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Effects of feeding polyphenols from pomegranate extract on health, growth, nutrient digestion, and immunocompetence of calves

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ABSTRACT

Objectives were to determine effects of feeding pomegranate extract (POMx) rich in polyphenols on performance, health, nutrient digestion, and immunocompetence of calves in the first 70 d of age. Holstein calves (n = 67), at 2 ± 1 d of age (d 0 = birth day) were randomly assigned to 0 (control), 5 (POMx5), or 10 g/d (POMx10) of pomegranate extract containing 16.9% gallic acid equivalent (GAE) to result in intakes of 0, 850 and 1,700 mg of GAE/d or an average of approximately 0, 15, and 30 mg of GAE/kg of body weight (BW) per day. All calves received colostrum during the first 24 h, pasteurized milk thereafter until 61 d of age, and grain was fed ad libitum for the first 70 d of age. Calves were housed in individual hutches, and grain intake, attitude and fecal scores, incidence and duration of health disorders, and treatments for health problems were evaluated daily. Body weight was measured on 2 consecutive days at 2, 30, and 70 d of age and averaged for each measurement. Concentrations of glucose and 3-hydroxybutyrate were measured in plasma. Nutrient digestion was measured using total fecal collection during a 3-d period. Neutrophil phagocytic and killing activities and antibody response to immunization with ovalbumin were measured. Peripheral blood mononuclear cells were cultured and cytokine production measured. Feeding POMx had no effect on intake or BW gain in the first 30 d of age, but after 30 d of age, both grain dry matter intake and BW gain decreased with increasing addition of POMx, which resulted in calves that were 1.8 and 4.3 kg lighter at 70 d of age for POMx5 and POMx10, respectively, compared with controls. Feeding POMx did not influence dry matter, organic matter, or starch digestibility, but it reduced crude protein and fat digestion. Plasma concentrations of glucose and 3-hydroxybutyrate were similar among treatments throughout the first 70 d of age. Measures of calf health such as fecal and attitude scores, risk of fever, and rectal temperature were not altered by treatments. Similarly, neutrophil phagocytic and killing activities did not differ among treatments. On the contrary, feeding POMx increased synthesis of interferon-γ and interleukin-4 by peripheral blood mononuclear cells and improved total immunoglobulin G responses to ovalbumin vaccination. These results suggest that feeding POMx top-dressed onto the grain suppresses intake of grain and digestibility of fat and protein, likely because of the high tannin content. Nevertheless, polyphenols from POMx enhanced mitogen-induced cytokine production and response to vaccination, which might benefit immune competence of calves and potentially health. Additional studies are warranted to minimize the effect of POMx on intake and digestibility and to better understand the mechanisms by which polyphenols improve immune response of calves.

Key words: calf, health, polyphenols, pomegranate

INTRODUCTION

Diseases of the digestive tract such as gastrointestinal infections and subsequent diarrhea and dehydration account for the majority of health problems affecting calves during the preweaning period and are the primary reason for death and poor development in the first 60 d of age (Davis and Drackley, 1998; NAHMS, 2007). Minimizing gastrointestinal diseases is not only important to reducing mortality, but calves suffering from diarrhea during the first 3 mo of life produce less milk in their first lactation (Svensson and Hultgren, 2008).

In the most recent report of the National Animal Health Monitoring System (NAHMS, 2007), 7.8% of unweaned heifers died on dairy farms in the United States. As the young calf matures and shifts from a liquid diet to a diet based on cereal grains and forages, the risk for diarrhea tends to decline (Davis and Drackley, 1998). To reduce the risk of diarrhea and other diseases, it is critical that calves receive adequate

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intake of colostrum in the first few hours of life (Davis and Drackley, 1998; Weaver et al., 2000), and benefits to colostrum feeding go beyond provision of adequate passive transfer. The National Dairy Heifer Evaluation Project (NDHEP, 1991) observed that over 40% of calves had failure of transfer of passive immunity based on serum IgG <1.0 g/dL (NDHEP, 1991). More recently, failure of passive transfer affected over 30% of the calves in dairy farms based on serum total protein <5.0 g/dL (Tyler et al., 1998) and 43% of bull calves in veal operations based on serum total protein <5.5 g/dL (Wilson et al., 2000). Despite inadequate passive transfer, calves can be successfully raised, but risk of morbidity and mortality increase.

Young calves are often fed subtherapeutic doses of antimicrobials in the milk to minimize the prevalence of infections early in life; however, addition of penicillin to milk fed to calves increased resistance to antibiotics by gut bacteria (Langford et al., 2003). Because subtherapeutic use of antibiotics in food animals can reduce the efficacy of therapeutically used antibiotics and increase the risks for human infections caused by antibiotic-resistant bacteria (Phillips et al., 2004), an alternative method to improve animal performance is to provide feed additives that minimize the risk of diseases (Magalhães et al., 2008). These additives might alter colonization of the digestive tract by pathogenic agents, but can also influence the immune system. A reasonable assumption is that improving the immunocompetence of calves would reduce the risk of diseases during the preweaning period.

Making of pomegranate juice results in the production of pomegranate extract that is a rich source of polyphenols, presumably with potential health benefits. Pomegranate juice contains more than 100 phytochemicals (Seeram et al., 2006), but pomegranate extracts, one of the by-products of extracting the juice from pomegranate, contain polyphenolic compounds, primarily punicalagin and ellagitannins, which have been shown to possess antimicrobial, antioxidant, antiinflammatory, antimitotic, and immunomodulatory properties both in vivo and in vitro (Adams et al., 2006; Jayaprakasha et al., 2006; Rosenblat and Aviram, 2006). In rats induced to have diarrhea by oral administration of castor oil, feeding extract from pomegranate seeds decreased defecation and gastrointestinal motility, presumably by reducing fluid pooling into the intestine (Das et al., 1999). In addition, the antimicrobial properties of pomegranate extract might directly reduce gastrointestinal infections, which in turn could reduce the risk of diarrhea. Finally, the antioxidant and immunomodulatory properties of pomegranate extract might improve immune function, which could benefit health. Although polyphenolic compounds might improve animal health, they can also decrease proteolytic activity and, thus, compromise protein digestion (Broderick et al., 1991). Therefore, benefits to animal health should be weighed relative to potential decline on nutrient digestion and growth.

To our knowledge, the effects of feeding polyphenols from pomegranate extract on DMI, nutrient digestion, growth, and immune responses in preweaned calves are unknown. We hypothesized that feeding pomegranate extract to calves might enhance immune competence, which in turn can benefit health. Objectives were to determine the effects of feeding pomegranate extract top-dressed onto the grain during the first 70 d of age on intake, growth, nutrient digestion, health, and measures of humoral and innate immunocompetence of dairy calves.

MATERIALS AND METHODS

Animals, Housing, and Feeding

Calves were housed in individual hutches located at approximately 60 cm apart and assigned in sequence of 1 per treatment. All calves received 3 feedings each of 1.9 L of frozen-thawed colostrum in the first 24 h of life. Thereafter, calves were fed nonsaleable pasteurized whole milk (Table 1) originating from recently calved cows or cows in the hospital pen. Milk was collected twice daily and pasteurized once using a continuous-flow, commercial calf milk pasteurizer (Terminator T1000, Goodnature Products Inc., Orchard Park, NY) by flash pasteurization, in which milk temperature was elevated and held at 72°C for 15 to 30 s and then quickly cooled to 35°C. Milk was fed 3 times daily at 0530, 1200, and 1600 h in the first 21 d of life and twice daily thereafter, at 0530 and 1600 h, until 60 d of age, when calves were weaned from milk. Calves were offered 1.9 L in each feeding in bottles.

All calves were fed the same mixture of grains (Table 2) to meet or exceed nutrient requirements for a preweaned and early-weaned Holstein calf to achieve adequate growth as suggested by the NRC (2001) and others (Davis and Drackley, 1998). Grain was fed once daily in the morning, immediately after milk feeding, for ad libitum intake during the first 70 d of age.

Treatments and Measurements of Grain DMI and BW

Calves were randomly assigned to 1 of 3 treatments: 0 (control, n = 23), 5 (**POMx5**, n = 22), or 10 g/d (**POMx10**, n = 22) of a dried pomegranate extract rich in polyphenols containing 16.9% gallic acid equiva-

Table 1. Nutrient composition (mean ± SD) of pasteurized milk fed to calves[1]

Nutrient composition	As is	DM basis
DM, %	11.64 ± 1.18	—
Fat, %	3.30 ± 1.21	27.95 ± 8.71
True protein, %	3.23 ± 0.39	27.96 ± 3.78
Lactose, %	4.24 ± 0.55	36.61 ± 4.97
SNF, %	8.34 ± 0.93	72.05 ± 8.71
Ash, %	0.87 ± 0.17	7.45 ± 1.37
ME,[2] Mcal/kg	0.61	5.26
SCC, × 10^3/mL	1,334 ± 1,344	—
Bacteria, $\log_{10}$ cfu/mL	1.72 ± 1.18	—

[1]Average of samples collected 4 times weekly throughout the study (n = 30).

[2]ME calculated according to NRC (2001) based on the composition of milk using average values for DM, fat, true protein, and ash.

lent (**GAE**) to result in intakes of 0, 850, and 1,700 mg of GAE/d throughout the study. Based on expected mean BW of calves in the first 70 d of age of 56 kg (Magalhães et al., 2008), these amounts would result in intakes of 0, 15, and 30 mg of GAE/kg of BW per day. The dosages chosen for POMx5 and POMx10 were based on intakes of GAE/kg of BW extrapolated from studies with human subjects in which clinical benefits to consuming polyphenols from pomegranate juice were reported (Pantuck et al., 2006; Heber et al., 2007).

Table 2. Ingredient composition of the calf grain and nutrient content (mean ± SD) of grain and pomegranate extract (POMx)

Item	Calf grain	
Ingredient composition, DM basis		
Steam-rolled corn, %	30.0	
Steam-rolled oats, %	18.5	
Dried beet pulp, shreds %	20.0	
Pellet,[1] %	25.0	
Cane molasses, %	6.5	
Nutrient composition, DM basis	Calf grain[2]	POMx
DM, %	86.54 ± 1.11	92.56 ± 0.26
ME,[3] Mcal/kg	2.98	—
OM, %	92.34 ± 0.35	89.80 ± 0.16
CP, %	20.67 ± 0.79	1.11 ± 0.06
NDF, %	15.00 ± 0.74	0.60 ± 0.53
NDF insoluble CP, %	3.24 ± 0.22	—
Lignin, %	0.56 ± 0.13	—
NFC,[4] %	55.13 ± 3.10	87.99 ± 0.29
Fat, %	2.62 ± 0.19	0.11 ± 0.01
Ca, %	1.06 ± 0.08	—
P, %	0.74 ± 0.01	—
K, %	1.26 ± 0.04	—
Mg, %	0.39 ± 0.05	—
Na, %	0.44 ± 0.00	—
Cl, %	0.53 ± 0.03	—
S, %	0.29 ± 0.01	
Zn, mg/kg	91.5 ± 3.5	—
Cu, mg/kg	14.5 ± 0.7	—
Mn, mg/kg	72.5 ± 12.2	—
Monensin, mg/kg	30	—

[1]Pellet contained 18.20% Pro-Lak (blend of marine and animal by-products; H. J. Baker & Bro. Inc., Stamford, CT), 65.00% solvent extract soybean meal, 9.10% corn distillers grains, 1.80% calcium carbonate, 4.10% dicalcium phosphate, 0.55% sodium chloride, 0.55% magnesium oxide, 0.09% 4-Plex (Zinpro Corp., Eden Prairie, MN), 0.018% zinc sulfate, 0.01% manganese sulfate, 0.0003% sodium selenite, 0.40% of a mixture of iodine and vitamins A, D and E, and 0.07% Rumensin 80 (176 mg/kg of monensin; Elanco Animal Health, Indianapolis, IN).

[2]Average of 6 composited samples collected weekly.

[3]According to NRC (2001) for a young calf consuming 1.0 kg of DM.

[4]Calculated according to the following formula: OM – [CP + (NDF − NDF insoluble CP) + fat].

Pomegranate extract was top-dressed onto the grain immediately before feeding to ensure that calves would consume the full amount daily. Grain was offered once daily to allow for 5% orts and amounts offered and refused were measured daily. All calves were weighed on 2 consecutive days at 2 and 3, 30 and 31, and 70 and 71 d of age, and the average BW for each measurement was calculated.

Grain and Milk Sampling and Nutrient Analyses

Grain was sampled once a week and dried at 55°C for 48 h, and moisture content was recorded. Dried samples were ground to pass a 1-mm screen and samples were then composited for 2-mo periods and analyzed for contents of DM, OM, and ether extract (AOAC, 2000), and NDF (Van Soest et al., 1991). The N content of samples was analyzed using a nitrogen analyzer (FP-528 Nitrogen Determinator, Leco Corp., St. Joseph, MI), and CP was calculated by multiplying the N content by 6.25. Mineral content was analyzed at the Dairyland Laboratory (Arcadia, WI) using an inductively coupled plasma mass spectrometer (Thermo Jarrell-Ash, Franklin, MA).

Samples of pasteurized milk were collected 4 times weekly throughout the study and analyzed for concentrations of total solids, ash, lactose, fat, and true protein (Foss 303 Milk-O-Scan, Foss Foods Inc., Eden Prairie, MN) at the DHIA Laboratory in Tulare, California. Solids-not-fat were calculated by difference between total solids and fat. Composition of pasteurized milk (Table 1) was used to estimate the energy concentration in milk using NRC (2001) calculations. Samples of milk postpasteurization were also analyzed 4 times for bacterial contamination at the Milk Quality Laboratory at the Veterinary Medicine Teaching and Research Center, University of California–Davis, to determine bacterial counts (cfu/mL) of milk.

Attitude and Fecal Consistency Scoring

Attitude and fecal consistency were scored daily by the research team during the morning milk feeding using a 1 to 4 scale. For attitude, calves were categorized as 1 when alert and responsive, 2 when nonactive, 3 when depressed, and 4 when moribund. Fecal consistency was scored as 1 when firm, 2 when soft or of moderate consistency, 3 when runny or mild diarrhea, and 4 when watery and profuse diarrhea (Larson et al., 1977; Magalhães et al., 2008). Weekly averages of attitude and fecal scores were generated for individual calves for statistical analyses. Calves with fecal score >2 were used for analysis of risk of diarrhea.

Incidence of Health Disorders, Treatments, and Costs Associated with Treatments

Incidence of health disorders was recorded daily for individual calves. Rectal temperature was measured daily for the first 21 d in the study and in any calf displaying clinical signs of disease after d 21. Calves with rectal temperature >39.5°C were considered to be febrile.

Calves were evaluated for the presence of diarrhea, which was characterized by presence of watery feces using fecal score >2; pneumonia was characterized by presence of respiratory distress, increased respiratory frequency, and nasal discharge. The day when disease was first diagnosed was recorded and duration of each illness event was determined.

Calves with digestive and respiratory problems were treated by farm personnel according to protocols established by the herd veterinarian. Medication used (antibiotics, antiinflammatory, and antidiarrheic products), dosage, and duration of treatments were recorded for individual calves. Costs associated with health treatments were calculated based on currents costs for each product, daily dosage for each medication for individual calves, which was administered based on BW of animals, estimated time spent by personnel with individual treatments, and respective personnel wages.

Analyses of Total Protein and IgG in Serum and Metabolites in Plasma

Concentration of total protein was measured in serum from all calves on enrollment day using a clinical refractometer. Concentration of total IgG in serum was also measured in serum at enrollment by single radial immunodiffusion using a commercial kit (no. 240-60, Veterinary Medical Research and Development, Pullman, WA). Plasma collected from calves at 2, 14, 28, 42, 56, and 70 d of age were analyzed for concentrations of glucose by direct measurement using the YSI Model 2700 Select Biochemistry Analyzer (Yellow Springs Instrument Co. Inc., Yellow Springs, OH), and for BHBA using a commercial kit (Ranbut, D-3-hydroxybutyrate, Randox Laboratories Ltd., Antrim, UK) based on the enzymatic oxidation of BHBA to acetoacetate and concomitant reduction of NAD^+ to NADH (Williamson et al., 1962).

Evaluation of Humoral and Innate Immune Responses

Ovalbumin (**OVA**) solution was prepared by dissolving 0.5 mg of OVA (Type VII, Sigma Chemical Co., St. Louis, MO) and 0.5 mg of the adjuvant Quil-A (Ac-

curate Chemical, Westbury, NY) in 1.0 mL of PBS (0.1 *M*, pH = 7.4) . Calves received a 1.0-mL i.m. injection at 4, 21, and 42 d of age, and serum samples collected on the same days and again at 56 d of age for analysis of OVA-specific concentrations of total IgG. Concentrations of IgG to OVA were measured by ELISA as described by Wagter et al. (2000).

Neutrophil phagocytosis and intracellular killing of *Escherichia coli* ATCC 25922 were evaluated in calves at 35 ± 1 d of age. Neutrophils were harvested from 50 mL of jugular blood, and bacteria were prepared as described by Hogan et al. (1992). Suspensions of neutrophils and opsonized bacteria were incubated in a 1:3 ratio at 37°C and 100 rpm for 90 min in water bath. Following incubation, 50 μL of bacteria-neutrophil samples were mixed with 25 μL of acridine orange solution and 25 μL of crystal violet solution. Wet mount slides were prepared and neutrophils were evaluated under an epifluorescent microscope. Bacteria present in the cytoplasm of neutrophils were stained in either red (dead) or green (live), and the number of neutrophils phagocytizing bacteria and number of dead and live bacteria was counted in the first 50 neutrophils visible under the microscope.

Isolation and Culture of Peripheral Blood Mononuclear Cells and Cytokine Measurements

At 50 d of age, 30 mL of jugular blood was collected from each calf into evacuated tubes containing K_2 EDTA and immediately transported to the laboratory in ice. Tubes were centrifuged at 1,200 × *g* for 15 min and buffy coats were carefully removed. Buffy coats were placed into a 50-mL tube containing 18 mL of sterile PBS. Three tubes containing Histopaque were carefully overlaid each with 7 mL of buffy coat-PBS solution. Tubes were centrifuged at 450 × *g* for 40 min. The buffy coats were repipetted into 15-mL conical tubes, and then centrifuged at 900 × *g* for 15 min and the supernatant removed. Contaminating erythrocytes were lysed by resuspending the pellet in 10 mL of 0.03 *M* of NaCl solution for 60 s. Then, 2.67 mL of 0.63 *M* solution of NaCl was pipetted into each tube and mixed, and tubes were centrifuged at 900 × *g* for 15 min. The supernatant was removed and the pellet resuspended in 7 mL of cell culture medium containing 89% RPMI 1640 (Gibco, Invitrogen Corporation, Carlsbad, CA), 10% fetal calf serum (Sigma-Aldrich), and 1% antibiotic-antimycotic (Gibco, Invitrogen Corp.). The number of cells was counted using a hemacytometer and the concentration was corrected to 1.34×10^6 cells/mL by adding cell culture medium.

To determine the production of tumor necrosis factor-α (**TNF-α**), 150 μL of cell culture suspension (200×10^3 cells) was incubated with 50 μL of a solution containing 5 ng/mL of recombinant IFN-γ (Endogen, Rockford, IL) and 2.5 μg/mL of *E. coli* 0111:B4 LPS (Sigma-Aldrich), in triplicate for 24 h at 37°C. After incubation, ELISA was performed according to manufacturer instructions (Endogen). Similarly, to evaluate the production of IFN-γ and IL-4, incubation of 150 μL of cell culture suspension with 50 μL of phytohemagglutinin-P (10 μg/mL, Sigma-Aldrich) was performed in triplicate for each assay for 72 h at 37°C. After incubation, an ELISA was performed according to manufacturer instructions (Endogen).

Total-Tract Apparent Digestibility of Nutrients

In the last week of the experiment, 32 calves (10 control, 11 POMx5, and 11 POMx10) were subjected to total fecal collection during a 4-d period. Calves were offered grain twice daily and the amounts offered and refused were recorded for each calf. Samples of grain offered and refused were collected daily from each calf. Calves were fitted with fecal collection bags and feces were collected individually from bags twice daily and stored in sealed containers at −25°C. At the end of the 4-d collection period, samples of grain offered and refused and of feces from each calf were weighed and dried at 55°C for 48 h, and moisture disappearance was recorded. Dried samples were ground to pass a 1-mm screen and then analyzed for DM, OM, CP, fat, starch, and NDF as described previously. Intake of a given nutrient was calculated based on grain DM offered and the concentration of the nutrient in the grain offered minus the amount of grain DM refused and the concentration of the nutrient in the refused grain. Apparent total-tract digestibility of dietary nutrients was calculated based on intake of each nutrient subtracted from the fecal excretion of the respective nutrient, and then divided by the intake of the same nutrient.

Experimental Design and Statistical Analyses

The experimental design was a randomized incomplete block design. Calves at 2 d of age were blocked in groups of 3 according to sex and day of birth and, within each block, randomly assigned to treatments. Preplanned orthogonal polynomial contrasts were evaluated for each outcome to determine linear and quadratic responses to addition of polyphenols from pomegranate extract. Two previous pilot experiments were conducted with 30 calves each for 10 d. In those experiments, grain DM intake declined 5 to 8% in calves fed POMx10 from 32 to 42 d of age. Based on those values and the expected grain intake and SD for grain intake of calves (Magalhães et al., 2008), the sample

sizes were calculated to provide sufficient replicates to detect statistical significance when grain DM intake is reduced by 8%, when calves consume 0.8 to 1 kg/d of grain DM ($\alpha = 0.05$; $\beta = 0.20$).

Continuous variables were analyzed by ANOVA using the MIXED or GLM procedures of SAS (SAS Inst. Inc., Cary, NC) when repeated or single measurements for an experimental unit were taken, respectively. Variables with a single measurement during the study were analyzed with the fixed effects of treatment and sex of calf. Serum IgG concentrations were used as covariates in statistical models. For BW changes, initial BW at study enrollment was used as covariate. Variables with repeated measurements within the same calf were analyzed with the fixed effects of treatment, time of measurement (day or week), interaction between treatment and time, and the random effect of calf nested within treatment. The repeated statement was included and the covariance structure was chosen based on the smallest Schwarz's Bayesian criterion.

Daily fecal and attitude scores were averaged into weekly means and were normally distributed; therefore, data were analyzed by the MIXED procedure of SAS (SAS Inst. Inc.) with a model that included the effects of treatment, week, interaction between treatment and week, and calf nested within treatment as the random error. For the latter model, the covariance structure that best fitted the data was chosen based on the smallest Schwarz's Bayesian criterion. In addition, the proportion of days with scores above 2 or equal to 4 was analyzed by the GLM procedure of SAS (SAS Inst. Inc.).

Number of cases of health disorders per calf was analyzed by the Kruskal-Wallis nonparametric method to test equality of medians between treatments. Medians and mean rank were generated (SAS Inst. Inc.). Risk of fever and treatment for health disorders per 100 calf days at risk was calculated for each calf and then analyzed using the GLM procedure of SAS (SAS Inst. Inc.).

Least squares means and SEM are reported. Treatment differences with $P \leq 0.05$ were considered significant and $0.05 < P \leq 0.10$ were designated as tendency.

RESULTS

Microbiological analyses of pasteurized milk fed to calves showed a very low mean bacterial count (Table 1). In fact, the range for the 30 samples analyzed was 0 to 3,000 cfu/mL, with a median value of 200 cfu/mL. None of the samples analyzed had *Salmonella* spp. or *Mycoplasma* spp. detected.

Although the nutrient composition and IgG concentration of colostrum fed to each calf were not measured in this study, serum total protein and serum total IgG at 2 ± 1 d of age were quantified, which are related to colostral IgG intake and absorption. Serum total protein concentration on the day of study enrollment was similar ($P = 0.49$) among treatments and averaged 6.29, 6.50, and 6.40 g/dL for control, POMx5, and POMx10, respectively. Similarly, serum total IgG (3.02, 3.04, and 3.32 g/dL, respectively) and hematocrit (34.8, 34.8, and 31.7%, respectively) did not differ for control, POMx5, and POMx10. None of the calves had serum total protein <5.0 g/dL or serum total IgG <1.0 g/dL, which would characterize failure of passive transfer (Tyler et al., 1998; Weaver et al., 2000).

Grain DMI in the first 30 d of age was low and averaged 120 g/d, with no difference among treatments (Table 3). The low grain intake in the first weeks was likely caused by the thrice-daily feeding of milk in the first 21 d. Grain intake increased substantially after 30 d, but calves fed POMx experienced a linear reduction ($P = 0.05$) in grain DMI (Table 3). Overall, calves consumed (±SEM) 846 ± 34, 787 ± 34, and 753 ± 35 g/d in the first 70 d of age for control, POMx5, and POMx10, respectively. Body weight change followed a similar pattern as that for grain DMI. Feeding POMx had no effect on BW gain in the first 30 d of age, but reduced ($P = 0.02$) weight gain after 30 d, which resulted in calves that were 1.8 and 4.3 kg lighter ($P = 0.03$) at the end of the study for POMx5 and POM10, respectively, compared with control calves.

Total-tract apparent digestibility of DM, OM, and starch were not affected by feeding POMx (Table 4). However, feeding incremental amounts of polyphenols linearly reduced ($P = 0.01$) and tended to reduce ($P = 0.06$) CP and fat digestion, respectively. Concentrations of glucose and BHBA in plasma did not differ among treatments (Figure 1). No interaction between treatment and age of calves was observed for concentrations of glucose or BHBA. Concentrations of BHBA increased ($P < 0.001$) with age of calves, whereas those of glucose decreased ($P < 0.001$) in plasma of calves past 28 d of age.

Fecal scores, either the mean or the proportion of days with mild or watery diarrhea, did not differ among treatments. Fecal score averaged 1.84 ± 0.04, and on approximately 25 ± 1.8% of the study days calves had runny feces, and on 8.2 ± 0.9% of the days, watery feces. Fecal scores declined ($P < 0.001$) sharply after the second week of age, but no interaction between treatment and age of calves was observed for fecal scores. Similar to fecal scores, attitude score was unaffected by treatments and averaged 1.17 ± 0.02, but it decreased

Table 3. Effect of feeding polyphenols from pomegranate extract on performance and growth of calves

	Treatment (Trt)[1]			*P*-value		
Item	Control	POMx5	POMx10	Trt × Day	Linear	Quadratic
Grain intake, g/d						
4 to 30 d of age	123 ± 11	116 ± 12	117 ± 12	0.99	0.73	0.79
31 to 70 d of age	1,374 ± 54	1,278 ± 55	1,212 ± 57	0.34	0.05	0.82
BW change, g/d						
2 to 30 d of age	380 ± 19	375 ± 20	378 ± 20	—	0.96	0.89
30 to 70 d of age	830 ± 30	783 ± 30	721 ± 32	—	0.02	0.82
BW, kg						
d 30 of age	51.0 ± 0.5	50.6 ± 0.5	50.9 ± 0.5	—	0.94	0.62
d 70 of age	83.9 ± 1.3	82.1 ± 1.4	79.6 ± 1.4	—	0.03	0.84

[1]Control = 0 g/d of pomegranate extract; POMx5 = 5 g/d of pomegranate extract containing 16.9% gallic acid equivalent top-dressed onto the grain; POMx10 = 10 g/d of pomegranate extract containing 16.9% gallic acid equivalent top-dressed onto the grain.

($P < 0.001$) with age of calves, and no interaction between treatment and age was observed. Both fecal and attitude scores peaked at 2 wk of age. The risk of calves to develop fever was similar among treatments, and averaged 1.16, 1.16, and 1.19 d/100 calf-days for control, POMx5, and POMx10, respectively. The rectal temperatures throughout the first 21 d of age did not differ. Nevertheless, treatment cost per calf tended ($P = 0.10$) to increase with increasing dose of POMx ($8.24 ± 1.29 vs. 9.09 ± 1.32 vs. 11.38 ± 1.35) because of a tendency ($P = 0.06$) for increased treatments/100 calf-days (control = 6.99 ± 0.98 vs. POMx5 = 7.98 ± 1.00 vs. POMx10 = 9.69 ± 1.02).

Phagocytic and killing activities of neutrophils against *E. coli* did not differ among treatments. The proportion of neutrophils phagocytizing at least one bacterium (62.6 ± 4.6%), the number of bacteria phagocytized per neutrophil (4.9 ± 0.5), the percentage of neutrophils with killing activity (55.5 ± 4.5%), and the percentage of phagocytized bacteria killed (73.0 ± 4.0) were all not different among control, POMx5, and POMx10. When peripheral blood mononuclear cells were cultured in vitro, those originating from calves fed POMx had similar production of TNF-α compared with those from control calves. Nevertheless, increasing the consumption of POMx increased ($P = 0.05$) INF-γ and IL-4 production by peripheral blood mononuclear cells (Table 5). Humoral responses to vaccination against OVA by measuring anti-OVA total IgG in serum of calves was enhanced ($P = 0.004$) with increasing the amount of POMx consumed (Figure 2). Concentrations of anti-OVA total IgG in serum increased ($P < 0.001$) with age and sequential immunizations of calves with OVA. Responses were similar in the first 21 d, but anti-OVA serum titers increased after anamnestic responses on d 42 and 56 of age (treatment by age interaction, $P = 0.04$).

DISCUSSION

Newborn calves are usually at high risk for development of digestive diseases, particularly diarrhea, and dietary methods that minimize the risk of digestive problems without the need for antimicrobial therapy are desired (Magalhães et al., 2008). The immune system provides a biological marker to evaluate the potential health benefits of dietary supplements in food animal production. Pomegranate juice has potent antioxidant properties attributed to its high content of polyphenols (Rosenblat and Aviram, 2006). The close relation be-

Table 4. Effect of feeding polyphenols from pomegranate extract on total-tract apparent nutrient digestion of calves

	Treatment[1]			*P*-value	
Digestibility, %	Control	POMx5	POMx10	Linear	Quadratic
DM	87.7 ± 2.4	86.8 ± 2.3	84.8 ± 2.3	0.40	0.85
OM	88.4 ± 1.5	87.7 ± 1.5	88.4 ± 1.5	0.98	0.70
CP	93.8 ± 0.9	92.5 ± 0.9	89.5 ± 0.9	0.01	0.42
Fat	94.8 ± 3.2	92.6 ± 3.1	85.8 ± 3.2	0.06	0.55
Starch	97.9 ± 2.1	97.8 ± 2.0	94.0 ± 2.0	0.19	0.47

[1]Control = 0 g/d of pomegranate extract; POMx5 = 5 g/d of pomegranate extract containing 16.9% gallic acid equivalent top-dressed onto the grain; POMx10 = 10 g/d of pomegranate extract containing 16.9% gallic acid equivalent top-dressed onto the grain.

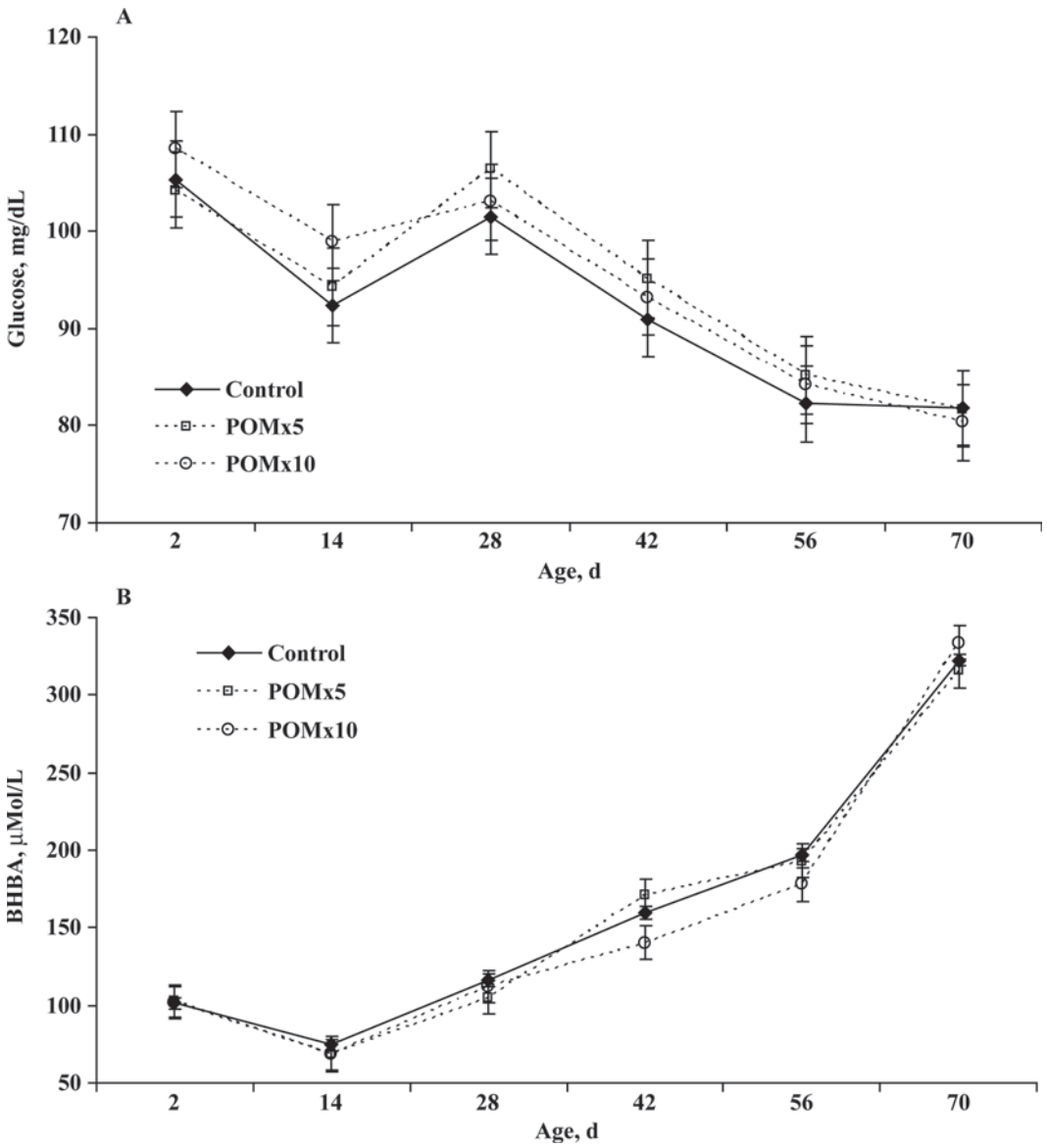

Figure 1. Plasma concentrations of A) glucose and B) BHBA in calves fed 0 (control), 5 (POMx5), or 10 g/d (POMx10) of pomegranate extract containing 16.9% gallic acid equivalent. For glucose, treatment ($P = 0.65$), age ($P < 0.001$), interaction between treatment and age ($P = 0.97$). Orthogonal responses to treatment: linear ($P = 0.41$); quadratic ($P = 0.70$). For BHBA, treatment ($P = 0.55$), age ($P < 0.001$), interaction between treatment and age ($P = 0.69$). Orthogonal responses to treatment: linear ($P = 0.28$); quadratic ($P = 0.92$).

tween oxidative stress and the immune system implies that the balance between oxidants and antioxidants in immune cells is critical, because they need to produce reactive oxygen species to carry out their functions (Álvarez et al., 2006).

Most research on the effects of polyphenols on immune competence used in vitro models (Middleton, 1998; Serrano et al., 1998; Miles et al., 2005). The majority of data from those studies indicated that polyphenols inhibit both phagocyte and lymphocyte functions. However, in the present study, neither polymorphonuclear neutrophil function nor mononuclear cell proinflammatory cytokine synthesis were altered when calves were supplemented with polyphenols from a pomegranate extract. Furthermore, mitogen-induced lymphocyte cytokine synthesis and the secondary humoral response were increased in calves fed the pomegranate extract, and the effect was linear with respect to the dose of pomegranate extract in the calf starter. These data indicate a dichotomy between in vitro and in vivo effects of polyphenols on immune cell functions. Further supporting this notion, increasing the consumption of polyphenol-rich foods in vivo from cereal grains, purple sweet potato leaves, or fruit juices improved

Table 5. Effect of feeding polyphenols from pomegranate extract to calves on cytokine production by peripheral blood mononuclear cells

	Treatment[1]			*P*-value	
Cytokine	Control	POMx5	POMx10	Linear	Quadratic
Tumor necrosis factor-α, pg/10^6 cells	1,036.6 ± 99.1	932.6 ± 108.0	1,060.0 ± 101.6	0.85	0.27
IFN-γ, pg/10^6 cells	102.2 ± 80.9	200.0 ± 89.6	296.8 ± 77.0	0.05	0.99
IL-4, pg/10^6 cells	270.9 ± 94.2	431.8 ± 103.0	497.4 ± 91.9	0.05	0.64

[1]Control = 0 g/d of pomegranate extract; POMx5 = 5 g/d of pomegranate extract containing 16.9% gallic acid equivalent top-dressed onto the grain; POMx10 = 10 g/d of pomegranate extract containing 16.9% gallic acid equivalent top-dressed onto the grain.

lymphocyte functions in mice, rabbit, and human subjects (Gracious et al., 2001; Chen et al., 2005; Álvarez et al., 2006). Gracious et al. (2001), evaluating *Punica granatum* fruit rind powder at dose of 100 mg/kg, found stimulation in the cell-mediated and humoral components on the immune system in rabbits. Similarly, Chen et al. (2005) observed improvements in mitogen-induced lymphocyte proliferation and synthesis of both IL-2 and IL-4 in human subjects supplemented 902 mg/d of GAE from purple sweet potato leaves. Furthermore, salivary IgA secretion was increased in those subjects supplemented with polyphenols, which indicated a positive effect on mucosal immunity. In addition to positive effects on lymphocyte function, Álvarez et al. (2006) also reported improved lymphoproliferative response, IL-2 release, phagocytic efficiency, and oxidative burst capacity of polymorphonuclear neutrophils from premature mice following 5-wk supplementation with polyphenol-rich cereals. However, the dose of polyphenols (as total GAE/kg of BW) was 100-fold greater than in the present study. The exact reason for the differential effects of polyphenols on immune competence when studied in vivo and in vitro is unknown, but it could be related to compounds produced from the metabolism of polyphenols in vivo or the doses used in vitro, which are usually greater than can be achieved in plasma. Regardless, these data indicate that supplementing polyphenols from pomegranate extracts to preweaned dairy calves improves lymphocyte function, which may improve humoral and cell-mediated immunity as the calf undergoes active immunization.

Pomegranate extracts show promising antibacterial, antifungal, antiviral, and antidiarrheal activities, primarily because of they are rich in phenols, tannins, flavonoids, and alkaloids. In the present study, pomegranate extract had no detectable effects on reduction of diarrhea. Results are consistent with a previous study evaluating hydrolysable tannin extract of sweet chestnut wood fed to pigs, which provided no evidence to inhibit the survival of gastrointestinal bacteria (Van Parys et

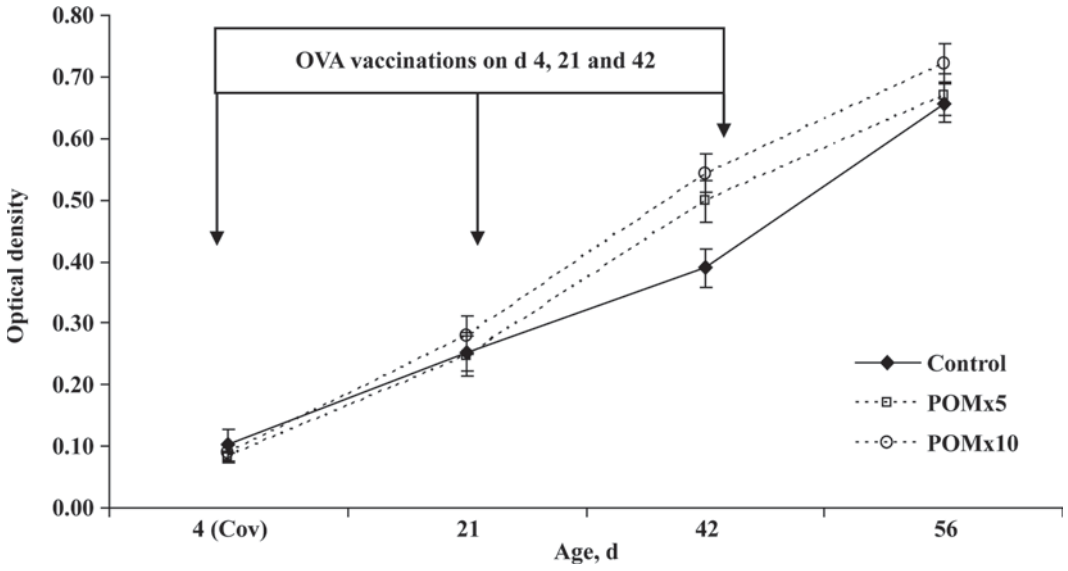

Figure 2. Anti-ovalbumin (OVA) IgG titers in serum (optical density) of calves fed 0 (control), 5 (POMx5), or 10 g/d (POMx10) of pomegranate extract containing 16.9% gallic acid equivalent. Measurement on d 4 served as covariate (Cov). Effect of treatment ($P = 0.02$), age ($P < 0.0001$), and interaction between treatment and age ($P = 0.04$). Orthogonal polynomial responses to treatment: linear ($P = 0.004$); quadratic ($P = 0.93$).

al., 2010). In contrast, Das et al. (1999) reported that *P. granatum* seed extract in doses from 100 to 600 mg/kg reduced diarrhea in rats by inhibiting gastrointestinal motility and PGE-2–induced enteropooling. The authors indicated that tannins denature proteins and the resulting protein tannate formed in the intestinal lumen reduces secretion by the intestinal mucosa (Das et al., 1999). Although they did not show the characterization of seed extract used, the chemical components of seed are different from those present on juice (Lansky and Newman, 2007). In in vitro studies, Reddy et al. (2007) and Duman et al. (2009), using pomegranate juice extract and aril extracts, respectively, confirmed the antimicrobial potential of *P. granatum*. These discrepancies between in vivo and in vitro responses might be explained by the fact that a portion of tannins can be hydrolyzed in the digestive tract and their products could affect its microbial capacities; Van Parys et al. (2010) found a strong action of hydrolyzable tannin against *Salmonella* spp. in vitro but not in vivo. Also, the differences in dosage used in the current experiment compared with in vitro studies might partially explain the lack of effect observed with calves.

Calves have low grain intake in the first few weeks after birth, when diarrhea is prevalent (Magalhães et al., 2008). The low intake of grain could have limited the intake of POMx in the first days of the study, when disease was more prevalent. Clinical signs of disease decreased sharply after 2 wk of age; therefore, the effects of POMx when top-dressed onto grain might be limited by the low intake of calves and, therefore, not influence health when diseases are most prevalent. Furthermore, the lack of an effect on phagocyte function, which is imperative in controlling the early growth of pathogens upon primary and repeat exposures, indicates that supplementing polyphenol-rich pomegranate extracts to preweaned calves at the doses used in the present study would not improve disease resistance during the first 2 wk of life. Improvements in lymphocyte functions, specifically cell-mediated and humoral responses, would not manifest until secondary antigen exposure. Therefore, the health benefits of supplementing pomegranate extracts to dairy calves would not be apparent until later in life. Importantly, during the preweaned period, calves are undergoing active immunization from both environmental pathogens as well as vaccines.

High concentrations of tannins may reduce feed intake, digestibility of protein and carbohydrates, and animal performance through their negative effects on palatability and digestion (Broderick et al., 1991; Reed, 1995). In the present study, 460 and 920 mg of polyphenols were fed per day provided by POMx5 and POMx10, respectively, and these amounts affected grain intake after 30 d of age, which reduced BW gain. These effects caused a marginal reduction in weaning BW on d 70 for calves fed POMx5, and a 5% reduction in weaning BW for calves fed POMx10. The decline in BW gain for calves fed POMx might have been a combination of less grain intake after d 30 associated with reduced total-tract apparent digestibility of CP and fat, although apparent total-tract digestion of DM, OM, and starch were similar among treatments. The reduction of protein digestibility is explained by the strong interaction between tannins and dietary proteins (Broderick et al., 1991), and the effects of tannins on protease activity (Van Leeuwen et al., 1995).

The reductions in grain intake and BW gain after 30 d of age contrast with results observed in rats by Cerdá et al. (2003). The authors found that the inclusion of pomegranate extract reduced intake and growth in the first 15 d of feeding, after which supplemented rats had improved growth rate. When older calves, approximately 11 mo of age, were fed fresh pomegranate peels in addition to a standard TMR during an 8-wk experiment, peel intake increased linearly during the feeding experiment without an adverse effect on average daily gain (Shabtay et al., 2008). Several factors influence feed intake, and it is possible that the astringent properties of POMx when fed dry could have influenced the palatability of the grain and, therefore, intake. Also, in the first 30 d of age, grain intake was very low because most of the DM consumed was provided by milk, which would limit a potential effect of POMx influencing intake. As grain intake increased and the amount of milk offered decreased after 21 d of age, calves were forced to consume more grain to meet their nutrient needs. At this point, it is likely that the astringency sensation caused by the formation of complexes between tannins and salivary glycoproteins might have inhibited grain intake.

CONCLUSIONS

Incorporation of pomegranate extract rich in polyphenols top-dressed onto the grain of calves from 2 to 70 d of age did not affect grain DMI or BW gain in the first 30 d of age when intake was low. After 30 d of age, feeding pomegranate extract reduced grain intake and BW gain, which ultimately resulted in calves that were 1.8 and 4.3 kg lighter at 70 d of age, or a 2.1 and 5.1% decrease in BW for POMx5 and POM10, respectively, compared with control calves. Feeding pomegranate extract did not influence concentrations of metabolites in plasma, fecal and attitude scores of calves, and neutrophil phagocytic and killing activities. Yet, calves fed pomegranate extract had blood mononuclear cells that produced more lymphocyte-derived cytokines in vitro and responded with increased titers against antigen vac-

cination. These data indicate that despite inhibitory effects on grain intake and fat and protein digestion when pomegranate extract was top-dressed onto the grain, its phytochemical content influenced lymphocyte function. Further studies are warranted to minimize the effect of pomegranate extract on intake and digestibility of nutrients and to better understand the mechanisms by which polyphenols improve immune response of calves. Ultimately, studies need to be designed to determine whether changes in immune responses from feeding pomegranate extract reflect improvements in health of calves during the pre- and postweaning periods.

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