Brief Communication

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Enhanced Bactericidal Activity against *Escherichia coli* in Calves Fed *Morinda citrifolia* (Noni) Puree

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Background: Although adequate colostrum intake and properly used antibiotics can provide much protection for the bovine neonate, increased antibiotic scrutiny and consumer demand for organic products have prompted investigations of natural immunomodulators for enhancing calf health. One plant-based immunomodulator, *Morinda citrifolia* (noni) fruit, is a well-recognized natural product that has a broad range of immunomodulatory effects.

Hypothesis: Neonatal calves fed noni puree would demonstrate whole blood phagocytic capacity in Gram-negative and Gram-positive in vitro assays.

Animals: Blood samples from 18 neonatal Holstein bull calves.

Methods: Calves were divided into 2 groups: Group 1 comprised control calves, whereas Group 2 received 30 mL of noni puree twice a day in milk replacer. Day 0 blood samples were obtained between 36 and 48 hours of age before the first feeding of puree. Ethylenediaminetetraacetic acid anticoagulated blood was collected from each calf on days 0, 3, 7, and 14. Bactericidal assays were performed to estimate the percentage killing of *Escherichia coli* and *Staphylococcus epidermidis*.

Results: Blood samples from noni puree-fed calves displayed significantly more *E. coli* bacterial killing than did controls on day 14, and although differences were not significant on days 0, 3, and 7, bacterial killing progressively increased over time. There was no significant difference between the groups for *S. epidermidis* killing.

Conclusions and Clinical Importance: The immunomodulatory effect of noni puree may prove valuable in the future as production animal antibiotic use becomes more restricted. Additional clinical trials are warranted to investigate the clinical application of noni puree in promoting calf health.

Key words: Immunomodulator; Neonatal; Phagocytic assay; Production medicine.

The importance of adequate passive transfer (APT) of immunoglobulins in minimizing calf morbidity and mortality in the first few weeks of life is well established. Most recently, it has been indicated that APT of the dam's immunoglobulins, colostral leukocytes, and cytokines is important to neonatal adaptive and innate immunity. Despite the immunomodulatory effect of APT in the health and development of the neonate, developmental immaturity of the immune system remains an important factor in increased susceptibility to infectious diseases. These factors include decreased antibody production, decreased secretion of cytokines, and decreased neutrophil function.[1]

One management strategy to reduce morbidity and mortality in neonates, especially in colostrum-deprived or -deficient calves, has been the administration of antibiotics in milk replacer or by injection. However, increased scrutiny of antibiotics in animal production and increased consumer demand for organic products have prompted investigation of plant-based immunomodulators to enhance calf health and production.

The development and validation of a safe, effective, organic, and relatively inexpensive immune modulator for preweaned dairy replacement calves has potential economic benefits for the producer as well as for the broader dairy and beef industries by producing more cost-efficient products that are more marketable.[2] In this context, immunomodulators have attracted interest as potential supplements to enhance the immune defense of neonates, with a suggested role as immunomodulators that may decrease morbidity and mortality. Their increased use could slow the development of antibiotic-resistant pathogens, enhance calf health, and provide safer products for human consumption as well.

One of the effects of some immunomodulators is to enhance immune stimulation during the innate immune response to bacterial pathogens. The health of young calves with an immature immune system depends on rapid and potent innate immunity. Therefore, modulation of innate immunity may substantially impact a calf's ability to respond to a wide spectrum of pathogens. Interestingly, nutritional supplementation by itself, although having a positive effect on increased neonatal calf growth rate, has not increased adaptive immune responses and actually leads to decreased lymphocyte viability.[3] Several types of immunomodulators have been identified and tested, including peptides, lipopolysaccharides (LPSs), glycoproteins, lipid derivatives, proteins, and other substances isolated from microorganisms, some of which have failed to produce clinically relevant effects in the clinical setting. Immunomodulators from botanical sources may be a novel alternative given their proposed immunomodulatory effects, relatively low toxicity, and bioavailability. Juice made from the *Morinda citrifolia* fruit (noni) is a well-recognized natural herbal product

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that reportedly has a broad range of immunomodulatory effects, including antibacterial, anti-inflammatory, antitumorigenesis, and antioxidant activity.[4–7] Among others, acubin, L-aperuloside, alizarin, and the recently discovered polysaccharide arabinogalactan are compounds within the noni fruit responsible for antibacterial activity.[8,9] The effect of arabinogalactan on macrophages, as shown in peritoneal murine cells, is based on mimicking the innate response of phagocytes to bacterial arabinogalactans.[9] For this experiment, a commercially available noni puree for bovine neonates[a] was used. The commercial product currently has a suggested feeding regimen spanning the first 21 days of life. The objective of this study was to evaluate the effect of feeding calves noni puree on bacterial killing in the first 2 weeks of life.

Materials and Methods

Animals

Animals for this project were obtained from 6 local dairies. The study and procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Wisconsin-Madison. Eighteen newborn Holstein bull calves that had already received 4.0 L of pooled colostrum by 12 hours of age arrived in pairs at the Veterinary Medical Teaching Hospital within 12 hours of birth and were housed in individual pens without nose-to-nose contact. The animals were assigned to treatment groups and housing in the order in which they were removed from the calf trailer by hospital personnel who had no further involvement with the study.

Upon arrival, every animal received a physical examination, followed by daily examinations, including temperature, ease of cough induction, fecal consistency, and presence and severity of ocular or otic abnormalities. Calf health evaluations, which were recorded as calf health scores (Table 1), were overseen by internists boarded in the American College of Veterinary Internal Medicine and blinded to treatment groups. Any calf receiving a total health score ≥ 5 for 3 consecutive days was treated by a veterinarian accordingly and subsequently removed from the study. APT (immunoglobulin [Ig]G > 1,000 mg/dL) was confirmed for all calves with the IgG Midland Quick Test Kit[b] at 24 hours of age.

Calf pairs consisted of 1 noni puree-fed and 1 control calf. Calves were bottle fed 2 L of CALF GLO nonmedicated milk replacer[c] reconstituted according to the manufacturer's label twice daily for the first 7 days and 2.5 L twice daily from days 8 to 14. Noni puree-fed calves received 30 mL of noni puree twice a day in milk replacer. Calves had access to 125 g calf starter and 4 L of fresh water per day.

Bactericidal Assay

Anticoagulated ethylenediaminetetraacetic acid whole blood was collected in vacutainer tubes[d] from the jugular vein of each calf on days 0, 3, 7, and 14. Day 0 samples were obtained from each calf between 36 and 48 hours of age and before the first feeding of puree. A modified in vitro bactericidal assay was performed using whole blood.[10] Briefly, isolates of *Escherichia coli* and *Staphylococcus epidermidis* from clinical cases of calf septicemia and mastitis, respectively, were inoculated onto trypticase soy agar with 5% sheep blood[e] and incubated at 37 °C for 18–24 hours by a technician blinded to treatment groups. Before assay, colonies of each bacterium were resuspended in tryptic soy broth (TSB). For each sample, an equal aliquot of blood was added to 500 μL Roswell Park Memorial Institute (RPMI) 1640[f] media + 50 μL bacteria (number of bacteria was determined by a 0.5 McFarland standard at 1.5×10^8 colony-forming units per milliliter [cfu/mL]). Bacterial growth controls were prepared by adding 50 μL diluted bacteria to 950 μL RPMI. Experimental samples and bacterial controls were incubated for 2 h at 37 °C. Ten-fold serial dilutions in TSB of the incubated samples and controls were made, plated in duplicate on blood agar plates, and incubated overnight at 37 °C, which yielded 5–500 colonies. Based on previously cited techniques and for consistency and accuracy, plates with 30 and 300 colonies were counted and adjusted for dilution, and cfu/mL were determined to calculate percentage killing.[10,11] If both plates contained between 30 and 300 colonies, they were averaged. To determine the percentage killing, the following formula was used:

$$\frac{\text{Bacterial control (cfu/mL)} - \text{sample (cfu/mL)}}{\text{Bacterial control (cfu/mL)}} \times 100 = \text{percent bacteria killed.}$$

Statistical Analysis

Data were analyzed by PROC UNIVARIATE and PROC FREQ in SAS.[g] The differences between the percentage killing for each calf pair (noni puree-fed − control) were computed for both *E. coli* and *S. epidermidis* bacteria at each of 4 time points (0, 3, 7, and 14 days). Normal quantile plots of the differences indicated that they were not normally distributed, and Wilcoxon–signed rank tests were used to compare the median differences between the groups. A Friedman's test was performed to determine whether the differences between noni and control groups differed over time. The experiment-wise error rate was controlled at the 5% level within each bacteria type using a Bonferroni *P*-value correction.

Results

Of the 9 calf pairs, only 3 pairs had differing health scores among calves on day 0. In 2 pairs, the score was 1 unit higher for the control calf, and in 1 pair it was 1 unit higher for the noni-fed calf. No calves were removed from the study because of health reasons.

The results indicated that the median difference in *E. coli* bacterial killing between noni puree-supplemented calves and control calves increased over time. Noni-sup-

Table 1. Calf health scoring criteria

Score	Temperature	Cough	Nasal Discharge	Eyes or Ears	Fecal Score
0	37.8–38.3 °C	None	Normal serous discharge	Normal	Normal
1	38.3–38.8 °C	Induce single cough	Small amount of unilateral, cloudy discharge	Small amount of ocular discharge	Semiformed, pasty
2	38.9–39.4 °C	Induced repeated cough or occasional spontaneous cough	Bilateral, cloudy, or excessive mucus discharge	Moderate amount of discharge from both eyes or slight ear drop	Loose but enough consistency to stay on bedding
3	≥ 39.4 °C	Repeated spontaneous coughing	Copious, bilateral, mucopurulent nasal discharge	Head tilt or both ears dropped	Watery, sifts through bedding

plemented calves showed significantly more killing power at day 14 compared with control ($P = .0215$). Although the added benefit of noni increased over time, the difference did not increase significantly (Friedman's test, $P = .0534$). There were no differences between the groups for *S. epidermidis* bactericidal activity at any of the time points or between time points (Fig 1).

Discussion

Although colostral leukocytes are essential in promoting neonatal immunity, their effects are far surpassed by a mature immune system. Menge et al[12] reported that neutrophils from neonatal calves have decreased phagocytic capacity against *E. coli* when compared with older calves. Consequently, efforts to improve phagocytic function in the first 14 days of life may help lower morbidity and mortality rates in pathogen-challenged neonates. Our results suggest that noni puree may help neonatal calves by enhancing the innate immune response. Noni puree supplementation was associated with significantly diminished *E. coli* growth but not a reduction in *S. epidermidis* bactericidal activity. This finding may have relevance in the bovine neonate because Gram-negative septicemia associated with failure of passive transfer and Gram-negative enteric diseases such as colibacillois and salmonellosis that may induce bacteremia are very important causes of morbidity and mortality.[13,14] Septicemia associated with *E. coli* infection and infection by specific invasive or pathogenic antigenic forms of enteric *E. coli* tend to occur during the first 7 days of life, and the results of our study indicated a significant difference only at day 14. The question remains as to whether this enhanced Gram-negative growth inhibition will occur early enough to be of clinical relevance in calves, and whether these ex vivo studies will translate into improved survival and decreased morbidity on the farm. Based on the preliminary results reported here, larger field studies are currently being conducted to investigate these questions. The predominance of Gram-negative infection in septicemic, enteric, and pneumonic disease during the first few weeks of life in calves should not be taken to imply a lack of importance for Gram-positive disease in calves. The lack of ex vivo bactericidal effect versus a marker Gram-positive species may have implications for general neonatal health as well as more specific relevance for umbilical infections and several Gram-positive contagious and environmental mastitis pathogens that can be acquired by calves in early life. The discrepancy between inhibition of Gram-negative growth and phagocytosis of Gram-positive bacteria and the precise mechanism of Gram-negative inhibition are not explained by the current study and also are important areas for further study.

Bactericidal activity of *E. coli* could occur by 3 distinct pathways: (1) pattern recognition of the LPS component of the outer membrane by pattern-associated molecular patterns, (2) direct phagocytosis of *E. coli* by neutrophils, and (3) helper T-cell recognition of *E. coli* antigens expressed on major histocompatibility complex class-II molecules by macrophages and B cells. Unlike the response observed with *E. coli*, calves supplemented with noni puree did not demonstrate enhanced *S. epidermidis* bactericidal activity ex vivo. Although the limited scope of this study precludes a definitive explanation, some plausible mechanisms should be noted. First, acquired colostral antibodies to LPS, by natural exposure or vaccinal stimulation of the dam, may have had a positive interaction with components of the noni puree that were absent with respect to passively acquired antibody to *S. epidermidis*. Passively acquired antiLPS antibody most likely was present at some titer in all calves in this study because the calves were from farms with Gram-negative core mutant vaccine protocols in dry cows and heifers as

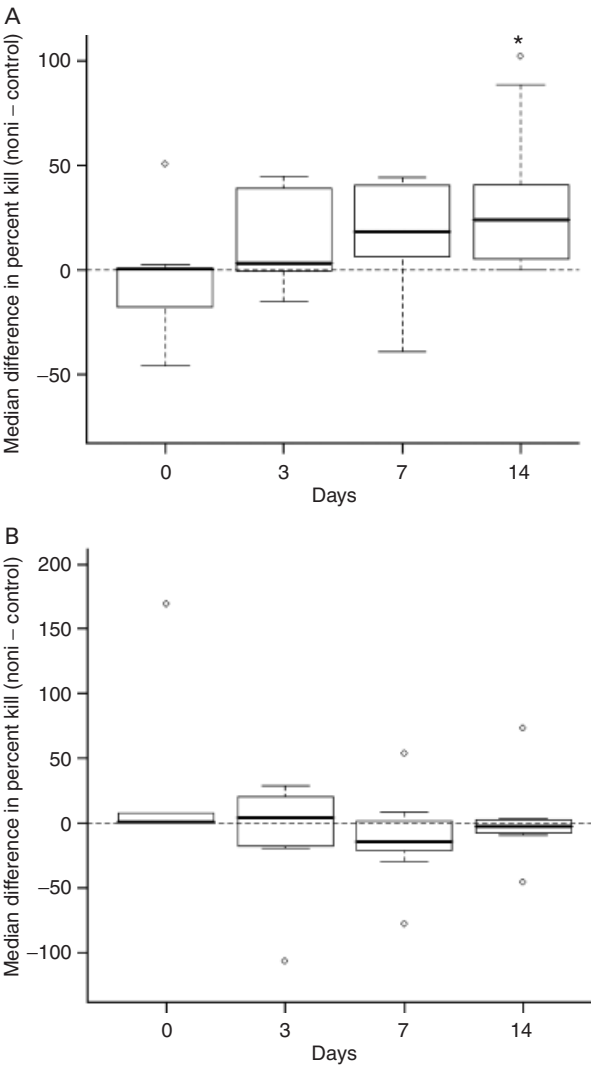

Fig 1. Percent *Escherichia coli* and *Staphylococcus epidermidis* killed median differences between control and 2 noni-fed calves. Median differences of calf pairs (noni – control; n = 9) in percent killing of (A) *E. coli* and (B) *S. epidermidis*. Noni-supplemented calves had progressively greater phagocytic killing of *E. coli* and were significantly different at day 14 when compared with control. There were no significant differences between treatment groups in percent *S. epidermidis* killing. The dark line indicates the median; the box contains the middle 50% of the data points; the whiskers extend out to include any point within 150% of the interquartile range (IQR); a circle indicates a point outside of 1.5 × IQR. $^{*}P < .05$.

part of their mastitis control programs. To what extent pre-existent antibodies to Gram-positive organisms were present is unknown. Furthermore, as yet poorly elucidated interactions between cytokines and puree components may selectively enhance the response to Gram-negative, but not Gram-positive, organisms. Noni puree either may stimulate the innate immunity of the calf, resulting in enhanced bactericidal activity, or may potentiate immune function by enhancing colostral-acquired innate immu nity. With respect to the latter, the role of colostral leukocytes and cytokines in enhancing neonatal immunity recently has been an area of active research. Yamanaka et al[15] suggested that colostral cytokines (interleukin [IL]-1β, tumor necrosis factor [TNF]-α, interferon [IFN]-γ) play a role in enhancing neonate immunity by upregulation of IL-2 mRNA and mature IL-2 receptors (CD25), perhaps in part because of an increased sensitivity of peripheral blood mononuclear cells (PBMC) to colostral cytokines. Pretreatment of PBMC from newborn calves with IL-1β significantly enhanced CD25 expression and promoted a mitogenic response to concanavalin A whereas IL-1α was an inhibitor.[16]

Given the recent advances in phytochemical immunopharmacology and most specifically the role of polysaccharides as immunomodulators, noni puree potentially may stimulate the acquired innate immune response by modulating colostral cells, neonatal mononuclear cells, or both.[9] Larger clinical trials are warranted to test the clinical application of noni puree in promoting calf health and production and in decreasing morbidity and mortality in a Gram-negative endotoxin or live pathogen challenge model.

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Footnotes

[a] MorindaMax, CalfBoost, Morinda Agriculture, Provo, UT
[b] Midland Bioproducts Corporation , Boone, IA
[c] Vita Plus Corporation, Madison, WI
[d] Becton Dickinson, Franklin Lakes, NJ
[e] BBL, Becton Dickinson, Sparks, MD
[f] Invitrogen Corp, Carlsbad, CA
[g] SAS Institute, Cary, NC

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