterson et al. (*42*) also conducted an experiment investigating the use of a vaccine containing type III secreted proteins from *E. coli* O157:H7. This experiment tested the following four treatments: 1) no vaccination, 2) one dose on day 42, 3) two doses: one on day 0 and one on day 42, and 4) three doses: one each on days 0, 21, and 42. Receiving one, two, or three doses of vaccine resulted in cattle that were respectively 68, 66, and 73% less likely to shed the pathogen when compared to controls. Some of the cattle penned with those given the vaccine were given a placebo treatment. Placebo-treated steers were less likely to shed *E. coli* O157:H7 when compared to external controls that were not housed with vaccinated animals. The researchers concluded that this was the result of reduced shedding by the vaccinated penmates.

Feeding Practices to Reduce Acid Resistant *Escherichia coli* Before Slaughter

Some research has indicated that feeding a high concentrate diet such as those fed in feedyards may contribute to the survival of acid resistant *E. coli*. Diez-Gonzalez et al. (*20*) conducted experiments to investigate this by feeding cattle diets consisting of 0, 45, or 90% grain. The researchers observed that as the percentage of grain increased in the diet so did the acid resistant *E. coli* count. In response to these findings they investigated whether a transition to a hay diet would decrease these populations. After 5 days on a hay diet the *E. coli* population decreased by 10^6-fold.

The experiment by Diez-Gonzalez et al. (20) has been reported as contradictory in its results. The *in vivo* work in the experiment by Diez-Gonzalez (20) tested acid resistance of generic non-serotyped *E. coli*. The *in vitro* work included in the experiment tested *E. coli* O157:H7 and generic *E. coli*. Grauke et al. (2003) observed that generic *E. coli* and pathogenic *E. coli* O157:H7 have different reactions to diet and acid shock tests. Their experiments indicated that a hay or grain diet did not affect the acid resistance of *E. coli* O157:H7. On the other hand there were higher populations of generic coliforms in grain-fed versus hay-fed cattle.

Sodium Chlorate Supplementation to Reduce *Escherichia coli* O157:H7

The nitrate reductase activity of *E. coli* O157:H7 also reduces chlorate to chlorite which is toxic to the organism (*57*). Calloway et al. (*12*) tested the effect of chlorate supplemented drinking water on cattle experimentally infected with

three strains of *E. coli* O157:H7. They observed that the treatment reduced the pathogen by two logs (10^4 to 10^2) in the rumen and three logs (10^6 to 10^3) in the feces. In another animal experiment with sheep Callaway et al. (*13*) observed that chlorate decreased *E. coli* O157:H7 in experimentally infected animals when compared to the control group.

The Use of Direct-fed Microbials to Control the Shedding of *Escherichia coli* O157:H7

In vitro experiments with lactic acid bacteria have shown inhibition of pathogens in laboratory growth media as well as ruminal fluid and manure (*10*). The success of these experiments led to cattle experiments in order to assess the effects of the direct-fed microbials on pathogens *in vivo*. Brashears et al. (*9*) conducted a study in which steers were fed a 90% concentrate diet with one of three treatments. The control treatment had added lactose which is the carrier for the direct-fed microbials. The experimental treatment animals either received 1×10^9 CFU of *Lactobacillus acidophilus* NPC 747 or *Lactobacillus crispatus* NPC 750. When averaged over time, the NPC 747 treatment showed decreased *E. coli* O157:H7 positives and was more effective than NPC 750. There also was a significant decrease in *E. coli* O157:H7 on the hide samples. Such reductions can result in less contamination of meat products.

A similar direct-fed microbial experiment was conducted by Peterson et al. (*41*). A total of 448 steers (192 steers in 2002 and 256 steers in 2003) were randomly assigned to the treatment group that would receive *L. acidophilus*

NP51 as a daily supplement or a control group that would receive no supplementation. They developed a regression model to predict the likelihood for shedding *E. coli* O157:H7. Over the 2 year experiment they observed that the cattle in pens treated with NP51 were 35% less likely to shed the pathogen than those steers in the control pens.

The Use of Direct-fed Microbials in Livestock

Probiotics or direct-fed microbials have been shown to provide an array of potential health benefits for both humans and domestic animals. A popular definition was coined by Fuller (*25*) who stated that probiotics are “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”. As a dietary supplement for humans they have been shown to be effective for lactose maldigestion, anti-carcinogenic properties, control of serum cholesterol, control of intestinal pathogens, and enhanced immunity. Probiotic cultures for animals and livestock have been investigated and are now more widely used. Some applications for animals include inhibition of pathogens, enhanced immunity, increased milk yields, and improved growth and performance. The following will discuss possible improvements in feed efficiency, milk yield, and immune responses in cattle. The use of direct-fed microbials to inhibit pathogens in cattle was discussed in the previous section.

Direct-fed Microbial Supplementation for Calves

Cruywagen et al. (*16*) fed forty Holstein-Friesian bull calves either milk replacer with no additives or milk replacer with 1 ml (5×10^7) of *L. acidophilus*. The calves were assigned to one of the two treatments at two days of age and each treatment was given twice daily. At seven days of age the calves were allowed ad libitum access to a commercial calf starter pellet. The researchers measured weight, feed intake, and fecal consistency as an indication of scours. There were no significant differences in body weight between treatments. Average daily gain was significantly different between treatments during the 2nd week of life for the calves. The control group lost 4% of their initial body weight in the first two weeks while the treatment group lost only 0.8% of initial body weight. This loss of body weight is often seen in calves fed milk replacer. The treatment had no effect on scours or dry matter intake. The researchers concluded that the use of probiotics for calves during the first two weeks of life may be beneficial. Probiotics may be especially beneficial for calves fed milk replacer.

A similar experiment with dairy calves was conducted by Abu-Tarboush et al. (*1*). Twenty-four Holstein bull calves were assigned to one of three treatments at five days of age. The treatments included: 1) control treatment with 50% whole milk plus 50% milk replacer, 2) whole milk and milk replacer with added *L. acidophilus* and *L. plantarum* (mixed lactobacilli treatment), and 3) whole milk and milk replacer with added *L. acidophilus* 27SC. The calves received their liquid diet plus treatments twice daily. In addition calves were offered a pelleted

starter diet ad libitum. The researchers observed that the average daily gains were not significantly different between treatments. Although not significant they did observe that the calves fed the mixed culture of lactobacilli had the greatest body weight gains during weeks 7-9 of the experiment. The group receiving only *L. acidophilus* 27SC had the highest gains during weeks 10-12. There was a significant difference between treatments when looking at the scour index for all sampling periods. The scour index used was: 1 = watery, 2 = moderate, and 3 = normal. The group receiving *L. acidophilus* 27SC had the scouring result closest to normal at 2.76. The mixed culture of lactobacilli treatment group had the next highest at 2.69 while the control group had the lowest score of 2.55.

Improved Feed Efficiency with Direct-fed Microbial Supplementation

Rust et al. (*50*) conducted a research project to test the effects of Bovamine^{TM} rumen culture on the performance of feedlot steers. There were 320 steers that were blocked by weight and assigned to one of four treatments. The four treatments included: 1) water, no bacteria (control), 2) 10^9 cfu/g *Propionibacterium freudenreichii* (PF24) and 10^6 cfu/g of *L. acidophilus* (LA45), 3) 10^9 *P. freudenreichi* (PF24) plus 10^6 cfu/g of *L. acidophilus* LA45 and 10^6 cfu/g of *L. acidophilus* LA51, and 4) 10^9 *P. freudenreichi* (PF24) plus 10^8 cfu/g of *L. acidophilus* LA45 and 10^8 cfu/g of *L. acidophilus* LA51. The four treatments were applied to the diet in the amount of 1000 ml/pen/day and then mixed. Each pen received feed once daily. The duration of the experiment was 115 days with the cattle being weighed and orts collected at 28 day intervals. After 115 days the

cattle were harvested and the quality and yield grades were recorded. Cattle receiving any of the three microbial treatments had a 6.9% faster weight gain than the control group. There were no significant differences among carcass weights or dry matter intake when comparing treatments.

Feeding increasing doses of *L. acidophilus* (strain NP 51) combined with a single dose of *P. freudenreichii* (strain NP 24) was tested in two feeding experiments by Vasconcelos et al. (*61*). Two hundred forty steers were selected for each experiment based on body frame and body weight. The control treatment contained only the lactose carrier while the other three treatments all had 1×10^9 cfu/(steer·day) of *P. freudenreichii* (strain NP 24) plus either a low, medium, or high dose of *L. acidophilus* (strain NP 51) which had 1×10^7, 1×10^8, or 1×10^9 cfu/(steer·day) respectively. These treatments were used in both experiments but the method of feeding the treatments differed. In experiment 1 the freeze-dried direct-fed microbials were suspended in water and mixed with the diet. In experiment 2 the freeze-dried packet of culture was mixed with 1 kg of air-dried corn before adding and mixing with the diet. Blocks of cattle were harvested when both body weight and visual appraisal indicated that about 60% of the cattle would grade USDA choice. The data from both experiments were pooled. Overall the average daily gain and dry matter intake were not significantly different between treatments and control. The treatments with the low (1×10^7) and high (1×10^9) doses of *L. acidophilus* NP 51 showed an improvement of about 2 to 3% of live-weight gain:feed. The medium dose (1×10^8) did not show as much of an effect on gain:feed when compared to the

control group. The treatments did not affect carcass characteristics when compared to controls. The researchers concluded that both the strains of direct-fed microbials as well as the doses need to be investigated further.

Other studies on the use of direct-fed microbials have had results that were not as positive as the two previous studies mentioned. Beauchemin et al. (*6*) conducted two different cattle experiments. The first experiment tested *Enterococcuss faecium* in eight cannulated steers. The second experiment tested the same culture as the first experiment with added yeast in eight steers with both ruminal and duodenal cannulas. The two studies looked at many parameters including feed intake, body weight, ruminal pH and fermentation, microbial counts from rumen and fecal samples, digestion, and blood chemistry. This experiment points to the importance of bacterial species selection and their interaction with the diet fed. Supplementing the cattle with *E. faecium* caused an increase in propionate but with that came some undesirable metabolic changes. The addition of yeast to the *E. faecium* decreased the undesirable changes but overall there was not much difference between the treatment and control groups. The culture used in their experiment proved to have little effect on reducing acidosis in cattle on a high-grain diet. The researchers stated that *E. faecium* with or without yeast might possibly have some benefit in cattle that are not already adapted to high grain diets.

Improved Milk Yields in Dairy Cattle from Direct-fed Microbial Supplementation

There have been many studies conducted to investigate the effects of direct-fed microbials on milk production by dairy cattle. Nocek and Kautz (*38*) studied the effects of direct-fed microbials on forty-four multiparous Holstein cows. The cows were assigned to one of two treatment groups 21 +/- 3 days before their expected calving date. The experiment continued through 10 weeks post-calving. The control treatment consisted of a total mixed ration (TMR) with no direct-fed microbial supplementation prepartum or postpartum. The experimental group received TMR prepartum and TMR postpartum plus an added 2 g of direct-fed microbials/cow/day. The direct-fed microbial treatment was a commercial product called Probios TC (Chr. Hansen) which is comprised of 5×10^9 cfu of yeast and 5×10^9 cfu of two strains of *Enterococcus faecium*. The treatment was mixed with corn meal and then added to the feed. The control group received corn meal with no added cultures. The cows receiving the direct-fed microbials had increased milk production and dry matter intake when compared to controls. The experimental group had a lower milk fat percentage than the control cows.

Stein et al. (*56*) looked at the effect of *Propionibacterium* P169 (P169) supplementation on milk yields, milk components, ruminal parameters, and reproductive measures of dairy cows. Nineteen primiparous and 19 multiparous Holstein cow were assigned to treatments two weeks before calving. The treatments consisted of the following: 1) control group received a lactation TMR, 2) low-dose group received the control TMR with 6×10^{10} cfu/cow of P169, and

3) high-dose group received the control TMR with 6 x 10^{11} cfu/cow of P169. The P169 treament was top-dressed on 4.5 kg TMR once per day. The daily milk production for each cow was recorded and milk samples were collected two times each week for analysis. Blood samples were taken once weekly to determine progesterone concentrations. Estrous behavior of the cows was also monitored. Rumen fluid samples were analyzed for concentrations of volatile fatty acids (VFA). The milk production was averaged across primiparous and multiparous cows. Both the low and high-dose treatment cows had significantly higher milk production than controls. The percentage of fat in the milk was significantly higher for the low-dose and control groups. The high-dose P169 supplement resulted in an increase in propionate which is the most metabolically efficient VFA. There were no differences observed between treatments when considering the reproductive measures. It should be noted that the dosages of the direct-fed microbials in this experiment were exceptionally high.

The direct-fed microbial experiments for dairy cattle often use a variety of different cultures and doses. Studies also differ in stage of pregnancy or lactation for which the supplements are administered. This makes it difficult to determine whether direct-fed microbials as a whole provide consistent results. The two previously mentioned dairy cattle experiments administered probiotic supplements prior to calving and for either 10 weeks (*38*) or 30 weeks (*56*) postpartum. In contrast to this Raeth-Knight et al. (*45*) administered one of three treatments to Holstein cows in midlactation. The treatments used were 1) 1 x 10^9 cfu/d *L. acidophilus* LA747 and 2 x 10^9 cfu/d *P. freudenreichii* PF24 (DFM1), 2) 1

x 10^9 cfu/d *L. acidophilus* LA747, 2 x 10^9 cfu/d *P. freudenreichii* PF24, and 5 x 10^8 cfu/d *L. acidophilus* LA45 (DFM2), and 3) lactose as the control treatment. There were two simultaneous studies with these treatments. Experiment one was a lactation study with 39 multiparous and 18 primiparous Holstein cows. Experiment two was a rumen fermentation study with three rumen-fistulated multiparous Holstein cows. Experiment one resulted in no significant differences among treatments for dry matter intake, milk yields, or feed efficiency. The results of experiment two showed no significant treatment differences in rumen pH, ammonia, or VFA. The researchers concluded that the supplementation of mid-lactation cows under their experimental conditions was not beneficial when compared to control treatments. The comparison of these studies provides further indications that the benefits of direct-fed microbials may be largely affected by many factors.

Improved Immune Response of Cattle Fed Direct-fed Microbials

There is very little literature available discussing the impact of direct-fed microbials on the immune system of cattle. These types of studies have been done on other species. Lessard and Brisson (*35*) observed that pigs consuming a fermented product with *L. bulgaricus*, *L. casei*, and *S. thermophilus* had higher IgG levels than controls when challenged with a transmissible gastroenteritis virus vaccine. Shu and Gill (*51*) showed that *L. rhamnosus* HN001 (DR20^{TM}) significantly increased anti-*E. coli* IgA titers in mice challenged with *E. coli* O157:H7.

Because of the growing interest in direct-fed microbials for cattle, Emmanuel et al. (*22*) conducted a cattle experiment to investigate immune responses from these supplements. The researchers had two consecutive experiments with 8 cannulated steers. The design was a 2 x 2 Latin square with 2 periods and 2 diets in each experiment. In experiment 1 the treatments were either a top-dressed control treatment or *E. faecium* EF212 treatment. Experiment 2 was very similar except the treatment group received *E. faecium* EF212 plus *Saccharomyces cerevisiae* (yeast). There were 21 days in each test period which could be broken down into 10 days of adaptation and 11 days of measurement. Blood samples were taken on days 17 and 21. The blood parameters tested were serum amyloid A (SAA), lipopolysaccharide binding protein (LBP), haptoglobin, and alpha$_1$-acid glycoprotein (α_1-AGP). These compounds are acute phase proteins which are released by the immune system due to inflammation, infection, or injury. Experiment 1 resulted in no significant treatment effects for steers fed *E. faecium* when looking at plasma SAA, LBP, haptoglobin, and α_1-AGP. In experiment 2 the steers receiving *E. faecium* and yeast had higher plasma SAA levels than control steers. This may be relevant because it is thought that SAA is involved in binding and removal of endotoxin. The treatment steers also had elevated LBP and haptoglobin which also indicates the presence of an inflammatory response. The researchers concluded that the mechanisms for the increase in these proteins should be further studied. Whether these acute phase proteins are beneficial to the host would also require further investigation.

Characteristics of Immunoglobulins in the Ruminant System

The following information is an overview of the location and functions of IgA, IgG, and IgM in the ruminant system. Immunoglobulins are produced in response to antigens that are perceived as foreign by the host organism. Production of antibodies represents the humoral immune system (*26*). Serum immunoglobulins are made up mostly of IgG. The structure of IgG consists of two gamma heavy chains and two kappa or lambda light chains. Of all of the immunoglobulins, IgG is the most effective at complement fixation, bacterial agglutination, toxin neutralization, virus neutralization, and precipications (*26*). Bovine IgG can be divided into the subclasses IgG_1 and IgG_2. Bovine IgG differs from IgG in other species in its distribution in external secretions. Immunoglobulin A is the more abundant immunoglobulin in the external secretions including colostrum and milk for humans, rats, and mice (*37*). Cattle and other ruminants have more IgG_1 in mammary secretions (*26, 37*) as well as other excretions excluding those for saliva and tear production (*37*). Both bovine IgG_1 and IgG_2 have been observed to be identical in the ability to fix bovine complement. They do however differ in some other functions. The IgG_2 is responsible for inducing both adherence and phagocytosis by neutrophils and monocytes (*37*). In non-ruminants the intestinal secretions contain mostly IgA. In ruminant intestinal systems IgA comprises a large amount of the immunoglobulins but IgG is also present in substantial amounts (*33*).

Secretory IgA is made up of an IgA dimer which is connected by a covalently bonded J chain. The dimeric IgA-J-chain complex has a strong

attraction for the secretory component. The secretory component is a sizeable transmembrane protein made by glandular epithelial cells. The free form of the secretory component is proteolytically cleaved while a portion of it remains in the membrane. The part left in the membrane is a receptor for the dimeric IgA-J-chain complex. Once the secretory component becomes part of the complex it is moved across the glandular epithelial cell. The secretory IgA is then released in the lumen of the secretory organ (*33*). A secretory peptide remains with the IgA and is responsible for the resistance of secretory IgA to digestive enzymes. This resistance makes secretory IgA able to stay intact in transit through the gut (*26*).

Secretory IgA and serum IgA apparently are produced by different cellular systems. The B cells of the lamina propria produce secretory IgA. Secretory IgA appears to be of great importance for protection against pathogens because it can be detected on mucous membranes and most secretions. Secretory IgA is dimeric while serum IgA is in the monomeric form. Serum IgA is not a very effective opsonin and does not activate the complement system except by an alternate pathway. In rodents the hepatobiliary route is documented as a means to clear IgA-antigen immune complexes from serum. It could be possible that this is also the case for cattle (*26*).

The first antibody to appear in the primary immune response is IgM. It is nicknamed the “ancestral antibody” because it was the first antibody to appear phylogenetically as well as the first to appear as an animal matures from birth to adulthood. Serum IgM is orientated as a circular shaped pentamer with five functional bivalent units. It contains ten heavy and light chains with a small J

chain in the center. It contains a total of ten antigen-binding regions and responds to most antigens as the most efficient complement activator. It also is the most efficient at agglutinating antigens. In some cases it can also lyse or damage pathogens (*26*).

Butler (*11*) has put together the most extensive review of bovine immunoglobulins to date. Butler (*11*) states that bovine immunoglobulins are fundamentally the same as those found in other mammals and he has compiled the amounts of immunoglobulins in serum and many other bovine sampling sites. The values included are from studies, however with no standardization of methods. The differing ages and breeds of cattle used in the studies may also influence the results.

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CHAPTER III

DETERMINATION OF THE MECHANISM(S) BY WHICH DIRECT-FED MICROBIALS CONTROL *ESCHERICHIA COLI* O157:H7 IN CATTLE

Lacey M. Guillen and Stanley E. Gilliland

Food and Agricultural Products Research and Technology Center

and

Department of Animal Science

Oklahoma State University

Stillwater, Oklahoma 74074

ABSTRACT

This experiment was conducted to determine if immune enhancement might be responsible for the success of the direct-fed microbials, *Lactobacillus acidophilus* and *Propionibacterium freudenreichii*, in reducing the carriage of *Escherichia coli* O157:H7 in live cattle. Ten steers fed a pelleted growing diet ad libitum were assigned to one of two treatments. Five of the steers received the direct-fed microbials (DFM) (1×10^9 CFU each) while the control group received only lactose (carrier). Treatments were fed once daily for 21 days after which the animals were transported to a bovine BSL-2 barn, rectally incolulated with *E. coli* O157:H7 ATCC 43894 (10 ml of 2.0×10^7 cfu/ml), and housed in separate pens. The once daily treatments and ad libitum feeding were continued. After inoculation the previously mentioned samples were taken every 12 hours for 48 hours, daily until 7 days post-inoculation, and weekly until the conclusion of the experiment. Feed consumption and weekly body weights were used to determine overall performance measures. Performance, immunological, and microbial plating data were analyzed using Proc Mixed ANOVA, repeated measures ANOVA, and Fischer's exact test, respectively. A $P<0.10$ was considered significant. The control group had a higher dry matter intake ($P=0.06$). There were no differences in average daily gain ($P=0.60$) or gain to feed ratio (0.97) between the two groups. The immunological results of the experiment showed higher levels of serum IgA for the DFM group for all post-challenge sample times. There were treatment ($P<0.10$) and day effects ($P=0.0001$) for serum IgA. Granulocytes and granulocytes as a percentage of

white blood cells showed a significant treatment x day interaction (P=0.07 and P=0.06, respectively) with the DFM group being higher for both. Several serum blood chemistry parameters had significant treatment x day interactions including: BUN (P=0.02), BUN:creatinine (P=0.01), anion gap (P=0.03), total protein (P=0.09), albumin (P=0.02), and aspartate aminotransferase (P=0.05). Serum alanine aminotransferanse and alkaline phosphatase both had treatment effects (P=0.07 and P=0.02 respectively). There were no significant differences between treatments for numbers of animals that were negative or positive for *E. coli* O157:H7 throughout the experiment. These results suggest that feeding the direct-fed microbial product, Bovamine®, may have a potential to enhance the immune response of cattle inoculated with *E. coli* O157:H7.

INTRODUCTION

Escherichia coli O157:H7 first became a pathogen of concern in the early 1980's when it was linked to outbreaks of foodborne illness from the consumption of ground beef (*52*). Cattle have been deemed one of the main reservoirs for the transmission of *E. coli* O157:H7 (*15*). In the decades following the early 1980's outbreak there have been a wide array of different and unanticipated situations involving the transmission of *E. coli* O157:H7. Many subsequent outbreaks also were linked to beef products, although in 1991 some were associated with fresh produce (*50*). *Escherichia coli* O157:H7 had the ability to survive in organic wastes that are used as fertilizers (*3*). Contaminated runoff water also may inadvertently pollute fields of crops with the pathogen (*1*). Amazingly outbreaks have even been linked to buildings at county fairs (*20, 61*) and petting zoos (*12*).

All of these various outbreaks reinforce the need for strategies to decrease and/or eliminate sources of *E. coli* O157:H7. Because cattle have been linked directly or indirectly to sources of contamination they appear to be an important species to establish prevention strategies. There has been a surge of research to investigate different methods to reduce *E. coli* O157:H7 in live cattle. Some of the strategies studied include vaccines against *E. coli* O157:H7 (*45, 47*), transition from a grain to hay diet prior to slaughter (*17*), sodium chlorate treatment (9*, 10, 55*), and direct-fed microbials for pathogen reduction (5*, 44*).

The focus of this experiment was to investigate the effect of a commercially available direct-fed microbial product containing a species of *Lactobacillus* and one *Proprionibacterium* species on the fecal shedding of *E. coli*

O157:H7 by ten steers that were rectally inoculated with the pathogen. Five of the steers were fed a control treatment while the other five received the direct-fed microbials. The direct-fed microbials used had been shown to cause significant reduction in the incidence of *E. coli* O157:H7 in feedlot cattle (5). Fecal samples and rectal swabs were tested for *E. coli* O157:H7 to determine fecal shedding throughout the experiment. Blood serum and fecal extracts were used to test IgG, IgA, and IgM at each sampling time. Blood samples were also tested for serum blood chemistry and a complete blood count. Dry matter intake, average daily gain, and gain efficiency also were calculated for each animal throughout the experiment.

MATERIALS AND METHODS

Source and Maintenance of Cultures

Escherichia coli O157:H7 ATCC 43894 was from the food microbiology culture collection at Oklahoma State University. It was subcultured weekly using a 1% inoculum into 10 ml of sterile tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI) and incubated at 37℃ for 18 hrs. It was subcultured at least three times on consecutive days just prior to use. The direct-fed microbial (DFM) product, marketed as Bovamine®, contained *L. acidophilus and P. freudenreichii*, and was obtained from Nutrition Physiology Corp. (Guymon, OK). Lactose was the carrier for the cultures in the DFM. The culture packets (individual daily doses) were stored in a freezer at -20℃. Microbial populations in the product were confirmed by plating serial dilutions of packets of the DFM on MRS agar (Difco Laboratories, Detroit, MI).

Feeding Experiment

Ten 9-month-old Angus-cross steers were randomly allocated to one of two treatments. The starting body weight of the control and the direct-fed microbial (DFM) groups were 604.0+/-13.95 and 607.6+/-13.95, respectively. Five of the steers received the DFM. The five remaining steers received a control treatment of lactose (an amount equal to that in the DFM), which is the carrier for the DFM. All of the steers were cared for and handled in accordance with the guidelines set by the Oklahoma State University Institutional Animal

Care and Use Committee (protocol # AG-07-7). Day 0 baseline samples were taken prior to administration of the treatments. The samples included the following: body weight, blood samples, rectoanal mucosal swabs, and fecal samples.

The steers were housed in separate pens and individually fed a pelleted growing diet (Table 1) ad libitum with treatments given once per day. The treatments were top-dressed on the feed. Samples were taken on day 7 and day 14 after the start of treatments. During the third and final week of the adjustment period the steers were transported to the BSL-2 bovine isolation barn at the Center for Veterinary Health Sciences, Oklahoma State University. The steers were in the BSL-2 barn for three days before inoculation with *E. coli* O157:H7 ATCC 43894.

On day 21 steers were sampled before they were rectally inoculated with *E. coli* O157:H7 ATCC 43894 as described by Sheng et al. (*54*) with a few minor changes. Ten milliliters of a cell suspension of *E. coli* O157:H7 ATCC 43894 (2.0×10^7 CFU/ml) was asceptically poured onto a sterile sponge in a sterile Whirl Pak bag (Nasco, Fort Atkinson, WI) and placed on ice for transport to the barn. The sponge absorbed all of the liquid culture. One sponge was prepared for each animal. After manual removal of feces with a gloved hand (Nitrile gloves, VWR, West Chester, PA) the sponge was inserted using a gloved hand into the terminal rectum of each steer. The culture was gently squeezed out of the sponge inside the rectum and rubbed onto the rectoanal mucosa. The tail of

each animal was then held down for 10 minutes to prevent defecation and allow the pathogen to associate with the rectoanal mucosa.

After inoculation samples were taken at 12 hour intervals including days 21.5, 22, 22.5, and 23. Daily samples were taken on days 24 through 28. Samples were then taken weekly on days 35, 42, 49, 56, and 63 which marked the conclusion of the experiment. All steers were reinoculated on day 35 with a higher dose of 10 ml of 1.0×10^9 cfu/ml *E. coli* O157:H7 ATCC 43894. An additional sample was taken on day 36 after the second inoculation.

At the conclusion of the experiment all 10 steers were cleared of *E. coli* O157:H7. A combination of ceftiofur (1 ml per 100 lb of a 50 mg/ml ceftiofur product subcutaneously; Naxcel, Pfizer, Inc., New York, NY) and neomycin (22 mg/kg orally; Agrilabs, St. Joseph, MO) was administered for three consecutive days. After 3 days of antibiotics fecal samples and rectal swab samples were analyzed to confirm that every animal was negative for *E. coli* O157:H7.

Steer Weights and Feed Data

Weekly body weights of steers were taken prior to each sampling. Weights of feed offered and refused were used to calculate daily dry matter intake. Gain to feed ratios and average daily gains were also calculated. The overall performance data were analyzed for the experiment. (Steer number 184 was eliminated from these calculations due to chronic bloating.)

Processing of Fecal and Swab Samples

Fecal samples were collected by manual stimulation or free catch, placed in a sterile Whirl-Pak bag (Nasco, Fort Atkinson, WI) and put on ice until processing. Rectoanal mucosal swab samples (RAMS) were collected with a sterile foam-tipped swab (VWR, West Chester, PA). The swab was inserted 3 to 5 cm into the anus and the mucosal surface was rapidly swabbed with an in and out motion. Swabs were immediately placed into 10 ml of GN broth supplemented with antibiotics and stored on ice.

Swabs and fecal samples were processed using a similar procedure to that of Brashears et al. (*5*). Briefly, ten grams of feces were asceptically added to 90 ml of GN-VCC broth (GN broth, Difco Laboratories, Detroit, MI), with 8 µg/ml vancomycin (Sigma-Aldrich, St. Louis, MO), 50 ng/ml cefixime (USP, Rockville, MD), and 10 µg/ml cefsulodin (Sigma-Aldrich, St. Louis, MO). Each rectoanal mucosal swab was added to a separate tube containing 10 ml of GN broth supplemented with the same concentrations of antibiotics used for the fecal samples. Both sets of samples were incubated for 6 hours at 37℃. Rather than immunomagnetic separation the enriched samples were immunoconcentrated using the Vidas I.C.E. coli O157 kit in the mini Vidas system (Biomérieux, Durham, NC). Each immunoconcentrated sample was spread plated in duplicate onto sorbitol MacConkey agar (Difco Laboratories, Detroit, MI) supplemented with 4-methylumbelliferyl-β-D-glucuronide (5 mg/ml) (Sigma-Aldrich, St. Louis, MO), cefixime (50 µg/ml) (USP, Rockville, MD), and potassium tellurite (2.5 µg/ml) (MP Biomedicals, Solon, OH) which will be referred to as CT-SMAC +

MUG. The immunoconcentrated samples also were spread plated onto Chrom O157 agar (Chromagar Microbiology, Paris, France) supplemented with cefixime (0.025 µg/ml) (USP, Rockville, MD), cefsulodin (5 µg/ml) (Sigma-Aldrich, St. Louis, MO), and potassium tellurite (2.5 µg/ml) (MP Biomedicals, Solon, OH). Presumptive positive *E. coli* O157:H7 colonies on CT-SMAC + MUG appear colorless and non-fluorescent under UV light while positive colonies on Chrom O157 agar appear to be a mauve color. The identifications of positive colonies were confirmed by using the GNI+ kit in the Vitek 32 system (Biomérieux, Durham, NC) and the RIM™ *E. coli* O157:H7 Latex Test (Remel, Lenexa, KS) to confirm the O157 and H7 antigens.

Another portion of fecal samples taken both pre- and post-inoculation was used to make a fecal extract in order to analyze immunoglobulins. The fecal extraction method was performed as described by Peters et al. (*43*). The following immunoglobulins were measured: IgA, IgG, and IgM. An enzyme linked immunosorbent assay (ELISA) quantitation kit for each bovine immunoglobulin was used (Bethyl Laboratories, Inc., Montgomergy, TX). The appropriate dilutions were determined and the manufacturer's instructions were followed with one exception. Bovine serum albumin (BSA) was eliminated from the reagents for measuring IgM due to cross-reactivity problems (eliminating BSA was suggested by Bethyl Laboratories). The intra assay coefficient of variation for fecal IgA, IgG, and IgM were 4.23%, 4.30%, and 4.02% respectively. The inter assay coefficient of variation for fecal IgA, IgG, and IgM were 3.31%, 4.28%, and 6.53% respectively.

Processing of Blood Samples

Blood samples were collected from each steer via the jugular vein at each sampling time. Sterile vacutainers (BD vaccutainer, Franklin Lakes, NJ, red top) were used to collect the blood. Blood samples were placed on ice after collection and then refrigerated overnight. The samples were centrifuged at 3,000 x g for 10 minutes to separate the serum. Transfer pipets were used to transfer blood serum into labeled cryogenic vials. Serum samples were stored at -80℃ until further processing.

A second blood sample also was taken in a vacutainer (BD vaccutainer, Franklin Lakes, NJ, purple top) containing EDTA (anti-coagulant) for a complete blood count. These blood samples were analyzed using either a VetScan®HM2 (Abaxis, Inc., Union City, CA) or an Abbott Cell-dyn 3500 (GMI, Inc., Ramsey, MN). Complete blood counts were conducted only for the post-challenge period. A complete blood count for every time period post-challenge is also not available. The day 21 sample is just prior to the challenge with *E. coli* O157:H7 so it will serve as a baseline.

One vial of serum for each animal and sampling time was submitted to the Stillwater Medical Center for a complete blood panel using Vitros 5,1 FS system (Ortho Clinical Diagnostics, Raritan, NJ). The complete blood panel was tested for: glucose, blood urea nitrogen (BUN), creatinine, BUN:creatinine, sodium, potassium, chloride, carbon dioxide, anion gap, calcium, total protein, albumin, globulin, albumin:globulin, aspartate aminotransferase (AST), alanine aminotransaminase (ALT), alkaline phosphatase, and total bilirubin.

Blood serum was also analyzed for IgA, IgG, and IgM. An ELISA kit (Bethyl Laboratories, Inc., Montgomergy, TX) for each bovine immunoglobulin was used. The manufacturer's directions were followed except that bovine serum albumin (BSA) was not added to the reagents used for measuring IgM due to cross-reactivity problems (eliminating BSA was suggested by Bethyl Laboratories). The intra assay coefficient of variation for serum IgA, IgG, and IgM were 5.93%, 4.04%, and 5.86% respectively. The inter assay coefficient of variation for serum IgA, IgG, and IgM were 9.68%, 6.00%, and 5.05% respectively.

Statistical Analyses

Blood chemistry data as well as serum and fecal immunoglobulin data were analyzed using a repeated measures analysis of variance (ANOVA) with the SAS software (*53*). The first-order autoregressive covariance structure was adopted. Differences were considered significant at the $p<0.10$ level. The slice option was used when there was a significant treatment or treatment x time interaction ($p<0.10$). Pairwise differences were not needed to separate Least squares means because there were only two treatments.

Performance data including dry matter intake, gain:feed, and average daily gain were analyzed using Proc Mixed ANOVA with the SAS software (*53*). Differences were considered significant at the $p<0.10$ level. The slice option was used when there was a significant treatment or treatment x time interaction ($p<0.10$). Pairwise differences were not needed to separate least squares

means because there were only two treatments.

The Fischer's exact test was used to analyze microbial plating data that indicated whether each steer was positive or negative for *E. coli* O157:H7. Data from both CT-SMAC + MUG agar and Chrom O157 agar were used for this analysis.

RESULTS

Detection of *Escherichia coli* O157:H7

Pre-enriched fecal and swab samples were plated on both CT-SMAC+MUG and Chrom 0157 agars. A steer was considered positive if a confirmed positive was observed on either CT-SMAC+MUG or Chrom 0157. For the most part the positive and negative results for the fecal samples and swabs were the same for both types of media. A steer was considered positive if the fecal samples and/or rectoanal swab sample was confirmed positive. Prior to challenge, no steers in either group were positive for *E. coli* O157:H7. During the day following the challenge, all five control steers were positive on one or more sampling days. In the treatment group four of the five steers were positive on one or more days. Due to small sample sizes, the Fischer's exact test to analyze the positive and negative plating data was unable to detect any differences between the two treatments. Positive and negative plating results for *E. coli* O157:H7 are included Table A1 in Appendix A.

Serum Immunoglobulins

The immunoglobulin levels were measured throughout the three week adjustment period (pre-challenge). After *E. coli* O157:H7 inoculation the following sampling schedule was used: 12-Hour sampling intervals for day 21.5 (12 hours post-challenge), 22, 22.5, and 23, daily sampling for days 24 through 28, and weekly sampling for the remainder of the experiment including days 35,

42, 49, 56, and 63. Results for serum immunoglobulins A, G, and M are in table 2.

Serum IgA

Overall serum IgA levels were different between the treatments (P<0.10) and days (P=0.0001). There was no treatment x day interaction (P=0.33). Although the interaction was not significant overall, the DFM group had higher serum IgA levels for all post-challenge sampling days except day 23 (Figure 1). Serum IgA increased post-challenge for both groups but there was a much more dramatic increase for the DFM group. Serum IgA was significantly higher in the DFM group on days 21.5 (P=0.08), 22.5 (P=0.05), 28 (P=0.08), and 35 (P=0.06).

Serum IgG

There were no overall differences between treatments (P=0.71) for serum IgG. There was a day effect (P=0.002) but there was not a treatment x day interaction (P=0.64). The control group's serum IgG seemed to peak at day 21.5 post-challenge with a higher level than the steers fed the DFM. The DFM group's serum IgG peaked at day 22.5. Each group's IgG levels began to decrease slightly after they reached their highest point during the post-challenge 12-hour sampling period (Figure A1 Appendix A).

Serum IgM

Overall there were no treatment (p=0.88) or treatment x day (P=0.66) effects for serum IgM. There was a significant day effect (P<0.0001). There was a sharp increase in serum IgM for both groups shortly after the challenge (Figure A2 Appendix A).

Fecal Immunoglobulins

Results for fecal immunoglobulins A, G, and M are in Table 2. Fecal IgA had a day effect (P=0.03) with no treatment (P=0.15) or treatment x day (P=0.90) effects (Figure A3 Appendix A). Fecal IgG also had a day effect (P=0.01) with no treatment (P=0.51) or treatment x day (P=0.82) effects. There were differences between treatments (P=0.01) and days (P=0.03) for IgM, although there was no treatment x day effect (P=0.90). The control group had higher IgM levels on day 7 (pre-challenge) (P=0.01) and day 28 (P=0.09)

Complete Blood Count

The complete blood count results are in Table 3. There were day (P=0.001) and treatment x day (P=0.07, Figure 2A) effects for granulocytes. However, there was not an overall treatment effect (P=0.88). The DFM group had significantly higher total granulocytes on days 22.5 (P=0.01) and 25 (P=0.08). The DFM group had its highest level of granulocytes on day 25 while the control group peaked on day 42. Granulocytes as a percentage of white blood cells had day (P=0.001) and treatment x day (P=0.06, Figure 2B) effects. The treatments did not differ overall (P=0.92); however, the DFM group was significantly higher on day 23 (P=0.01). Both groups increased from day 21 to day 63. The DFM group peaked at day 23 while the control group peaked at day 63.

There were day (P=0.04) and treatment x day (P<0.10, Figure 3A) effects for total lymphocytes. Overall there were no treatment differences (P=0.95) for lymphocytes. The lymphocytes for both groups followed similar trends except the DFM group had a sharp increase from day 23 to day 25 followed by a sharp decrease from day 25 to day 28. Lymphocytes as a percentage of white blood cells had day (P=0.01) and treatment x day (P=0.02, Figure 3B) effects. Overall there were no treatment differences (P=0.85); however, the control group had significantly higher levels on days 23 (P=0.02) and 28 (P=0.08). The DFM group had decreased lymphocytes as a percentage of white blood cells until day 23 after which they increased until the end of the experiment. The results for the control group showed sharp increases and decreases between days 22 to 49.

The percentage of hematocrit had day (P=0.08) and treatment x day (P=0.06, Figure A6 Appendix A) effects but not treatment (P=0.11) effects. The two groups diverged from similar decreasing percent hematocrit trends from days 28 to 42 and days 42 to 49. The control group increased from day 28 to 42 while the DFM group decreased. The DFM group increased from day 42 to 49 while the control group increased from day 49 to 56. Both groups decreased from day 56 to 63. The control was significantly higher at days 42 (P=0.01), 56 (P=0.02), and 63 (P=0.01). Additional complete blood count parameters that were not statistically different between groups are in Figures A10 to A17 in Appendix A. Reference values for bovine complete blood count parameters are included in Table A2 in Appendix A.

Serum Blood Chemistry

Overall statistical results for blood chemistry are included in Table 4. Blood urea nitrogen (BUN) did not differ (P=0.13) for treatments. There were day (P<0.0001) and treatment x day (P=0.02, figure 4A) effects. Blood urea nitrogen for the DFM group remained constant from day 22.5 to day 63. The BUN of the control group had a much sharper increase from days 28 to 63. The control group had significantly higher BUN on days 35 (P=0.07), 42 (P=0.07), 49 (P=0.004), and 63 (P=0.01). Blood urea nitrogen:creatinine levels had treatment (P=0.002), day (P<0.0001), and treatment x day interaction (P=0.01, Figure 4B) effects. The trends were similar to the BUN results. Prior to challenge, both groups increased from day 0 to day 14; then decreased until day 21.5 for the control and day 22 for the DFM group. Each group exhibited different degrees of increase in BUN post-challenge. The DFM group leveled out at about day 25. The control group continued to increase with some fluctuation for the remainder of the experiment. The control group had higher BUN:creatinine on days 21 (P=0.02), 22 (P=0.07), 26 (P=0.09), 27 (P=0.02), 35 (P=0.003), 42 (P=0.01), 49 (P=0.0002), 56 (P=0.02), and 63 (P=0.001).

The anion gap had day (P<0.0001) and treatment x day (P=0.03, Figure A7 Appendix A) effects. There was not a treatment effect (P=0.96). The serum anion gap for both groups decreased throughout the experiment. The control group had significantly higher anion gap levels on day 21(P=0.08) while the DFM group was significantly higher on days 22.5 (P=0.04) and 25 (P=0.005).

Serum total protein had day (P<0.0001) and treatment x day (P=0.09, Figure A8 Appendix A) effects. There was no overall treatment difference (P=0.32). The control group had significantly higher total protein on day 14 (P=0.04). Serum albumin had significant day (P<0.0001) and treatment x day (P=0.02, Figure A9 Appendix A) effects. Serum albumin had no overall treatment significance (P=0.44). Both groups had fluctuating serum albumin but there was an overall decrease throughout the experiment. The control group had significantly higher serum albumin on day 14 (P=0.09).

The enzyme aspartate aminotransferase had treatment (P=0.03), day (P=0.03), and treatment x day (P=0.05, Figure 5) effects. The aspartate aminotransferase levels were similar for both groups until day 35, at which time the control group had a sharp increase. After day 35 the control group's levels of this enzyme decreased but the last measurement taken on day 63 was still higher than all measurements taken before day 35. The control group had statistically higher aspartate aminotransferase on days 35 (P<0.0001), 42 (P=0.004), and 49 (P=0.01).

Serum alanine aminotransferase had treatment (P=0.07, Figure 6) and day (P<0.0001) effects. There was not a treatment x day interaction effect (P=0.14). The control group had statistically higher levels on days 21.5 (P=0.03), 22 (P=0.06), 35 (P=0.04), and 63 (P<0.10).

Serum alkaline phosphatase had both a treatment (P=0.02, Figure 7) and day (P=0.07) effect but no treatment x day interaction effect (P=0.52). The control group had statistically higher levels on days 23 (P=0.08), 24 (P=0.05), 25

(P=0.07), 26 (P=0.08), 27 (P=0.06), 28 (P=0.07), 42 (P=0.005), and 49 (P<0.10). Additional serum blood chemistry parameters that were not statistically different between treatments are in Figures A18 to A27 in Appendix A. Reference values for bovine blood chemistry analytes are included in Table A2 in Appendix A.

Steer Performance

The control group had a higher dry matter intake from the beginning to the end of the experiment (P=0.06). There was no difference in overall average daily gain between the two groups (P=0.60). There was also no difference in overall gain efficiency between groups (P=0.97).

DISCUSSION

The direct-fed microbial containing *L. acidophilus* and *P. freudenreichii* used in the current experiment has previously been shown to reduce fecal shedding of *E. coli* O157:H7 in cattle. Brashears et al. (*5*) observed that cattle fed *L. acidophilus* NPC 747 were 49.0% less likely to be positive for the pathogen compared to control animals. These decreases contributed to significantly less positive hides when comparing control animals to treatment animals. This strain of *L. acidophilus* is the same strain that is included in Bovamine® (based on information from the supplier, Nutrition Physiology Corporation, Guymon, OK). The history of this strain is included in the Appendix B.

Lactobacillus acidophilus NP51 is yet another alias for the *L. acidophilus* culture included in Bovamine®. Younts-Dahl et al. (*66*) observed that cattle fed *L. acidophilus* NP51 at a level of 10^9 CFU per steer resulted in the largest decreases in shedding *E. coli* O157. This experiment also included *Propionibacterium freudenreichii* (NP24) at 10^9 CFU per steer for each treatment. The Bovamine® product includes this same strain of *P. freudenreichii* that was used in the experiment by Younts-Dahl et al. (*66*) (personal communication with Dr. Mindy Brashears, Texas Tech University).

The decision to inoculate the steers rectally with *E. coli* O157:H7 came about based on recent research that indicates that the pathogen preferentially colonizes the rectoanal junction (RAJ) (*40*). Prior to these findings the colon had been identified as the principle site of colonization for *E. coli* O157:H7 (*30*). The

protocol for inoculating the steers in the present experiment was adapted from an experiment by Sheng et al. (*54*). The rectal inoculation of the experiment by Sheng et al. (*54*) resulted in long-term colonization of the cattle. In the current experiment all of the steers started out negative for *E. coli* O157:H7. The steers were first inoculated with a 10 ml cell suspension (2.0×10^7 cfu/ml) of the same strain of *E. coli* O157:H7 used in some experiments included in an experiment by Sheng et al. (*54*). We did not observe the uniform pathogen colonization as was described by Sheng et al. (*54*). Both fecal samples and rectoanal mucosal swabs (RAMS) were tested for the presence of this pathogen. There were differences observed in the numbers of positive animals between the two sample types but overall the results from fecal and swab samples were similar. Experiments have been conducted solely to compare RAMS and fecal samples for detecting *E. coli* O157:H7. These experiments indicate that RAMS are more sensitive for detection (*51*). This supports the findings that *E. coli* O157:H7 preferentially colonizes the RAJ (*40*). Naylor et al. (*40*) observed high levels of tissue-associated *E. coli* O157:H7 proximal to the RAJ. The uneven distribution of the pathogen in the fecal samples of these animals led them to conclude that the bacteria had only coated the surface of the fecal material as it was excreted. Another factor may be that RAMS are not inundated with fecal material that contains many other types of bacteria (*51*). Rice et al. (*51*) also sampled naturally colonized animals with RAMS and fecal samples. They observed that some animals had positive RAMS but negative fecal samples. This suggests that the animals may be harboring the pathogen but not shedding it. Similar

situations may have occurred in the present experiment resulting in inconsistent occurrence of positive samples.

The differences in culture positive steers in the current experiment and that of Sheng et al. (*54*) may be explained by differences between the two experiments. Sheng et al. (*54*) had a low-dose group (1.0×10^7 CFU *E. coli* O157:H7 ATCC 43894) and a high-dose group (10^9 CFU of a four-strain mixture with equal parts of: ATCC 43894, WSU 180, WSU 400, and WSU 588). In the current experiment, we used only *E. coli* O157:H7 ATCC 43894. Strain ATCC 43894 was isolated from an outbreak of hemmorhagic colitis in Michigan (*63*). Strains WSU 180, WSU 400, and WSU 588 used by Sheng's group were cultured from cattle (*54*). Sheng et al. (*54*) made no conclusions concerning the use of a 1.0×10^7 CFU dose of a single culture compared to the use of a 1.0×10^9 CFU dose a four-strain mixture. They also made no mention of any effects due to the origin of the strain. Wells et al. (*64*) identified ground beef products as the culprit in causing the outbreak of hemmorhagic colitis in Michigan. It could then be presumed that the ATCC 43894 strain actually may have originated from cattle since it appeared in the ground beef. Therefore in this case the origin of the strain may not have necessarily differed as to influence the colonization of the cattle. It might be helpful in future research to investigate if and why using a high dose of multiple strains of *E. coli* O157:H7 affects successful long-term rectal colonization of cattle.

The sampling periods for the experiments were also very different. Sheng et al. (*54*) sampled days 0, 7, 14, 22, 29, 36, 44, 58, and 66 for the high-

dose group and days 0, 6, 13, 20, 27, 34, 41, 48, and 55 for the low-dose group. In the current experiment a more intense sampling schedule was used because of efforts to detect changes in immune response. It might be argued that sampling this often could skew results because too much of the pathogen could be manually removed during the sampling and limit its ability to maintain colonization. This seems unlikely though because most fecal samples in the present experiment were collected by a free catch method and did not have to be invasively collected. It is also unlikely that the swabs used for sampling would interfere with pathogen persistence throughout the experiment. Sheng et al. (*54*) tested the effects of repeated rectal swabbing on colonization and observed that it had no effect.

The two experiments also differed somewhat in the methods for detecting *E. coli* O157:H7. In the present experiment plating non-enriched samples yielded no *E. coli* O157:H7 positive colonies on the CT-SMAC + MUG or Chrom O157 agars. Sheng et al. (*54*) were able to detect *E. coli* O157:H7 by direct plating for many samples. When they were not able to detect by direct plating they used an enrichment procedure that differed slightly from the present experiment. Sheng et al. (*54*) used TSB broth supplemented with cefixime, potassium tellurite, and vancomycin while the present experiment used GN broth with added vancomycin, cefixime, and cefsulodin. GN broth, Hajna is used as an enrichment medium to recover enteric Gram-negative bacilli (*18*). In both studies enrichment cultures were plated on SMAC agar but the added antibiotics differed somewhat. Sheng et al. (*54*) added cefixime, potassium tellurite, vacomycin, and

MUG to the media while the present experiment used SMAC agar with added MUG, cefixime, and potassium tellurite. Cefixime is used to inhibit *Proteus* spp. which are sorbitol non-fermenters like *E. coli* O157:H7 (*13*). Vancomycin inhibits the growth of Gram-positive bacteria (*39*). Potassium tellurite is included in selective media because it is well documented that most strains of *E. coli* O157:H7 contain one or more copies of a tellurite resistance gene (*58*). In both experiments the addition of MUG served as a differential agent for colonies negative for glucuronidase. *Escherichia coli* O157:H7 is negative for both sorbitol fermentation (*42*) and glucuronidase (*23*). Therefore the colonies appear colorless and do not fluoresce under a UV light when plated on SMAG agar with added MUG. The slight differences in enrichment and culturing procedures should not be enough to affect the overall results.

Inconsistencies in direct-fed microbial studies previously reported make it difficult to compare the immunoglobulin analyses in the present experiment. Previous direct-fed microbial experiments have mainly evaluated cattle performance, carcass characteristics, and carriage of *E. coli* O157:H7. These types of experiments rarely look at immune parameters. Recently the area of immune enhancement related to direct-fed microbials has been receiving increased attention. This is due to the thought that an increased immune response is possibly the mode or one of the modes of action by which direct-fed microbials decrease pathogen carriage in cattle. Elam et al. (*21*) measured serum IgA in one of the two experiments included in their study. The blood samples to measure serum IgA were collected at the time of slaughter and at no

other time period. The researchers used the same basic ELISA method that was used in the present experiment but observed no differences. In the present experiment there was not a significant treatment x day interaction but the DFM group had higher serum IgA levels for all post-challenge sampling times. This included a post-challenge increase of a much higher magnitude than the control group which would suggest an increased immune response.

The serum immunoglobulin results for IgA and IgM obtained in the present experiment fall within the range among the mean values compiled from the various studies included in the review by Butler (*8*). Interestingly the serum IgM levels in the present experiment peaked on day 22. This is the only time period that the values rise slightly above the previously mentioned range among means values in Butler's (*8*) report. This peak may have been observed relatively soon after the challenge with *E. coli* O157:H7 because IgM has been shown to be the first immunoglobulin to appear during an immune response (*27*). Of the serum immunoglobulins, IgG is the most abundant followed by IgM and the least abundant serum immunoglobulin is IgA. A table in the review by Butler (*8*) includes the range among the mean values of immunoglobulin concentrations from the studies included. This same table includes the amounts of IgG1 and IgG2 in biological fluids. In the present experiment the ELISA procedure used made no differentiation between the two subclasses of IgG. The total serum IgG from the present experiment corresponds to the amounts listed for serum in the table when the values for IgG1 and IgG2 are combined.

The serum IgG and IgM levels observed in the present experiment are similar to the value listed by Gershwin et al. (*27*). The serum IgA levels were similar for the most part but in several instances they were lower than the value reported by Gershwin et al. (*27*). As previously mentioned any differences could very well result from the values being obtained from different breeds of cattle as well as age and sex variations and methods of immunoglobulin analyses.

In the present experiment fecal immunoglobulins were measured in efforts to get an idea of influence of the direct-fed microbial on intestinal or mucosal immunity. There are different opinions on the use of fecal extracts to measure mucosal immunity. Ferguson et al. (*24*) reported that the use of fecal extracts to quantify immunoglobulins can be inaccurate and misleading. The researchers came to this conclusion by comparing fecal extract immunoglobulins to immunoglobulins measured by using whole gut lavage fluid. The inconsistencies observed in using fecal extracts led the researchers to conclude that gut humoral immunity should only be measured by whole gut lavage, segmental gut perfusion, intestinal fluid aspiration, and biopsy.

McNeilly et al. (*38*) conducted an experiment in which they determined some effective methods for measuring bovine mucosal immunity. The researchers determined that both nasal secretions and rectal swabs are effective in sampling for mucosal immunity. They also determined that these same samples could be used for antigen-specific immunoglobulins such as the H7 flagellin-specific antibodies detected in their experiment.

In the present experiment the fecal immunoglobulin ELISA raw data results appeared to be quite variable. However, the standard errors for the overall stastical analysis of the fecal immunoglobulins were not particularly high. There were no statistical differences for fecal IgA and IgG. There was an overall treatment difference for fecal IgM. The two sampling days on which these differences occurred do not appear to be the result of an immune response. The control group had significantly higher fecal IgM on day 7 which was prior to the *E. coli* O157:H7 challenge. The other difference occurred on day 28 post-challenge. Immunoglobulin M is the first antibody to respond in an immune response (*27*). The serum immunoglobulin results indicated an increase immediately after the challenge. The same might be expected from measures of intestinal immunity since the pathogen mainly colonizes at the terminal rectum (*40*). It is difficult to verify whether or not the results correspond to any previous research due to the many different methods and sample preparations used in the limited research available. The fecal immunoglobulin results from the present experiments are much lower than the rectal swab immunoglobulins reported by McNeilly et al. (*38*). The fecal IgA and IgG results are 10 to 100-fold and 100 to 1000-fold lower respectively than the results for the rectal swabs by McNeilly et al. (*38*). In retrospect it may have been more useful in the present experiment to have a more accepted and verified method for measuring mucosal immunity in cattle. In addition to this it also may have be useful to test for immunoglobulins that are antigen-specific to the pathogen used for the challenge.

The complete blood count results indicated that total granulocytes were significantly higher in the DFM group on days 23 and 25. Granulocytes as a percentage of white blood cells were significantly higher in the DFM group on day 23. The more dramatic increase in granulocytes post-challenge in the DFM group may be in efforts to control the inoculated *E. coli* O157:H7. Granulocytes and mononuclear cells are part of a group of cell types called leukocytes or white blood cells. Granulocytes can be broken down into neutrophils, eosinophils, and basophils (*34*). Research has shown that bovine granulocytes lack lysozyme. Granulocytes can phagocytize invading microorganisms. *In vitro* studies with lysates from bovine granulocytes have been shown to kill *E. coli* and *Staphylococcus aureus* (*26*). Neutrophils make up the highest number of circulating granulocytes. They are phagocytic and generally first to respond to an inflammation site (*60*).

Lymphocytes had a slightly significant treatment x day interaction but there were no significant differences between treatments at each sampling day. The biggest difference between the groups occurred on day 25. The DFM group was higher than the control group although not significant. The increase in lymphocytes for the DFM group on day 25 was the highest increase post-challenge for either group. This may be an indication of an increased immune response in the DFM group post-challenge. The total lymphocytes for the two groups were fairly similar for all other sampling times. There was no significant treatment x day interaction for white blood cells but lymphocytes as a percentage of white blood cells were significantly higher for the control group on days 23 and

28. The DFM group had a sharp increase in total white blood cell count on day 25 that corresponded with increases in granulocytes and lymphocytes on the same day. This makes sense because both granulocytes and lymphocytes are types of leukocytes or white blood cells. With the exception of lymphocytes all white blood cell types are produced and mature in bone marrow. Bone marrow stem cells produce lymphocytes which mature and replicate in lymphoid tissues (*34*). Lymphocytes and macrophages are the two cell types that are implicated in an immune response and make up what is termed the lymphoreticular system. Lymphocytes and the cell subpopulations they give rise to have specific immune responses such as production of humoral antibodies for targeted agents and specified responses to foreign tissue antigens. Both B cells and T cells are derived from the differentiation of lymphocytes (*27*). Assays to quantify B and T cell proliferation may be useful in future work of this type.

The control group had significantly higher percentages of hematocrit on days 42, 56, and 63 but the values were not out of the reference range of 24-46% (*34*). It does not appear that the percent hematocrit differences were clinically significant because they were well within the reference range and cannot be readily explained by other factors.

The mean square estimates for each blood chemistry parameter for both groups were compared to reference ranges for normal bovine blood analytes. The blood analytes that had significant treatment and/or treatment x day effects will be discussed in more detail than those that were not. Those

values that were not significant will be discussed only if they were outside of the reference ranges.

Blood serum glucose for both groups exceeded the reference range of 45-75 mg/dl (*49*) during the adjustment period. The values were also higher than the reference range for a majority of sampling times post-challenge. Serum albumin was within 2.1-3.6 g/dl (*49*). Serum albumin had a significant treatment x day interaction but only day 14 during the adjustment period was significantly different. Both groups showed roughly the same trends for serum albumin. These results appear to be clinically insignificant. Globulin levels exceeded the reference range of 3.0-3.5 g/dl (*36*) for all sampling times. The albumin to globulin ratio was only slightly lower or higher than the range of 0.84-0.94 (*36*) at a few sampling times. Total protein stayed within the range of 5.7-8.1 g/dl (*49*). Total protein was only significantly different between the groups on day 14 of the adjustment period. This makes sense because the serum albumin was also only significantly different at this sampling day. Serum albumin is the most abundant of the serum proteins (*35*).

The BUN:Creatinine levels were very similar between the two groups during the adaptation period. The BUN:Creatinine levels were significantly higher for the control group on day 21 just prior to *E. coli* O157:H7 inoculation. The DFM group had a lower ratio of BUN to creatinine throughout the remainder of the experiment. It was significantly lower post-challenge on days 22, 26, 27, 35, 49, 56, and 63. Blood urea nitrogen measurements were significantly lower in the DFM group on days 49 and 63. The values were not outside the reference

range of 6.0-27 mg/dl (*49*) at any sampling time. Blood urea nitrogen and serum creatinine measurements are used as an indication of nitrogen waste retention in the kidneys. Ruminant microflora may utilize more urea than the microflora of non-ruminants. This should be taken into consideration when interpreting BUN levels and the subsequent BUN to creatinine ratio (*25*). A possible explanation for the differences in BUN and BUN:creatinine in the present experiment may be the lower feed intake of the DFM group. Both of these measures began to be increasingly different towards the end of the experiment. It was also towards the end of the experiment that the dry matter intake of the two treatments began to noticeably differ.

The anion gap was significantly higher for the control group on day 21 and higher for the DFM group on days 22.5 and 25. The anion gap is measured as the difference between the cations, sodium and potassium, and the anions, chloride and bicarbonate. This measure can be useful in diagnosing acid-base disorders of animals (*11*). In the present experiment the difference in anion gap is not clinically significant.

Alanine aminotransferase (ALT) for both treatment groups exceeded the reference range of 11-40 units/L (*49*) starting at the day 7 pre-challenge sample. Both groups continued to be higher than the reference range for all time points until the DFM group fell slightly below the upper limit of the range on day 63. Day 63 is the last sampling time for the experiment. The control group had higher ALT levels on days 21.5, 22, and 35. Alanine aminotransferase is the enzyme responsible for the transamination of L-alanine to pyruvate and 2-

oxoglutarate to glutamate. The transamination is reversible and occurs in the cell cytoplasm (*37*). Alanine aminotransferase is used for diagnosing hepatic necrosis in small animals and primates. It is not used in larger animals because their livers contain such small amounts of ALT (*14*).

Aspartate aminotransferase (AST) was significantly higher in the control group on days 35, 42, and 49. From day 28 to day 35 the control group's levels for this enzyme had a sharp increase. This enzyme is responsible for catalyzing the reaction to convert L-aspartate and 2-oxoglutarate to oxaloacetate and glutamate respectively through transamination (*37*). The serum levels of AST serve as a marker for soft tissue damage because the enzyme is found in so many tissues (*4*). Creatine kinase is a more specific marker for muscle damage. Today there are organ-specific enzymes that are used more often than AST. As mentioned before, AST is found in many tissues so therefore it cannot be used as an organ-specific enzyme (*37*). A reference range for bovine serum AST is 78-132 units/L (*49*). In the present experiment all of the values were within this range. It should be noted that the control group closely approached this upper limit on day 35. There are no readily apparent reasons for why these values differed between groups towards the end of the experiment.

Serum alkaline phosphatase levels differed on days 23, 24, 25, 26, 27, 28, 49, and 56 with the control group being higher. Alkaline phosphatase enzymes are isoforms that hydrolyze phosphate esters. The dephosphorylation of ATP is catalyzed by alkaline phosphatase. Animals have both tissue-unspecific and steroid-induced hepatic isoforms of alkaline phosphatase. In the

serum of animals the tissue-unspecific alkaline phosphatase predominates. Measured alkaline phosphatase values in horses and ruminants may be of little significance because of the broad range of reference values available (*37*). The values observed in the current experiment were well within the wide reference range of 0-500 units/L (*49*). Adawi et al. (*2*) conducted an experiment to investigate *Lactobacillus* supplementation with and without arginine in an acute liver injury model in rats. Some of the treatments resulted in a large decrease in AST and ALT when compared to the acute liver injury control group. Alkaline phosphatase was also decreased in some treatment groups but to a lesser extent than AST and ALT. The rat model may not be directly comparable to a bovine model but more investigation into the possible decrease of liver enzymes with *Lactobacillus* supplementation may be needed. This may or may not be a possible explanation for the decreases in these enzymes in the present experiment. As mentioned previously serum ALT, AST, and alkaline phosphatase are not useful markers of liver damage in cattle (*13*, *36*, *37*).

In the current experiment the control group had a higher overall dry matter intake. No differences between groups were observed for overall average daily gain and gain efficiency. Elam et al. (*21*) conducted two direct-fed microbial studies with varying combinations of treatments and days on feed. Both experiments used different combinations of *L. acidophilus* NP51, *L. acidophilus* NP45, and *P. freudenreichii* NP24. *Propionibacterium* species use lactate and glucose to produce acetate and propionate (*28*). Daily supplementation of lactate utilizing with or without lactate producing direct-fed microbials may

improve feed efficiency and average daily gains for cattle fed high concentrate rations (*28*). This is also the same strain of *P. freudenreichii* that is included in Bovamine®. The cattle in experiments in their study were on feed for 104-170 days. They noted that there were no significant differences in overall dry matter intake (DMI), average daily gain (ADG), or gain:feed.

Vasconcelos et al. (*61*) also observed no differences in DMI or ADG in a cattle experiment that used the same direct-fed microbials as the current experiment. The researchers included *P. freudenreichii* strain NP24 in each of the three experimental treatments at 1×10^9 cfu/(steer·day). The difference in the three treatments was an increasing dose of *L. acidophilus* NP51: low = 1×10^7, medium = 1×10^8, and high = 1×10^9 cfu/(steer·day). They did find a significant difference in gain efficiency (live weight basis) when they averaged results from the three doses of *L. acidophilus* and compared them to the control. The extremely small sample sizes and relatively short days on feed most likely limited a thorough look at the performance possibilities in the current experiment.

In recent years there has been much research on different colonization factors for *E. coli* O157:H7 in cattle. The exact mechanism by which cattle become transient carriers is unknown. Determining these colonization requirements will greatly advance the progress for developing successful vaccines and other intervention strategies. This knowledge will also aid in developing more successful colonization models for cattle. Pruinboom-Brees et al. (*48*) investigated whether tissues from cattle contain the Shiga toxin (Stx) receptor globotriaosylceramide (Gb_3). Thin layer chromatography of calf tissues

failed to detect Gb_3 in the gastrointestinal tract. Gb_3 was detected in kidney and brain tissues. Immunohistochemical methods were used to detect Stx1 and Stx2 binding in a variety of tissue samples in calves and adult cattle. These methods did not detect Stx binding in ileum and large intestine samples.

Naylor et al. (*40*) conducted studies with different colonization models including inoculation via stomach tube, oral inoculation, and a naturally colonized animal from a commercial beef operation. The orally inoculated steer was used as a "Trojan" steer to expose other steers to the pathogen in a group pen. The researchers hypothesize that an interaction between a bacterial adhesin and an epithelial cell receptor may be responsible for the bacterial tropism for the RAJ. Close analysis of the RAJ showed high concentrations of lymphoid follicles. Other *Enterobacteriaceae* such as *Salmonella typhimurium*, *Listeria monocytogenes*, and *Shigella flexneri*, also invade lymphoid follicles but the tropism is for those in Peyer's patches via M cells (*33*). Research indicates that intimin which is expressed by enterohemmorhagic *E. coli* O157 is required for attachment to human Peyer's patches (*46*). Intimin is also a requirement for the attachment to epithelial cells and the formation of attachment/effacing lesions in cattle (*15, 16*).

An experiment by Hoffman et al. (*31*) indicated that shiga-toxigenic *E. coli* O157:H7 infection can suppress antigen-specific cellular immune responses in cattle. These findings may better explain the colonization and carriage of the pathogen by cattle. A reduced or delayed antigen-specific cellular immune response may help support colonization of the pathogen in cattle. Further

research is needed to determine how the suppression of these cellular immune responses effects the duration and amount of shedding of *E. coli* O157:H7.

Future studies on direct-fed microbials might involve using quantitative real-time PCR-based methods to quantify the amount of *E. coli* O157:H7 in artificially colonized or naturally colonized animals. The results of this could be used to verify the success of colonization and serve as additional information for more traditional media-related detection methods. Quantitative real-time PCR may be useful when the pathogen is only detectable by enrichment methods. This would be useful because enrichment methods only give the result for whether or not the pathogen is present. It may also be beneficial to quantify direct-fed microbial strains in fecal or other biological samples using specific primers designed for the strain to be detected. This information could verify the success of colonization in the animal from ingesting the direct-fed microbials.

The advent of PCR-based methods to determine the components of complex microbiological systems has certainly expanded the field of knowledge for microbial diversity. Identification of microorganisms in complex systems such as bovine rumen fluid has previously been limited by microorganisms that are unculturable by standard methods. This problem may also make it difficult to accurately describe the distributions of the rumen and gut microbiota. The uses of targeted genes that come mostly from ribosomal RNA greatly increased the ability to examine these population distributions (*59*). Several other methods such as restriction fragment length polymorphisms have been developed since this type of work first began. Despite these great achievements no method is

without limitations. Wintzingerode et al. (*65*) have summarized many of the steps in procedures that could lead to biased and inaccurate results. Some of these crucial steps include sample collection, storage, and preparation. Cells must be lysed in order to extract DNA for PCR experiments. Gram-positive bacteria require more meticulous methods to disrupt their thick cell wall than Gram-negative bacteria. In a mixed sample the more severe methods to lyse Gram-positive cells can lead to fragmented nucleic acids from Gram-negative cells. These fragments can lead to erroneous results. On the other hand if the lysing methods are not rigorous enough then there may be insufficient lysing of many microorganisms present. Cells that do not lyse or lyse insufficiently will bias the final population distribution results (*65*). This point became evident in some preliminary lab work related to the present experiment. One of the Bovamine® cultures, *P. freudenreichii*, proved to be very difficult to lyse in pure culture. This led to doubts that this culture could be effectively lysed in cattle fecal samples for any PCR-based analysis in the future. Special attention would need to be paid to issues such as this so that an effective method may be developed.

Other future work in this area could include the measurement of antibodies to Esp and Tir proteins that are secreted by *E. coli* O157:H7. These proteins are a critical part of the formation of A/E lesions. Antibodies to these proteins and to O157 LPS have been detected in adult beef cattle that were inoculated with *E. coli* O157:H7 (*6*). Peripheral blood mononuclear cell (PBMC) stimulation assays can give an indication of the lymphocyte proliferation in response to infection (*31*). Measuring these antibodies and PBMC proliferation

in inoculated cattle with and without direct-fed microbials may help to explain the mode of action for the direct-fed microbials.

The current experiment did suggest a tendency toward increased immune response in the DFM group. This was indicated by the statistically significant increase in granulocytes and the continued incidence of serum IgA being more elevated in the DFM group after challenge with *E. coli* O157:H7. In addition to this there also were some tendencies for serum immunoglobulins to be higher in the DFM group even though not significant. The marked increase in lymphocytes and total white blood cells may also be an indication of a heightened immune response, although not statistically significant. As mentioned previously, a well-designed and verified method for testing mucosal immunity would be much better for more accurately testing mucosal immunoglobulins. Conducting a similar experiment with a much larger sample size should bring out treatment differences more clearly. The ability to find differences in this experiment was definitely limited by its small sample size. Conducting a large scale experiment of this type would need to be carefully designed because the cost of such an experiment could be immense. Certainly a much larger experiment involving a challenge with *E. coli* O157:H7 would be very difficult because of the necessity of using BSL-2 facilities. More research in this area is needed because *E. coli* O157:H7 can not only be found as a beef contaminant but also as a contaminant in fresh fruits, vegetables, and water. Determining effective procedures to minimize this is paramount. There is not one

definitive intervention for this problem but rather the implementation of several preventative measures is necessary.

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Figure legends

FIGURE 1. Effects of Direct-fed microbials (DFM) and *Escherichia coli* O157:H7 on serum IgA levels of steers. Days 0, 7, 14, and 21 represent pre-challenge samples. Values plotted represent Least squares means ± standard error of the mean (5 animals per treatment). [a,b]Superscripts between treatments on the same day are significantly different (Trt P<0.10).

FIGURE 2A. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 on total blood granulocytes levels of steers. Day 21 represents pre-challenge sample. Values plotted represent Least squares means ± standard error of the mean (5 animals per treatment). [a,b]Superscripts between treatments on the same day are significantly different (P<0.10).

FIGURE 2B. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 on granulocytes as a percentage of white blood cells in steers. Day 21 represents pre-challenge sample. Values plotted represent Least squares means ± standard error of the mean (5 animals per treatment). [a,b]Superscripts between treatments on the same day are significantly different (P<0.10).

FIGURE 3A. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 on total blood lymphocytes levels of steers. Day 21 represents pre-

challenge sample. Values plotted represent Least squares means ± standard error of the mean (5 animals per treatment).

FIGURE 3B. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 on lymphocytes as a percentage of white blood cells in steers. Day 21 represents pre-challenge sample. Values plotted represent Least squares means ± standard error of the mean (5 animals per treatment). [a,b]Superscripts between treatments on the same day are significantly different (P<0.10).

FIGURE 4A. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 on serum blood urea nitrogen (BUN) levels in steers. Days 0, 7, 14, and 21 represent pre-challenge samples. Values plotted represent Least squares means ± standard error of the mean (5 animals per treatment). [a,b]Superscripts between treatments on the same day are significantly different (P<0.10).

FIGURE 4B. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 on serum BUN:creatinine in steers. Days 0, 7, 14, and 21 represent pre-challenge samples. Values plotted represent Least squares means ± standard error of the mean (5 animals per treatment). [a,b]Superscripts between treatments on the same day are significantly different (P<0.10).

FIGURE 5. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 on serum aspartate aminotransferase levels in steers. Days 0, 7, 14, and 21 represent pre-challenge samples. Values plotted represent Least squares means ± standard error of the mean (5 animals per treatment). [a,b]Superscripts between treatments on the same day are significantly different (P<0.10).

FIGURE 6. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 on serum alanine aminotransferase levels in steers. Days 0, 7, 14, and 21 represent pre-challenge samples. Values plotted represent Least squares means ± standard error of the mean (5 animals per treatment). [a,b]Superscripts between treatments on the same day are significantly different (P<0.10).

FIGURE 7. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 on serum alkaline phosphatase levels in steers. Days 0, 7, 14, and 21 represent pre-challenge samples. Values plotted represent Least squares means ± standard error of the mean (5 animals per treatment). [a,b]Superscripts between treatments on the same day are significantly different (P<0.10).

Figure 1

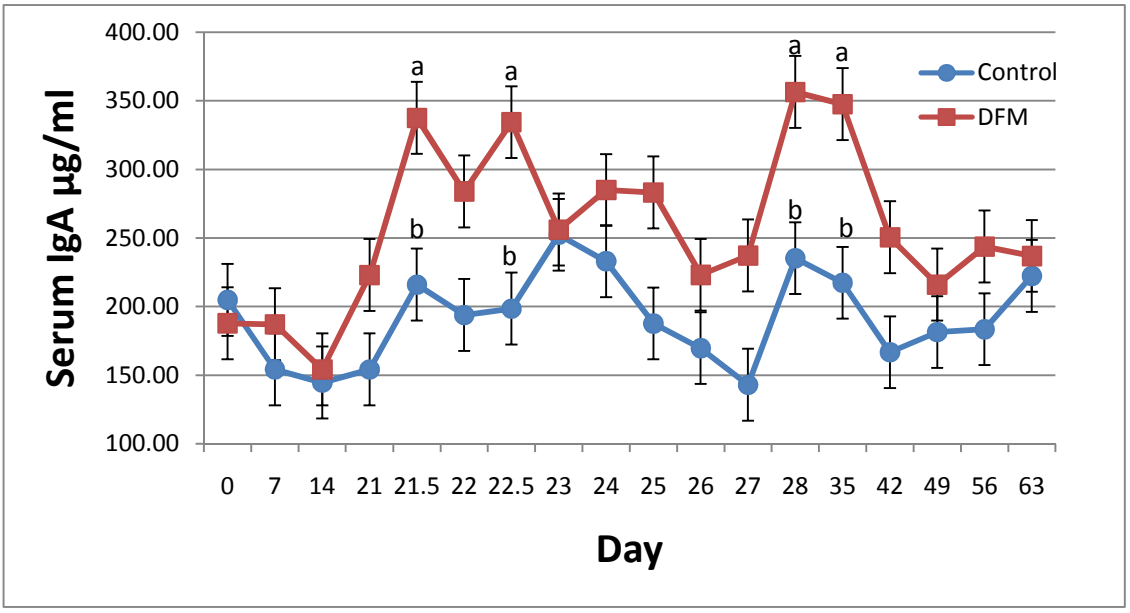
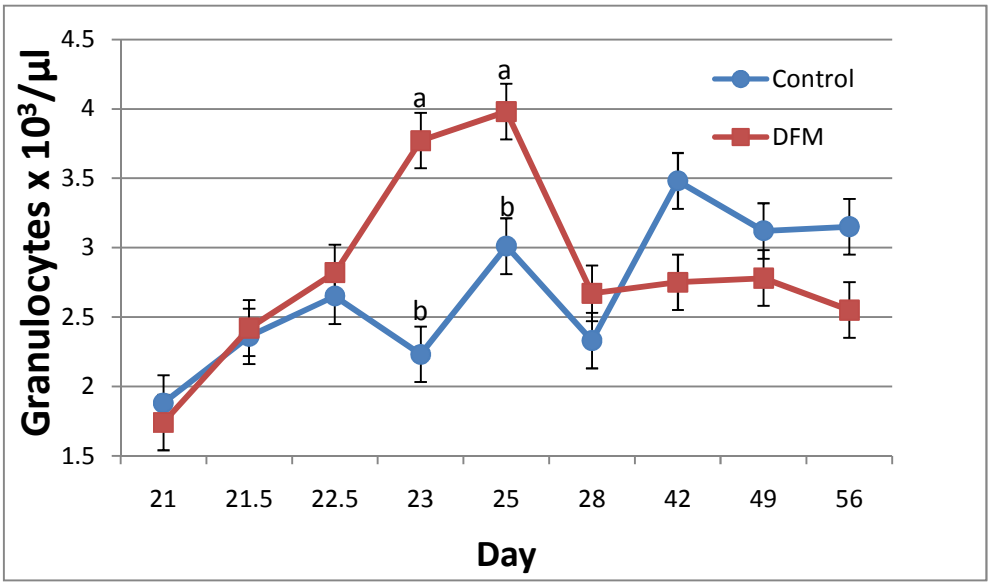

Serum IgA µg/ml
400.00
350.00
300.00
250.00
200.00
150.00
100.00
0
7
14
21
21.5
22
22.5
23
24
25
26
27
28
35
42
49
56
63
Day
Control
DFM
a
b

Figure 2

A


Granulocytes x 10³/µl
4.5
4
3.5
3
2.5
2
1.5
a
a
b
b
Control
DFM
21
21.5
22.5
23
25
28
42
49
56
Day

B

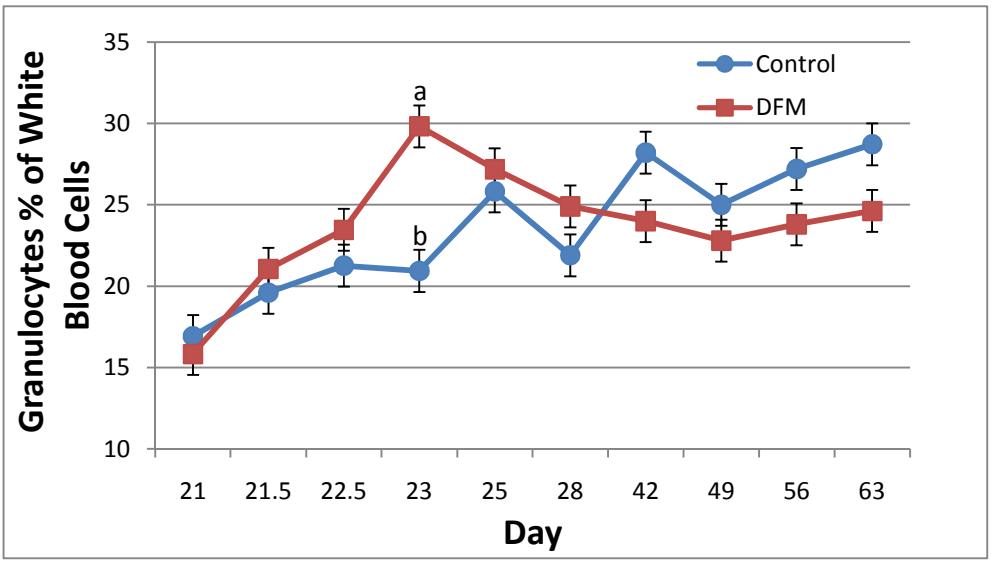
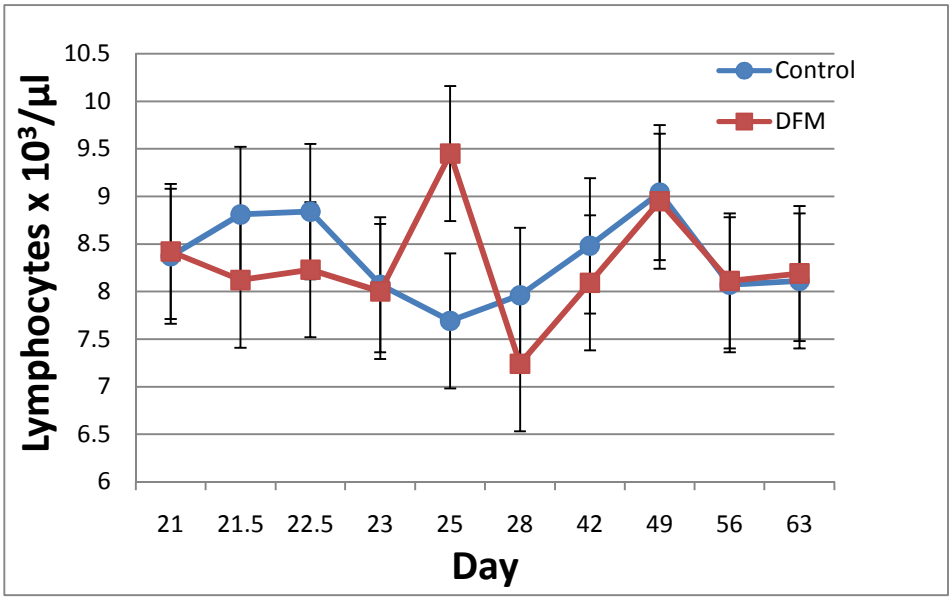

Granulocytes % of White Blood Cells
35
30
25
20
15
10
a
b
Control
DFM
21
21.5
22.5
23
25
28
42
49
56
63
Day

Figure 3

A


Lymphocytes x 10³/µl
10.5
10
9.5
9
8.5
8
7.5
7
6.5
6
Control
DFM
21
21.5
22.5
23
25
28
42
49
56
63
Day

B

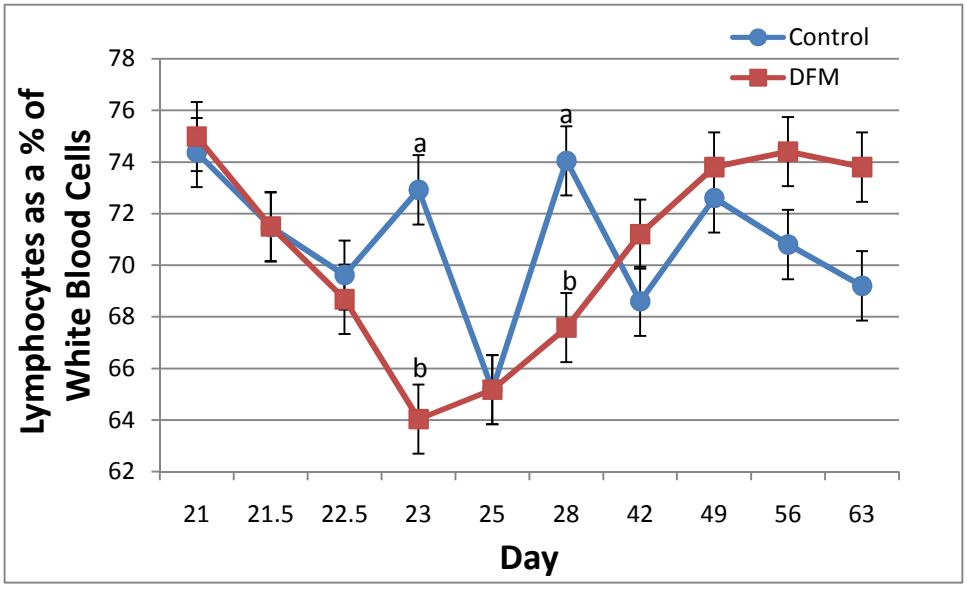
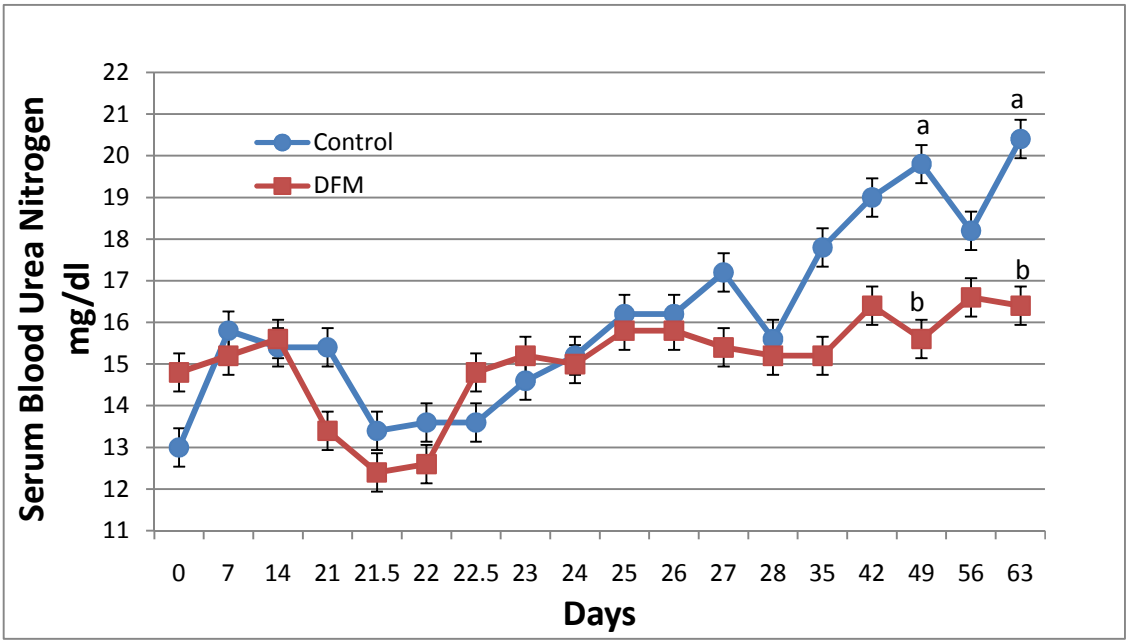

Lymphocytes as a % of White Blood Cells
78
76
74
72
70
68
66
64
62
Control
DFM
a
b
a
b
21
21.5
22.5
23
25
28
42
49
56
63
Day

Figure 4

A


Serum Blood Urea Nitrogen mg/dl
Control
DFM
Days
0 7 14 21 21.5 22 22.5 23 24 25 26 27 28 35 42 49 56 63

B

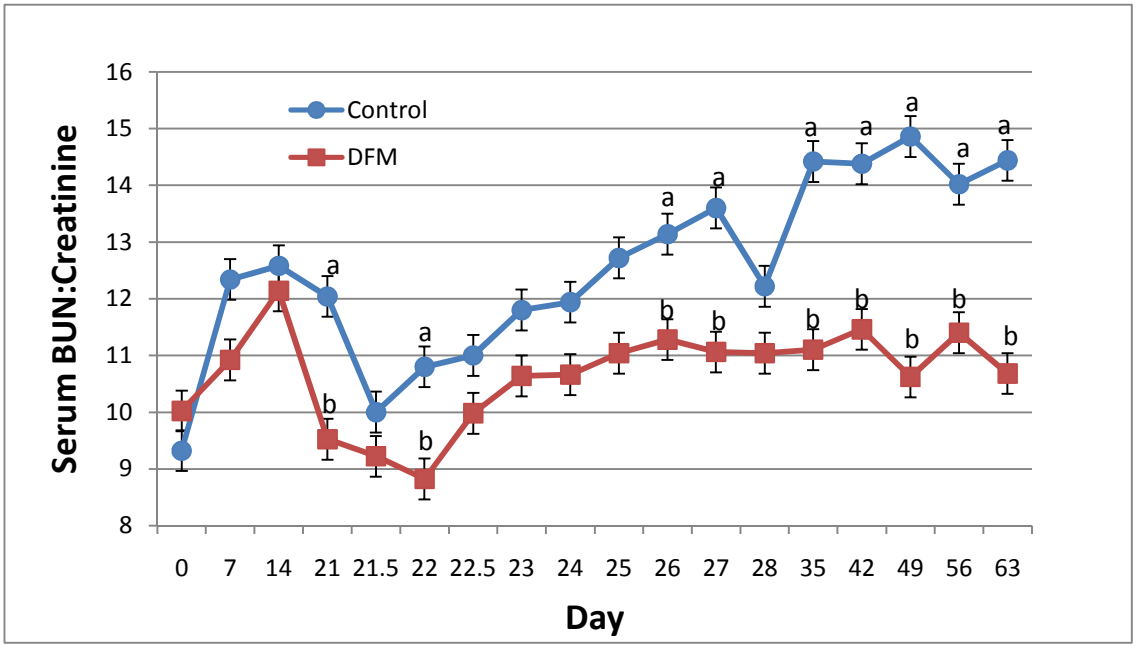

Serum BUN:Creatinine
Control
DFM
Day
0 7 14 21 21.5 22 22.5 23 24 25 26 27 28 35 42 49 56 63

Figure 5

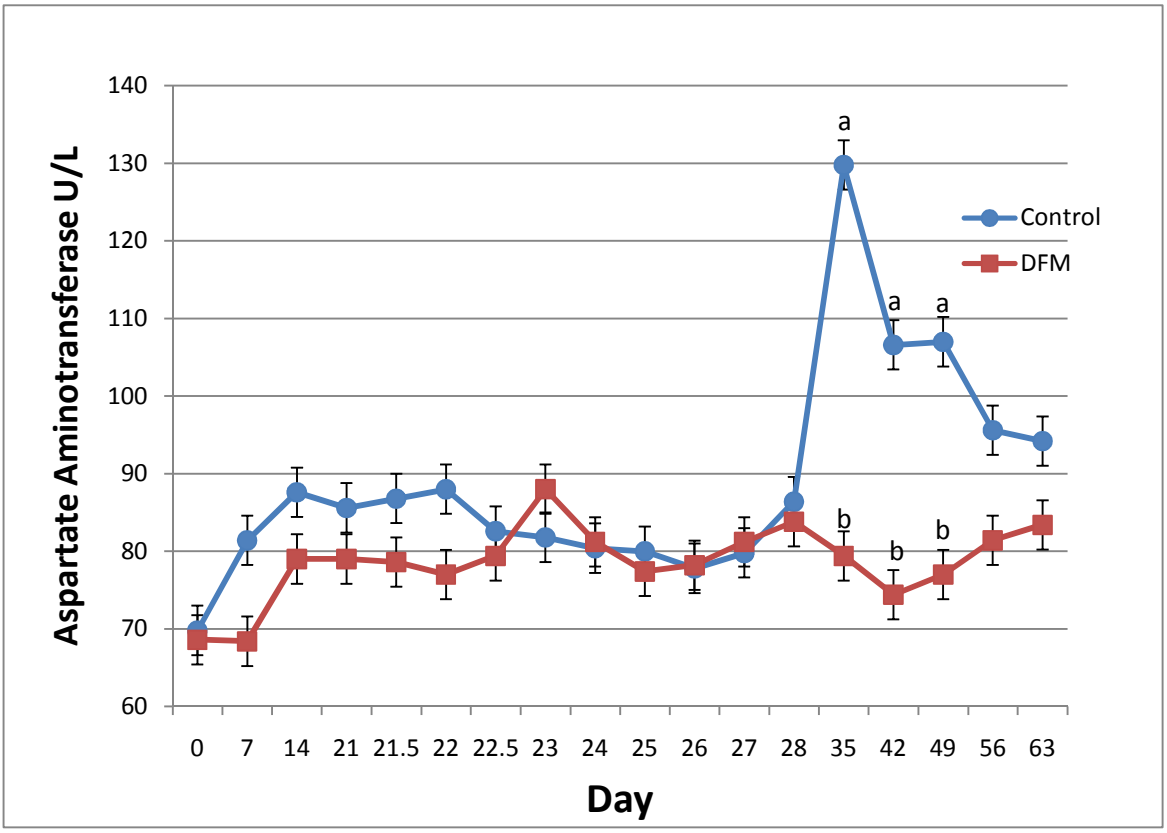

Aspartate Aminotransferase U/L
140
130
120
110
100
90
80
70
60
0
7
14
21
21.5
22
22.5
23
24
25
26
27
28
35
42
49
56
63
Day
Control
DFM
a
a
a
b
b
b

Figure 6

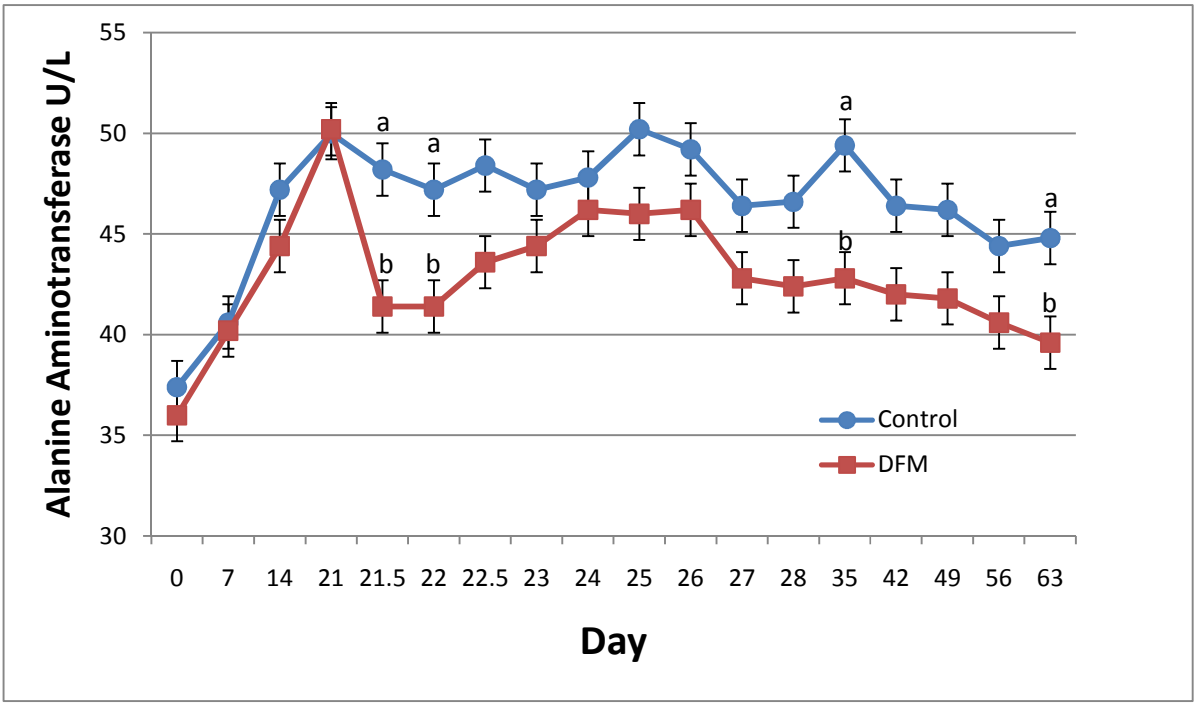

Alanine Aminotransferase U/L
55
50
45
40
35
30
0
7
14
21
21.5
22
22.5
23
24
25
26
27
28
35
42
49
56
63
Day
a
a
a
a
b
b
b
b
Control
DFM

Figure 7

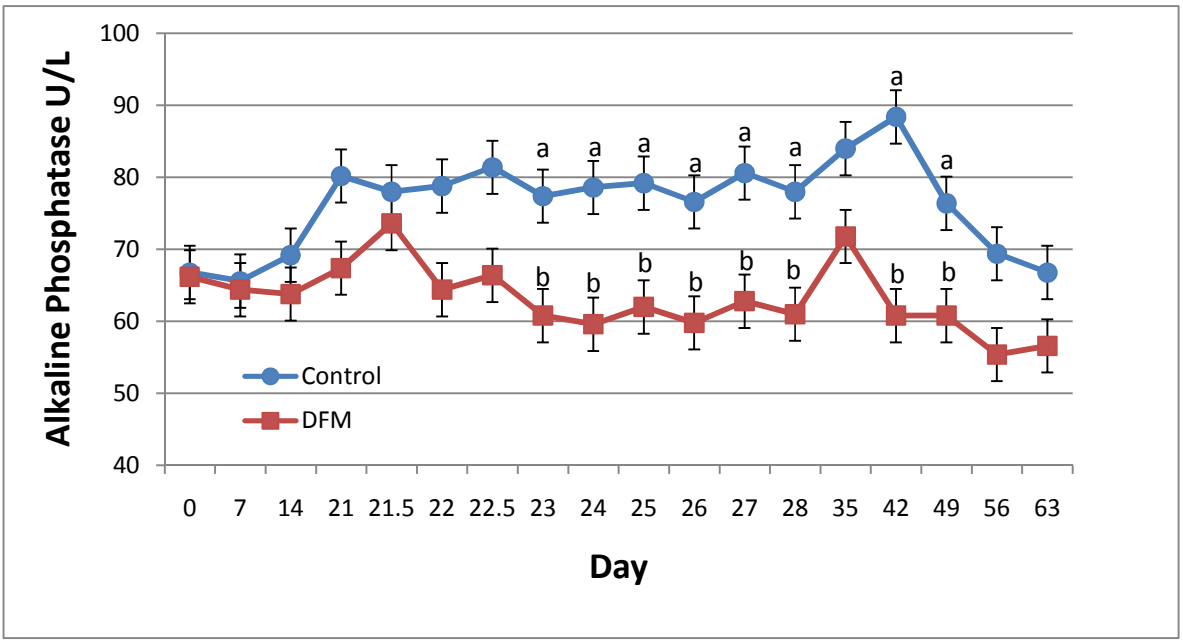

Alkaline Phosphatase U/L
100
90
80
70
60
50
40
0
7
14
21
21.5
22
22.5
23
24
25
26
27
28
35
42
49
56
63
Day
Control
DFM
a
b

Table 1. Composition of diet used in cattle feeding experiment.

Component	Percentage on a dry matter basis
Wheat midds	37.6363
Alfalfa pellets	25.0000
Cottonseed hulls	15.0000
Soybean hulls	15.0000
Soymeal 47.7	3.0000
Cane molasses	3.0000
Salt	0.2500
Calcium carbonate	0.5500
Ammonium sulfate	0.5000
Selenium 600	0.0380
Vitamin A-30,000	0.0203
Vitamin E-50%	0.0027
Zinc sulfate	0.0022
Zinc oxide	0.0005

Nutrient	Mcal/kg
Net energy maintenance	1.4780
Net energy gain	0.8172

Crude protein, % (x100)	0.1618
Calcium, % (x100)	0.0079

Table 2. Effect of Direct-fed Microbials and *Escherichia coli* O157:H7 Challenge on Serum and Fecal IgA, IgG, and IgM in Steers

Immunoglobulin	Treatment		SEM[2]	P-values		
	Control[1]	DFM[1]		Trt	Day	Trt x Day
Serum IgA (µg/ml)	192.15	258.01	26.20	0.10	0.0001	0.33
Serum IgG (mg/ml)	27.40	27.9	1.00	0.71	0.002	0.64
Serum IgM (mg/ml)	3.36	3.31	0.20	0.88	<0.0001	0.66
Fecal IgA (µg/ml)	1.19	0.88	0.15	0.15	0.03	0.89
Fecal IgG (ng/ml)	50.72	42.57	8.70	0.51	0.01	0.82
Fecal IgM (ng/ml)	191.13	135.25	14.61	0.01	0.03	0.90

[1]Least squared means; control = no direct-fed microbials, DFM = direct-fed microbials

[2]Standard error of the mean

Table 3. Effects of Direct-fed Microbials and *Escherichia coli* O157:H7 challenge on Complete Blood Count in Steers.

Measurement	Treatment		SEM[2]	P-values		
	Control[1]	DFM[1]		Trt	Day	Trt x Day
White blood cells (WBC) (x10^3/µl)	11.75	11.78	0.84	0.98	0.001	0.27
Granulocytes (x10^3/µl)	2.75	2.80	0.2	0.88	0.001	0.07
Granulocytes % of WBCs	23.56	23.75	1.29	0.92	0.001	0.06
Lymphocytes (x10^3/µl)	8.34	8.28	0.71	0.95	0.04	0.10
Lymphocytes % of WBCs	70.88	70.52	1.34	0.85	0.01	0.02
Mean platelet volume	7.10	6.86	0.15	0.29	0.17	0.98
Monocytes (x10^3/µl)	0.66	0.68	0.07	0.88	<0.0001	0.63
Monocytes % of WBCs	5.61	5.73	0.53	0.87	<0.0001	0.39
Red blood cell (RBC) count	7.98	8.21	0.22	0.47	0.02	0.88
Platelet count (10^3/µl)	0.55	0.37	0.09	0.19	<0.0001	0.52
Platelet count % of RBCs	0.36	0.20	0.08	0.21	<0.0001	0.55
Platelet distribution width	36.09	34.78	0.54	0.12	0.87	0.78
Hemoglobin (g/dl)	11.67	11.14	0.18	0.06	<0.0001	0.58
Mean corpuscular hemoglobin concentration(g/dl)	31.28	30.65	0.5	0.40	0.64	0.76
% hematocrit	35.33	34.28	0.44	0.11	0.08	0.06

[1]Least squared means; control = without direct-fed microbials, DFM = direct-fed microbials
[2]Standard error of the mean

Table 4. Effects of Direct-fed Microbials and *Escherichia coli* O157:H7 Challenge on Blood Chemistry in Steers.

Measurement	Treatment		SEM[2]	P-values		
	Control[1]	DFM[1]		Trt	Day	Trt x Day
Glucose (mg/dl)	76.47	75.66	1.09	0.6100	<0.0001	0.4661
BUN (mg/dl)	16.13	15.08	0.46	0.1300	<0.0001	0.0246
Creatinine (mg/dl)	1.30	1.42	0.07	0.2900	0.0500	0.1213
BUN:Creatinine	12.53	10.64	0.36	0.002	<0.0001	0.01
Sodium (mmol/L)	139.66	139.81	0.22	0.61	<0.0001	0.89
Potassium (mmol/L)	5.27	5.33	0.06	0.44	<0.0001	0.28
Chloride (mmol/L)	102.39	102.22	0.27	0.66	<0.0001	0.92
Carbon dioxide (mmol/L)	23.91	24.37	0.25	0.21	<0.0001	0.25
Anion Gap	18.57	18.55	0.21	0.96	<0.0001	0.03
Calcium (mg/dl)	10.25	10.19	0.10	0.70	<0.0001	0.55
Total Protein (g/dl)	7.07	6.96	0.07	0.32	<0.0001	0.09
Albumin (g/dl)	3.29	3.24	0.05	0.44	<0.0001	0.02
Globulin (g/dl)	3.78	3.73	0.05	0.50	<0.0001	0.67
Albumin:Globulin	0.87	0.87	0.01	0.92	0.02	0.38
Aspartate aminotransferase (U/L)	88.96	78.63	3.18	0.03	0.03	0.05
Alanine aminotransferase (U/L)	46.53	42.89	1.31	0.07	<0.0001	0.14
Alkaline Phosphatase (U/L)	76.41	63.20	3.69	0.02	0.07	0.52
Total Bilirubin (mg/dl)	0.32	0.20	0.06	0.12	0.03	0.11

[1]Least squared means; control = no direct-fed microbials, DFM = direct-fed microbials
[2]Standard error of the mean

Table 5. Effect of Direct-fed Microbials and *Escherichia coli* O157:H7 Challenge on Overall Steer Performance

Measurement	Treatment		SEM[2]	P-value
	Control[1]	DFM[1]		Trt
Dry Matter Intake (kg)	9.24	8.06	0.39	0.06
Average Daily Gain (kg)	1.42	1.24	0.25	0.60
Gain to Feed Ratio	0.15	0.15	0.03	0.97

[1]Least squared means; control = no direct-fed microbials, DFM = direct-fed microbials

[2]Most conservative standard error of the mean; control n=5, DFM n=4.

APPENDIX A

Additional Results Tables and Figures

Table A1. Microbial testing of fecal and swab samples from each steer to detect *Escherichia coli* 0157:H7

	Control steers[1,2]					Direct-fed microbial steers[1,2]				
Day	34	98	190	300	647	173	184	203	651	881
0	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative
7	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative
14	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative
21	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative
21.5	negative	negative	negative	positive	positive	negative	positive	negative	positive	positive
22	positive	negative	negative	negative	positive	positive	negative	negative	positive	positive
22.5	negative	positive	negative	negative	positive	positive	negative	negative	positive	positive
23	positive	positive	negative	negative	positive	positive	negative	negative	positive	positive
24	negative	positive	negative	negative	positive	positive	negative	negative	negative	positive
25	negative	positive	negative	negative	positive	positive	negative	negative	negative	positive
26	negative	positive	negative	negative	positive	positive	negative	negative	negative	positive
27	negative	positive	negative	negative	positive	positive	negative	negative	negative	positive
28	positive	positive	negative	positive	positive	positive	negative	negative	negative	positive
35	positive	positive	positive	positive	positive	positive	positive	negative	negative	positive
42	positive	positive	negative	negative	positive	negative	positive	negative	negative	positive
49	negative	positive	negative	negative	positive	negative	positive	negative	negative	positive
56	negative	negative	negative	negative	negative	negative	negative	negative	negative	positive
63	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative

[1]positive for fecal and/or swab samples

[2]Positives confirmed using the Vitek 32 system (Biomérieux, Durham, NC) RIM™ E. coli O157:H7 Latex Test (Remel, Lenexa, KS)

Table A2. Normal Bovine Blood Chemistry and Complete Blood Count Values	
Blood Parameter	Reference Value
Glucose (mg/dl)[1]	45-75
BUN (mg/dl)[1]	6.0-27
Creatinine (mg/dl)[1]	1.0-2.0
Sodium (mmol/L)[1]	132-152
Potassium (mmol/L)[1]	3.9-5.8
Chloride (mmol/L)[1]	95-110
Carbon dioxide (mmol/L)[2]	21.2-32.2
Anion Gap (mEq/L)[1]	14-26
Calcium (mg/dl)[1]	9.7-12.4
Total Protein (g/dl)[1]	5.7-8.1
Albumin (g/dl)[1]	2.1-3.6
Globulin (g/l)[2]	30.0-34.8
Albumin:Globulin[2]	0.84-0.94
Aspartate aminotransferase (U/L)[1]	78-132
Alanine aminotransferase (U/L)[1]	11-40
Alkaline Phosphatase (U/L)[1]	0-500
Total Bilirubin (mg/dl)[2]	0.01-0.5
White blood cells (WBC) (per µl)[1]	4,000-12,000
Neutrophil (per µl) (type of granulocyte)[3]	600-4,000
Eosinophil (per µl) (type of granulocyte)[3]	0-2,400
Basophil (per µl) (type of granulocyte)[3]	0-200
Lymphocytes (per µl)[1]	2,500-7,500
Lymphocytes % of WBCs[3]	45-75
Monocytes (per µl)[1]	25-800
Red blood cell count (RBC) ($x10^6$/µl)[1]	5.0-10.0
Platelet count (10^3/µl)[3]	100-800
Hemoglobin (g/l)[2]	80-150
% hematocrit or packed cell volume (%)[3]	24-46

[1]Radostits et al. 2007. A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs, and Goats.

[2]Kaneko, J. J. (ed.). 1989. Clinical Biochemistry of Domestic Animals

[3]Feldman, B.F., J.G. Zinkl, and N.C. Jain (Eds). 2000. Shalm's Veterinary Hematology

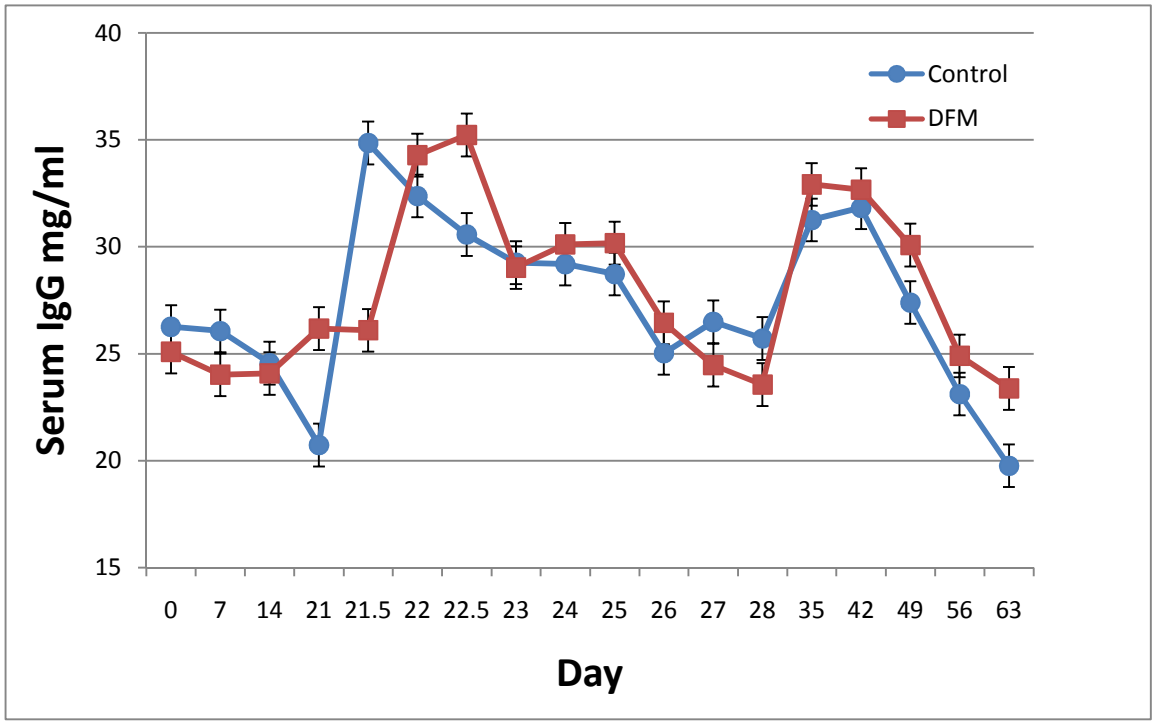


Figure A1. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Serum IgG Levels in Steers (Days 0, 7, 14, and 21 are pre-challenge).

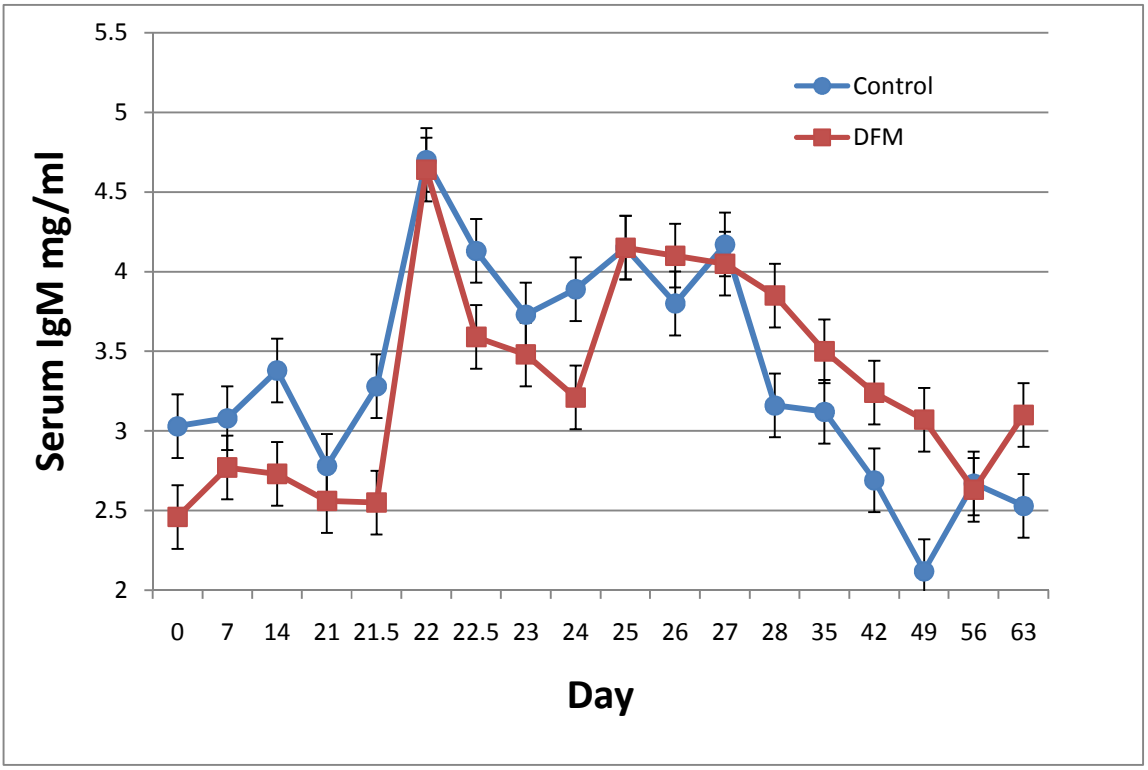


Figure A2. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Serum IgM Levels in Steers (Days 0, 7, 14, and 21 are pre-challenge).

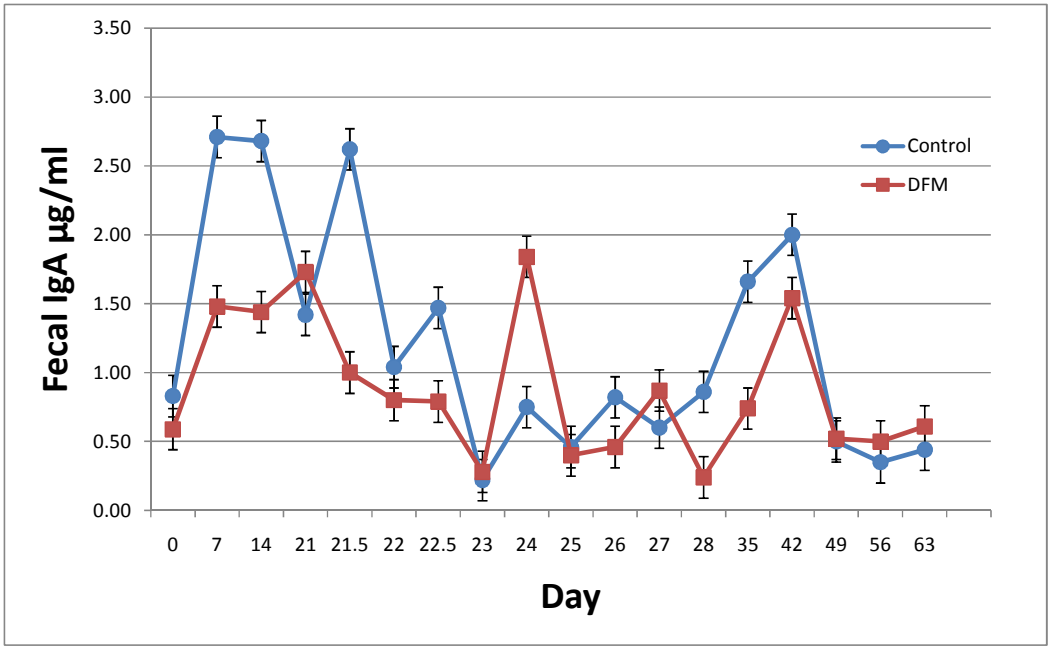


Figure A3. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Fecal IgA Levels in Steers (Days 0, 7, 14, and 21 are pre-challenge).

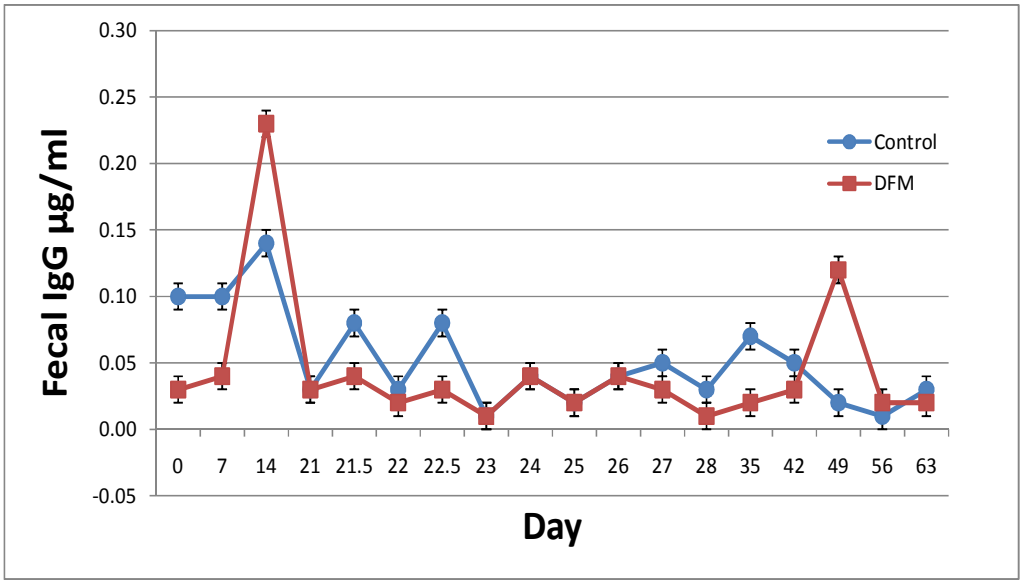


Figure A4. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Fecal IgG Levels in Steers (Days 0, 7, 14, and 21 are pre-challenge).

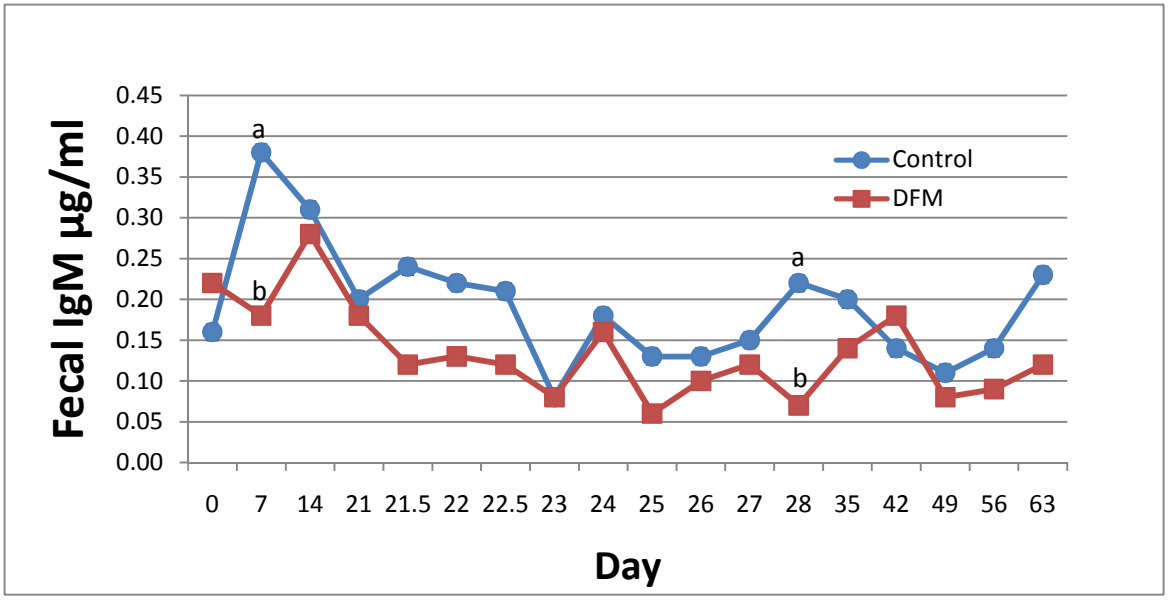


Figure A5. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Fecal IgM Levels in Steers (Days 0, 7, 14, and 21 are pre-challenge). ^{a,b}Superscripts between treatments on the same day are significantly different (Trt P<0.10).

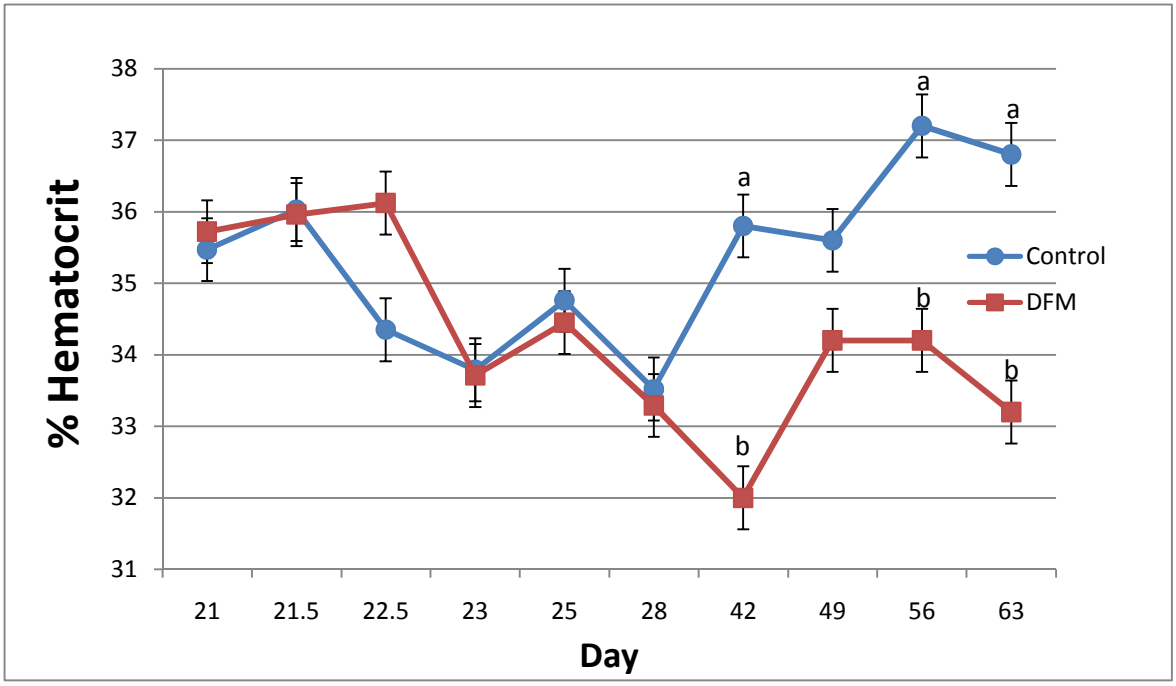


Figure A6. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Percent Hematocrit in Steers (Days 21 is pre-challenge). [a,b]Superscripts between treatments on the same day are significantly different (Trt P<0.10).

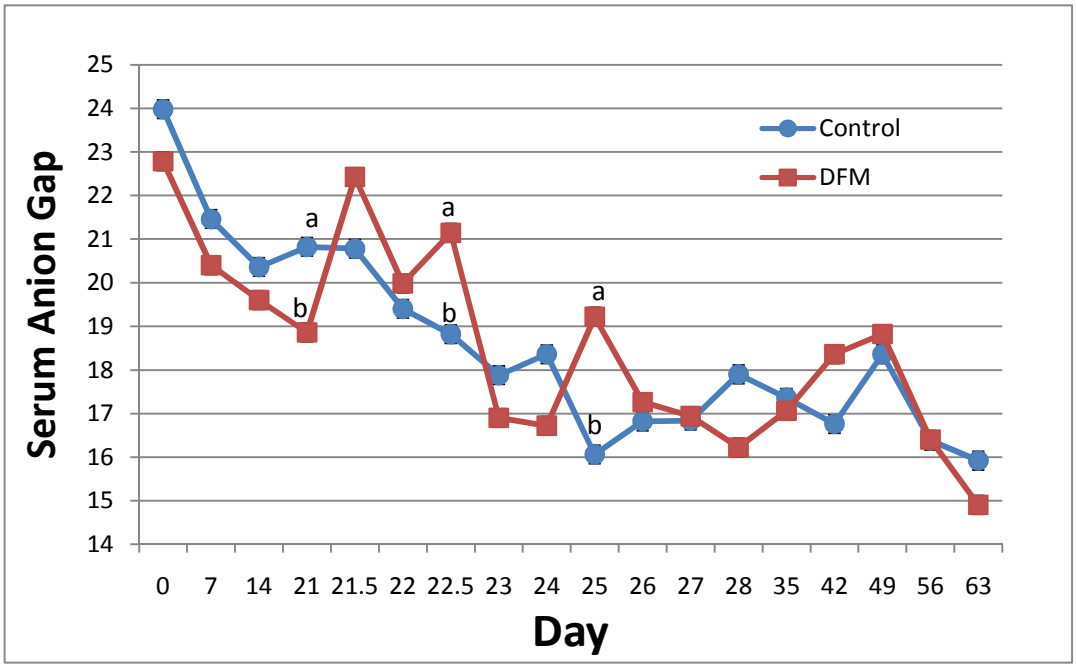


Figure A7. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Serum Anion Gap in Steers (Days 0, 7, 14, and 21 are pre-challenge). [a,b]Superscripts between treatments on the same day are significantly different (Trt P<0.10).

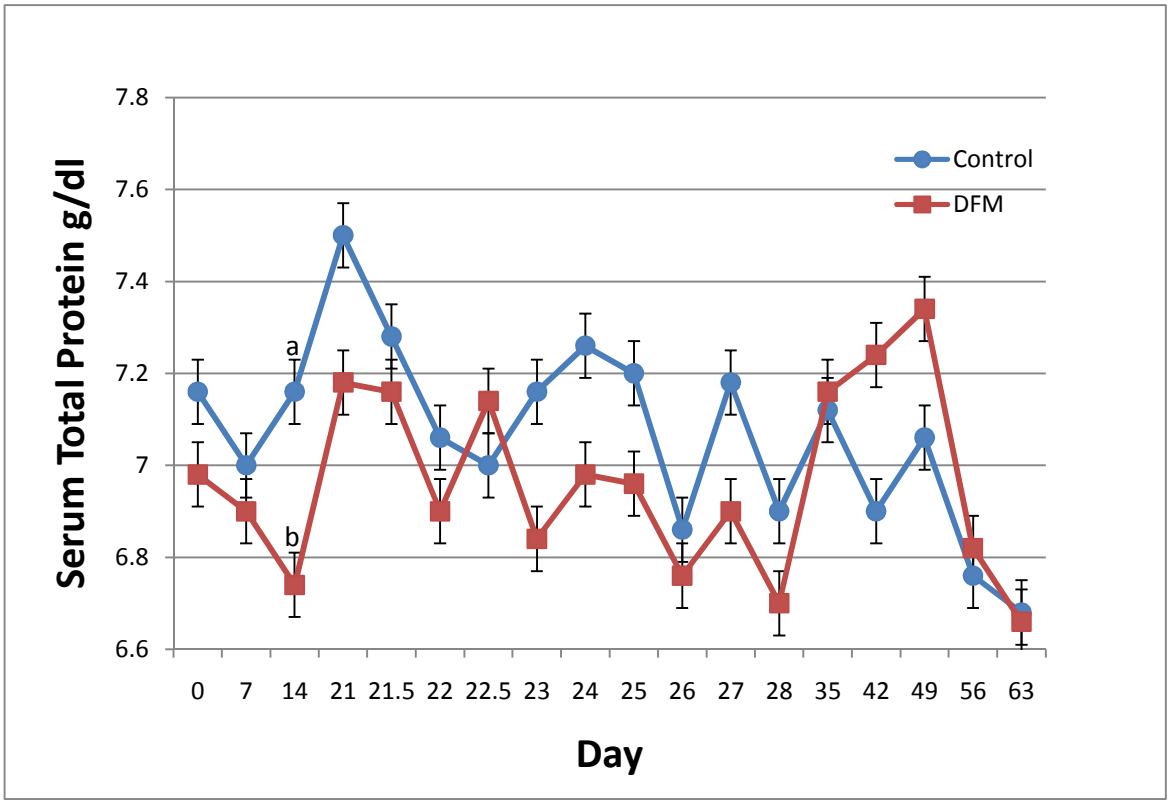


Figure A8. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Serum Total Protein in Steers (Days 0, 7, 14, and 21 are pre-challenge). [a,b]Superscripts between treatments on the same day are significantly different (Trt P<0.10).

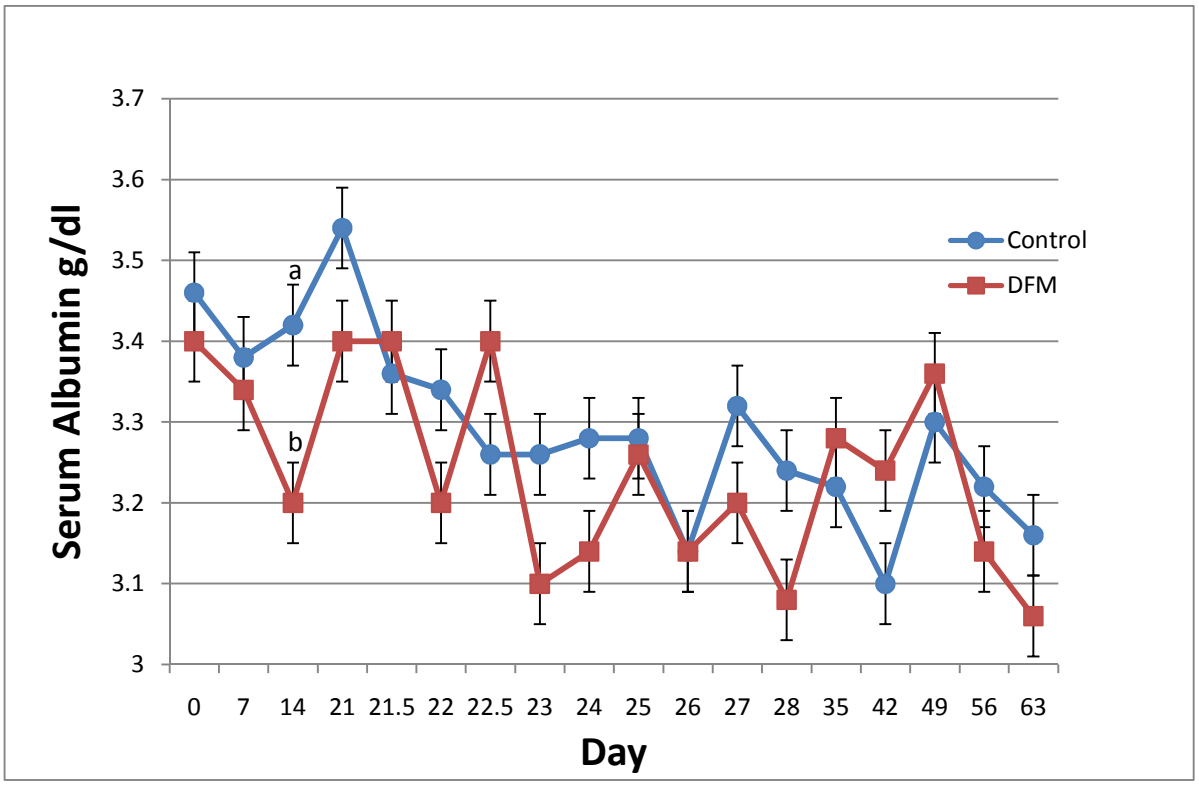


Figure A9. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Serum Albumin in Steers (Days 0, 7, 14, and 21 are pre-challenge). [a,b]Superscripts between treatments on the same day are significantly different (Trt P<0.10).

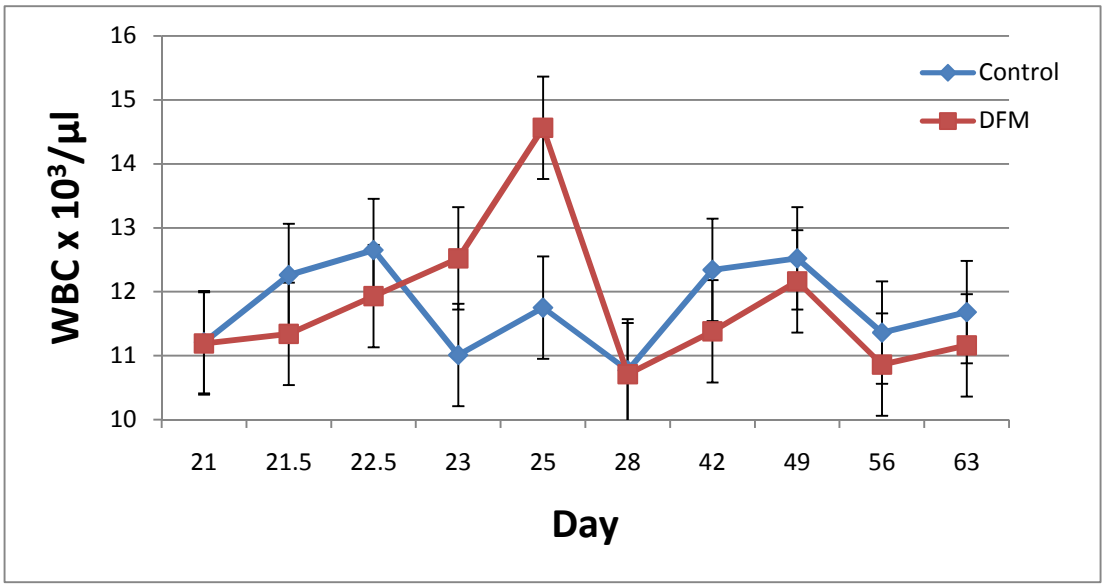


Figure A10. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Total White Blood Cell Count (WBC) in Steers (Day 21 is pre-challenge).

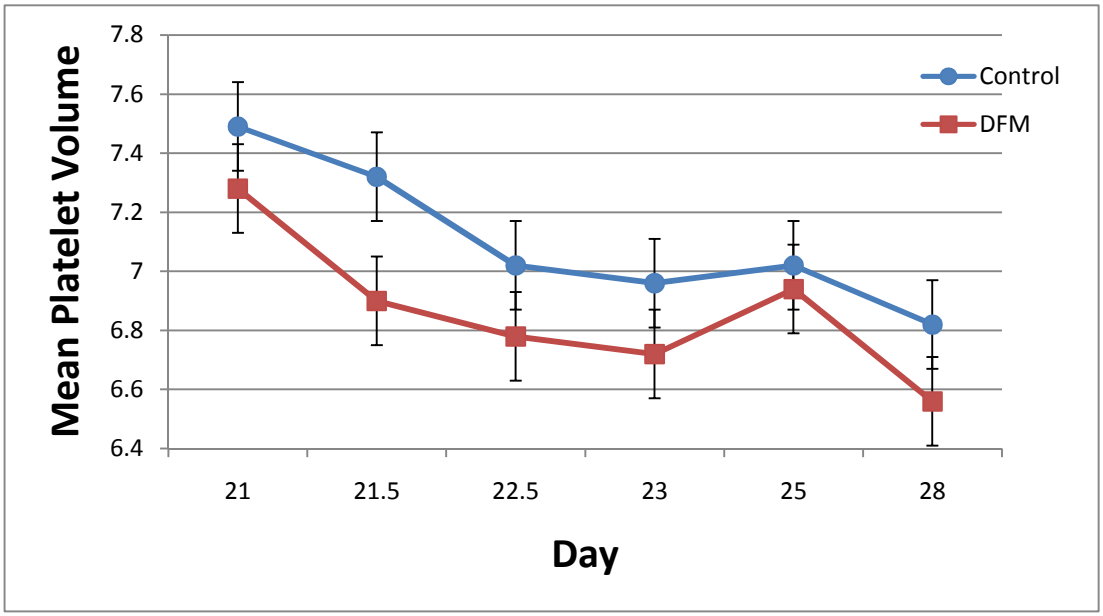


Figure A11. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Mean Platelet Volume in Steers (Day 21 is pre-challenge).

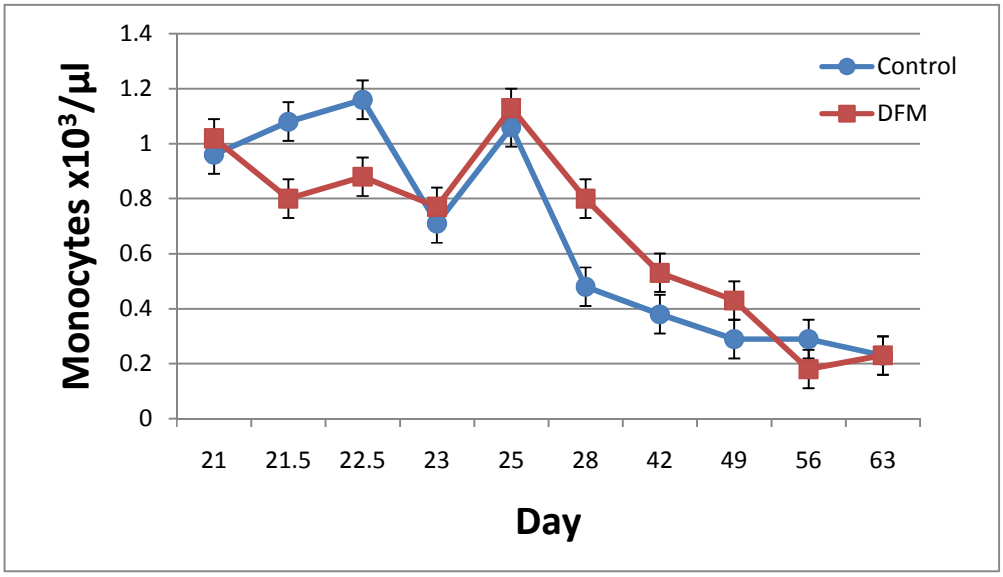


Figure A12A. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Total Blood Monocytes in Steers (Day 21 is pre-challenge).

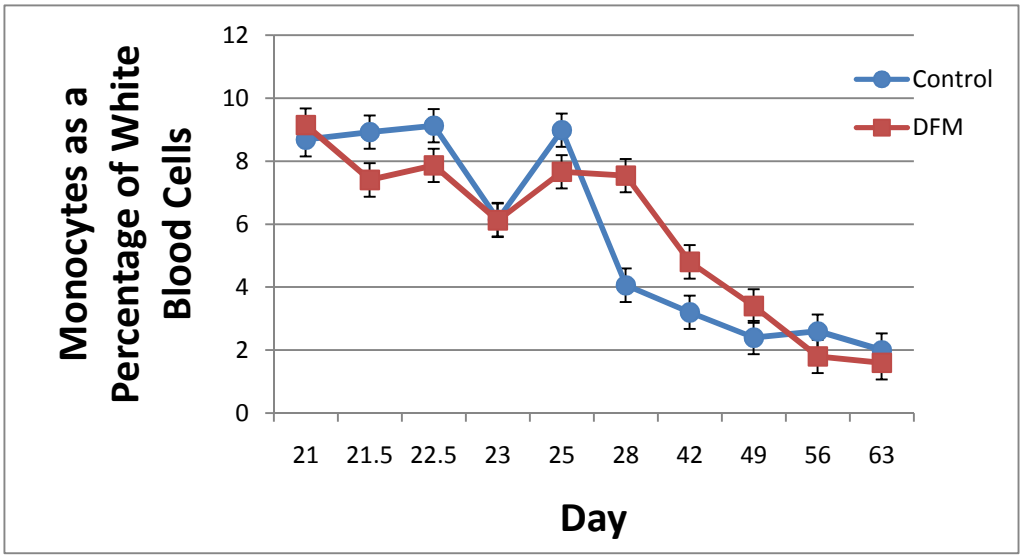


Figure A12B. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Monocytes as a percentage of white blood cells in Steers (Day 21 is pre-challenge).

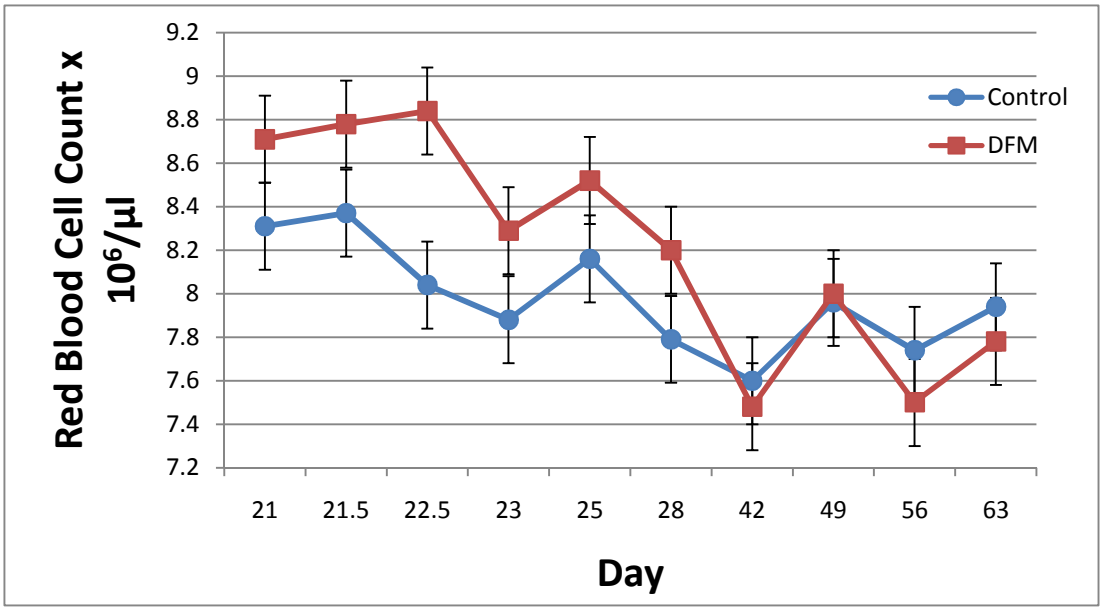


Figure A13. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Total Red Blood Cell Count in Steers (Day 21 is pre-challenge).

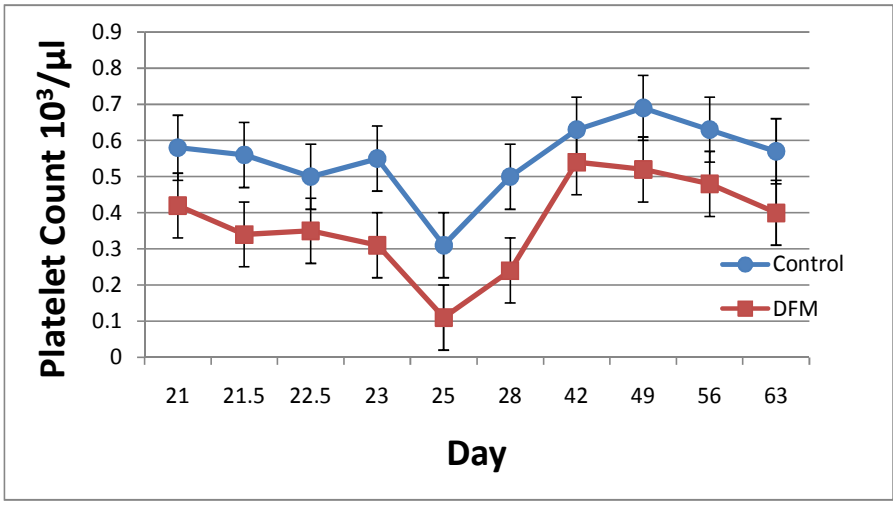


Figure A14A. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Platelet Count in Steers (Day 21 is pre-challenge).

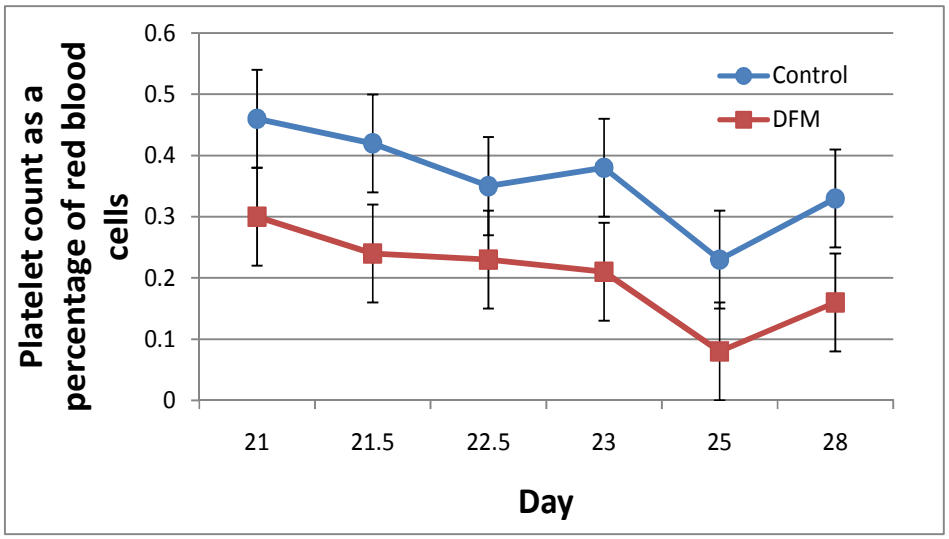


Figure A14B. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Platelet Count as a Percentage of Red Blood Cells in Steers (Day 21 is pre-challenge).

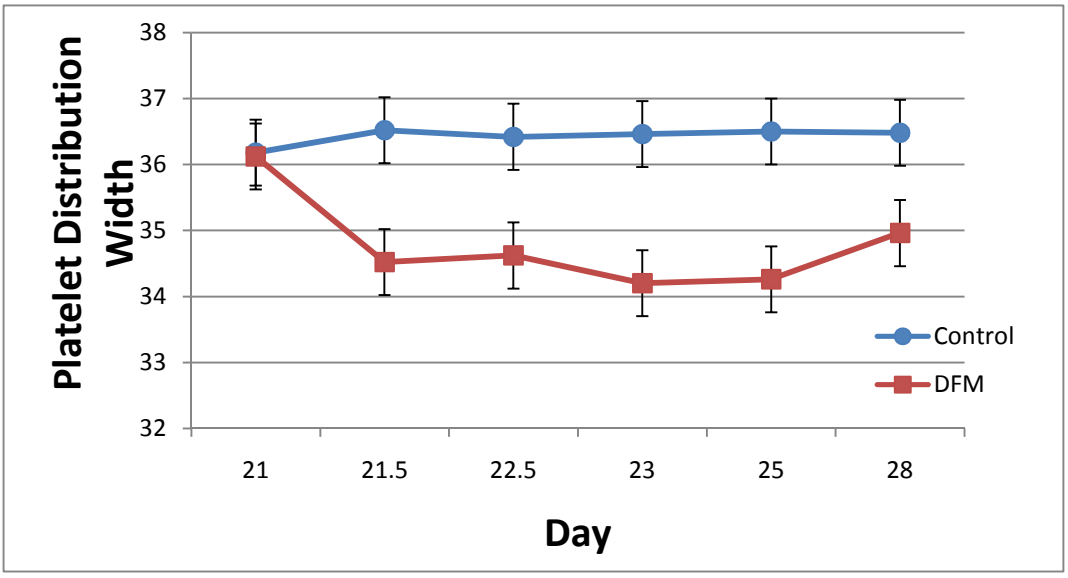


Figure A15. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Platelet Distribution Width in Steers (Day 21 is pre-challenge).

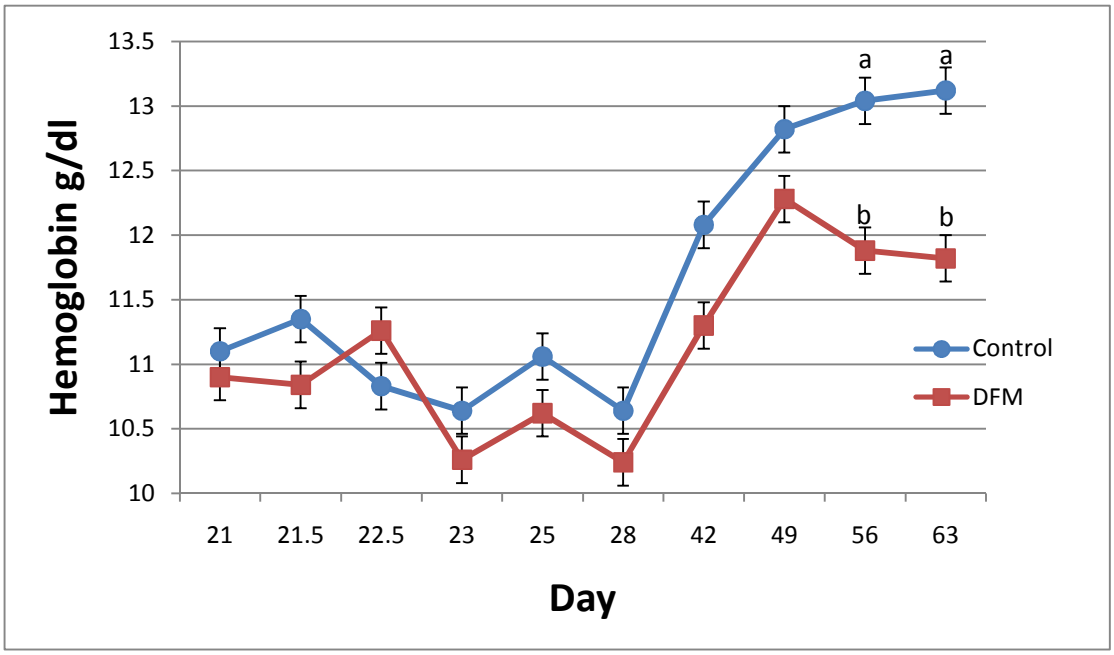


Figure A16. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Hemoglobin in Steers (Day 21 is pre-challenge). [a,b]Superscripts between treatments on the same day are significantly different (Trt P<0.10).

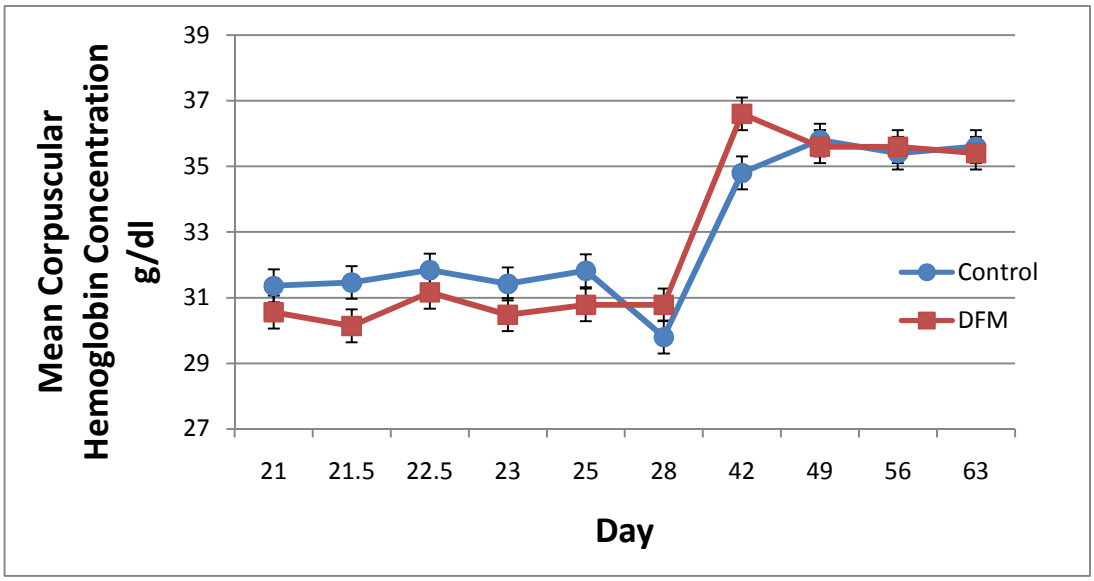


Figure A17. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Mean Corpuscular Hemoglobin Concentration in Steers (Day 21 is pre-challenge).

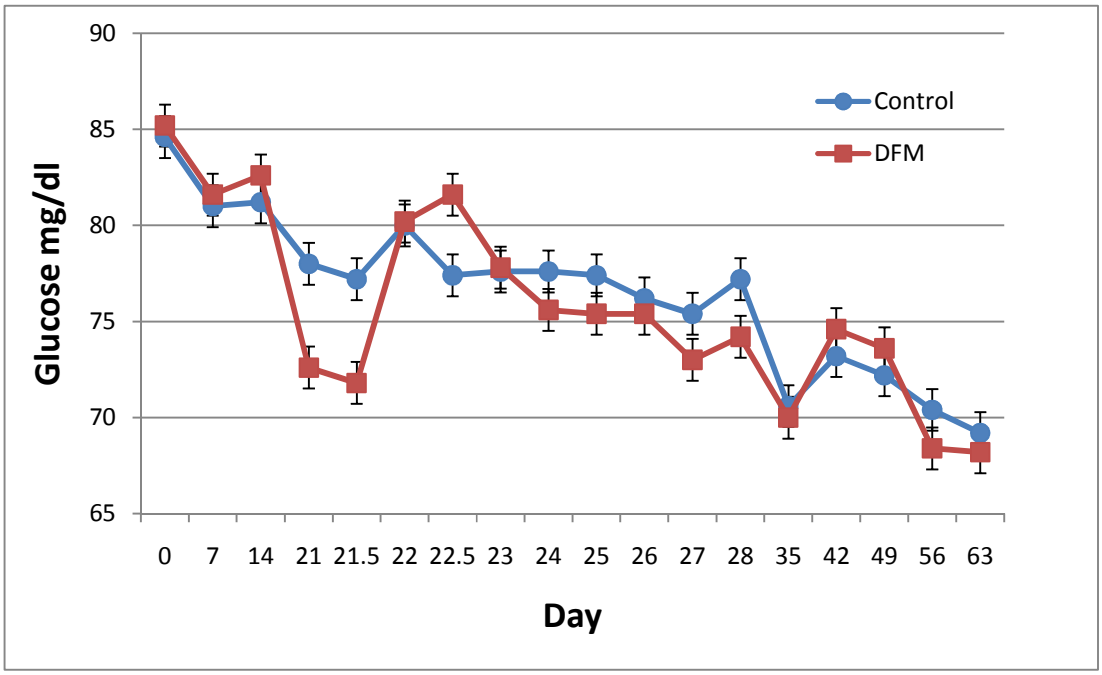


Figure A18. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Serum Glucose in Steers (Days 0, 7, 14, and 21 are pre-challenge).

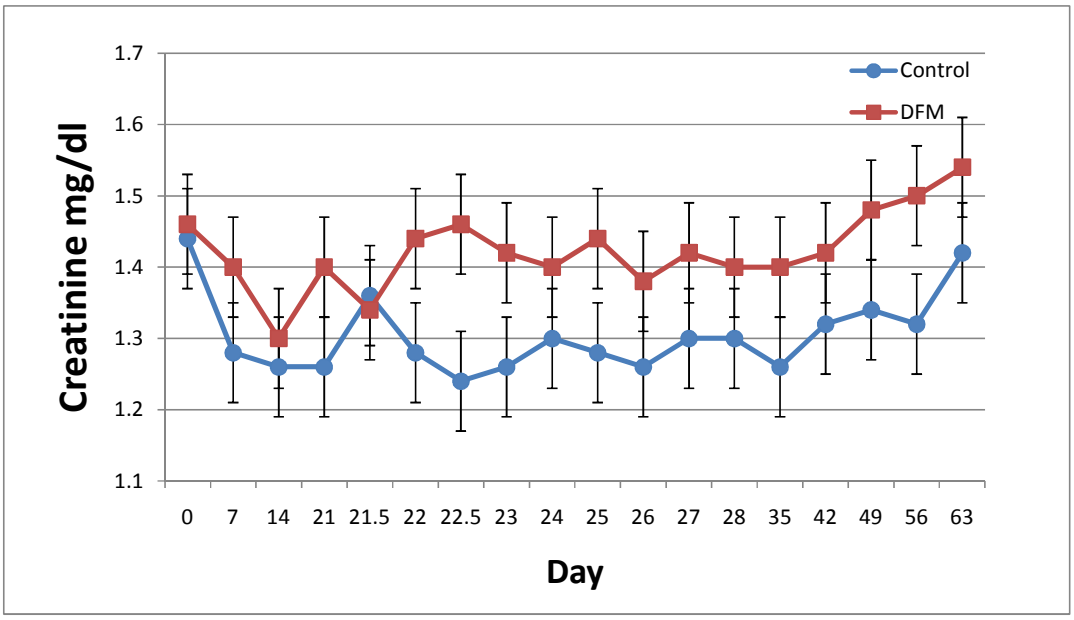


Figure A19. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Serum Creatinine in Steers (Days 0, 7, 14, and 21 are pre-challenge).

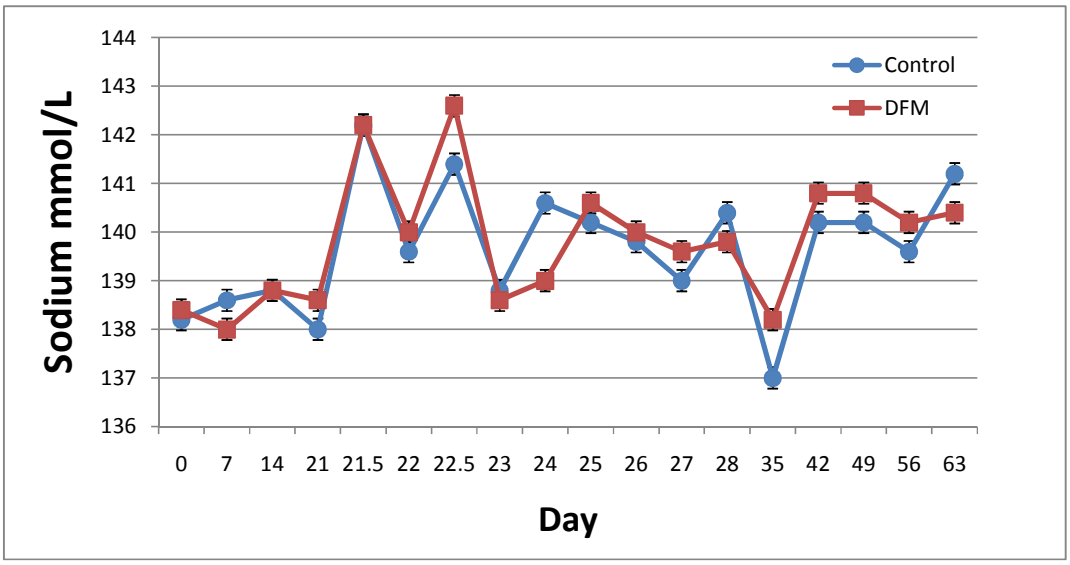


Figure A20. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Serum Sodium in Steers (Days 0, 7, 14, and 21 are pre-challenge).

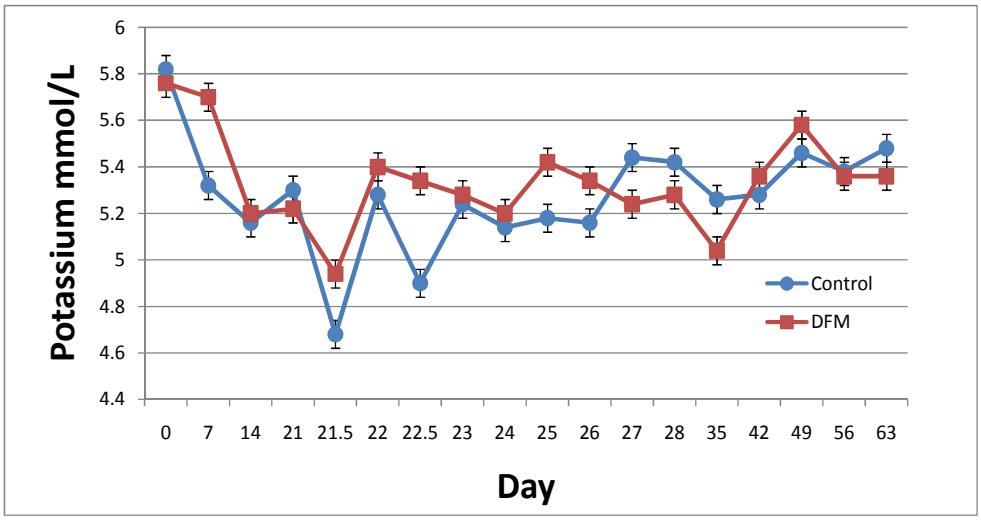


Figure A21. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Serum Potassium in Steers (Days 0, 7, 14, and 21 are pre-challenge).

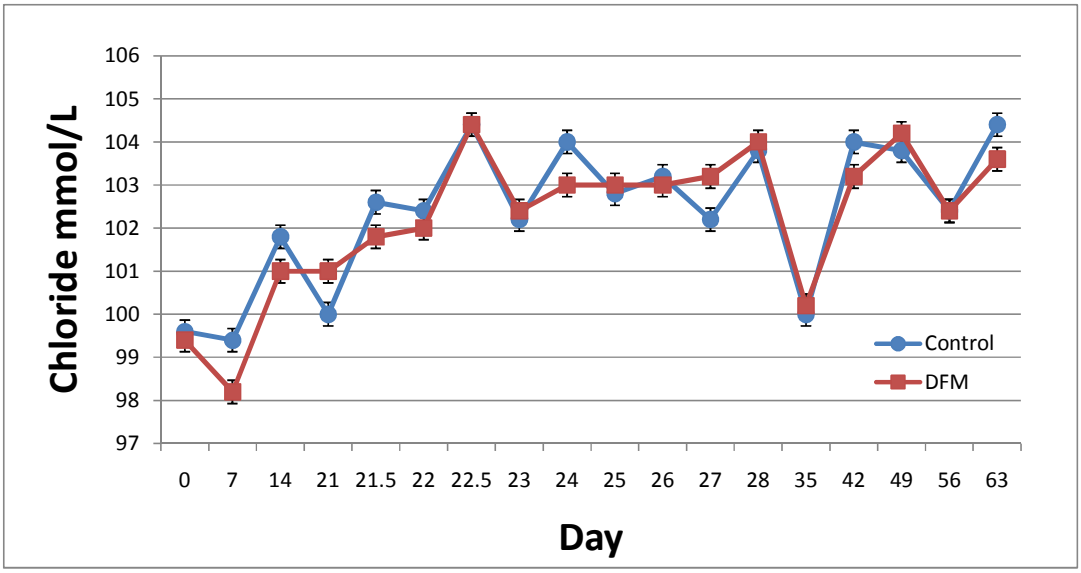


Figure A22. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Serum Chloride in Steers (Days 0, 7, 14, and 21 are pre-challenge).

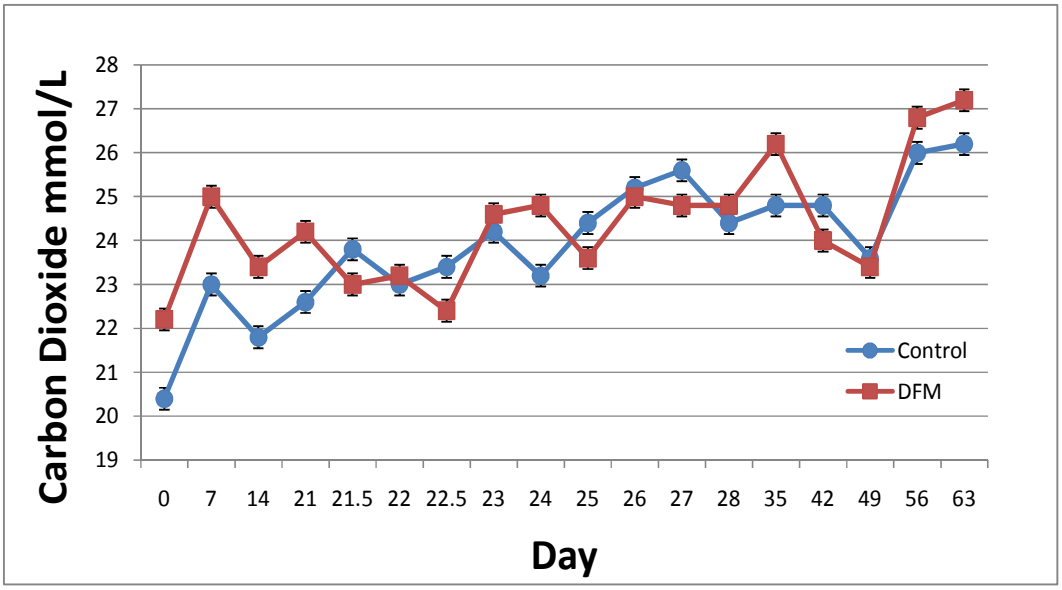


Figure A23. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Serum Carbon Dioxide in Steers (Days 0, 7, 14, and 21 are pre-challenge).

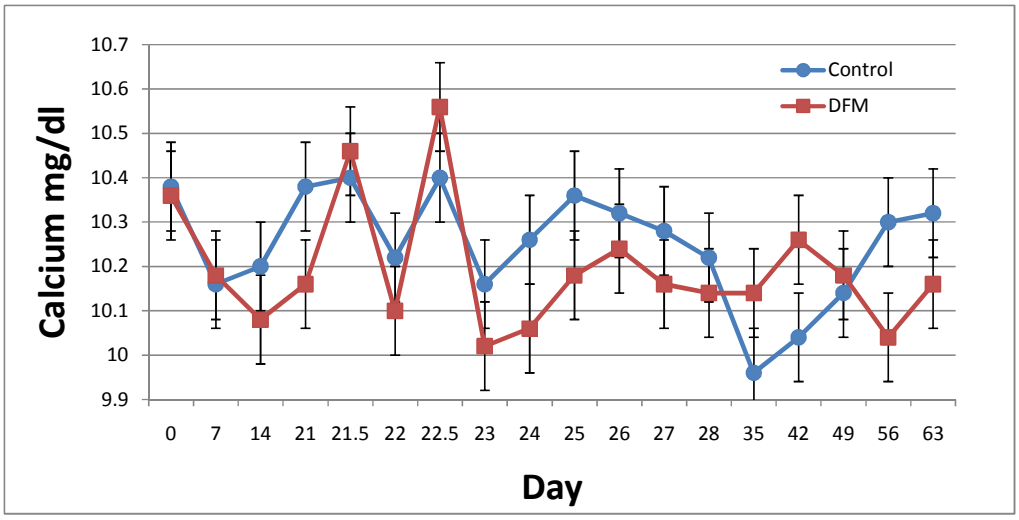


Figure A24. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Serum Calcium in Steers (Days 0, 7, 14, and 21 are pre-challenge).

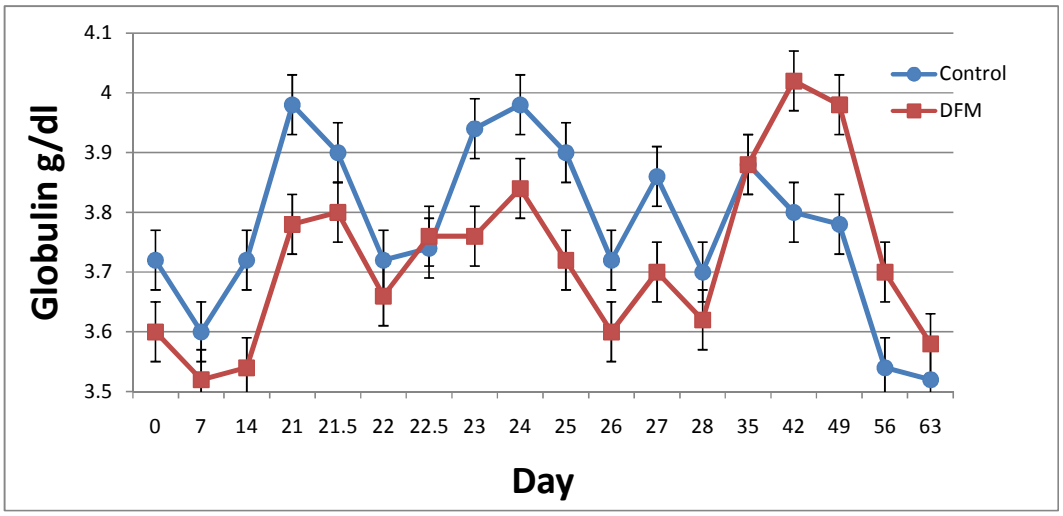


Figure A25. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Serum Globulin in Steers (Days 0, 7, 14, and 21 are pre-challenge).

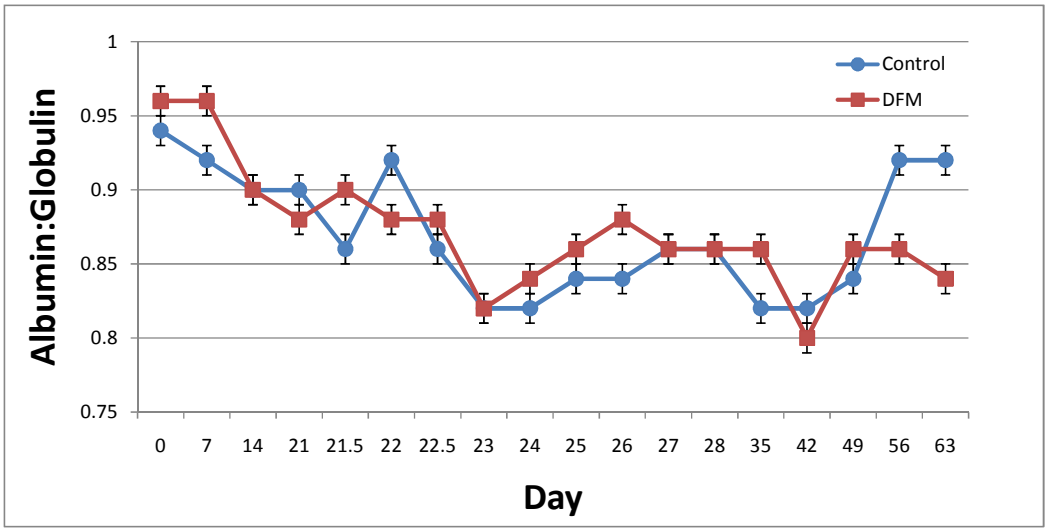


Figure A26. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Serum Albumin to Globulin Ratio in Steers (Days 0, 7, 14, and 21 are pre-challenge).

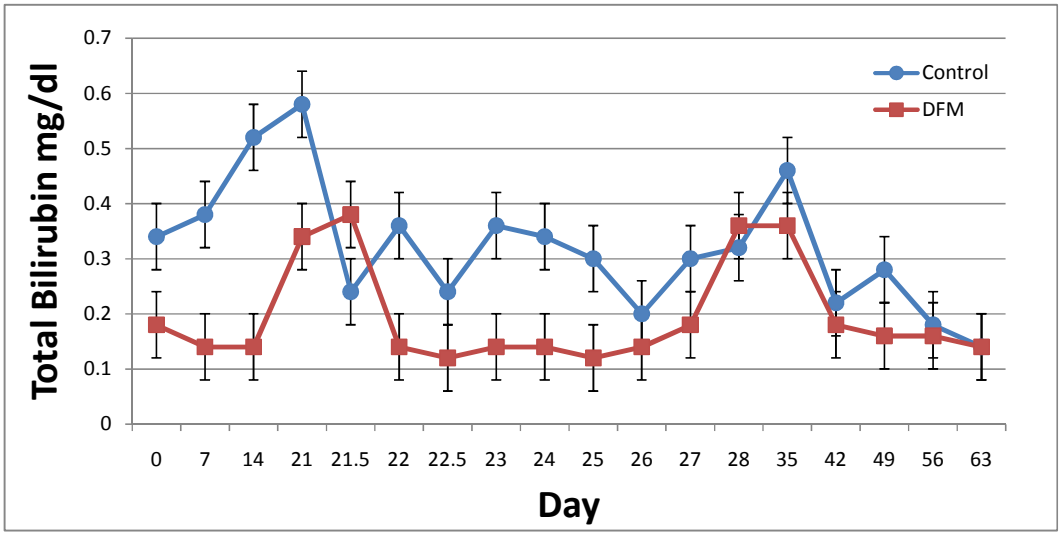


Figure A27. Effects of Direct-fed Microbials (DFM) and *Escherichia coli* O157:H7 Challenge on Blood Serum Total Bilirubin in Steers (Days 0, 7, 14, and 21 are pre-challenge).

APPENDIX B

History of *Lactobacillus acidophilus* strain in Bovamine®

History of the isolation of the *Lactobacillus acidophilus* strain in Bovamine®

This particular *L. acidophilus* culture was isolated in 1978 at Oklahoma State University. The source of the culture was the ileum of a Holstein calf. At that time the strain was designated *L. acidophilus* 381-IL-28. It has been licensed to Nutrition Physiology Corporation for their exclusive commercial use. Other strain designations used in journal articles include *L. acidophilus* 747 and NP51 (Based on information supplied by Nutrition Physiology Corporation, Guymon, OK).

VITA

LACEY MICHELE GUILLEN

Candidate for the Degree of

Doctor of Philosophy

Dissertation: DETERMINATION OF THE MECHANISM(S) BY WHICH DIRECT-FED MICROBIALS CONTROL *ESCHERICHIA COLI* O157:H7 IN CATTLE

Major Field: Food Science

Biographical:

Personal Data: Born in Amarillo, TX, December 4, 1980, the daughter of Mike and Jenny Smith

Education: Received the Bachelor of Science degree in Food Technology from Texas Tech University in August, 2003; completed requirements for the Master of Science degree at Oklahoma State University in December, 2006

Experience: Student lab assistant at Texas Tech University, 2001-2003; Research Assistant/Teaching Assistant at Oklahoma State University 2003-present.

Professional Memberships: Institute of Food Technologists, International Association for Food Protection, American Dairy Science Association, Sigma Xi, Gamma Sigma Delta

_____

Name: Lacey Michele Guillen Date of Degree: May, 2009

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: DETERMINATION OF THE MECHANISM(S) BY WHICH DIRECT-FED MICROBIALS CONTROL *ESCHERICHIA COLI* O157:H7 IN CATTLE

Pages in Study: 125 Candidate for the Degree of Doctor of Philosophy

Major Field: Food Science

Scope and Method of Study: The purpose of this experiment was to investigate the possible mechanisms responsible for the success of the direct-fed microbials, *Lactobacillus acidophilus* and *Propionibacterium freudenreichii* (included in and marketed as Bovamine®), in controlling *Escherichia coli* O157:H7 in cattle. In order to do this an *E. coli* O157:H7 challenge was conducted with ten steers. Five were fed the direct-fed microbials and five were fed the control treatment, lactose (direct-fed microbial carrier). Immunoglobulins, blood chemistry, complete blood counts, performance measures, and carriage of *E. coli* O157:H7 were measured throughout the trial.

Findings and Conclusions: An increased level of serum IgA was observed in the direct-fed microbial group post-challenge. The direct-fed microbial group also showed an increased amount of blood granulocytes and granulocytes as a percentage of white blood cells post-challenge. The direct-fed microbial group had a higher post-challenge increase in total lymphocytes while the control group had higher lymphocytes as a percentage of white blood cells post-challenge. The control group had higher dry matter intakes. Both groups exhibited similar levels of all measurements prior to the challenge.

ADVISER'S APPROVAL: Dr. Stanley Gilliland