

ETHNOVETERINARY APPLICATION OF *MORINDA CITRIFOLIA* FRUIT PUREE ON A
COMMERCIAL HEIFER REARING FACILITY WITH ENDEMIC *SALMONELLOSIS*

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Abstract

We have previously reported that *Morinda citrifolia* (noni) puree modulates neonatal calves developmental maturation of the innate and adaptive immune system. In this study, the effect of noni puree on respiratory and gastrointestinal (GI), health in preweaned dairy calves on a farm with endemic *salmonellosis* was examined. Two clinical trials were conducted whereby each trial evaluated one processing technique of noni puree. Trials 1 and 2 tested noni versions A and B, respectively. Puree analysis and trial methods were identical to each other, with the calf as the experimental unit. Calves were designated to 1 of 3 treatment groups in each trial and received either: 0, 15 or 30 mL every 12 hr of noni supplement for the first 3 weeks of life. Health scores, weaning age, weight gain from admission to weaning, and weaned by 6 weeks, were used as clinical endpoints for statistical analysis. In trial 1, calves supplemented with 15 mL noni puree of version A every 12 hr had a higher probability of being weaned by 6 weeks of age than control calves ($P = 0.04$). In trial 2, calves receiving 30 mL of version B every 12 hr had a 54.5% reduction in total medical treatments by 42 days of age when compared to controls ($P = 0.02$). There was a trend in reduced respiratory (61%), and GI (52%) medical treatments per calf when compared to controls ($P = 0.06$ and 0.08, respectively). There were no differences in weight gain or mortality for any treatment group in either trial.

Key words: *Morinda citrifolia*, natural products, neonatal calf, noni, dairy

Introduction

The globally emergence of new and resistant pathogens in dairy herds in conjunction with the improper use of antibiotics is challenging the success of herd health management programs to maintain biosecurity and international economic trade. This perspective is supported by The European Union's ban of antibiotics and related drugs on livestock for the purpose of growth promotion (Commission Press Room, 2003) and the Food and Drug Administration and World Health Organization support of similar policies being adopted by the United States (FDA, 2000; Ferber, 2003).

The animals most at risk for contracting disease on modern dairy farms are neonatal calves as their immune system is developmentally immature and incapable of mounting an adequate defense against many infectious pathogens, such as *Salmonellosis* (Chase et al., 2008). While adequate colostrum intake and properly used antibiotics can provide much protection (Berge et al., 2005), increased antibiotic scrutiny and consumer demand for organic products has prompted investigations of ethnoveterinary practices for enhancing dairy production by improving calf health and well-being through disease prevention (Gakuya et al., 2011; Akerreta et al., 2010; Dilshad et al., 2010; Bonet and Valles, 2007). Improving calf health through the validation of safe, effective and relatively inexpensive ethnoveterinary remedies to enhance preruminant dairy calf immunity has economic benefits for the producer as well as the broader dairy and beef industries by producing more cost efficient products that are more marketable (Matsabisa et al., 2012; Lans et al., 2007; Ahmadu et al., 2007; Blecha, 2001).

While the neonatal calf is capable of mounting an immune response at birth, its response is best characterized as immune-naïve. The role of diet and nutrition in maintaining a well-balanced immune system are well recognized and recent findings demonstrating immune modulation with bioactive food components and ingredients, such as pre- and probiotics, β -glucans and fungal immunomodulatory proteins, support the notion that natural products can potentially replace the use of prophylactic antibiotics in pre-ruminant animal health and well-being. The *Morinda citrifolia* (noni) fruit is a natural product with global equatorial distribution and established validated bioactive compounds that support its ethnoveterinary applications (Razafimandimbison et al., 2011; Kinghorn et al., 2011; Deng et al., 2010a; West et al., 2010; Razafimandimbison et al., 2010; Pawlus and Kinghorn, 2007).

The noni fruit has a broad range of validated immune enhancing effects including: antibacterial, anti-inflammatory, anti-cancer and anti-oxidant activity (Nitteranon et al., 2011, Kusirisin et al., 2009; Akihisa et al., 2007; Yang et al., 2007, Pawlus et al., 2005). The Iridoid and polysaccharide fractions of noni has been shown to induce the release of several immune mediators, many of which have beneficial stimulatory effects and may aid in the maturation of the neonatal immune system (Deng et al., 2010b; Bui et al., 2006); Hirazumi and Furusawa, 1999).

Previously, we examined the effects of feeding calves noni puree for the first 2 weeks of life on a parameter of innate immunity. Bacterial killing was evaluated via an *ex vivo* whole blood bactericidal assay (Schäfer et al., 2008). Noni supplemented calves showed significantly more killing power at day 14 when compared to control calves. Additionally, the added benefit of noni increased over time from day 3 to 14.

To determine if the immune enhancing effect of noni was broad based, including both innate and adaptive responses, we investigated the effects of feeding calves noni puree for the first 2 weeks of life on T cell activation. We evaluated mitogen-induced T cell activation via expression of interleukin-2 receptor (IL-2r, CD25) (Brooks et al., 2009). Results showed noni puree-fed calves had increased increase in CD25 expression on CD4⁺ and CD8⁺ T cells on day 3 of the study or approximately 4 to 5 days postpartum, as well as an up-regulatory effect over time, (0 to 14 days), for CD8⁺ T cells.

Taken together, *in vitro* and *in vivo* studies demonstrate immune modulating function of noni fruit puree in calves. As such, we postulated that these enhanced immune responses may translate into increased health and well-being for newborn Holstein dairy calves raised in a gram negative bacterial challenged environment on a commercial operation, thus validating it's ethoveterinary application in a clinical study. To address this, two clinical trials were performed, each to analyze one version of a noni fruit puree supplement for calves (Morinda Agricultural Products, Inc., Provo UT, USA). A low and high dosage of each version was tested to investigate potential dose-effects. The trials were performed on a commercial heifer rearing facility with a confirmed history of endemic *salmonellosis*. The effects on weaning age, weight gain, incidence of disease events and mortality in neonatal calves were analyzed through weaning.

Materials and methods

Noni puree products

Each clinical trial evaluated either version A (trial 1) or B (trial 2). Differences between version A and B were the result of ingredient processing changes related to emulsion quality of the solution. There were no differences between version A and B in nutritional content or guaranteed analysis (Table 1). Methods used for product analysis follow the Association of Analytical Communities International (AOAC) and/or United States Pharmacopeia (USP) guidelines: pulp by centrifugation and gravimetric analysis; mineral content by ICP analysis against standards of known concentration; fat by acid hydrolysis followed by extraction with hexane; fat-soluble vitamins by saponification, extraction and reverse-phase HPLC with standards of known concentration; water-soluble vitamins by extraction and reverse-phase HPLC with standards of known concentration; moisture by microwave oven; fiber by standard method using enzymatic treatment and pH control; brix by refractometer; ash by gravimetric analysis. Protein content was determined by Standard Kjeldahl method (Sapan et al., 1999), which is used for the validation of analytical processes for the determination of protein concentration. The energy density found in a 15 mL aliquot of either version is minimal (10 calories or less). The product was stored at room temperature until use and added to small batches of freshly prepared milk replacer, which were consumed within 15 min of preparation.

Animals

The trials and procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison. The trials were conducted on a commercial calf-raising farm in Central Wisconsin. Microbiologic testing (UW-School of Veterinary Medicine, Clinical Microbiology) of fecal and post mortem derived samples from at least one pre-weaned calf from each of the calf barns prior to, and during, the trials confirmed the presence of multiple drug resistant *Salmonella dublin* and *Salmonella newport* on this farm. At capacity, the farm can accommodate approximately 280 preweaned calves, 60 weaning calves, and 220 weaned calves. Historically, the average heifer calf on this farm began weaning on day 49, when they were moved into group housing and no longer offered milk replacer. For the purposes of this study calves received 2.25 L of milk replacer that had been reconstituted according to the manufacturer's instructions (Table 2, Akey Inc., Lewisburg OH, USA) twice a day in a bucket until weaning. Calf starter (Table 2, Vita Plus, Madison WI, USA), was offered daily at the farm from first arrival until weaning, which was defined by calves consuming 0.9 kg (2 lb) per day for 3 consecutive days in addition to their milk replacer. Between feedings, calves had access to 2 L of fresh water or BlueLite electrolytes (Table 3, TechMix, Inc., Stewart MN, USA), which alternated every other day.

Starter refusals were discarded and replaced with 0.9 kg starter once daily for each calf. Trial 1 enrolled 112 calves and 114 calves were enrolled in trial 2. All calves were Holstein heifers and were followed to weaning. Calves arrived from a 4,000 cow commercial dairy in North-Central Wisconsin where they had been housed individually on straw and transported within 3 days of birth. Travel time to the trial site was approximately 2 h. At the trial farm, calf pens, approximately 1.2 m × 2 m in size, were located in 4 separate barns. The pens were separated by metal fencing, aligned in 2 rows and bedded with straw. Fresh kiln dried shavings were added to pens weekly; no bedding was removed until the calf was moved into group

housing for weaning. Fence spacing did allow for calves to come in direct contact with their adjoining neighbors, which often occurred after feeding.

Treatment Allocation

Upon arrival at the trial farm, calves were weighed immediately after being unloaded and randomly assigned to 1 of the 3 treatment groups. Final weights were measured the day the calves were moved to group housing. Both weights were taken between the morning and evening feedings. Randomization of treatment assignment order for a total population of 250 calves, to include all enrollees of trials 1 and 2, was generated with R (R Foundation for Statistical Computing, ver.2.4.1. Vienna, Austria). How many calves were in each group? For trials 1 and 2, Group 1 served as a control (n=34 and 39), whereas groups 2 (n=35 and 38), and 3 (n=38 and 32) received 15 mL every 12 hr or 30 mL every 12 h, respectively, of a noni fruit puree-based supplement in their milk replacer for 21 d. Those responsible for feeding used a color-coding system to differentiate the noni treatment groups. Technicians overseeing the trials were blinded to treatments, as they were not present during feedings. Calves were assigned to barns by completely filling an available barn before moving to the next. The restricted randomization of the treatments upon admission insured that each barn had approximately the same proportion of calves assigned to each treatment group.

Inclusion Criteria

Blood samples were taken from each animal in 5 mL monoject tubes (Tyco Healthcare Group LP, Mansfield MA, USA) for total serum protein and IgG analysis upon arrival at the trial farm to determine if the calf had received adequate passive transfer through the ingestion of colostrum. Blood samples were allowed to clot for 18 hr at room temperature before the serum was removed and stored at 4° C. Serum samples were analyzed by the University of Wisconsin, School of Veterinary Medicine's clinical pathology laboratory. Calves with IgG levels ≥ 1000 mg/dL (Weaver et al., 2000; Schäfer et al., 2008), and total protein levels ≥ 5.0 g/dL were considered to have adequate passive transfer, and calves with lower levels were classified as having failure of passive transfer and subsequently removed from the trial. Calves were also removed from the trial if their starting weight did not fall between 32 and 54.5 kg, or if they died within 48 hr of arrival at the calf-raising farm. A total of 5 calves were removed from each trial for failing to meet these criteria.

Experimental Procedure

Calf health was evaluated through daily physical evaluations, which were recorded as health scores as previously described (Schäfer et al., 2008). Every animal received daily physical examinations, including observations on rectal temperature, ease of cough induction by manual compression of the trachea, fecal consistency, nasal discharge and presence and severity of ocular or otic abnormalities, which were aligned with specific respiratory, gastrointestinal (GI), and pyrexia disease events and subsequent predetermined medical treatment regimens. Technicians blinded to noni treatment groups and recorded daily as calf health scores, which dictated whether the calf required medical treatment, performed calf health evaluations (Table 4). Calves receiving a combined respiratory score (temperature, cough, nasal discharge and eyes or ears) ≥ 5 , a fecal score ≥ 2 or a temperature $\geq 39.4^{\circ}\text{C}$ were classified as having an incidence of disease and subsequently treated by farm personnel according to the established farm protocol (Table 5). Medical treatments were divided into respiratory disease related antibiotics, gastrointestinal disease related antibiotics, non-steroidal fever reducers and electrolytes. Calves that experienced a second respiratory disease event after receiving the first full antibiotic treatment, received the second respiratory antibiotic. Likewise, calves that experienced a third or subsequent respiratory disease incidence, received the third respiratory antibiotic.

Statistical Analysis

All statistical analysis was performed in SAS (SAS Institute, Cary NC, USA). Each trial was analyzed separately, with the calf as the experimental unit. Health scores (fecal, respiratory, and total), weaning age, weight gain from admission to weaning, and whether the calf was weaned by 6 weeks, were used as clinical endpoints for statistical analysis. All models were corrected for body weight at admission (a continuous variable) and the barn a calf was housed in (a categorical variable). Dosage of version A and B for calves (0, 15 or 30 mL every 12 h) was treated as a 3-level factor rather than as the actual numerical dose because preliminary graphs of the data indicated a possible non-linear relationship between dose and the endpoints of interest. Health scores were analyzed with Poisson regression models using Proc GENMOD, using the likelihood ratio test to evaluate the overall effect of each variable. Ages at weaning and weight gain were analyzed with linear models in Proc MIXED, using F-tests to evaluate overall effects. Residuals for the linear models were checked and verified to meet the necessary assumptions of normality and constant variance across treatment groups. Whether the calf was weaned by 6 weeks or not was analyzed with a logistic regression model using Proc GENMOD, again using the likelihood ratio test to evaluate overall effects and additionally using odds ratios with Wald *P*-values to look at pairwise comparisons of the doses after a significant likelihood ratio test. Treatment effects were considered significant at $P \leq 0.05$ and trends were identified at $P < 0.10$.

Results

Results of trial 1 revealed that supplementing calves with version A had an effect on the probability of being weaned by 6 weeks of age compared to control (Table 6). Calves receiving 15 mL noni puree every 12 hr of version A (low dose) had a higher probability of being weaned by 6 weeks of age than non-supplemented (control) calves (odds ratio 2.97; $P = 0.04$). In trial 2, calves receiving 30 mL every 12 hr of version B had a 54.5 % ($P = 0.02$), reduction in total respiratory and GI health scores when compared to controls (31 vs. 68, respectively). Individually, there was a trend in reduced respiratory ($P = 0.06$), and GI ($P = 0.08$), health scores for the same group of calves when compared to controls (7 vs. 18, 61% and 24 vs. 50, 52%), respectively. There was no difference in weight gain and average daily gain for any treatment group in either trial.

Table 1: Analysis and ingredients of Noni fruit puree for trial 1 (version A), and trial 2 (version B).

Parameter	Version A ^a	Version B ^a
Protein, %	2.55	2.65
Fat, %	2.67	1.47
Ash, %	0.54	0.54
Moisture, %	86.57	82.94
Insoluble Fiber, %	0.67	1.52
Soluble Fiber, %	1.67	1.95
Viscosity, centipoise	3560	4180
pH	4.03	4.01

^aGuaranteed Analysis: Crude Protein (not less than) 2.0%, Crude Fat (not less than) 0.5%, Crude Fiber (not more than) 3.0%, Vitamin E (not less than) 400 IU/30 mL. Ingredients: *Morinda citrifolia* (noni) fruit, water, dl-alpha-tocopheryl acetate (vitamin E), soy lecithin, xanthan gum, flaxseed oil, vegetable oil, mixed tocopherols, and rosemary extract.

Table 2: Milk replacer and starter information as provided from Manufacturer labels.

Milk Replacer ^a Active Drug Ingredients	
Oxytetracycline: 0.134 g/kg Neomycin Sulfate: 0.382 g/kg	
Guaranteed Analysis:	
Crude ProteinMin. 26.0% Crude FatMin. 17.0% Crude FiberMax. 0.1% Calcium (Ca).....Min. 1.0%; Max. 1.0%	Phosphorous (P).....Min. 0.6% Vitamin AMin. 51,480 IU/kg Vitamin D3Min. 11,000 IU/kg Vitamin E.....Min. 220 IU/kg
Calf Starter ^b Guaranteed Analysis:	
Crude ProteinMin. 17.5% Crude FatMin. 5.0% Crude FiberMax. 0.8% Calcium (Ca).....Min. 1.3%; Max. 1.0% Phosphorous (P).....Min. 0.5%	Salt.....Min. 0.4%; Max. 0.7% Selenium0.6 ppm Vitamin AMin. 51,480 IU/kg Vitamin D3Min. 11,000 IU/kg Vitamin EMin. 220 IU/kg

^aAkey Inc., Lewisburg OH, USA

^bVita Plus, Madison WI, USA

Table 3: Electrolyte replacement information as provided from Manufacturer labels.

Electrolytes ^c Guaranteed Analysis:
Ingredients: Dextrose, Sucrose, Lactose, Fructose, Citric Acid, Dipotassium Phosphate, Potassium Chloride, Sodium Chloride, Calcium Lactate, Magnesium Gluconate, Sodium Bicarbonate, Glycine, L-Lysine Monohydrochloride, Zinc Methionine Complex, Vitamin A Acetate, d-Activated Animal Sterol (source of Vitamin D3), dl-Alpha Tocopheryl Acetate (source of Vitamin E activity), Choline Bitartrate, Niacin Supplement, Ascorbic Acid, d-Calcium Pantothenate, Riboflavin Supplement, Pyrioxine Hydrochloride, Thiamine Mononitrate, Folic Acid, d-Biotin, Vitamin B12 Supplement, Artificial Flavors, FD&C Certified Color Added.

^cTechMix, Inc., Stewart MN, USA

Table 4: 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	Semi-formed, 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

Table 5: Established farm protocol. Calf health scores derived through from physical evaluations dictated calf medical treatment regimens.

Health Score	Treatment	Dose	Tx # ^a	Route
Fecal = 2	Electrolytes ^b	2 L	1	oral
Fecal = 3	Electrolytes	2 L	2	oral
Fecal = 2 + blood	Electrolytes	2 L	1	oral
	Gentamicin ^c	2 cc	1	oral
Fecal = 3 + blood	Electrolytes	2 L	2	oral
	Gentamicin	2 cc	1	oral
Total respiratory score of ≥ 5	1 st Ceftriaxone ^d	2 cc	1	s.c.
	2 nd Tulathromycin ^e	2 cc	1	i.m.
	3 rd Trimethoprim Sulfa ^f	960 mg	6	oral
Temperature ≥ 39.4°C	Flunixin meglumine ^g	2 cc	1	i.m.

i.m., Intramuscular; s.c. Subcutaneous; ^aNumber of treatments (Tx) given for the disease incidence.

^bTechMix, Inc., Stewart MN, USA; ^c, ^gIntervet/Schering-Plough Animal Health, The Netherlands

^d, ^ePfizer Inc., New York NY, USA; ^fMutual Pharmaceutical Co. Inc., Philadelphia PA, USA

Table 6: Summary of calf production data for trail 1 (version A) and trial 2 (version B).

	Trial 1 with Version A			Trial 2 with Version B		
	Control	Low dose	High dose	Control	Low dose	High dose
Calves (n)	34	35	38	39	38	32
Initial age (d)	1.97 ± 0.20*	2.43 ± 0.20	1.95 ± 0.18	2.10 ± 0.21	2.18 ± 0.21	2.06 ± 0.20
Weaning age (days)	43.15 ± 0.86	40.91 ± 0.67	42.61 ± 0.69	39.90 ± 0.77	39.21 ± 0.77	38.69 ± 0.67
Initial BW	40.6 ± 0.85	41.05 ± 0.64	40.71 ± 0.94	40.51 ± 0.73	41.0 ± 0.66	41.32 ± 0.71
Weaning BW	80.5 ± 1.54	78.16 ± 1.35	78.45 ± 1.32	79.74 ± 1.12	80.85 ± 1.11	81.07 ± 1.41
ADG (kg/d)	0.89 ± 0.03	0.91 ± 0.02	0.89 ± 0.02	0.98 ± 0.03	1.03 ± 0.03	1.03 ± 0.03
Weaned by 6 weeks of age (%)	47.10	74.29†	47.40	84.62	81.58	87.50
Total respiratory	36	27	39	18	19	7

health score						
Total GI health score	35	32	47	50	36	24
Total respiratory & GI health scores	71	59	86	68	55	31†

*, mean $\pm$ sem; BW, Body weight (kg); ADG, Average daily gain; Tx, Treatments; †, $P < 0.05$.

Discussion

Many of the statistics and conclusions in our study were principally based upon variables that were measured at weaning. We intentionally tried to mimic what is common practice on dairy farms, that is to base weaning on starter intake; such that calves were weaned when they had consumed 0.9 kg of starter for 3 consecutive days as confirmed by farm personnel. We were anxious not to intrude on the daily workings of the farm, and also to mimic what is a common place practice for heifer raisers nationally when investigating the impact of a product on weaning. It is highly relevant to point out that both the farm personnel and the technicians were blinded as to treatment groups and as such their quantification of grain intake and therefore weaning date were independent and unbiased with respect to supplementation with noni puree. Two clinical trials were performed to analyze the effect of a noni fruit puree-based liquid supplement for calves on a commercial heifer rearing facility with a confirmed history of endemic *Salmonella dublin* and *Salmonella newport* infection. The results of these trials demonstrated that calves receiving noni puree had a reduction in total medical scores (treatments), required relative to controls.

Infection with multiple drug resistant enteric pathogens is an increasing challenge to the dairy industry, and often conventional veterinary therapeutic approaches are thwarted by their emergence. Our previous work has demonstrated improved bactericidal activity against gram negative organisms (Schäfer et al., 2008) and although our trials made no attempt to compare *Salmonella* infection rates or *Salmonella*-associated morbidity in the controls or treated calves, the increasing prevalence of resistant gram-negative enteric pathogens on heifer rearing facilities in the US perhaps argues well for the use of noni puree as an ethnoveterinary application to a commercial calf-raising setting.

While it is tempting to attribute our observed results of earlier weaning and decreased disease incidences with noni puree supplementation, there are other potential influencers. The noni puree contains Vitamin E, which is a recognized immunomodulator that promotes a T helper1 cell responses (Maggini et al., 2007; Webb and Villamor 2007; Samanta et al., 2006). While the control calves were also supplemented with Vitamin E, which was present in the milk replacer and calf starter, it is possible that the net effect of the noni puree group induced a more profound immune response. However, two previous studies demonstrated enhanced phagocytosis of *E. coli* (Schäfer et al., 2008), and increased activation of CD4⁺ and CD8⁺ T cells (Brooks et al., 2009), in calves supplemented with noni puree without any additional ingredients.

The animals most at risk for contracting infectious disease on modern dairy farms are neonatal calves as their immune system is developmentally immature and incapable of mounting an adequate defense against many infectious pathogens. Although adequate colostrum intake and properly used antibiotics can provide much protection (Berge et al., 2005), increased antibiotic scrutiny and consumer demand for organic products has prompted investigations of natural immunomodulators for enhancing calf health and production. Improving calf health through the development and validation of a safe and effective immunomodulator for pre-weaned dairy calves has potential economic benefits for the producer as well as a broader global benefit to dairy and beef industries by reducing the broad based use of antimicrobials.

Conclusion

The results of these trials validate the ethnoveterinary application of noni fruit puree. They demonstrate that a natural, plant-based product is available to enhance immunity of neonatal calves and potentially long term health, provide an alternative to antibiotic use in calves and enable producers to raise healthier animals that require less time and treatment in the preweaning period. Further studies are warranted to identify bioactive components in noni puree responsible for modulating immune function. Other Rubiaceae family species indigenous to Africa may be investigated for ethnoveterinary applications.

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