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**Effect of Direct-Fed Microbials and Enzyme Supplementation in Prepartum Holstein Cows
on Colostrum and Calf Immunity**

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Honors Thesis

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Abstract:

In cows, colostrum is composed of several antibodies and nutrients to provide immunity and energy to the calf. Feeding calves high quality colostrum has been shown to improve calf health, leading to reduced mortality in calves and greater milk production in cows. The addition of direct-fed microbials (**DFM**) to cow diets has been theorized to improve feed efficiency and milk production, with studies showing mixed results. However, few experiments have studied the effect of feeding DFM on colostrum quality. In this experiment two treatments were given, 1) DFM and 2) DFM and enzymes (**DFME**). Colostrum was analyzed to determine if yield, composition, and immunoglobulin (IgG and IgA) concentration were affected. Calf serum IgG and IgA concentrations were analyzed to determine if 24 h concentrations and apparent efficiency of absorption (**AEA**) were affected. There were no differences with regard to yield or IgA concentration. The percent of ash showed a positive trend, indicating a higher percentage with the treatments ($P = 0.067$). The treatments had no effect on the additional components analyzed. The results for the IgG concentration were not significant although an increase was observed from 79.1 mg/mL in the control to 91.1 mg/mL in the DFME treatment. Neither treatment had an effect on calf immunoglobulin concentration or AEA. Based on the results, feeding DFM or DFME improves percent ash and might increase IgG concentration, but further research is necessary.

Introduction:

Colostrum is produced by mothers to be the first source of food a newborn will ingest. The concentrations of nutrients such as protein, fat, and carbohydrate vary greatly between species but are genetically controlled to provide suitable amounts for the survival of offspring (Langer, 2009). In general, fat and carbohydrates are metabolized for energy while proteins aid in cellular function or protect against pathogens as antibodies. Colostrum is initially synthesized in the weeks just prior to birth and stored in the developing mammary glands. After birth, colostrum will gradually transition to milk over hours or days, depending on the species.

In cows, colostrum is analyzed for a variety of nutrients. The most common measurements are percent and yield of: fat, true protein (**TP**), total solids (**TS**), ash, and lactose. Compared to milk, colostrum contains less lactose but higher amounts of all other nutrients, most notably TP (Parrish et al., 1950). True protein consists only of proteins, rather than non-protein nitrogen sources; in colostrum, TP is composed mainly of casein and immunoglobulins. Immunoglobulins distinguish colostrum from milk, with significantly higher amounts of IgG, specifically IgG₁, in addition to relatively smaller amounts of IgA and IgM (Kehoe et al., 2007).

The effect of colostrum intake in neonates has been studied across multiple species. Studies on neonatal puppies and kittens have found inadequate colostrum intake leads to failure of passive immune transfer (**FPT**; Claus et al., 2006; Mila et al., 2014). Similar results have been shown in neonates born agammaglobulinemic, including piglets and calves, where colostrum is the only source of functional immunoglobulins, including the primary immunoglobulin, IgG (Tyler et al., 1998; Rook and Bland, 2002). Failure of passive immune transfer, where serum antibody concentrations in the neonate are below experimentally derived levels, leaves the neonate without adequate protection against infections. Puppies (Mila et al., 2014) and calves

(Tyler et al., 1998) with FPT had a greater risk of mortality than those with successful transfer. Besides mortality, there is evidence that dairy calves with FPT may have stunted growth rates as heifers (Robison et al., 1988) and lower milk production for 1st lactations (DeNise et al., 1989). Thus, colostrum has a considerable influence on the health and future productivity of the neonate.

In order to improve passive transfer rates, several approaches have been studied. The timing of colostrum feeding prior to gut closure is generally accepted as the greatest source of passive immunity in ungulates. Calves fed colostrum 6 h after birth had significantly higher serum immunoglobulin concentrations than those first fed 24 h or later, at which point gut closure occurs and the majority of colostral immunoglobulins are lost in feces (Matte et al., 1982). However, the quality of colostrum ingested must also be considered. Nocek et al. (1984) found calves fed high quality colostrum, with over 60 mg/mL IgG, had greater weight gain and improved health compared to calves fed lower quality colostrum. A similar study on goat kids found higher concentrations of immunoglobulins in atomized colostrum paste caused increased absorption and serum immunoglobulin levels (Rodríguez et al., 2009). With a higher concentration of immunoglobulins, neonates are more likely to absorb greater amounts.

Improving colostrum quality, with respect to component and immunoglobulin concentration, may be accomplished through the addition of direct-fed microbials (**DFM**) to maternal diets. Direct-fed microbials are live cultures of microorganisms chosen to improve the digestion, performance, and immunity of animals (McAllister et al., 2011). Some DFM affect the availability of nutrients to the animal. For example, the use of yeast, *Saccharomyces cerevisiae*, may improve fiber digestion by maintaining more desirable pH levels (Nocek et al., 2002; McAllister et al., 2011). With more nutrients available, animals can divert excess nutrients to

production rather than maintenance. Other microbes may serve to upregulate the immune system. While several DFM compete with pathogenic bacteria in the digestive tract, others stimulate an immune response (McAllister et al., 2011). Further, some DFM products contain bacteria that produce enzymes (**DFME**) to enhance digestion of starch and cellulose. However, most studies feeding DFM to prepartum cows have focused on the nutrition and health of the dam and her milk production postpartum, rather than the impact to colostrum and calf health (Nocek and Kautz, 2006; Oetzel et al., 2007; Boyd et al., 2011; AlZahal et al., 2014). To fully understand the action of DFM, all aspects must be considered, including any benefits to the calves through colostrum.

To determine if DFM has an impact on colostrum or calf health, two hypotheses were proposed. The first concerned colostrum. Not only have DFM had a positive impact on nutrition, but the microbes may increase immunoglobulin production. Studies in piglets have shown that feeding of DFM containing strains of *Enterococcus faecium* (Szabo et al., 2009) or *S. cerevisiae* with *Pediococcus acidilactici* (Lessard et al., 2009) increase pathogen-specific IgA concentrations. We hypothesized that feeding DFM or DFME would increase colostrum quality compared to cows fed a prepartum diet alone. Thus, higher concentrations of IgG, the major immunoglobulin in cow colostrum, and IgA should be seen in the colostrum of treated cows. Further, if DFM improve nutrition, higher amounts of colostrum components were expected in treated cows.

The effective absorption of immunoglobulins by calves is the ultimate goal of higher quality colostrum. Calves fed maternal colostrum from DFM or DFME treated cows should ingest colostrum with greater concentrations of IgG and IgA. Thus, calves from DFM treated dams were expected to have higher serum Ig concentrations than calves from dams fed the

prepartum diet only. In addition, if DFM affect colostral components regulating absorption, higher serum concentrations should be seen due to an increase in apparent efficiency of absorption (**AEA**) in the calves.

Materials and Methods:

Thirty six multiparous Holstein cows housed at the UNH Fairchild Dairy Teaching and Research Center were studied. Cows were placed in one of 12 blocks based on expected calving date. Within a block, each cow was randomly assigned one of three treatments: 1) control (**C**), designated pre-partum diet only; 2) DFM only (**T**), designated pre-partum diet with 27.24 g/d of Tri-Lution®; or 3) DFM and enzyme (**T+Z**), designated pre-partum diet with 27.24 g/d of Tri-Lution® and 18.16 g/d of Zy-mend™. Tri-Lution® contains approximately 1.3×10^{12} cfu/kg of both *Enterococcus faecium* and *Saccharomyces cerevisiae*. Zy-mend™ is composed of approximately 9×10^{11} cfu/kg of *E. faecium* and *S. cerevisiae*, in addition to 4.4×10^6 cfu/kg *Bacillus subtilis* and 4.4×10^5 cfu/kg *Trichoderma longibrachiatum*. Cows began the study 21 d prior to their expected calving date. Each was kept in a tie stall bedded with mattresses and kiln-dried sawdust from the start of the study until 3 d prior to the expected calving date, at which point they were moved to a maternity pen. Cows were fed separately in wooden tubs in tie stalls and metal tubs in maternity pens. Each cow had unlimited access to automated water bowls. One cow on the DFME treatment aborted and was removed from the study. In addition, one cow on the DFME treatment was fed the DFM treatment instead.

After calving, calves were removed as soon as possible to prevent nursing and dams were milked within 60 min to collect colostrum. The colostrum was weighed and analyzed for quality using either a colostrometer (Precision Hydrometer; Biogenics; Mapleton, OR) or refractometer

(Sugar/Brix Refractometer 300001; SPER Scientific; Scottsdale, AZ). Two 40 mL samples were taken for further analysis. One was stored at -20°C for IgG and IgA radial immunodiffusion (**RID**) analysis (Triple J Farms, Bellingham, WA). The second was preserved with 2-bromo-2-nitropropane-1,3-diol and shipped to DairyOne Cooperative, Inc. (Ithaca, NY) for analysis of fat (method 989.05, AOAC 2006), TP (method 991.20, AOAC, 2006), TS (method 990.20, AOAC, 2006), ash (method 942.05) and lactose (calculated by the following equation: % TS - % fat - % TP - % ash). Two to 4 L of the remaining maternal colostrum was fed to the calf or calves within the first 2 h after birth.

Calves:

After being removed, calves were weighed using a platform scale (Salter Scales, Fairfield, NJ). Each was then placed in a separate 1 × 2.15 m stall with a mat and kiln-dried sawdust. All were navel dipped with 7% iodine, and heifer calves received required Bovine Rota-Coronavirus (Calf Guard; Zoetis Inc.; Kalamazoo, MI) and *Escherichia coli* vaccinations (Bar-Guard-99; Boehringer Ingelheim; St. Joseph, MO). Blood samples at 0 h and 24 h were taken from the jugular vein using 22 gauge needles and 10 mL vacutainer tubes (Covidien, LLC, Mansfield, MA). Samples were then clotted and centrifuged at 4° C for 20 min at 4,261 × g (5430 R Eppendorf, Hauppauge, NY), 2,323 × g (5810R, Eppendorf, Hauppauge, NY) or 1,704 × g (Servall Lynx 4000, Thermo Fisher Scientific, Chelmsford, MA). The serum was frozen at -20° C until IgG and IgA RID assays were performed (Triple J Farms, Bellingham, WA).

After the 0 h blood sample was collected, each calf was fed 4 L of colostrum via bottle or stomach tube. 10 calves did not receive the full 4 L of maternal colostrum. Six of these 10 calves had trouble bottle feeding and stomach tubing the full amount. The other four calves did not

receive enough because their dam produced less than 4 L of colostrum. In addition, 3 calves received no maternal colostrum due to the dam being leukosis positive; these 3 calves did not have blood samples taken at 24 h as they were fed either frozen colostrum from the farm and/or colostrum replacer.

Radial Immunodiffusion for IgG and IgA:

Plates were obtained for RID analysis of the IgG and IgA concentrations in colostrum and calf serum. Distilled water and three standards (IgA: 53, 194, 387 mg/dL; IgG: 196, 1402, 2748 mg/dL) were used to determine the concentration. For the colostrum samples, samples were thawed and mixed prior to pipetting. Colostrum samples were diluted with distilled water 1:4 for IgG and 1:3 for IgA. Five μ L of each sample was pipetted into the corresponding well, with two samples per cow to be averaged. For blood serum samples, samples were thawed and mixed prior to pipetting. Serum was directly pipetted into the corresponding well, with 2 samples per calf to be averaged. All plates were left at room temperature in the resealed packages for 48 h. After 48 h, plates were read using a digital RID plate reader (Binding Site, Birmingham, UK). Standards were graphed and the averaged sample diameters compared with the graph to determine concentrations. Samples were rerun if the diameters of the two samples from the same animal differed by 10mm or greater. Samples with diameters greater than the largest standard were rerun at a higher dilution.

Statistical Analysis:

Data was analyzed using the MIXED procedure in SAS (SAS 9.4, Cary, NC.) Means for each treatment and standard errors were reported. Parity was used as a covariate for calf immunoglobulin concentration at 24 h and AEA. The covariate was removed from the model if $P > 0.25$. Significance was defined as $P \leq 0.05$. Trends were defined as $0.05 < P \leq 0.1$.

Results:

For colostrum, there were few differences between treatments. Cows on the control diet with no DFM had the highest yield (10.7 ± 1.49 kg) while the DFM treatment of Tri-Lution[®] only had the lowest (6.56 ± 1.45 kg); however, there was no difference ($P > 0.05$) in yield between any treatments (Table 1). Cows fed DFME had the highest concentrations of IgG and IgA in colostrum compared to the other treatments, but again were not significantly higher ($P > 0.05$). Although cows in the control group produced colostrum with the lowest concentration of IgG (79.1 ± 5.54 mg/mL), those cows also yielded the most IgG of all treatments (836.8 ± 105.9 g). IgG concentrations for all treatment groups ranged from 46.7 to 134.2 mg/mL.

Table 1. Descriptive statistics of treatments for colostrum yield, immunoglobulin concentration, and colostrum composition in cows fed control, DFM, or DFME treatments 21 d prior to expected parturition.

Variable	Treatment ¹			SE	P value
	C	T	T + Z		
Colostrum (kg)	10.7	6.56	9.40	1.6	0.159
IgG (mg/mL)	79.1	88.2	91.1	6.0	0.338
IgG (g)	836.8	562.0	782.3	116.5	0.186
IgA (mg/mL)	6.30	6.07	6.97	0.6	0.475
IgA (g)	63.31	37.22	62.83	8.8	0.054
Fat (%)	5.76	5.14	4.90	0.9	0.752
Fat (kg)	0.66	0.37	0.52	0.13	0.237
TP (%)	13.7	16.1	16.1	1.0	0.134
TP (kg)	1.42	1.06	1.33	0.2	0.408
TS (%)	26.0	25.3	24.9	2.2	0.931
TS (kg)	2.72	1.70	2.20	0.4	0.128
Ash (%)	1.07	1.16	1.25	0.05	0.067
Ash (kg)	0.11	0.06	0.08	0.02	0.245
Lactose (%)	5.48	2.85	2.58	1.6	0.352
Lactose (kg)	0.47	0.19	0.26	0.12	0.181

¹Treatment: C = No Tri-Lution[®] or Zy-Mend[™]; T = 27.24 g/d Tri-Lution[®]; T + Z = 27.24 g/d Tri-Lution[®] and 18.16 g/d Zy-Mend[™].

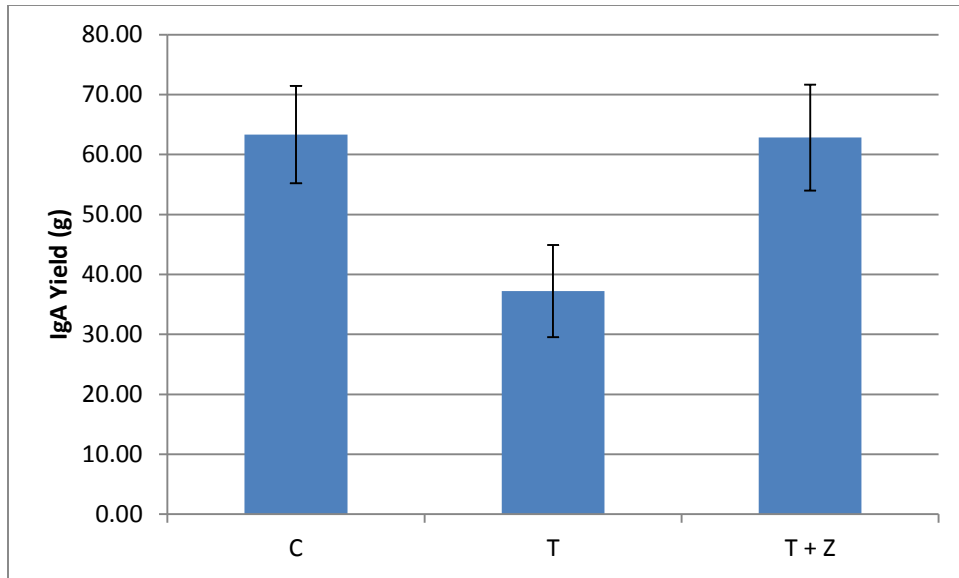


Figure 1: Colostral IgA yield for control (C), DFM (T) and DFME (T+Z) treatments, $P = 0.054$.

There was a trend in IgA yield ($P = 0.054$). The amount of IgA in the DFM only treatment was lower (37.22 ± 7.69 g) than either the control or DFME treatments (63.31 ± 8.13 ; 62.83 ± 8.84 g, respectively) (Fig. 1). There was no effect on the concentration of IgA in colostrum ($P > 0.05$), although the DFM treatment was again lower than the other treatments.

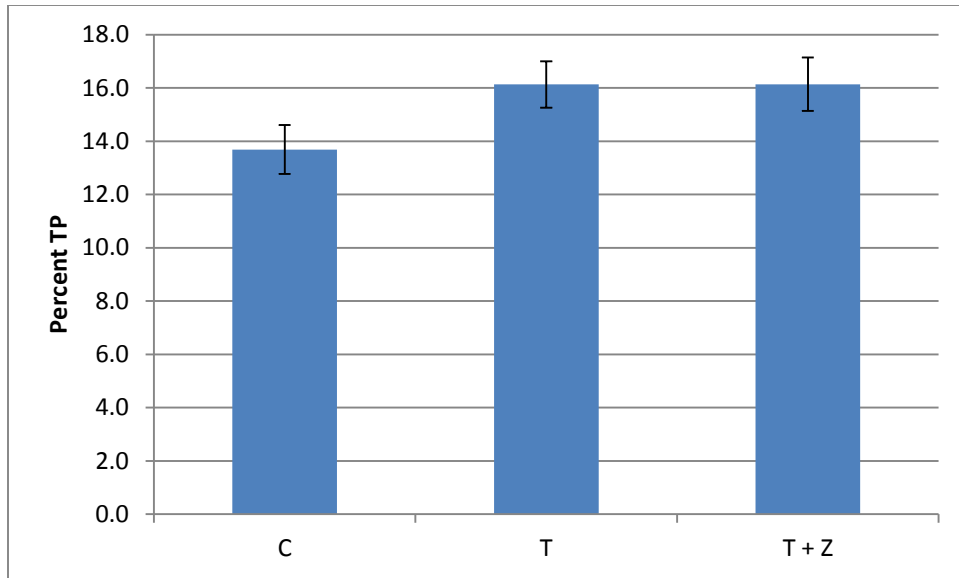


Figure 2: Percentage of true protein (TP) in colostrum for control (C), DFM (T) and DFME (T+Z) treatments, $P > 0.05$.

The treatments also had few effects on the composition of components in colostrum. There was no significant effect of DFM or DFME on yield or percentage for all colostrum components measured. Cows in the control group produced higher percentages of fat, TS, and lactose than DFM treated cows, with DFME cows producing colostrum with the lowest percentage of these components (Table 1). Although not different ($P = 0.13$), percent of TP in colostrum from DFM and DFME treated cows was greater (16.1%) than in control cows (13.7%) (Fig. 2).

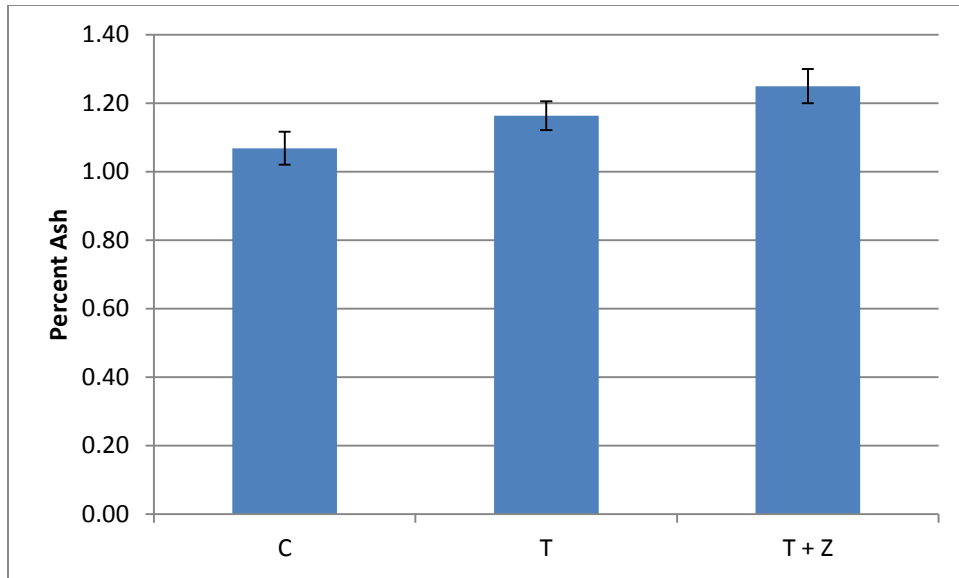


Figure 3: Percentage of ash in colostrum for control (C), DFM (T) and DFME (T+Z) treatments, $P = 0.067$.

In addition, there was one trend ($P = 0.067$) regarding ash percent. Cows fed DFME showed a positive increase in ash percentage ($1.25 \pm 0.05\%$), followed by DFM treatment ($1.16 \pm 0.04\%$), compared to the control group ($1.07 \pm 0.05\%$) (Fig. 3).

For calves, there was no difference between treatments. Calf body weight (**BW**) at birth was not affected by DFM treatment (Table 2). Most calves started with no serum IgG or IgA at 0 h, although 9/37 calves had negligible amounts of IgG present (Fig. 4 and 5). Calves absorbed both IgG and IgA; however, there was no difference in serum concentrations at 24 h or AEA between control and treatment groups. Serum IgG concentrations at 24 h for all treatment groups ranged from 12.8 to 42.7 mg/mL.

Table 2. Descriptive statistics of calf body weight at birth, serum immunoglobulin concentrations, and apparent efficiency of absorption after ingestion of maternal colostrum.

Variable	Treatment ¹			SE	P value
	C	T	T + Z		
BW (kg)	44.1	44.7	45.8	2.0	0.826
IgG 0 h (mg/mL)	1.7	0.3	1.2	1.0	0.575
IgG 24 h (mg/mL)	24.6	25.9	26.3	3.4	0.920
IgA 0 h (mg/mL)	0.03	0.00	0.00	0.02	0.442
IgA 24 h (mg/mL)	1.43	1.54	1.49	0.15	0.509
AEA IgG ² (%)	34.1	31.1	33.5	3.4	0.800
AEA IgA ³ (%)	29.3	28.1	29.5	2.9	0.928

¹Treatment: C = No Tri-Lution® or Zy-Mend™; T = 27.24 g/d Tri-Lution®; T + Z = 27.24 g/d Tri-Lution® and 18.16 g/d Zy-Mend™.

²AEA IgG: ((Serum IgG [g/L] * BW[kg]*0.09)/IgG Intake[g])*100

³AEA IgA: ((Serum IgA [g/L] * BW[kg]*0.09)/IgA Intake[g])*100

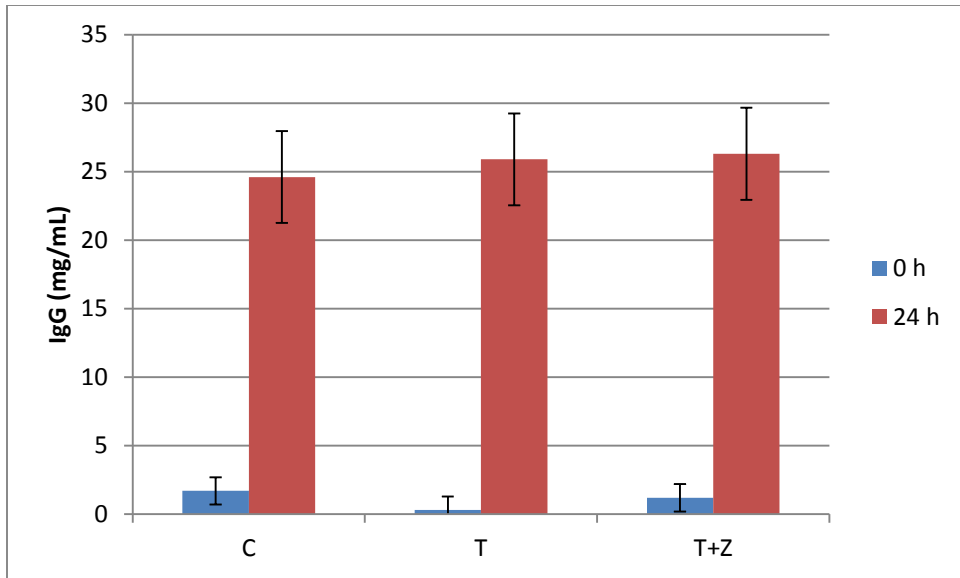


Figure 4: Serum IgG concentration in calves at 0 h and 24 h after birth. Calves were grouped as control (C), DFM (T) or DFME (T+Z) based on the treatment group of the dam. $P > 0.05$ for both 0 h and 24 h.

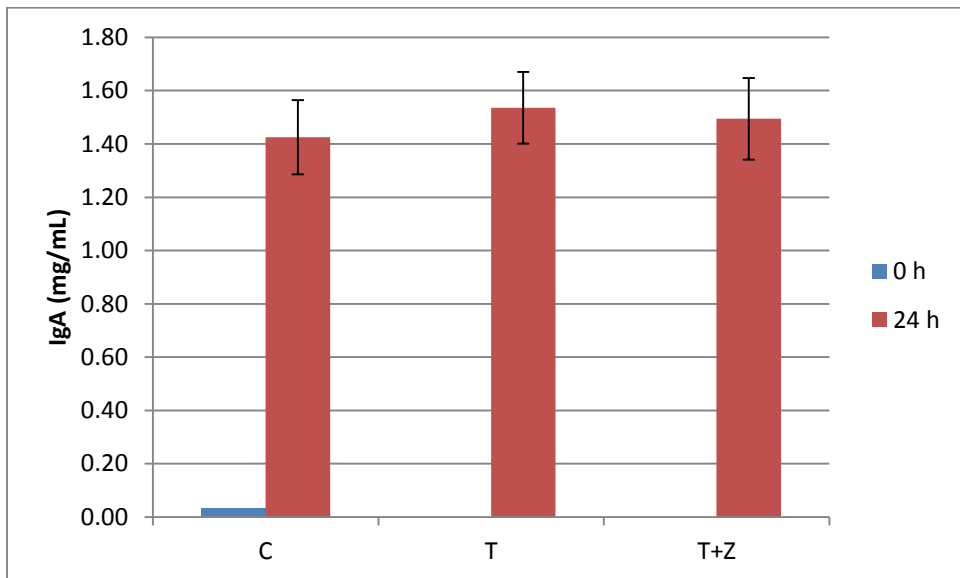


Figure 5: Serum IgA concentration in calves at 0 h and 24 h after birth. Calves were grouped as control (C), DFM (T) or DFME (T+Z) based on the treatment group of the dam. $P > 0.05$ for both 0 h and 24 h.

Discussion:

The addition of DFM or DFME to prepartum cow diets had few effects on the quality or composition of colostrum. Similarly, there was no difference with regard to the calf variables measured.

Considering colostrum yield, there was no difference between treatments, although cows in the control group produced more than either treatment group. There is a lack of data on the effect of DFM on colostrum yield as the majority of studies have focused on milk yield. Previous studies have shown mixed results regarding milk yield after treatment with DFM, with some reporting improved yields (Nocek and Kautz, 2006; Boyd et al., 2011) while others showed no difference (Oetzel et al., 2007; AlZahal et al., 2014). Caution must be used comparing milk to colostrum, however, as milk yields may not predict colostrum yields (Cabral et al., 2016).

The concentration of either IgG or IgA was not affected by treatment. For yield, cows on the DFM treatment produced the least immunoglobulin, specifically IgA. This most likely reflects their lower colostrum yield, as all groups had similar immunoglobulin concentrations. The concentration of IgG is comparable to the average of 74.2 mg/mL for colostrum from Holsteins (Morrill et al., 2012). Few studies in cattle have been conducted to determine if immunoglobulin concentrations are affected by DFM. One DFM study using Angus-cross steers challenged with *E. coli* O157:H7 found no change in serum or fecal concentration of either IgG or IgA, although levels in the DFM group tended to be higher (Guillen, 2009). Guillen (2009) noted that granulocyte levels were significantly higher with DFM treatment, in addition to other indications of an increased immune response. Another study in day-old chicks found increased levels of IgG and IgA in serum and intestinal secretions with DFM treatment; although several mechanisms were proposed, none could be determined as causing the increase (Haghighi et al.,

2006). While the mechanisms behind the proposed action of DFM on immunoglobulin production remains unclear, the DFM fed in this experiment did not affect the uptake of IgG or IgA by the mammary gland for colostrum.

Besides the effect of DFM, colostrum quality can be affected by yield at first milking. Pritchett et al. (1991) described an inverse effect of first milking yield on IgG concentration, where yields greater than 8.5 kg began showing dilution of IgG. In contrast, this experiment found no negative impact, as both control and DFME groups had high quality colostrum with yields over 8.5 kg (Table 1).

Although not significant, there was an increase in percentage of TP with the DFM treatments. For Holsteins, the average percentage of colostrum protein is 12.5%, which is close to the control group but noticeably lower than the DFM/DFME treatment groups (Morrill et al., 2012). As the immunoglobulin concentrations were not affected by treatment, other proteins contributed to the increase. Oetzel et al. (2007) found an increase in milk protein percentage during the early stage of lactation feeding two strains of *E. faecium* and *S. cerevisiae*. In contrast, Nocek and Kautz (2006) found no difference in milk protein percentage feeding *E. faecium* alone. For further study, TP should be analyzed further to determine which proteins may be increased with DFM supplementation.

There was a trend regarding ash percentage. Groups fed DFM had the highest percentages, meaning the colostrum contained more minerals. Ash percentage values were similar to previous values for colostrum from Holsteins (Parrish et al, 1950; Oyeniya and Hunter, 1978). The various minerals, especially Ca and P, are vital to the proper growth and metabolism of calves. No previous DFM studies have analyzed colostrum or milk for ash content. Based on

these results, DFM may improve bioavailability or uptake of minerals. Again, further analysis of the ash composition may reveal if DFM influence some or all minerals in colostrum.

While DFM had mixed results on colostrum quality, there was no effect seen in calves. Neither serum concentrations at 24 h after birth nor AEA differed between groups. The average calf in all groups absorbed acceptable levels of IgG, reaching a recommended concentration above 10 mg/mL (Quigley and Drewry, 1998). Several factors serve a role in absorption of immunoglobulins, such as age and colostrum quality (Quigley and Drewry, 1998). Regarding quality, Stott and Fellah (1983) found AEA increased with higher IgG concentration, resulting in less colostrum required for adequate absorption. As all groups had similar, high quality colostrum, it is unsurprising AEA and serum concentration were unaffected. If DFM cause an increase in proteins or compounds aiding immune function, as proposed, there does not appear to be an effect on calf gut absorption of immunoglobulins (McAllister et al., 2011).

For future studies, more work should be devoted to improving colostrum, as there appear to be more possibilities for feeding of DFM. For example, individual cows may react differently to DFM. Nocek and Kautz (2006) found that cows supplemented with DFM had lower milk fat percentages than the control group. However, the researchers noted that DFM did not depress fat production and that cows in the treatment group tended to produce less fat based on their previous lactation (Nocek and Kautz, 2006). This difference may have accounted for differences between groups. Future experiments should consider following cows over two pregnancies, starting on either a control or DFM treatment diet and switching treatments between pregnancies to determine if DFM has an individual effect on colostrum quality.

Another consideration is timing of DFM supplementation. In order to cause effects, the DFM must first colonize the rumen, which may not occur immediately. Notable increases in

IgG₁ in colostrum secretions may begin as early as 5 wk prior to parturition, with most substantial colostrogenesis starting between 2 and 3 wk prior (Brandon et al., 1971). In this experiment, feeding of DFM did not begin until 3 wk prior to expected parturition, meaning that immunoglobulins already present were being sequestered in colostrum as DFM began colonizing the rumen. A longer period between the start of DFM treatment and parturition may allow additional effects to be seen.

Finally, other DFM products should be studied, as Tri-Lution[®] and Zy-Mend[™] contain only four of hundreds of potential microbes. All have various modes of action; some may prove more effective at improving colostrum than others. As more than 60% of all colostrum produced in the United States is poor quality, below 50 mg of IgG/mL, any improvement is welcome to prevent development of FPT in calves (Morrill et al., 2012). Further studies must determine whether DFM supplementation aids dairy farmers in improving colostrum quality.

Conclusions:

Feeding DFM or DFME to prepartum cows improved ash percentages in colostrum. However, supplementation had no effect on the IgG or IgA concentration of colostrum, nor on the additional colostrum components. Calf body weight, 0 and 24 h serum concentrations of IgG and IgA, and AEA of both immunoglobulins were not affected by DFM treatment, either prior to parturition or by feeding of maternal colostrum from DFM supplemented dams. Future studies must investigate the usefulness of feeding DFM for the purpose of improving colostrum by determining the mechanisms and best microbial species to feed. Understanding how DFM may improve colostrum could prove useful in providing more calves the benefits of high quality colostrum.

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