Effect of conventional and intensified milk replacer feeding programs on performance, vaccination response, and neutrophil mRNA levels of Holstein calves

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ABSTRACT

This study compared conventional and intensified milk replacer feeding regimens on growth, intake, respiratory and fecal scores, vaccination response, and neutrophil mRNA levels. Holstein calves were randomly assigned to a 10-wk study on d 2 of life. Treatments were conventional (CON; $n = 8$) and intensified (INT; $n = 7$) milk replacer feeding programs. Conventional calves were fed a 20.8% crude protein and 21.0% fat milk replacer at 1.25% of birth body weight (BW) from wk 1 to 6 of life and 0.625% of birth BW during wk 7. A 29.3% crude protein and 16.2% fat milk replacer was fed to INT calves at 1.5% of birth BW during wk 1, 2% of current BW from wk 2 to 6, and 1% of current BW during wk 7. All calves were given milk replacer twice daily during wk 1 to 6, once daily during wk 7, and were weaned completely during wk 8. Calf starter intake was measured daily through wk 8. Body weight and withers height were measured weekly. Fecal and respiratory scores were recorded twice daily at feeding. Calves were vaccinated against ovalbumin at the end of wk 1, 3, and 5. Blood samples were collected at the end of wk 1, 3, 5, and 8 for analysis of serum anti-ovalbumin IgG concentration and for isolation of neutrophils. Quantitative PCR was used to measure neutrophil mRNA levels of 7 functionality genes. Treatment did not affect total DMI or anti-ovalbumin IgG response. Intensified milk replacer feeding increased average daily gain, protein intake, fat intake, and feed efficiency compared with the CON feeding program. Compared with CON calves, INT calves had greater fecal scores, indicating looser feces and greater respiratory scores, indicating more respiratory problems. Calves assigned to the INT treatment had increased neutrophil mRNA levels of L-selectin, and at wk 8, neutrophil cytosolic factor 1 was increased and toll-like receptor 4 tended to be increased

compared with CON calves. This suggests greater activation of neutrophils in INT calves postweaning, but differences were relatively small and levels of the other 4 genes were unaffected. An INT milk replacer feeding program increased growth, fecal scores, and respiratory scores preweaning, increased mRNA levels of 2 neutrophil genes postweaning, and did not affect vaccination response.

Key words: calf, milk replacer, neutrophil, growth

INTRODUCTION

Typical calf feeding programs limit-feed milk replacer to supply DM at a rate of 1 to 1.5% birth BW per d through weaning (Davis and Drackley, 1998). Nutrient composition typically ranges from 18 to 24% CP and 10 to 22% fat (Davis and Drackley, 1998). Although restricted feeding of milk replacer reduces rates of gain and feed efficiency, the rationale for this practice is to promote starter consumption for earlier weaning and rumen development (Davis and Drackley, 1998; Khan et al., 2011). Intensified milk replacer feeding programs use milk replacers with greater CP percentage, lower fat percentage, and fed at greater rates to calves compared with conventional programs. When weaned at the same age, calves raised using intensified feeding programs have greater rates of gain and are heavier and taller at weaning than calves raised using conventional programs (Hill et al., 2008; Raeth-Knight et al., 2009).

Malnutrition negatively affects immune function of dairy calves (Griebel et al., 1987), and researchers have evaluated the relationship between milk replacer feeding level and calf health. These studies have shown that increased rates of milk replacer feeding generally either result in poorer measures of calf health or have no effect. Pollock et al. (1994) found that calves fed increased amounts of milk replacer had decreased antibody titer response to vaccinations against keyhole limpet hemocyanin and horse erythrocytes. Some studies have also shown that calves fed using intensified milk replacer feeding programs were medicated more often than control calves (Cowles et al., 2006; Hill et al.,

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2006; Quigley et al., 2006; Raeth-Knight et al., 2009). However, other studies did not find consistent positive or negative effects of level of milk replacer feeding on measured aspects of health or adaptive immune function (Nonnecke et al., 2003; Davis Rincker et al., 2011).

Previous studies have focused on evaluating effects of milk replacer feeding level on aspects of health and adaptive immune function, but few have measured effects on the innate immune system. Neutrophils serve an essential function in the initial response of the innate immune system because they are among the first cells to arrive at the site of infection (Burton et al., 2005; Tizard, 2009). During inflammation, L-selectin (**SELL**) expressed on the neutrophil surface promotes neutrophil infiltration into infected tissue (Tizard, 2009). Once the neutrophils arrive at the site of infection, toll-like receptors (**TLR**) expressed on neutrophil surfaces recognize and bind pathogens (Kuijpers and Roos, 2004). Neutrophils phagocytize pathogens and then destroy them by mechanisms including respiratory burst. Components of respiratory burst pathways include neutrophil cytosolic factor 1 (**NCF1**, a component of the NADPH oxidase enzyme complex) and superoxide dismutase (**SOD**; Robinson, 2009). Neutrophils also produce a variety of antimicrobial compounds, including bactericidal/permeability-increasing protein (**BPI**; Schultz and Weiss, 2007). Cytokines, such as tumor necrosis factor (**TNF**)- α and IL-1, produced by tissues and immune cells, can bind to receptors on neutrophils to regulate neutrophil function (Tizard, 2009). In an ideal situation, neutrophils use these tools to destroy invading pathogens quickly and prevent illness. Little data is available on expression of these factors in calves fed using either conventional or intensified milk replacer feeding programs.

The objective of this study was to evaluate performance, vaccination response, and neutrophil mRNA levels of dairy calves assigned to conventional or intensified milk replacer feeding regimens. We hypothesized that, compared with a conventional milk replacer program, increased nutrition provided by an intensified milk replacer program would increase growth as well as enhance innate immune function, as indicated by mRNA levels of neutrophil function genes. Based on previous reports, we expected the response to ovalbumin vaccination to either be unaffected by treatment or decreased in response to intensified milk replacer feeding.

MATERIALS AND METHODS

Animals and Feeding

All procedures were approved by the University of Delaware Agricultural Animal Care and Use Commit-

tee. Fifteen Holstein calves (10 male and 5 female) from the University of Delaware (Newark) dairy herd were used in this study. Calves were housed in individual hutches bedded with straw and wood shavings. Hutches were equipped with water and grain buckets, and water was replaced twice daily to allow for ad libitum water consumption. At birth, calves were removed from their dams, weighed, and placed in a hutch. Navels were dipped in iodine, and calves were administered an intranasal respiratory vaccine (TSV-2; Pfizer Animal Health, New York, NY). Calves were given a total of 3.8 L of good-quality colostrum (>50 g of IgG/L, as measured by a colostrometer) divided into 2 feedings within the first 6 and 18 h after birth. Usually, the calf was given its dam's colostrum, but when IgG concentrations were less than 50 g/L, colostrum previously collected and stored at -20°C was used.

On d 2 of life, Holstein calves were blocked by sex and date of birth and randomly assigned to the control (**CON**) or intensified (**INT**) treatment. Twin calves of the same sex were assigned to the same block and administered opposite treatments, whereas opposite sex twins were not included. The final heifer block contained only 1 heifer calf assigned to CON due to an extensive gap (~ 1 mo) until the birth of the next eligible heifer. Heifer birth weight ranged from 35 to 48 kg (mean = 41, SD = 5) and bull birth weight ranged from 37 to 54 kg (mean = 44, SD = 6).

Calves assigned to CON ($n = 8$) were fed a conventional 20% fat, 20% protein milk replacer (Advance Calvita Supreme; Milk Specialties Global, Carpentersville, IL) at 1.25% of birth BW per day, divided into 2 daily feedings from wk 1 through 6 of life. Calves assigned to INT ($n = 7$) were fed an intensified 28.5% protein, 15% fat milk replacer (Advance Excelerate; Milk Specialties Global) at 1.5% of birth BW per day during wk 1 and 2% of current BW per day from wk 2 through 6 of life, divided into 2 daily feedings. During wk 7, CON and INT calves were fed milk replacer once daily (0.625% of birth BW and 1% of current BW, respectively). All calves were completely weaned at the beginning of wk 8 (d 50 of life). Milk replacers were reconstituted in water to achieve 12.5 and 15% TS for CON and INT treatments, respectively. The amount of milk replacer fed to INT calves was adjusted weekly based on weekly BW measurements. For both treatments, twice-daily feedings occurred at 0700 and 1500 h, whereas once-daily feedings occurred only at 0700 h.

Throughout the milk replacer feeding period, a fat supplement (Advance Milk Energizer; Milk Specialties Global) was added to both milk replacers to provide additional energy when temperatures were lower than 10°C (28 g for temperatures -1 to 10°C and 56 g for temperatures $< -1^{\circ}\text{C}$). The amount of fat supplement

Figure 1. Ambient temperatures during the milk replacer feeding period. The first calf enrolled in the study was fed milk replacer during study d 1 through 49. The final calf enrolled in the study was fed milk replacer during study d 31 through 79. AM = morning feeding; PM = afternoon feeding.

fed was dependent only on temperature and was not adjusted for calf weight or treatment. Temperatures recorded at each milk replacer feeding time are presented in Figure 1.

Calves were offered starter ad libitum starting on d 3 of life. Starter ingredient composition is presented in Table 1. All ingredients, except steam flaked corn and barley, were provided as a 4-mm pellet. Calves were initially offered 0.23 kg of starter daily, with amounts offered increasing by 0.23 kg as starter intake increased. Remaining starter was weighed and discarded once daily. Milk replacer and starter samples were collected weekly, composited by month, and sent to Cumberland Valley Analytical Services (Hagerstown, MD) for wet chemistry analysis. Nutrient composition of feeds is presented in Table 2.

The final once-daily milk replacer feeding occurred on d 49 of life, and treatments ceased on d 50. Measurements continued through wk 10 (d 70 $\pm$ 3 d) to evaluate posttreatment responses. Calves continued to be housed individually through 56 d of age to allow for measurement of starter intake. At the beginning of wk 9 (57 d of age), calves from both treatments were comingled in a single group pen, and growth data were collected during wk 9 (d 63 $\pm$ 3 d) and 10 (d 70 $\pm$ 3 d). The pen consisted of a 3-sided shed with an 85-m² straw-bedded pack interior and a 105-m² fenced-in dirt

lot along the open side of the shed. An automatic waterer was inside the shed. Starter grain was allocated to the group at 2.7 kg/head per day, split between 2 daily feedings and was fed in a 3 $\times$ 0.6-m trough. Orchard

Table 1. Calf starter ingredient composition

Ingredient	% of DM
Steam-flaked corn	40.0
Soybean meal	24.0
Barley, steam crimped	11.0
Molasses	5.0
Soybean meal, heat treated	4.9
Limestone	3.5
Cottonseed meal	2.8
Distillers dried grains with solubles	2.0
Sodium chloride	1.5
Macrominerals ¹	1.4
Soybean oil	1.0
Wheat middlings	0.8
Corn gluten meal	0.7
Binders	0.7
Trace minerals and vitamins ²	0.6
Minor ingredients ³	0.1

¹Contained 13.3% Mg, 12.3% S, 9.8% K, 6.8% P, and 4.8% Ca.

²Met or exceeded requirements for Co, Cu, Fe, I, Mn, Zn, and vitamins A, D, and E.

³Contained 40.4% monensin sodium (Rumensin 80; Elanco, Greenfield, IN), 30.8% yeast (Diamond V Mills Inc., Cedar Rapids, IA), and 28.8% flavoring agents (Verus Animal Nutrition Inc., Winnipeg, MB, Canada).

Table 2. Nutrient composition of experimental feeds

Nutrient, % of DM unless otherwise indicated	Milk replacer ¹		Milk replacer energy supplement ²	Calf starter	Grass hay
	CON	INT			
DM, %	97.7	98.1	98.0	87.3	87.4
CP	20.8	29.3	7.1	24.3	5.6
Fat	21.0	16.2	61.2	4.5	2.3
NDF	—	—	—	17.5	60.9
Starch	—	—	—	39.8	3.8
Lactose ³	49.0	46.2	25.6	—	—
Ash	9.2	8.2	6.1	7.9	3.5
Calcium	1.3	1.0	0.2	1.1	0.2
Phosphorus	0.8	0.7	0.2	0.7	0.2
NE _M , Mcal/kg ⁴	4.0	4.0	5.6	2.4	1.3

¹CON = control treatment; INT = intensified treatment.

²Manufacturer-reported nutrient content (Advance Milk Energizer; Milk Specialties Global, Carpentersville, IL).

³Calculated as 100 – protein – fat – ash.

⁴Net energy for maintenance calculated according to NRC (2001).

grass hay was available ad libitum in a 1.2-m-long hay feeder mounted on an interior wall. Calves were removed from the pen after wk 10, and the number of calves in the pen during the trial ranged from 2 to 8.

Growth Measurements and Sampling

Body weight and withers height were measured once weekly on the same day each week through the end of wk 10 of life ($d 70 \pm 3$). Body weight was measured using a portable scale (Nasco, Fort Atkinson, WI). Withers height was measured using a measuring stick with a sliding crossbar (Nasco). Fecal and respiratory scores were recorded twice daily as measures of digestive and respiratory health, respectively. Fecal scores were recorded as 1 = firm, well-formed; 2 = soft, pudding-like; 3 = runny, pancake batter; and 4 = liquid, splatters. Respiratory scores were recorded as 1 = normal, 2 = runny nose, 3 = heavy breathing, 4 = cough-moist, 5 = cough-dry, and 6 = fever. All health disorders were treated according to herd protocol and recorded.

On $d 7 \pm 3$ (wk 1), 21 ± 3 (wk 3), and 35 ± 3 (wk 5) of life, calves were given a 1-mL i.m. injection that contained 0.5 mg of ovalbumin (Sigma-Aldrich, Saint Louis, MO) and 0.5 mg of Quil-A adjuvant (Accurate Chemical & Scientific Corp., Westbury, NY). Blood was collected by jugular venipuncture on $d 7 \pm 3$ (wk 1), 21 ± 3 (wk 3), 35 ± 3 (wk 5), and 56 ± 3 (wk 8) of life. During wk 1, 3, and 5, the ovalbumin vaccination was administered within 5 min after blood sampling. Blood was collected into a 10-mL serum tube (BD Vacutainer Systems, Franklin Lakes, NJ) for measurement of anti-ovalbumin IgG concentration and into two 10-mL K₂ EDTA tubes (BD Vacutainer Systems) for neutrophil isolation and measurement of white blood

cell (WBC) concentration. Blood collected in EDTA tubes was placed on ice. Blood collected in the serum tubes was allowed to clot at 21°C for approximately 4 h before being centrifuged at $1,000 \times g$ for 30 min at 21°C. Serum was stored at –20°C until analysis of anti-ovalbumin IgG concentration.

For measurement of WBC concentration, 10 μ L of blood from the EDTA tubes was pipetted into 10 mL of diluent (Isoton II; Beckman Coulter Inc., Fullerton, CA). Cell concentration was measured using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter Inc.) following addition of 4 drops of a commercial red blood cell lysing reagent (Zap-Oglobin II Lytic Reagent; Beckman Coulter Inc.). Only whole blood was measured and populations of WBC were not differentiated. Neutrophils were isolated from remaining blood based on the procedures of Carlson and Kaneko (1973). Specifically, blood was centrifuged at $1,000 \times g$ for 45 min at 4°C, and then plasma, buffy coat, and two-thirds of the red blood cell pack were removed. Red blood cells in the remaining sample were lysed by adding 12 mL of 4°C hypotonic solution (10.6 mM Na₂HPO₄ and 2.7 mM NaH₂PO₄) and mixing for 90 s. Then, 6 mL of 4°C hypertonic solution (10.6 mM Na₂HPO₄, 2.7 mM NaH₂PO₄, and 430 mM NaCl) was added, and tubes were mixed to restore isotonicity. Samples were centrifuged at $800 \times g$ for 5 min at 4°C. The pellet was washed twice in 10 mL of Hank's Balanced Salt Solution (HBSS; Mediatech Inc., Manassas, VA), resuspended in 2 mL of HBSS, and split into two 1.5-mL microcentrifuge tubes. The tubes were centrifuged at $9,600 \times g$ for 15 min at 4°C, the supernatant was pipetted off, and 500 μ L of TRIzol reagent (Invitrogen Corp., Carlsbad, CA) was added. Samples were stored at –80°C until RNA isolation. Neutrophil viability was

97.5% as determined by Trypan blue exclusion, and cells were 87.5% neutrophils, as determined by staining (HEMA 3 Stain Set; Fischer Scientific Co., Kalamazoo, MI).

Anti-Ovalbumin IgG

Serum levels of anti-ovalbumin IgG were measured using an ELISA (do Amaral et al., 2011). Briefly, 96-well plates were coated with ovalbumin before adding 1/50 and 1/200 diluted samples or controls to duplicate wells. Positive and negative controls were obtained by pooling serum samples from all calves for wk 8 and 1, respectively. Plates were then treated with conjugated antibody (rabbit anti-bovine IgG alkaline phosphatase; Sigma-Aldrich) and ρ -nitrophenyl phosphate liquid substrate system (Sigma-Aldrich) before being read at 405 nm. Correction factors for each plate were calculated from controls, and relative ovalbumin IgG concentration is presented in arbitrary units of optical density after application of correction factors. Intraassay coefficient of variation averaged 9.4% and interassay coefficient of variation averaged 7.4%.

Neutrophil mRNA Isolation and Quantification

Ribonucleic acid was isolated from neutrophils (Pure-Link Micro to Midi Total RNA Purification System; Invitrogen Corp.), and 1 μ g of RNA was DNase treated (New England Biolabs Inc., Ipswich, MA) and used to create 160 μ L of cDNA (Promega Reverse Transcriptase System; Promega Corp., Madison, WI). Neutrophil mRNA levels of *SELL*, *TLR-2*, *TLR-4*, *NCF1*, *SOD1*, *BPI*, *TNF- α R*, *IL-1R1*, *RPS9*, and β -actin (*ACTB*) were quantified using quantitative real-time reverse transcription PCR. Primers from Nebzydoski et al.

(2010) were used for *SELL*, *ACTB*, and *RPS9*, and primers from Moyes et al. (2010) were used for *SOD1* and *TLR-2*. Primers for the remaining neutrophil functionality genes (Table 3) were developed using Primer Express software (Applied Biosystems Inc., Foster City, CA) and published bovine sequences from the National Center for Biotechnology Information (NCBI, Bethesda, MD). For PCR, 10- μ L reaction mixes contained 5 μ L of SYBR green master mix (Applied Biosystems Inc.), 4 μ L of cDNA, 0.4 μ L of each of the left and right primers (10 mM), and 0.2 μ L of water. Messenger RNA levels were analyzed in triplicate wells. Each sample was quantified using a relative standard curve obtained by serial dilution of pooled calf neutrophil cDNA. The relative mRNA level for each neutrophil functionality gene was divided by the geometric mean mRNA level of 2 housekeeping genes, *RPS9* and *ACTB*.

Statistical Analysis

The MIXED procedure of SAS was used for data analysis (SAS Institute, 2006). Intake, fecal scores, and respiratory scores were averaged for each week before statistical analysis. Fixed effects were treatment, week, sex of the calf, and all 2- and 3-way interactions. Calf within block and block within sex were included as random effects. The Kenward-Roger adjustment for denominator degrees of freedom was used. Week was included as a repeated measurement using the autoregressive (1) covariance structure and the subject was calf nested within treatment. White blood cell concentration and mRNA data were $\log_{10}$ transformed before analysis to obtain normal distribution of residuals. For ease of presentation, relative mRNA levels were multiplied by 10 before log transformation. Significance was declared at $P < 0.05$, whereas trends were declared at $P < 0.10$.

Table 3. Primers used for PCR analysis

Gene ¹	Primer sequence	Product length, bp	NCBI accession number ²
<i>TLR-4</i>	5'-TCAGAAACCTCCGCTACCTTG 3'-TTCTGAAAAGAGTTGCCTGCC	118	NM_174198.6
<i>NCF1</i>	5'-TCCTCAACTTCTTCAAGGTGCG 3'-CAGCGTTGTTCTTGCCATCTTT	108	NM_174119.3
<i>BPI</i>	5'-AGCCTTATTTCCAGACGCTGC 3'-ATCCAGGTTATTGGCTGTGGC	104	NM_173895.2
<i>TNFα-R</i>	5'-GTGCAGTGCGTGTGTTTGTC 3'-ATCTTCGCAACCACTGCCTTG	110	NM_174674.2
<i>IL-1R1</i>	5'-GTTTACGGCATTAGCGCCTT 3'-TTGTTGATCTTCTCTGAACCGC	106	XM_593695

¹*TLR-4* = toll-like receptor 4; *NCF1* = neutrophil cytosolic factor 1; *BPI* = bactericidal/permeability-increasing protein; *TNF α -R* = tumor necrosis factor α receptor; *IL-1R1* = IL-1 type 1 receptor.

²National Center for Biotechnology Information (NCBI, Bethesda, MD) database accessed November 2008.

RESULTS AND DISCUSSION

Growth and Intake

Growth and intake results are presented in Table 4. We expected to observe increases in BW and ADG with the INT treatment based on results of other studies (Hill et al., 2008; Raeth-Knight et al., 2009). However, no main effect of treatment was observed on wk 10 BW or on BW measured throughout the trial (Table 4). Body weight was affected by week and the interaction of treatment $\times$ week. The interaction was due to INT calves being heavier than CON calves during wk 5 to 7, with a tendency to be heavier during wk 8 and 9 (Figure 2A). The loss of difference in BW at wk 10 is in contrast to the results of Raeth-Knight et al. (2009) who found that BW differences were maintained both immediately postweaning and at 6 mo of age. However, interpretation of performance data in the current study is limited by the relatively small numbers of animals used. Others have found that increased milk replacer feeding levels did not result in BW differences that were sustained postweaning (Cowles et al., 2006; Morrison et al., 2009; Davis Rincker et al., 2011).

Average daily gain was affected by treatment, week, and their interaction (Table 4). The treatment effect was due to INT calves gaining an average of 0.12 kg/d more than CON calves ($P = 0.01$). However, the interaction of treatment $\times$ week demonstrated that INT increased ADG only during wk 2 and 3, with a tendency during wk 5 (Figure 2B). No benefit of INT on ADG was observed during wk 1, 4, or 6 of milk replacer feeding, the week of once-daily milk replacer feeding (wk 7), or postweaning (wk 8 to 10). These results are similar

to those of others who reported increased rates of gain with intensified milk replacer feeding programs during the milk feeding period but either similar or reduced rates of gain during the weaning and postweaning period compared with control calves (Hill et al., 2006, 2007; Morrison et al., 2009; Davis Rincker et al., 2011).

Withers height did not differ between INT and CON calves ($P = 0.65$; Table 4). Withers height was affected by week ($P < 0.001$) and tended to be affected by treatment $\times$ week ($P = 0.08$); this tendency was due to numeric differences at some weeks, particularly postweaning (Figure 2C). Other studies have found that intensified milk replacer feeding programs increase withers height and other measures of stature, including body length, hip height, hip width, and heart girth (Cowles et al., 2006; Hill et al., 2008; Raeth-Knight et al., 2009; Davis Rincker et al., 2011). The small number of animals used in this trial likely contributed to the lack of significance.

Total DMI did not differ between treatments ($P = 0.15$; Table 4). In accordance with treatment design, milk replacer intake was affected by treatment, week, and their interaction ($P < 0.001$), with greater milk replacer intake during wk 1 through 7 in INT compared with CON calves (Figure 3A). Intake of the energy supplement added to both milk replacers when ambient temperatures were $\leq 10^{\circ}\text{C}$ did not differ between treatments but was affected by week ($P < 0.001$) due to increasing temperatures as the experiment progressed (Figure 1). Starter intake was affected by treatment, week, and their interaction ($P < 0.01$; Figure 3B). Overall, INT calves consumed 0.31 kg/d less of starter DM than CON calves, with differences observed dur-

Table 4. Effect of conventional (CON) and intensified (INT) milk replacer feeding programs in Holstein calves on measures of growth through wk 10 and intake through wk 8

Item	Treatment ¹		SEM	P-value		
	CON	INT		Treatment	Week	Treatment $\times$ week
BW, kg						
Birth	43.5	41.1	2.7	0.54	—	—
Final, 10 wk	94.6	99.8	2.9	0.22	—	—
Overall, 0–10 wk	63.1	68.1	2.5	0.18	<0.001	<0.001
ADG, kg/d	0.74	0.86	0.04	0.01	<0.001	0.003
Withers height, cm	84.1	84.8	1.0	0.65	<0.001	0.08
DMI, kg/d						
Total	1.22	1.36	0.07	0.15	<0.001	0.16
Milk replacer	0.43	0.85	0.02	<0.001	<0.001	<0.001
Milk energy supplement	0.035	0.035	0.002	0.84	<0.001	0.59
Starter	0.75	0.48	0.06	0.006	<0.001	<0.001
Protein	0.28	0.37	0.02	0.002	<0.001	0.003
Fat	0.15	0.18	0.01	0.002	<0.001	<0.001
ADG/total DMI	0.51	0.61	0.03	0.02	0.46	0.24

¹Conventional calves were fed 20.8% CP and 21.0% fat milk replacer at 1.25% of birth BW/d during wk 1 to 6 and 0.625% of birth BW/d during wk 7; INT calves were fed 29.3% CP and 16.2% fat milk replacer at 1.5% of birth BW/d during wk 1, 2% of current BW/d during wk 2 to 6, and 1% of current BW/d during wk 7.

Figure 2. Effect of conventional (CON, —●—) and intensified (INT, - -□- -) milk replacer feeding programs on BW (A), ADG (B), and withers height (C). Error bars indicate standard error of the difference for the interaction of treatment and week. Probability that INT differs from CON: † $P < 0.10$; * $P < 0.05$.

ing wk 4 through 7. The greatest difference in starter DMI between treatments of 0.67 kg/d was observed during wk 6, the last week of twice-daily milk replacer feedings. The difference in starter intake between treatments was 0.55 kg/d during wk 7 of once-daily milk replacer feedings and was no longer present during wk 8 following complete weaning.

Reduced starter intake during the milk feeding period is commonly observed when calves are fed increased amounts of milk (Huber et al., 1984; Jasper and Weary, 2002) or milk replacer (Cowles et al., 2006; Quigley et al., 2006; Hill et al., 2008; Davis Rincker et al., 2011). Postweaning intake measurements are a bit more variable among studies. In studies using similar treatments to those in the present study, Raeth-Knight et al. (2009) found no difference in starter intake measured 1 wk postweaning, whereas Cowles et al. (2006) and Hill et al. (2006) found that calves fed using an intensified milk replacer feeding program consumed less starter postweaning than conventional calves. Other studies have found that increased feeding levels of milk or milk replacer reduced postweaning intake (Terré et al., 2007) or had no negative effects (Jasper and Weary, 2002; Hill et al., 2010).

Although total DMI did not differ between treatments, intakes of protein and fat were greater for INT than CON calves ($P = 0.002$), and intakes of both protein and fat were affected by week ($P < 0.001$) and the interaction of treatment and week ($P \leq 0.003$; Table 4). Compared with CON, calves assigned to the INT treatment consumed more protein during wk 1 to 6 (Figure 3C) and more fat during wk 2 to 7 (Figure 3D). Research has shown that both increased milk replacer feeding level and increased milk replacer protein percentage independently increased rates of gain when no starter was fed (Diaz et al., 2001; Blome et al., 2003; Bartlett et al., 2006). The increased BW and ADG observed at certain weeks in INT compared with CON calves was likely due to greater intake of both protein and total calories.

Efficiency of growth (ADG/total DMI) was increased in INT calves ($P = 0.02$), but was not affected by week or the interaction of treatment and week (Table 4). Similar improvements in gain over feed have been found by others and are due to increased caloric intake, resulting in dilution of maintenance energy costs (Diaz et al., 2001; Raeth-Knight et al., 2009; Davis Rincker et al., 2011).

Health

Calf health disorders were treated according to herd protocol. One male INT calf was given 1 cc i.m. of flunixin meglumine (Banamine; Schering-Plough Animal

Figure 3. Effect of conventional (CON, —●—) and intensified (INT, - -□- -) milk replacer feeding programs on DMI of milk replacer (A), starter (B), protein (C), and fat (D). Error bars indicate standard error of the difference for the interaction of treatment and week. Probability that INT differs from CON: * $P < 0.05$; ** $P < 0.01$.

Health, Kenilworth, NJ) on d 1 of age and received physical therapy to stretch contracted tendons in the front legs. The same calf was also given 5 cc i.m. of ceftiofur sodium (Naxcel; Pharmacia & Upjohn Co. LLC, Kalamazoo, MI) for respiratory illness on d 12 and 13 of life. Two INT heifers (26 and 30 d old) and 1 CON heifer (22 d old) were treated for coughing and diarrhea with 5 cc i.m. of ceftiofur sodium for 5 d. Another INT bull calf (40 d old) was treated for diarrhea with 5 cc i.m. of ceftiofur sodium for 5 d. Overall, more treatments were given for INT than CON calves (4 vs. 1). Increased rates of milk replacer feeding has been shown to increase incidence of respiratory illness, diarrhea, or days medicated in some studies (Cowles et al., 2006; Hill et al., 2006; Quigley et al., 2006) but not others (Nonnecke et al., 2003; Davis Rincker et al., 2011). In a model using stressed calves, morbidity and mortality increased with increased milk replacer feed-

ing level (Quigley et al., 2006). Some of the differences between studies in health responses of calves to milk replacer feeding level may be due to differences in environmental stressors.

Overall, respiratory scores were low, indicating few problems with respiratory disease. Respiratory scores were higher for INT calves than CON calves ($P = 0.05$; Table 5). Although the interaction of treatment and week was not significant, the greatest differences between treatments were found at wk 2 ($P = 0.02$) and wk 7 ($P = 0.02$; Figure 4A). Week 2 was the first week of 2% BW milk replacer feeding and wk 7 was the week of once-daily milk replacer feeding, suggesting that transition periods may have caused more respiratory stress in INT calves. Respiratory scores also tended to be higher for INT calves during wk 5 ($P = 0.09$). Nonnecke et al. (2003) similarly found that calves fed using an intensified milk replacer feeding program had increased respi-

Table 5. Effect of conventional (CON) and intensified (INT) milk replacer feeding programs in Holstein calves on health measures and neutrophil mRNA levels

Item	Treatment ¹		SEM	P-value		
	CON	INT		Treatment	Week ²	Treatment × week
Respiratory score	1.01	1.05	0.01	0.05	0.76	0.26
Fecal score	1.65	1.74	0.15	0.02	0.04	0.11
White blood cells, log ₁₀ cells/mL	7.11	7.13	0.03	0.59	0.37	0.73
Anti-ovalbumin IgG, log ₁₀ OD ³	0.26	0.30	0.03	0.38	<0.001	0.57
Neutrophil gene ⁴	Relative mRNA level, log ₁₀					
<i>SELL</i>	0.73	0.95	0.06	0.02	0.04	0.42
<i>TLR-2</i>	1.03	1.04	0.04	0.80	0.18	0.74
<i>TLR-4</i>	1.00	1.10	0.05	0.12	0.16	0.24
<i>NCF1</i>	0.93	1.02	0.06	0.22	0.28	0.13
<i>SOD1</i>	1.02	0.91	0.05	0.11	0.12	0.35
<i>BPI</i>	0.54	0.66	0.10	0.34	0.004	0.94
<i>TNF-αR</i>	0.91	0.93	0.06	0.75	0.05	0.47
<i>IL-1R1</i>	0.69	0.75	0.10	0.59	0.23	0.43

¹Conventional calves were fed 20.8% CP and 21.0% fat milk replacer at 1.25% of birth BW/d during wk 1 to 6 and 0.625% of birth BW/d during wk 7; INT calves were fed 29.3% CP and 16.2% fat milk replacer at 1.5% of birth BW/d during wk 1, 2% of current BW/d during wk 2 to 6, and 1% of current BW/d during wk 7.

²Respiratory and fecal scores were measured twice daily and averaged by week. All other measures were collected at a single time during wk 1, 3, 5, and 8 of life.

³OD = optical density.

⁴*SELL* = L-selectin; *TLR-2* = toll-like receptor 2; *TLR-4* = toll-like receptor 4; *NCF1* = neutrophil cytosolic factor 1; *SOD1* = superoxide dismutase 1; *BPI* = bactericidal/permeability-increasing protein; *TNF-αR* = tumor necrosis factor α receptor; *IL-1R1* = IL-1 type 1 receptor.

ratory scores compared with a conventional program. Across their observational period, respiratory scores were 1.55 for calves fed using an intensified program compared with 1.10 in control calves. Both the present study and that of Nonnecke et al. (2003) indicate that increased rates of milk replacer feeding increase measures of respiratory distress.

Feces were softer for INT than CON calves ($P = 0.02$; Table 5) and became firmer over time for both treatments ($P = 0.04$ for week effect). Although no significant interaction of treatment and time occurred, the treatment effect was driven by differences during the weeks of greatest milk replacer feeding. Differences in fecal score were significant during wk 3 ($P = 0.005$), 4 ($P = 0.004$), and 6 ($P = 0.02$), and tended to occur during wk 5 ($P = 0.09$; Figure 4B). Softer feces in response to increased milk replacer feeding is commonly reported (Nonnecke et al., 2003; Hill et al., 2006; Raeth-Knight et al., 2009; Davis Rincker et al., 2011), although it was not found by Daniels et al. (2008). Raeth-Knight et al. (2009) found that scouring was increased by intensified milk replacer feeding and, in the present study, 2 INT calves and 0 CON calves were treated for diarrhea.

Treatment did not affect WBC concentration or response to ovalbumin vaccination (Figure 4C and 4D). For both treatments, ovalbumin vaccinations were administered on wk 1, 3, and 5, and anti-ovalbumin IgG increased in response to time ($P < 0.001$ for week ef-

fect). Results from a study conducted by Pollock et al. (1994) found that calves fed milk replacer at a greater level had decreased anti-keyhole limpet hemocyanin IgG₂ and IgA responses and decreased anti-horse erythrocyte titers compared with calves fed milk replacer at a lower level.

Neutrophil mRNA results are presented in Table 5 and Figure 5. Genes upregulated during neutrophil activation were evaluated to assess different aspects of neutrophil function. Across all times, treatment was significant only for *SELL*, with mRNA levels being greater for INT than CON calves ($P = 0.02$). Week was significant for *SELL*, *BPI*, and *TNF-αR* (Table 5), and during the period of study, *SELL* increased while *BPI* and *TNF-αR* decreased. Neutrophil mRNA data from each week are presented in Figure 5. The interaction of treatment and week was not significant for any gene, but several minor differences between treatments were observed at individual weeks. At wk 1, CON calves had greater mRNA levels of *SOD1* compared with INT calves ($P = 0.02$; Figure 5E). By comparing reverse-transformed least squares means, wk 1 *SOD1* was 85% greater in CON than INT calves. At wk 8, INT calves had increased mRNA levels of *SELL* ($P = 0.02$; Figure 5A) and *NCF1* ($P = 0.01$; Figure 5D) and tended to have increased *TLR-4* ($P = 0.06$; Figure 5C) compared with CON calves. Reverse-transformed means corresponded to 153% greater *SELL*, 129% greater *NCF1*, and 95% greater *TLR-4* at wk 8.

Figure 4. Effect of conventional (CON, —●—) and intensified (INT, - -□- -) milk replacer feeding programs on respiratory score (A), fecal score (B), $\log_{10}$ white blood cell (WBC) concentration (C), and serum anti-ovalbumin IgG (D). Error bars indicate standard error of the difference for the interaction of treatment and week. Probability that INT differs from CON: † $P < 0.10$; * $P < 0.05$; ** $P < 0.01$. OD = optical density.

During neutrophil response to infection, SELL expressed on the surface of neutrophils interacts with inflammatory markers expressed on the surface of endothelial cells to allow for neutrophils to exit the blood and enter infected tissue (Kansas, 1996). Reduced neutrophil SELL increases susceptibility to infection in rodent models and is proposed to contribute to periparturient cow diseases (Kansas, 1996; Burton et al., 2005). Toll-like receptor 4 expressed on the surface of neutrophils recognizes LPS from gram-negative pathogens and initiates an innate immune response. As reviewed by De Schepper et al. (2008), increased TLR-4 on leukocytes results in a more rapid response to *Escherichia coli* mastitis infections. Neutrophil cytosolic factor 1 is a component of NADPH oxidase that generates superoxide, a free radical that can damage engulfed and extracellular pathogens (Robinson, 2009). Treatment differences in SELL, as well as and potential biologically relevant differences in *NCF1* and *TLR-4* at wk 8,

suggest an increase in activation status of neutrophils from INT calves and a moderately increased ability of neutrophils to migrate into tissues and destroy pathogens. It is possible that a more activated neutrophil population may have contributed to the increase in respiratory scores in the INT calves.

CONCLUSIONS

Compared with CON calves, INT calves had greater ADG but reduced starter DMI. Respiratory and fecal scores were greater in INT than CON calves, indicating greater incidence of respiratory ailments and softer feces. No treatment effect on ovalbumin vaccination response was observed. Neutrophils of INT calves had increased mRNA levels of SELL and the INT treatment may have resulted in greater activation of neutrophils postweaning.

Figure 5. Effect of conventional (CON, —●—) and intensified (INT, - -□- -) milk replacer feeding programs on neutrophil mRNA levels of L-selectin (*SELL*; A), toll-like receptor 2 (*TLR-2*; B), toll-like receptor 4 (*TLR-4*; C), neutrophil cytosolic factor 1 (*NCF1*; D), superoxide dismutase 1 (*SOD1*; E), bactericidal/permeability-increasing protein (*BPI*; F), tumor necrosis factor α receptor (*TNF- α R*; G), and IL-1 type 1 receptor (*IL-1R1*; H). Error bars indicate standard error of the difference for the interaction of treatment and week. Probability that INT differs from CON: † $P < 0.10$; * $P < 0.05$.

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