Applied Animal Science 36:118–123
https://doi.org/10.15232/aas.2019-01928

PRODUCTION AND MANAGEMENT: *Short Communication*

Short Communication: Bovine parainfluenza-3 antibodies in veal calves supplemented with cinnamaldehyde or lactoferrin

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ABSTRACT

Objective: Our objective was to investigate the effects of 2 immune modulatory compounds, cinnamaldehyde and lactoferrin, on potentiating the immune response to bovine parainfluenza-3 virus (bPI3) vaccination.

Materials: On arrival to the growing facility, veal calves were randomized to 1 of 3 treatments: control (no supplement), lactoferrin (1 g/d in milk replacer for 7 d), or cinnamaldehyde (1 g/d in milk replacer for 21 d). Plasma anti-bPI3 IgG response was assayed by indirect ELISA before (d 0) and 28 d after vaccination. Antibody titers were represented as sample-to-positive ratio, and a mixed linear regression analysis was used to compare titers between treatments, cohorts, and d 0 versus d 28.

Results and Discussion: Stress and crowding during transit leaves veal calves susceptible to respiratory viral pathogens. Bovine parainfluenza-3 virus is a principle etiological agent of bovine respiratory disease complex. Prophylactic antibiotic administration is a common practice to prevent diseases; however, this can lead to antibiotic resistance. Therefore, research on antimicrobial alternatives is warranted. Antibody titers against bPI3 were different between d 0 and 28 ($P < 0.0001$), but no effect was observed between treatments ($P = 0.21$) or treatment by day ($P = 0.74$); however, antibody titers were different from d 0 and 28 between cohorts ($P = 0.01$).

Implications and Applications: Antibodies detected are suspected to be of maternal origin. Further research is necessary to determine an optimal vaccination schedule to overcome maternal immunity and to estimate mucosal IgA antibodies. In addition, an appropriate dose, duration, and route of administration of cinnamaldehyde and lactoferrin must be considered.

Key words: feed additives, vaccine, immune response, stress

INTRODUCTION

Bovine respiratory disease (**BRD**) is a disease complex caused by numerous viral and bacterial pathogens in domestic cattle. Bovine parainfluenza-3 (**bPI3**) virus is a primary etiological agent in the BRD complex. The bPI3 virus is endemic in domestic cattle populations worldwide (Kapil and Basaraba, 1997; Ellis, 2010). Infection of bPI3 alone induces mild to moderate respiratory disease, although co-infection of other viruses and secondary bacterial infection can worsen the disease state. One study reported the association of BRD in veal calves to the level of immunoglobulin at their introduction to feedlot (Delabouglise et al., 2017).

Veal is a by-product of the dairy industry harvested from male calves. In the United States, veal is marketed as either bob (harvested at 2 to 10 d of age; Wilson et al., 2000) or special fed (harvested at 16 to 20 weeks; Terosky et al., 1997). Transportation from the dairy farm to growing facilities soon after birth exposes these young calves to stressors, such as transport, crowding, mixing with other calves, and nutrient deprivation, which increase disease risk (Taylor et al., 2010). Approximately 43% of veal calves have inadequate maternal immunity (Wilson et al., 2000), and veal calves with low immunoglobulins are more susceptible to respiratory disease (Pardon et al., 2015). In vivo prevalence of respiratory disease in veal calves is observed to be less than 7% (Brscic et al., 2012). However, postmortem prevalence of mild to moderate and severe respiratory disease has been reported to be 13.9 and 7.7%, respectively, and 21.4% of lungs showed signs of pleuritis (Brscic et al., 2012). Such disease results in decreased growth and lower carcass weight at slaughter, contributing to economic losses (Pardon et al., 2013). Prophylactic antibiotic therapy is common in veal production to reduce the risk of respiratory disease by combatting secondary bacterial infection (Renaud et al., 2017). How-

The authors have not stated any conflict of interest.
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ever, the overuse of antibiotics contributes to the evolution of antibiotic resistant bacteria. Therefore, finding alternatives to antimicrobial therapy that may enhance immunity to BRD pathogens and maintain antimicrobial efficacy is imperative.

Two antimicrobial alternatives, cinnamaldehyde and lactoferrin, have been studied in cattle. Cinnamaldehyde is a constituent of essential oils extracted from cinnamon plants, having antimicrobial and immunomodulatory properties (Upadhyay, 2010; De Cássia da Silveira e Sá et al., 2014). Studies of cinnamaldehyde in cattle have focused on nutrient metabolism, growth, milk production, and rumen microbiology (Chaves et al., 2008; Yang et al., 2010; Compiani et al., 2013; Vakili et al., 2013). Lactoferrin possesses a spectrum of biological properties including immunomodulatory and antimicrobial effects (Tomita et al., 2009; Abril Garcia-Montoya et al., 2012). Calves supplemented with lactoferrin had increased serum IgG response (Prgomet et al., 2007). In contrast, in some studies calves supplemented with lactoferrin had no improvement in health and performance (Robblee et al., 2003; Cowles et al., 2006; English et al., 2007; Habing et al., 2017), respiratory scores (English et al., 2007), or serum IgG levels (Dawes et al., 2004).

Thus, the objective of this study was to determine the effect of cinnamaldehyde and lactoferrin on the immune response (plasma bPI3-specific IgG) to vaccination in veal calves upon arrival to the growing facility. We hypothesized that veal calves supplemented with cinnamaldehyde or lactoferrin would have a greater increase in bPI3 antibody titers relative to calves that did not receive supplementation.

MATERIALS AND METHODS

Animals and Experimental Design

A total of 240 Holstein bull calves (approximately 3 to 7 d of age; actual age unknown) were enrolled in a randomized, controlled field study, in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee (Animal Use Protocol: 2015A00000131) of The Ohio State University. Calves were randomized to treatment upon arrival at the growing facility and remained in the study until 28 d after arrival. Calves arrived at the farm in 2 cohorts, and each cohort was housed in a different barn at the same facility (n = 120 per barn; cohort 1 and cohort 2). Calves were randomized by the authors using a complete block design (Microsoft Excel, Redmond, WA) into 1 of 3 treatment groups (n = 80 calves per treatment, 40 calves per treatment in each cohort): control (no supplement, **CON**), cinnamaldehyde (**CIN**; Healthy Aging, Columbus, IN), and lactoferrin (**LAC**; The Tatua Co-operative Dairy Company Ltd., Morrinsville, New Zealand). Calves receiving either cinnamaldehyde or lactoferrin were given 1 g/d manually mixed into the milk replacer at the evening feeding. Calves in the CIN group were supplemented for 21 d, and calves in the LAC group were supplemented for 7 d. Dose and duration of cinnamaldehyde supplementation was based on manufacturer recommendation (Healthy Aging). Dose and duration of lactoferrin supplementation was based on a previous study (Robblee et al., 2003). All calves were housed in individual wooden stalls (2.13 m × 0.61 m) with slatted flooring (Tenderfoot, Tandem Products Inc., Minneapolis, MN) and removable metal dividers with horizontal partitions that allowed visual and physical contact with neighboring calves. On arrival to the farm all calves, regardless of treatment, were given an electrolyte solution containing sulfamethoxazole. All calves were fed milk replacer (**MR**; 22% protein, 18% fat) twice per day (0500 and 1700 h) for the duration of the study. Starting at 220 g of MR powder reconstituted to 1.47 kg of MR per calf per feeding, milk replacer was gradually increased, and by 10 wk, calves got 709 g of MR powder reconstituted to 4.99 kg of MR per calf per feeding (9.98 kg/d).

Vaccination and Sampling

Blood samples were collected in 10-mL vacuum tubes containing EDTA liquid additive (Monoject Blood Collection Tubes, Covidien, Mansfield, MA) by jugular venipuncture at arrival to the facility just before vaccination (d 0) and 28 d after vaccination. Samples were immediately placed on ice and processed within 2 h of collection. Blood samples were centrifuged at 1,180 × *g* at 4°C for 15 min, 2 mL of plasma was decanted, and aliquots were stored in microcentrifuge tubes at −20°C until further use. All calves were vaccinated for bovine herpesvirus-1, bovine respiratory syncytial virus, and bPI3 with a commercial, trivalent modified-live vaccine by intranasal route (Inforce 3, Zoetis, Parsippany, NJ) on d 0 following blood collection.

Virus Culture

The SF-4 strain of bPI3 was procured from BEI resources (Manassas, VA). The virus was propagated in Vero cells as described previously (Durham and Hassard, 1990) with minor modifications. Briefly, cells were grown in T-175 flasks incubated at 37°C with 5% CO_2. Growth media for Vero cells contained Dulbecco's modified essential medium, 10% fetal bovine serum, 1% antibiotic-antimycotic, and 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (1 *M*). Infection media consisted of growth media with 5% lactalbumin hydrolysate and sterilized by a 0.2-μm liquid filter. Cells were inoculated with bPI3 at 0.1 multiplicity of infection using the stock virus 4.53 log tissue culture infective $dose_{50}$/mL to 50 to 60% confluent Vero cells monolayer. Virus titer was determined using the Reed-Muench method (Reed and Muench, 1938). After inoculation, culture supernatant was harvested once a visible cytopathic effect was observed. The virus-containing supernatant was collected every 48 h thereafter and replaced with fresh medium until the monolayer had been

destroyed. Collected supernatant was clarified by centrifugation at 600 × *g* for 20 min. The cell pellet was returned to the culture flask. Supernatant was pooled and frozen at −80°C until further use. Thawed virus supernatant from infected Vero cell cultures was subject to ultracentrifugation at 23,500 rpm (107,000 × *g*) with 20% sucrose cushion at 4°C for 3 h. The resulting pellet was soaked in 1× PBS overnight to dissolve. The solution was then sonicated at 40 kHz with 0.7-second pulse rate to dissociate protein aggregates. Protein estimation of the antigen was performed by Micro-BCA Assay (Bio-Rad, Hercules, CA).

ELISA

An indirect ELISA was standardized by checkerboard titration to measure anti-bPI3 IgG antibodies in calf plasma samples (CON: n = 78; CIN: n = 77; LAC: n = 78) as described previously (Durham and Hassard, 1990). Ten plasma samples (d 0: n = 2; d 28: n = 8) were screened by a diagnostic laboratory (Prairie Diagnostic Services, University of Saskatchewan, Canada) to determine the presence of bPI3 antibodies. Negative and moderate to high positive samples were used to validate the assay. Fetal bovine serum served as the negative control. Before plating, coating antigen was inactivated by 56°C water bath for 15 min (Marston and Vaughan, 1960; Singh and Cicy, 1967) and sonicated at 40 kHz with 0.7-second pulse rate. Polystyrene 96-well plates were coated with 25 μg/mL pretitrated antigen at 50 μL per well and incubated overnight at 4°C. Plates were washed and blocked with 5% skim milk powder containing 0.05% Tween-20 and incubated for 2 h at 4°C. Plates were washed using PBS 0.05% Tween-20. Plasma samples were added in duplicate wells at 1:25 dilution in 2.5% skim milk 0.05% Tween-20 and incubated for 2 h at room temperature. After washing, anti-bovine IgG secondary antibody HRP conjugate was added at pretitrated 1:5000 dilution in 2.5% skim milk solution containing 4% polyethylene glycol (6,000 MW) and incubated at room temperature for 2 h. Plates were washed, and a 1:1 mixture of 3,3′,5,5′-tetramethylbenzidine peroxidase and solution B peroxidase solution was added to each well and incubated for 30 min at room temperature. The reaction was stopped using 1 *M* phosphoric acid. Absorbances were read using an ELISA reader at 450 nm. Antibody titers were expressed as sample-to-positive ratio and calculated as follows:

$$(\text{net mean sample absorbance} - \text{net mean negative absorbance})/(\text{net mean positive absorbance} - \text{net mean negative absorbance}).$$

The negative absorbance was determined by averaging the absorbance values from negative controls in all plates assayed. The positive value was determined by averaging 10 highest net mean positive sample absorbance values from among the test samples assayed. Net means were calculated by subtracting the blank absorbance (antigen only) from all other values.

Statistical Analysis

Antibody titer data were not normally distributed, so the data were log-transformed before analysis. A mixed linear regression model (PROC MIXED, SAS v. 9.4, SAS Institute Inc., Cary, NC) with a random intercept was used to compare antibody titers of calves on each treatment; the model included treatment (CON vs. LAC vs. CIN), cohort (cohort 1 vs. cohort 2), day (d 0 vs. 28), cohort-by-day interaction, and cohort-by-treatment interaction. Calf was included as the subject. The sample-to-positive ratios are expressed as back-transformed LSM with 95% CI. Significance was determined at $P < 0.05$.

RESULTS AND DISCUSSION

There was no difference in bPI3-specific IgG between treatments (Table 1). However, bPI3-specific IgG differed by day, with higher antibody titers observed on d 0 compared with d 28. There was also a difference between cohorts, and there was a cohort by day interaction.

The difference in anti-bPI3 IgG titers between d 0 and 28 may indicate a lack of seroconversion in young veal calves; however, previous research has demonstrated a lack of seroconversion in calves with high serum neutralizing antibodies when vaccinated intranasally (Ellis et al., 2013). A typical humoral response in seronegative calves produces a 4-fold or greater increase in antibodies about 2 to 4 wk after antigen exposure (Ghram et al., 1989; Peters et al., 2004; Vangeel et al., 2009; Socha et al., 2013). The difference in this study may also be attributed to maternal antibody interference, a known phenomenon in calves (Chamorro et al., 2016). The pattern of maternal antibody decay in this study is similar to an earlier report (Dawson, 1966). Antibodies in offspring acquired from the mother can neutralize the antigen in a vaccine, preventing a serological response. Calves vaccinated in the presence of maternal antibodies have a 65% seroconversion rate when vaccinated with a live vaccine and 50% seroconversion rate with a killed vaccine, compared with a 100% seroconversion rate in calves without maternal antibodies (Van Donkersgoed et al., 1991). When administered the first vaccine dose in the presence of maternal antibodies, calves exhibit a greater antibody response when given a second dose compared with calves that received the first vaccine dose after maternal antibodies waned (Xue et al., 2010). Extrapolation of bovine respiratory syncytial virus, bovine herpesvirus-1, and bovine viral diarrhea virus have demonstrated induction of T-lymphocyte response and memory B-lymphocyte response when vaccinated in the face of maternal antibodies despite lack of seroconversion (Chamorro et al., 2016). Such cell-mediated immune response analyses should be considered in future investigations.

The management history of the calves in this study before arrival at the veal facility was unknown. Dairy producers in northeast Ohio were informally surveyed in person to gauge the practices associated with male dairy calves, including whether colostrum was fed, the amount of colostrum fed, preventive treatment, whether dams were vaccinated, and time of vaccination of dams. No standard practices were reported. Male calves may or may not receive colostrum or preventive disease treatment. Vaccination of dams also varied, either once annually or 6 wk before parturition, which may contribute to variation in the bPI3 antibodies in colostrum. The degree of failure of passive transfer by measuring total protein in these calves was determined previously (Pempek et al., 2017); 6% of calves had failure of passive transfer (<5.5 g/dL). Inconsistencies in management history may explain differences in anti-bPI3 IgG in these calves and contribute to the observed difference between cohorts in the present study. Future studies are needed to investigate the influence of postnatal management on bPI3-specific IgG in veal calves.

Supplementation of cinnamaldehyde or lactoferrin in veal calves after arrival to the growing facility did not influence bPI3 antibody titers, in contrast to our hypothesis. Serum neutralizing antibodies to bovine herpesvirus-1 in adult beef cattle fed an essential oil combination containing cinnamaldehyde, eugenol, and capsicum resulted in greater titers in the treated cattle versus control throughout the experimental period (Compiani et al., 2013). Other experimental models, both in vitro and in vivo, have demonstrated immunomodulatory functions of cinnamaldehyde, predominantly anti-inflammatory effects (De Cássia da Silveira e Sá et al., 2014). Cinnamaldehyde exerts inhibitory effects on mast cell activation through several signaling pathways (Hagenlocher et al., 2015) and inhibits NF-κB activation in macrophages in vitro (Reddy et al., 2004). Based on prior research on cinnamaldehyde, it may be expected that, at the optimal dose, duration and route of administration, pulmonary inflammation, and shedding of bPI3 will be decreased. The vaccine-induced humoral response in veal calves in our study was likely interfered by passive immunity. Without other immunologic measures, it is difficult to conclude whether cinnamaldehyde supplementation played any role.

Studies investigating the effects of lactoferrin against respiratory disease pathogens are limited. The effects of lactoferrin on systemic and local gastrointestinal immune function revealed total serum IgG was greater in calves supplemented with lactoferrin than control (Prgomet et al., 2007), but pro- and anti-inflammatory cytokine mRNA profiles varied by time point in lactoferrin-treated calves. When IgG was only assayed at 1 and 9 d of age, there was no difference in serum IgG concentrations in lactoferrin-treated versus nontreated calves (Dawes et al., 2004). Lactoferrin cannot pass through the gut barrier unless administered immediately postpartum (Dawes et al., 2004). When given orally, it is not absorbed into the blood stream but rather retained in the GI tract, and there, it exerts immunostimulatory (Tomita et al., 2009) and antimicrobial (van Hooijdonk et al., 2000; Abril Garcia-Montoya et al., 2012) effects. A study revealed that lactoferrin did not enhance IgG uptake in calves during the first 24 h and any intestinal development tested on the second day of life (Connelly and Erickson, 2016). Lactoferrin's potential to enhance respiratory immunity needs further investigation.

Future research should focus on understanding and overcoming maternal interference of vaccination to induce a humoral response influenced by feed supplements. Mea-

Table 1. Antibody titers of veal calves expressed as sample-to-positive ratio (±95% CI) by treatment (control, CON; lactoferrin, LAC; cinnamaldehyde, CIN), treatment × day, and cohort × day

Effect	Cohort	Treatment	Day	n	LSM[1]	95% CI	*P*-value
Treatment	—	CIN	—	154	1.40 (0.34)	1.36 to 1.45	0.21
	—	LAC	—	156	1.43 (0.36)	1.39 to 1.47	
	—	CON	—	156	1.48 (0.39)	1.44 to 1.52	
Cohort × day	1	—	0	117	1.26 (0.46)	1.22 to 1.30	0.01
	1	—	28	117	1.55 (0.32)	1.51 to 1.58	
	2	—	0	116	1.38 (0.44)	1.34 to 1.42	
	2	—	28	116	1.59 (0.23)	1.55 to 1.63	
Day × treatment	—	CIN	0	77	1.29 (0.42)	1.24 to 1.34	0.74
	—	LAC	0	78	1.31 (0.44)	1.26 to 1.36	
	—	CON	0	78	1.35 (0.49)	1.30 to 1.40	
	—	CIN	28	77	1.52 (0.26)	1.47 to 1.57	
	—	LAC	28	78	1.56 (0.27)	1.51 to 1.61	
	—	CON	28	78	1.62 (0.30)	1.58 to 1.67	

[1]Data where natural log-transformation was applied. The back-transformed LSM are presented with the log-transformed data in parentheses.

suring antibodies after calves receive a second vaccine dose is also needed. Other immunological measures also need to be considered, such as virus-specific lymphocyte response, mucosal antibody titers, and cytokines. Virus shedding and lung lesion scoring would be valuable to determine antiviral and anti-inflammatory effects in the respiratory tract. Studying adjunct supplementation of cinnamaldehyde and lactoferrin for potential synergy is also warranted. As such, based on our results, it is not recommended at this time to supplement veal calves with cinnamaldehyde or lactoferrin to enhance immunity to bPI3.

APPLICATIONS

In conclusion, we did not observe any effects of cinnamaldehyde or lactoferrin on vaccine-induced bPI3 antibody production in veal calves. Contrasting results were observed between cohorts, possibly due to seroconversion in cohort 1 at d 28 or differences in cohort history, though both cohorts had a similar pattern to the overall results. Though our results are not positive in inducing the expected antibody response to feed supplements, there are many confounding factors that may contribute to these study results, such as being conducted under field conditions as opposed to in a controlled laboratory.

ACKNOWLEDGMENTS

We thank Buckeye Veal Services LLC for allowing us to use their animals and facilities for this study. This work was supported by a competitive grant from USDA Animal Health Formula Funds (Grant No. 2015-16). Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University.

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