Effect of Formic Acid Treatment on Colostrum Quality, and on Absorption and Function of Immunoglobulins: A Randomized Controlled Trial in Holstein Dairy Calves

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Research Article

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Abstract

Background

Good quality colostrum is characterized by high immunoglobulin levels and low pathogen load. Techniques to decrease pathogens can damage immunoglobulins, potentially reducing both absorption and function. Objectives were to determine the effect of formic acid treatment on colostrum bacterial and immunoglobulin (IgG) levels before feeding, and serum immunoglobulin levels and neutralizing capabilities after feeding. Fifteen female Holstein calf pairs born < 12 h apart from different dams were randomly assigned to receive four liters of either untreated pooled (both dams) colostrum (MC) or colostrum acidified to pH 4.0-4.5 (AC). Colostrum characteristics evaluated; pH, bacterial load, IgG, and neutralization of Infectious Bovine Rhinotracheitis (IBRV), Bovine Viral Diarrhea (BVDV) Types 1 and 2. Blood collected on days 1, 3 and monthly for 6 months was analyzed for IgG, and both viral plus leptospiral neutralization.

Results

Compared to MC (mean 6.7, SD 0.4; median 6.8, range 6.0-7.3), AC pH was significantly reduced (mean 4.3, SD 0.2; median 4.3, range 4.0-4.5; $P < 0.001$). Total coliform count (cfu/mL) was also reduced (MC mean 149, SD 444; median 1, range 0-1,700; AC mean 8, SD 31; median 0, range 0-120; $P = 0.02$). Colostrum IgG levels were not significantly different between MC (mean 93.3, SD 39.7; median 92.80, range 36.7-164.4 g/L) and AC (mean 101.9, SD 36.7; median 108.3, range 33.8-164.4 g/L; $P = 0.54$). In calves, serum IgG peaked on day 3 (MC mean 26.1, SD 34.9; median 169.2, range 8.3-151.0 g/L; AC mean 30.24, SD 48.7; median 188.8, range 3.1-204.4 g/L; $P = 0.77$), and apparent efficiency of IgG absorption was not different between groups (MC mean 24.3, SD 11.4, median 25.3, range 8.6–51.3%; AC mean 22.6, SD 21.7, median 21.6, range 4.1-58.9%; $P = 0.65$). Thereafter, IgG levels declined but did not differ between groups. MC and AC serum neutralizing titers for IBRV, BVDV Types 1 and 2, or *Leptospira canicola*, *hardjo*, and *pomona* were not different.

Conclusions

Colostrum acidification significantly decreased bacterial load fed to newborn calves without affecting IgG concentration or virus neutralization. Nor did acid treatment impact serum IgG levels in calves or activity against common pathogens.

Background

According to the United States Department of Agriculture's National Animal Health Monitoring System (NAHMS) 2014 Dairy Survey, diarrhea and other gastrointestinal problems account for 56.4% and respiratory problems 24% of pre-weaned heifer deaths [1]. Additionally, respiratory disease is the predominant cause of mortality in weaned dairy heifers [1]. Because calves are born agammaglobulinemic and their capacity to mount an immune response de novo is limited, adequate colostrum intake that affords passive immunity is critical for disease prevention and their survival [2, 3]. The importance of absorbing an adequate quantity of immunoglobulins is unequivocal, but their functionality is also crucial to the health and welfare of dairy calves.

Economic losses associated with dairy calf diseases include implementation of prevention and treatment measures but should also consider potential impacts of calfhood disease on future productivity. For example, a single case of pneumonia during the first 2 months of life results in slower growth rates due to poor animal health and welfare, and decreased productivity as an adult [4, 5]; improved rate of average daily gain has been associated with a higher first lactation milk yield [6].

Normal colostrum provides a favorable medium for bacterial growth, contamination can increase the risk of pre-weaning morbidity and mortality and have a negative impact on the growth of dairy heifers [7]. Moreover, it is known that increased pathogen load in colostrum interferes with immunoglobulin absorption leading to poor passive transfer [8, 9, 10]. Limiting exposure of calves to colostrum contaminated with pathogenic organisms; bacteria, including *Escherichia coli* [11], *Salmonella* spp. [12], *Mycoplasma bovis* [13, 14], *Listeria monocytogenes* [15], and *Mycobacterium avium* ssp. *paratuberculosis* [16], and viruses (Bovine Leukemia Virus [17]), is one key factor in disease prevention.

Pathogen reduction by techniques such as heat treatment, pasteurization, irradiation (ultraviolet light), can be complicated and costly, and may result in an unacceptable product because of increased viscosity and feed refusal. A critical negative effect that might also occur with these techniques is denaturing of immunoglobulins leading to impairment of passive transfer [18, 19, 20]. The preservation of IgG may be dependent on the initial IgG concentration with loss being most apparent when initial concentrations are high [13]. Additionally, any structural changes likely limit pathogen neutralizing capabilities of those maternal antibodies that are transferred to the blood, thereby directly compromising both the quantity and quality of immunoglobulins in calves. For example, heat treatment has been shown to damage bovine immunoglobulins by changing their secondary structure causing them to lose immunoactivity [21, 22, 23]. Even widely adopted heat-treatment techniques such as 60 min at 60°C have been shown to decrease the abundance of immunoglobulins and other important components of colostrum likely play a role in gut maturation, glucose absorption and mucosal immunity [24].

As an alternative to the standard techniques such as heat treatment, the use of acids to acidify whole milk or milk replacers has recently gained attention in the dairy industry as a possible way to control the growth of bacteria in colostrum. Even though acid use has been anecdotally successful and is increasing in popularity it is not without potential consequences. While the effect of acidified colostrum on IgG absorption has been reported in abstract form [25], we are not aware of any formal publications in the standard literature describing possible functional effects on immunoglobulins. The current randomized controlled trial aimed to test the hypothesis that formic acid acidification could successfully reduce bacterial (pathogen) load without negatively impacting either the absorption or immunoactivity of colostral antibodies, thereby indicating that it could be an effective tool in colostrum management.

Results

Calf Characteristics

The power analysis indicated that 10 calves per group would be sufficient to detect a minimal difference in titers between the two groups of 1:291 with $\alpha = 0.05$ and power = 1.00. As 15 calves per group were used, the power of the study should have been sufficient to detect meaningful differences associated with feeding AC. A total of 15 calves were fed AC and 15 calves were fed MC, data collected from all 30 calves were included in the analysis. The mean birth weights ($\pm$ Standard Deviation, SD) for the 15 MC and 15 AC calves were 40.6 ± 2.4 kg and 40.7 ± 2.9 kg, respectively, with no significant difference between groups ($P = 0.95$).

Colostrum Characteristics

pH Reduction: Compared to MC (mean 6.7, SD 0.4; median 6.8, range 6.0-7.3), the pH of AC was significantly reduced (mean 4.3, SD 0.2; median 4.3, range 4.0-4.5; $P < 0.001$, Table 1).

IgG Concentration: The concentrations of IgG were not significantly different after acidification (MC mean 93.3 SD 39.7; range 36.7-164.4 g/L; AC mean 101.9, SD 36.7; range 33.8-164.4 g/L; $P = 0.54$; Table 1).

IgG Virus Neutralizing Titers: There were no significant differences between untreated and post-acidified colostrum in immunoglobulin levels against IBRV, BVDV Type-1, or BVDV Type-2 (Table 2).

Microbial Reduction: Compared to MC, total bacterial counts in AC were reduced (MC mean 10,700, SD 16,945; median 3,700, range 1,000-62,300; AC 2,973, SD 3,765; range 1,000-12,200) but this effect did not achieve statistical significance $P = 0.10$ (Figure 1). There was, however, a significant reduction in the total coliform count (MC mean 149, SD 444; range 0-1,700; AC mean 8, SD 31, range 0-120; $P = 0.02$; Figure 1). Generic *E. coli* were rarely detected and there was no significant difference between groups (MC mean 6, SD 18, range 0-70, AC not detected; $P = 0.48$).

(PLACE TABLE 1 HERE)

Serum Characteristics

Total Protein Concentration. At 3 days of age, total protein levels in calves fed MC (mean 6.5 mg/dL, SD 0.4, range 5.4-7.0) and those receiving AC (mean 6.3 mg/dL, SD 0.7, range 5.2-7.6) were not significantly different, $P = 0.38$ (Table 1).

IgG Concentration. With the exception of one calf in the MC group (4.7 g/dL), IgG levels on day 1 pre-colostrum were all less than 0.1 g/L. Peak IgG occurred on day 3 i.e., approximately 48 h after feeding (MC mean 26.1, SD 34.9, range 8.3-151.0; AC mean 30.2 SD 48.7, range 3.1-204.4; $P = 0.77$; day 3 regression coefficient 0.9, 95% CI -3.6-5.5; $P = 0.67$; Figure 2). Thereafter IgG levels slowly declined but they were never significantly different between groups at any time point. Regression analysis of the entire time course post-colostrum (i.e., days 3-180) also failed to demonstrate a significant difference between groups (coefficient 0.0005, 95% confidence interval [CI] -0.022-0.023 g/L, $P = 0.97$; Figure 2). On the most critical day i.e., day 3, apparent efficiency of IgG absorption (AEA) was not different between MC (mean 24.3, SD 11.4, median 25.3, range 8.6–51.3%) and AC (mean 22.6, SD 21.7, median 21.6, range 4.1-58.9%; $P = 0.65$; Table 1) groups (day 3, coefficient -1.3, 95% CI -8.6-5.9, $P = 0.71$).

IgG Serum Neutralizing Titers. There were no significant differences between MC and AC in immunoglobulin SNTs for IBRV, BVDV Type-1, or BVDV Type-2 at any point over the 180-day observation period (Table 2). Maternal antibody titers against IBRV, BVDV Types 1 and 2 tended to remain higher for longer in calves that received AC compared to calves that were fed MC, but this effect did not achieve statistical significance even when tobit regression was used to examine the overall time course (IBRV $P = 0.53$; BVDV Type-1 $P = 0.35$; BVDV Type-2 $P = 0.20$).

SNTs against *Leptospira grippotyphosa* and *icterohaemorrhagiae* were rarely observed and were not subject to analysis. *L. canicola* and *pomona* SNTs were infrequently detected in both groups and at no time were significantly different between those calves fed MC or AC (tobit regression over time course $P = 1.0$). SNT's against *L. hardjo* were measurable in all except one AC calf at day 3 and were still detectable in 37% of the calves at day 60, after which only one or two calves had measurable titers. There was no significant difference between MC or AC groups at any time point nor over the entire observation period (tobit regression days 3-180, $P = 0.39$).

(PLACE TABLE 2 HERE)

Discussion

Results of this study lend support to the use of acidification of bovine colostrum to improve colostrum quality through bacterial reduction. Acid is already being used to manipulate whole milk or milk replacer for calves and was chosen here partly because of its increasing popularity but also because it is a relatively simple and inexpensive technique accessible to most producers. Acidification has already been shown to reduce bacterial load including some specific pathogens [25, 26, 27, 28]. However, it has some important drawbacks including danger of handling, palatability of end-product (viscosity and taste), and potential damage to colostrum components, specifically immunoglobulins. Nevertheless, acidification offers an additional advantage to some other methods of colostrum treatment in that it may permit prolonged storage without the need for freezing or refrigeration. However, that would only be of value if it can be demonstrated that acid reduced bacterial load without negatively affecting the levels, absorption, and function of colostral immunoglobulins. Research into the effects of feeding acidified colostrum to newborn Holstein dairy calves is limited, particularly in terms of functional outcomes. This study showed that acidification of colostrum reduced bacterial load, potentially including pathogens (total coliforms), without negatively affecting immunoglobulins or their function after feeding to newborn calves.

Handling of acid should be done with care and eye and skin protection should always be used. United States Food and Drug Administration (FDA) regulations state that formic acid and formate salts from all added sources (if multiple sources of formic acid and its salts are used in combination) cannot exceed 1.2% of complete feed [29]. Although there are currently no guidelines for whole milk or colostrum, formic acid levels in all AC samples in this trial were $\leq 0.45\%$, i.e., approximately one third or lower than the established guidelines for livestock feeds. Additionally, observations made during this trial indicated that three factors chiefly contributed to successful colostrum acidification. First, it was important to chill the colostrum prior to addition of acid. Adding acid to colostrum with a temperature at or above 15-16°C (60°F) resulted in coagulation and increased viscosity, changes which make calves refuse to drink and essentially preclude esophageal tube feeding. Second, if already practicing acidification of milk, expect to use slightly more acid when acidifying colostrum. Compared to the same volume of whole raw milk, we used 15 mL more (40-45 vs 25-30 mL) of the 10% acid solution per 4 L of colostrum to reach the desired pH (4.0 to 4.5). Finally, the colostrum must be vigorously stirred while slowly adding the acid solution. The use of a drill attachment designed for mixing paint worked well to rapidly mix the acid and colostrum thereby preventing coagulation. The method we describe here resulted in colostrum that was low in bacteria, stable at room temperature for days (unpublished observations and a currently funded research project), and acceptable to calves via bottle or esophageal tube feeding. The main drawback found with formic acid acidified colostrum was the effect on palatability, which is likely true of all acids. We have observed calves to occasionally reject suckling or take longer to consume the desired amount of the acidified colostrum or milk when bottle fed. However, the decreased palatability can be overcome by esophageal tube feeding which is not hindered in any way. In the current study, to ensure complete intake of 4 L, colostrum (MC and AC) was administered to all calves by esophageal feeder.

To be considered excellent quality, colostrum should have a low bacterial (including pathogens) load, but the process of harvesting colostrum could result in bacterial contamination. Furthermore, cattle suffering from mammary gland infections at the time of calving could also contribute additional bacteria to the collected colostrum. In addition to bacterial pathogens, colostrum can also be a means of transfer for certain viruses [30]. The results of this study showed what might well be a clinically meaningful reduction in total bacteria and more specifically a significant reduction in total coliforms. Reduction of pathogens is a critical component of colostrum management because they are known to bind to free immunoglobulin in the gut lumen or block immunoglobulin uptake and transport across intestinal epithelial cells, thereby interfering with absorption. Decreasing bacteria to remove potential pathogens is permissive to enhanced passive transfer [8, 9, 10], and subsequent promotion of calf health. Although, from a microbial standpoint, the colostrum on our well-managed study farm was of very good quality from the outset, levels of bacteria and specifically coliforms were nonetheless reduced by acidification suggesting that routine use of acid might be a valuable tool in bacterial management. However, work with more heavily contaminated colostrum is necessary to definitively demonstrate this.

Evaluating the effect of acidification on immunoactivity of absorbed immunoglobulins was an essential part of this study. Colostrum quality has for the most part been judged by immunoglobulin concentration and bacterial load. The use of standard pathogen reducing techniques (heat treatment or true pasteurization, ultraviolet light irradiation, and pressure) are, under certain conditions, known to induce denaturing of immunoglobulins leading to reduced passive transfer and compromised serum immunoglobulin levels in calves [20, 21, 22, 23]. Even maternal antibodies that do reach the blood of calves may have decreased immunoactivity. The ability of absorbed, undamaged immunoglobulin to neutralize pathogens is a key component in disease prevention leading to healthier calves. It has been reported that free-access feeding of acidified milk replacer results in improved growth and overall health of both dairy and veal calves [31]. Here we show that formic acid acidification of colostrum decreased bacterial load and was neutral in terms of its effect on immunoglobulin absorption measured as serum total protein and IgG concentrations, and AEA. More importantly, the neutralizing capabilities of the absorbed immunoglobulins against both viruses (IBRV, and BVDV Types 1 and 2) and bacteria (serovars of *Leptospira* spp) were not different between MC and AC groups suggesting that the immunoglobulins remained functionally intact after acidification.

Information gained from this project could benefit the production animal through supplementing existing management strategies directed at maintaining animal health and well-being. Additional studies to investigate the combined effect of feeding acidified colostrum followed by the provision of acidified whole milk or milk replacer on health in dairy youngstock and their subsequent productivity could further elucidate the benefits of acidification to the dairy industry. Future work is planned to investigate another potential benefit of acidification in the preservation of colostrum with reduced bacterial load without the need for freezing or refrigeration.

Conclusions

Our results showed that acidification of colostrum decreased the bacterial load fed to newborn calves, including a significant reduction in total coliforms, which should aid in disease prevention. Acidification had no impact on absorption of immunoglobulins from the colostrum and neither did it negatively affect the neutralizing capability of maternal antibodies against common viral and bacterial pathogens.

Methods

Animal selection and colostrum collection

This study was conducted at a 520 milking Holstein dairy located in southeastern Pennsylvania that routinely fed 3.8 L of raw pooled colostrum. The study herd was selected based on relatively large size, excellent computerized records (DairyComp 305, Dairy One Cooperative Inc., Ithaca, NY, USA), well-trained personnel (including one author, SVC, who is a veterinarian employed full-time by the farm and was responsible for the day-to-day conduct of the field trial), and willingness of the owner to comply with study requirements. Cows were moved to the maternity area, an open straw bedded pack, within one week of their due date. Records and careful monitoring were used to identify those cows likely to calve within 12 h of one another and from among these animals fifteen pairs of female Holstein calves were enrolled in the study from May-August 2018. In this high producing herd (rolling herd average 14,800 Kg per cow annually; 4.1% fat and 3.2% protein), almost all cows routinely produce at least 8 liters of colostrum. However, if the total volume of colostrum pooled from both dams was < 10 liters the pair and their calves were not enrolled in the trial. Colostrum was harvested from both dams within 4 h of calving, collection was made over ice to rapidly cool to 15 - 20°C. Once cooled, the colostrum from both dams was pooled. Four liters of the pooled colostrum was acidified as described below. Colostrum characteristics including pH, bacterial load, and immunoglobulin G (IgG) concentration were evaluated in both untreated and acidified samples. Calves were removed from the dam immediately after birth and processed by navel dip, ear tagging, recording of body weight, and initial pre-colostrum blood draw. After processing, calf pairs were placed in a holding pen until being randomly assigned by coin-flip to receive via esophageal tube feeding 3.8 L of either untreated pooled maternal colostrum (MC) or the same pooled colostrum acidified to pH 4.0-4.5 with 10% formic acid (AC). Colostrum was not stored prior to feeding as all calf pairs received colostrum pooled and processed from their dams within 12 h of birth. After colostrum feeding, calves were housed in small groups (maximum 6); fed acidified whole milk twice daily and provided with water and a grain starter ration ad libitum until weaning at approximately 8 weeks of age. Post-weaning calves were housed in larger groups (maximum 15) and fed a grower ration before being transitioned onto a corn silage-based heifer mixed ration. In addition to day 1 pre-colostrum, blood samples were collected from each heifer pair on day 3 post-colostrum, and then monthly for 6 months. At no time during the study period did the enrolled calves receive any vaccinations. Blood was analyzed for IgG concentrations and neutralizing capabilities against viral Infectious Bovine Rhinotracheitis (IBRV), Bovine Viral Diarrhea Virus (BVDV) Type-1 and Type-2 and five serovars of bacterial *Leptospira*. For analysis of samples, BIS and the participating laboratories were not aware of how the samples were coded.

Colostrum acidification

Formic acid is available as an FDA approved food additive for livestock feed and drinking water (Code of Federal Regulations Title 21, 21CFR573.480, 2018) in clear liquid concentrated form and must be diluted to 10% with water before use as an acidifier. A 4 L aliquot of the cool pooled colostrum was acidified by slowly adding 10% formic acid while stirring vigorously until a pH between 4.0 to 4.5 was achieved. The volume of 10% formic acid required to reach the desired pH range was slightly variable but was between 40-45 ml/L and final concentration of formic acid in AC was $\leq 0.45\%$.

Colostrum Analysis

MC and AC were measured for immunoglobulin content by a colostrometer (Biogenics; Florence, OR) and a Brix refractometer (Cole-Parmer; Vernon Hill, IL) at the time of feeding i.e., immediately after pooling and processing of colostrum but within 12 h of the first calf in the pair being born. Measurement of pH also occurred at this time by means of Hydrion™ plastic indicator strips dipped into samples and read according to manufacturer's instructions (Micro Essential Laboratory Inc.; Brooklyn, NY).

The concentration of IgG in refrigerated MC and AC was quantitatively determined spectrophotometrically within 48 h of initial processing using a commercially available turbidimetric immunoassay (TIA) validated for bovine colostrum IgG (DVM Rapid Test™ II, Value Diagnostics™, MAI Animal Health; Elmwood, WI). Compared to the reference method of radial immunodiffusion (RID), performance of the same manufacturer's equine TIA test kit for measurement of immunoglobulins was good in quantifying equine serum IgG [32]. While equivalent TIA validation studies for bovine serum and colostrum IgG were not found, it is reasonable to expect that this methodology would perform just as well for measurement of bovine IgG.

Microbiological analysis of colostrum included counts (cfu/mL) for total bacteria, total coliforms, and generic *Escherichia coli*. A 30 ml aliquot of frozen MC and AC were shipped to the collaborating laboratory where they were thawed and inoculated onto aerobic and *E. coli* Petrifilm™ as described by the manufacturer (3M US; Minneapolis, MN). Colostrum was diluted in Butterfield's Buffer (3M US; Minneapolis, MN) at undiluted, 1:10, 1:100, 1:1,000, 1:10,000 and 1:100,000 dilutions before 1 mL aliquots of each dilution were plated onto room temperature aerobic and *E. coli* Petrifilm™. The film was placed onto the product agar and the sample distributed using a plate spreader. The agar was allowed to solidify for 1 min and then incubated at $32 \pm 2^\circ\text{C}$ for 48 ± 2 h. As a control, buffer alone was inoculated as described above to confirm diluent sterility. For total aerobic count, red colonies were enumerated from the aerobic Petrifilm™. For the total coliform count, all red and blue colonies with gas were enumerated from

the *E. coli* plate. For the *E. coli* count, only blue colonies with gas were enumerated from the *E. coli* Petrifilm™ and reported as colony forming units per mL. For full methodological description of measurement of colostrum neutralizing titers see Serum Analysis section below.

Serum Analysis

Serum samples were examined for total protein (TP, day 3 only), IgG concentration, serum neutralizing titers (SNTs) against IBRV, BVDV Types 1 and 2, and SNTs against five common serovars of *Leptospira* spp. (*canicola*, *grippotyphosa*, *hardjo*, *icterohaemorrhagiae* and *pomona*). Measurement of serum TP was by refractometry (Reichert®, Reichert Analytical Instruments; Seefeld, Germany).

The concentration of IgG in serum was determined spectrophotometrically by turbidimetric immunoassay for bovine serum/plasma analogous to the technique described above for colostrum. Apparent efficiency of IgG absorption (AEA) was calculated using the following equation [33, 34]:

$$AEA = \left\{ \frac{\text{Serum IgG concentration (g/L)} \times \text{BW (kg)} \times 0.09}{\text{Colostrum IgG concentration (g/L)} \times 4} \right\} \times 100$$

Where 0.09 = estimated plasma volume as % of body weight and 4 = the volume of colostrum administered in L; colostrum IgG concentration x volume fed = IgG intake in g.

Neutralizing titers were determined by virus neutralization (VN) assays to detect serum and colostrum antibodies directed against IBRV, and BVDV Types 1 and 2. After heat inactivation at 56°C, sera were diluted in duplicate across 96-well flat-bottomed microtiter plates (Corning Life Sciences DL; Corning, NY), resulting in dilutions ranging from 1:2 up to 1:4096. Diluted samples were incubated with an equal volume of virus at 200 x TCID₅₀ (tissue culture infectious dose 50) per 50 µl for 60 min. Viruses used included IBRV and cytopathic BVDV Types 1 and 2. A control plate was included in each run and contained known positive and negative sera, viral back-titer, and cell control. After initial incubation, a suspension of Madin-Darby bovine kidney (MDBK) cells was added to all wells. A further wash step was added for colostrum samples after 1 h. All plates were then incubated at 37°C and 5.5% CO₂ for 3 days. Plates were read on an inverted microscope. Endpoint titer was determined as the dilution at which neutralization of virus ended, as detected by viral cytopathic effect (CPE).

Serum neutralizing titers against leptospiral serovars were determined using *Leptospira interrogans* serovar *canicola* (strain Hond Utrecht IV), serovar *grippotyphosa* (strain Andaman), serovar *hardjo* (strain Hardjoprajitno), serovar *copenhageni* (serogroup Icterohaemorrhagiae; strain M20), and serovar *pomona* (strain Akiyami A) which were cultured and subcultured weekly in 10 mL of polysorbate 80-bovine albumin liquid broth (P80-BA, National Veterinary Service Laboratories; NVSL, Ames, IA) incubated at 28 ± 2°C. Weekly, approximately 100 µL was cultured on a blood agar plate (Becton Dickinson; Franklin Lakes, NJ) and grown at 35 ± 2°C for 48 h to check for contamination. Antigen for the *Leptospira* microagglutination (LeptoMAT) test were prepared as follows. *Leptospira* cultures were centrifuged at 600x g for 10-15 min, supernatant was transferred to an 18 x 150 mm glass tube and analyzed for transmittance using a spectrophotometer (Spectronic 20D, Milton Roy; Rochester, NY). Antigen density must range between 60 – 70% transmittance at a single-beam wavelength of 400 nm and can be adjusted using sterile P80-BA. Homologous reference positive control serum (NVSL) for each antigen/serovar was used at a 1:50 dilution in 0.01 M phosphate-buffered saline (PBS, pH 7.2 – 7.6) to confirm the specificity of the antigen. Serum to be tested was diluted initially 1:50 in PBS (50 µL) and serially diluted to 1:102,400 as needed in a flat-bottom 96-well microtiter plate. 50 µL of each antigen was added to each well of diluted sera or homologous reference positive control sera creating a 1:100 dilution in the first sample well as well as for the positive control serum. Micro-titer plates were incubated 1 h and 20 min to 2 h at room temperature. Each micro-titer plate was observed under the dark-field microscope (DM1000, Leica; Wetzlar, Germany) using 20x magnification with an indirect light source and the agglutination between antibodies and the free-swimming *Leptospira* was observed. Agglutination was semi-quantitatively assessed using the following metrics: 1) negative is no agglutination, 2) trace is 1-24% agglutination and up to 75% free-swimming *Leptospira*, 3) +1 agglutination is 25-49% agglutination, 4) +2 agglutination is 50-74% agglutination, 5) +3 agglutination is 75-99% agglutination and 6) +4 agglutination is 100% agglutination with no free-swimming *Leptospira* observed. Positive samples (2+, 3+, and 4+ agglutination) are reported according to their end-point titer. Reference positive control serum must obtain agglutination (+2, +3 or +4 agglutination) with the homologous serovar in at least the 1:3,200 dilution for the test to be valid. The negative control containing just *Leptospira* and PBS must be negative for each serovar.

Statistical Analysis

Prior to the start of the study a power calculation was performed focusing on the ability to detect differences in titers between groups. The criteria for the power calculation assumed only three time-sampling points and were set to detect a minimum titer difference of 1:291 between the MC and AC groups with alpha = 0.05 and power = 100. Data generated by the study were analyzed using STATA 17.0 (Stata Corp., College Station, TX). Descriptive statistics (mean, standard deviation, median, range, and interquartile range [IQR 25%-75%]) were calculated for all outcome variables. Normality was assessed by a Shapiro Wilk test. A t-test was used to evaluate associations between feeding MC or AC and outcome variables where data were normally distributed. Non-normal data were checked with a series of transformations but could not be normalized, hence the Wilcoxon rank sum or Kruskal Wallis tests, which make no assumptions about data distributions, were used to examine untransformed continuous or interval outcome variables, respectively. For serum IgG concentrations, regression analysis was used to compare the effect of MC and AC over the entire time course of days 3-180. Because a large number of SNT outcomes were below the detection limit of the various neutralization assays, where appropriate tobit regression was used. The latter estimates relationships between variables when upper and lower censoring occurs such as with tests

that have both lower and upper detection limitations. Inclusion of calf as a fixed effect and robust variance estimation were used to control for clustering in all regressions. Statistical significance was inferred when $P < 0.05$.

Abbreviations

IgG
immunoglobulin G
MC
untreated maternal colostrum
AC
formic acid acidified maternal colostrum
IBRV
infectious bovine rhinotracheitis virus
BVDV
bovine viral diarrhea virus
cfu
colony forming units
SD
standard deviation
AEA
apparent efficiency of IgG absorption
SNT
serum neutralizing titer(s)
FDA
United States Food and Drug Administration
NVSL
National Veterinary Service Laboratories
PBS
phosphate-buffered saline
IQR
interquartile range 25th -75th percentile.

Declarations

Ethics approval and Consent to participate

All procedures were conducted in accordance with the US Department of Agriculture's Animal Welfare Act and Animal Welfare Regulations, with the full informed consent of the animal owners, with approval from institutional review committees (Privately Owned Animal Protocol Committee and the University of Pennsylvania's Institutional Animal Care and Use Committee) and conducted under authority of IACUC protocol # 806697. The study and all methods are reported in accordance with ARRIVE guidelines.

Consent for publication

Not applicable.

Availability of data and materials

Open Science Framework (OSF) repository, accession number DOI 10.17605/OSF.IO/3XNZQ at the publicly accessible link <https://osf.io/3xnzq/>

Competing interests

The authors state that no conflicts exist.

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Authors' contributions

BIS was responsible for the whole project, generated the working hypothesis, worked with the collaborating laboratories for sample analysis, obtained financial support and participated equally with HWA in drafting, revising, and finalizing the manuscript.

SVC participated in study design, was responsible for on-farm sample and data collection and daily conduct of the trial, revised the initial draft of the manuscript.

HWA assisted with study design, conducted the statistical analysis and participated equally with BIS in drafting, revising, and finalizing the manuscript.

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Tables

Table 1
Calf and colostrum characteristics for groups receiving 4 L of untreated (MC) or acidified (AC) colostrum

Parameter	Untreated Colostrum (n = 15)	Acidified Colostrum (n = 15)
Birth Weight (kg)	40.6 (2.4)	40.7 (2.9)
Colostrometer (mg/mL)	103 (21)	103 (21)
Colostrum Brix (%)	24.8 (3.1)	24.6 (3.2)
Colostrum pH (post-treatment) [†]	6.8 (6.5-7.0)	4.3 (4.3-4.5)*
Colostrum IgG (g/L)	93.3 (39.7)	101.9 (36.7)
Serum Total Protein (day 3, mg/dL)	6.5 (0.4)	6.3 (0.7)
Apparent Efficiency of IgG Absorption (AEA day 3, %) [†]	24.3 (14.7-30.0)	22.6 (13.6-28.9)
[†] All values are shown as mean and standard deviation (SD) except for colostrum pH and AEA which were not normally distributed and are shown as median and interquartile range (IQR, 25-75%).		
* pH of acidified colostrum was significantly lower than untreated colostrum $P < 0.001$, Wilcoxon rank sum.		

Table 2
Colostrum and serum viral neutralizing titers over time for IBRV and BVD

Source	Day	IBRV Titer		BVDV Type-1 Titer		BVDV Type-2 Titer	
		Median (IQR*)		Median (IQR*)		Median (IQR*)	
		Untreated (n = 15)	Acidified (n = 15)	Untreated (n = 15)	Acidified (n = 15)	Untreated (n = 15)	Acidified (n = 15)
Colostrum	1	256 (64-1024)	256 (128-512)	8192 (4096-8192)	8192 (8192-8192)	8192 (4096-8192)	8192 (8192-8192)
Blood (serum)	1	1 (1-1)	1 (1-1)	1 (1-1)	1 (1-1)	1 (1-1)	1 (1-1)
	3	32 (16-128)	32 (16-128)	1024 (512-2048)	1024 (512-4096)	2048 (1024-2048)	4096 (1024-4096)
	30	8 (8-32)	16 (8-32)	256 (128-256)	512 (256-1024)	512 (64-512)	1024 (512-2048)
	60	8 (4-16)	8 (4-16)	64 (64-128)	128 (64-256)	128 (64-512)	256 (64-1024)
	90	2 (2-4)	4 (1-4)	32 (8-32)	32 (16-64)	32 (16-64)	64 (16-128)
	120	1 (1-2)	1 (1-2)	4 (2-8)	8 (4-16)	8 (4-16)	16 (8-64)
	150	1 (1-1)	1 (1-1)	2 (1-2)	2 (2-4)	4 (1-8)	4 (1-16)
	180	1 (1-1)	1 (1-1)	1 (1-2)	1 (1-1)	1 (1-2)	2 (1-4)
* IQR = Interquartile Range 25-75%							

Figures

Figure 1

Effect of acidification of colostrum on A) total and B) coliform bacterial counts (n = 15 per group)

Data are plotted as mean ± standard error but were analyzed using non-parametric methods which make no assumptions about data distributions.

* Total coliform count was significantly reduced in acidified compared to untreated colostrum $P = 0.022$, total bacterial count was also reduced but the effect was not statistically significant; Wilcoxon Rank Sum Test.

Figure 2

Box and whisker plot of median serum immunoglobulin (IgG) levels over time

Calves were fed 4 L of untreated (MC; n = 15) or formic acid acidified (AC; n = 15) colostrum at birth; the blood sample on day 1 was collected immediately prior to colostrum feeding