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Effects of Starter Protein Levels and Amounts of Milk Fed on Animal Health and Rumen Microbiota Changes in Holstein Male Calves

Research Article

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ABSTRACT

The aim of this study was to investigate the effect of two different levels of starter protein and amounts of milk fed on animal health, fecal score, immune responses and microbial population in the rumen of Holstein male calves. Two levels of starter crude protein (17 and 20% of starter dry matter (DM) and two levels of milk fed (7 and 10% of body weight) were combined in a 2 × 2 factorial experimental design. Forty newborn male Holstein calves (initial body weight 40±2 kg) were used for 75 days. Feeding the colostrum was performed immediately after birth for 3 days. Every calf consumed the probiotic mixture of protexin and *Saccharomyces cerevisiae* via milk (2 g/day). Water and calf starter were offered free choice. Individual fecal score was measured daily. Results showed that starter intake and weight gain were affected by days of age ($P<0.05$). But, there were no interactions between starter protein levels and amounts of milk fed on starter intake and weight gain ($P>0.05$). Starter protein levels, amounts of milk fed and their interactions had no effect on protozoa population, fecal consistency, general health score (GHS) and number of days with diarrhea ($P>0.05$). There were significant interactions between amounts of milk fed and starter protein levels on bacteria population ($P<0.05$). Also, amounts of milk fed and starter protein levels did not affect fecal fluidity, but interaction between them was significant ($P=0.046$). By increasing starter protein levels, the numbers of bacteria and protozoa decreased and increased, respectively. The effects of starter protein levels, amounts of milk fed and their interactions on immune response of calves were not statistically significant.

KEY WORDS calves, fecal score, general health score, milk fed, starter protein.

INTRODUCTION

Calves are faced with major stress events such as transportation, marketing, dietary changes, and exposure to a variety of infectious agents. Consequently, calves consume less milk (Loerch and Fluharty, 1999), are predisposed to loss of barrier function of the gut (Soderholm and Perdue, 2001), and may suffer from impaired immune function (Sheridan *et al*. 1994).

Moreover, the protective potential of the microbial gut flora tends to decrease (Cray *et al*. 1998). In addition, the antibiotics diminish not only the activity of the pathogenic flora, but also that of the protective flora. Improved health and performance is the main goal of calves rearing programs (Drackley, 2008).

Therefore, applying the appropriate strategy is the key factor to maximizing the daily weight gain while reducing the incidence of disease.

Over the last decade, there has been an improvement in nutrition of calves, especially in pre-weaning period (Hill *et al*. 2013). Ruminants depend on microbial populations which exist on their alimentary tract and their performance depends on the activities of their microorganisms to utilize the dietary feeds.

Rumen microbial ecosystem mainly consist of bacteria, protozoa and fungi. Their main function is fermentative process of feeds. Warner (1961) reported that some factors can influence the rumen microbial population. One animal at different times, or different animals on the same ration and dietary regime had very different rumen microbial populations. Therefore, calf performance can be affected. To establish a protective flora in veal calves, the use of probiotics is promising. Probiotics have been shown to improve anaerobiosis and supply nutrients to ruminal microbes (Chiquette *et al*. 2012). Probiotic consumption (for example: *Saccharomyces cerevisia*) is one of methods for controlling the fermentation process and can increase bacteria population in the ruminal fluid (Newbold *et al*. 1995). Therefore, in this study, we added a mix of probiotics to milk.

Also, amounts of milk fed and starter protein levels are important factors that affect calves performance. Milk feeding has a significant role in the health of calves before weaning, and it has positive effects on the future performance post-weaning (Khan *et al*. 2007). Therefore the calf feeding programs are required to be developed in early life. Requirements of protein of ruminants are calculated NRC (1989) and NRC (2001).

The NRC (1989) and NRC (2001) has recommended 18% crude protein (CP) in starter feed on dry matter basis (20% as-fed basis). Recently, some researchers reported that growth prediction models for protein needs of calves were not strong enough (Stamey *et al*. 2012; Hill *et al*. 2013). Hill *et al*. (2005) indicated that there were no differences in the performance of calves with starters containing 18 and 22% CP.

However, Drackley *et al*. (2002) found that performance of calves fed with starters containing 22% CP were more better than those fed 18% CP. Sekine *et al*. (2004) and Labussiere *et al*. (2008) showed that performance and feed intake of calves was not affected by CP concentration of the starters.

However, research during the last decade has shown the advantages of providing more milk or milk replacer on improving calf growth, welfare, and future productivity (Khan *et al*. 2011).

It is important to note that providing greater amounts of milk does not always improve performance, because of the challenges that arise during weaning period such as reducing starter intake and consequently, impair rumen development (Khan *et al*. 2011).

Several studies showed that there is no relationship between amount of milk fed and diarrhea, but diarrhea is caused related to maintenance, housing and hygiene (Ozkaya and Toker, 2012; Hammon *et al*. 2002).

The amount of milk intake and starter protein level were evaluated separately on calves' performance and different results have been achieved. But, it is necessary to re-evaluate the effects of two different levels of starter protein and amounts of milk fed on health and microbial population in the rumen fluid of calves, while we added probiotic to the milk (due to the positive effects of probiotics on the health of calves).

MATERIALS AND METHODS

Animals, housing and diets

This experiment was conducted at Phazil Agri-Animal Production Co. (Isfahan, Iran). Forty newborn male Holstein calves (initial body weight 40±2 kg) were separated from their dams immediately after birth, weighed and in a 2 × 2 factorial arrangements randomly (based on their initial body weight on d 3 of age) allocated to one of four treatments (n=10/group): 1) milk fed=7% of body weight (BW) and starter diet containing 17% CP, 2) milk fed= 10% of BW and starter diet containing 17% CP, 3) milk fed= 10% of BW and starter diet containing 20% CP and 4) milk fed= 10% of BW and starter diet containing 20% CP. Feeding the colostrum was performed immediately after birth for 3 days.

Calves were kept in individual hutches with straw bedding. Calves were fed by whole milk daily at 07:00 and 17:00. Water and calf starter were offered free choice. The composition and the analyzed nutrient content of offered feeds are provided in Tables 1 and 2. The rations were isoenergetic. Every calf consumed the probiotic mixture of Protexin and *Saccharomyces cerevisiae* via milk (2 g/d).

Microbial composition of protexin were *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Bifidobacterium bifidium*, *Enteroccus faecium*, *Streptoccus thermopphilus*, *Asperggillus oryzae* and *Candida pintolopsiie*.

Fecal scores

Individual fecal score was measured daily. Fecal output was observed and scored (fecal scoring: fluidity, 1= normal, 2= soft, 3= runny and 4= watery; consistency, 1= normal, 2= foamy, 3= mucus and 4= sticky (Mcleod *et al*. 2010). At the end of every week, the average of fecal scores were measured (Magalhaes *et al*. 2008).

Table 1 Ingredient and chemical composition of starter (percent in diet dry matter)

Composition (%)	Percenet	Chemical composition	Content
Corn grain	42	Dry matter, %	91.05
Barley grain	20	Energy (Mcal/kg)	2.95
Canola meal	3	Crude orotein, %	20.07
Soybean meal	26	Acid detergent fiber, %	6.93
Soybean whole roasted	5	Neutral detergent fiber, %	13.20
Sodium bicarbonate	0.7	Fat, %	4.00
Salt	0.5	Non- fiber carbohydrate, %	55.40
Calcium carbonate	0.8		
Vitamin premix[1]	1		
Mineral premix[2]	1		

[1] 1 kg vitamin premix contains: vitamin A: 13×10^5 IU; vitamin D_3: 8×10^4 IU; vitamin E: 6600 IU; vitamin B_1: 880 mg; vitamin B_{12}: 9.4 mg; vitamin C: 16500 mg; Riboflavin: 850 mg; Thiamin: 1740 mg; Pantothenic acid: 1345 mg; Pyridoxine: 870 mg; Folic acid: 76 mg and Biotin: 13.4 mg.
[2] 1 kg mineral premix contains: Copper: 0.1 g; Iron: 0.2 g; Manganese: 0.5 g; Zinc: 0.5 g; Magnesium: 0.8 g; Cobalt: 0.008 g; Selenium: 0.002 g and Iodine: 0.002 g.

Table 2 Ingredient and chemical composition of starter (percent in diet dry matter)

Composition (%)	Percent	Chemical composition	Content
Corn grain	34	Dry matter, %	93.05
Barley grain	25	Energy (Mcal/kg)	2.95
Wheat	10	Crude protein, %	17.03
Soybean meal	27	Acid detergent fiber, %	7.13
Sodium bicarbonate	0.7	Neutral detergent fiber, %	13.42
Salt	0.5	Fat, %	4.08
Calcium carbonate	0.8	Non- fiber carbohydrate, %	54.80
Vitamin premix[1]	1		
Mineral premix[2]	1		

[1] 1 kg vitamin premix contains: vitamin A: 13×10^5 IU; vitamin D_3: 8×10^4 IU; vitamin E: 6600 IU; vitamin B_1: 880 mg; vitamin B_{12}: 9.4 mg; vitamin C: 16500 mg; Riboflavin: 850 mg; Thiamin: 1740 mg; Pantothenic acid: 1345 mg; Pyridoxine: 870 mg; Folic acid: 76 mg and Biotin: 13.4 mg.
[2] 1 kg mineral premix contains: Copper: 0.1 g; Iron: 0.2 g; Manganese: 0.5 g; Zinc: 0.5 g; Magnesium: 0.8 g; Cobalt: 0.008 g; Selenium: 0.002 g and Iodine: 0.002 g.

General health score (GHS)

To monitor overall health in each group, a general health score (GHS) was designed. The incidence of diarrhea and therapeutic treatments for digestive, respiratory or other diseases were weighted and GHS was calculated using the formula suggested by Timmerman *et al.* (2005) as follows:

GHS per animal= 28 – 1 × total number of diarrheic days – 2 × the number of individual therapeutic treatments for digestive diseases – 3 × the number of individual therapeutic treatments for respiratory diseases − 2 × the number of individual therapeutic treatments for infections other than digestive or respiratory – 2 × the number of antibiotic treatments on a herd basis.

Bacteria counting

Rumen fluid (20 mL) was collected 3 hours after the morning feeding by stomach tube and were immediately poured into a bottle that containing co_2 and transferred to laboratory.

Then rumen fluid contents were mixed with dilution solution and added to culture medium (Table 3). For 30 second, the samples flushed with co_2 and then pipes doors were tightly closed and incubated at 39 ˚C. After 14 days, pH was measured.

When bottom of tube became dark and gray color, counting was done by most probable number (MPN) tables (Dehority, 2003).

Protozoa counting

In order to investigate the protozoa population, rumen contents (20 mL) were collected by means of esophagus tube on day 60. The samples were strained using of cheese cloth. Aldehyde solution used to fix the protozoa. Equal volumes (10 mL) of rumen fluid and formalin (10%) were mixed together. Then samples were stained with methylene blue, logol and brilliant green in darkness. The count was performed by means of a microscope with magnification of × 40. The counting results were reported as concentration (number per mL/ruminal fluid) (Ivan *et al*. 2001).

Skin test

Cell-mediated immune response was evaluated to determine skin thickness in response to phytohaemagglutinin (PHA) injection at 60 days after birth. Intradermal injection of PHA (150 µg of PHA in 0.1 mL phosphate buffer (PBS)) was performed on a top part shaved area of shoulder. The skinfold thickness was measured before injection and 2, 4, 6, 8, 12 and 24 h after injection using micrometric clippers (Roodposhti and Dabiri, 2012).

Table 3 Components of culture medium (percent)

Components	Percent
Mineral solution I* (V/V)	15
Mineral solution II** (V/V)	15
Resazurin 0.1% (V/V)	0.1
Rumen fluid (V/V)	40
Distilled water (V/V)	23.7
Glucose (W/V)	0.1
Cellobiose (W/V)	0.1
Maltose (W/V)	0.1
Xylose (W/V)	0.1
Cellolose suspension 3% (V/V)	0.75
Hemin solution 0.1% (V/V)	0.1
Trypticase (W/V)	0.2
Yeast Extract (W/V)	0.05
Volatile fatty acid (VFA) mixture*** (V/V)	0.45
Cystein-HCL-water 3% (V/V)	1.67
Sodium carbonate solution 12% (V/V)	3.33

* Contained: 3 g K_2HPO_4 per liter of distilled water.
** 3 g KH_2PO_4, 6 g NaCl, 0.6 g $MgSo_4$, 0.6 g CaCl, 6 g $(NH_4)_2S0_4$ per liter of distilled water.
*** 17 mL acid acetic, 6 mL propionic acid, 4 mL butric acid, 1 mL iso-butric acid, 1 mL N-valeric acid, 1 mL iso-valeric acid and 1 mL alpha methyle-butric acid.

Statistical analysis

Data were analyzed in a completely factorial (2×2) randomized design using the following statistical model:

$$X_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{(ijk)}$$

Where:
X_{ij}: dependent variable.
μ: overall mean.
α_i: effect of milk fed factor.
β_j: effect of starter protein level factor.
$\alpha\beta_{ij}$: interaction between milk fed and starter protein level.
$\varepsilon_{(ijk)}$: effect of error.

Data were analyzed by SAS software (SAS, 2002). Duncan's test was conducted to determine the differences between groups means. Significance was declared at $P \leq 0.05$. Body weight was considered as covariates.

RESULTS AND DISCUSSION

Starter intake and weight gain

Starter intake is one of factors that can affect microbial population in the rumen fluid (Warner, 1961). Results showed that increasing level milk feeding increased weight gain and decreased starter intake of calves. In the present study, starter intake and weight gain were affected by days of age (P<0.05). But, there were not interactions between starter protein levels and amounts of milk fed on starter intake and weight gain (P>0.05).

By decreasing starter protein levels, starter intake was numerically increased, but the differences were not significant (Table 4).

Bacteria and protozoa population in the rumen fluid

Results showed that interaction between milk fed and starter protein levels on bacterial population was significant (P=0.045). Treatment 1 (CP=17% and milk fed=7% of BW) and 4 (CP=20% and milk fed=10% of BW) had the highest and lowest bacterial population respectively (4.23×10^9 *vs.* 2.99×10^9/mL) and differences among treatments were significant (Table 6). The effect of none of main factors and their interaction on protozoa population were significant (P>0.05). By increasing starter protein levels, bacteria and protozoa populations decreased and increased respectively (Table 5). The rumen protozoa numbers in treatment 4 (CP=20% and milk fed=10% of BW) and 2(CP=17% and milk fed=10% of BW) was recorded respectively (3.40×10^6 *vs.* 2.59×10^6/mL), but there were no significant differences which have been observed between treatments (Table 6).

Ruminal bacteria have important role in food digestion, but balance need between the number of bacteria, protozoa and fungi for optimum fermentation and good performance (Wanapat and Pimpa, 1999).

The ruminal microbial population is not stable, physiological factors such as age, nutritional behaviors, production level, animal health, nature and relationships between microbial population and external factors such as the chemical composition of diet, nature and amounts of feed intake, frequency of feeding, season and other geographic factors can change the ratio of microorganisms in the rumen fluid.

Also, the relationship between microbial population (competition, cooperation, neutralization, interaction between species, hunt), different physiologies (growth rate, substrate, energy metabolism, resistance to acidity and toxic materials) affect establishment and development of microbial population (Russell *et al*. 1988). The existence of specific animal effects on microbial numbers found in rumen samples has been known for some time (Warner, 1961).

Also, the researchers showed that a large parts of the variation among animals in the numbers of bacteria and protozoa in rumen samples is due to the tendency of individual animals to maintain different bacteria/protozoa ratios in the rumen (Teather *et al*. 1984).

The amounts of feed intake influences microorganisms population in the rumen. Therefore, composition of diet and amounts of feed intake can change microbial population.

By increasing feed intake, the energy expended for the maintenance of microorganisms decreases. Because their passage rate increased and retention time will be reduced in the rumen (Van Soest, 1994).

Syncornization between amounts of energy and protein affect the number of rumen microorganisms. Some of nutrients are favorable for some of microorganisms, but for some of microorganisms are not (Browe *et al*. 2005).

Table 4 Effect of treatments on average of starter intake (gr/day) and and weight gain (g/day)

Time	Experimental treatments				SE	P-value						
	1	2	3	4		Protein	Milk	Day	Protein × milk	Protein × day	Milk × day	Protein × milk × day
Starter intake (g/day)												
First month	212.21	177.95	140.17	144.99	12.47	NS	NS	*	NS	NS	NS	NS
Second month	700.59	664.65	624.10	606.85	13.85	NS	NS	*	NS	NS	NS	NS
Total period	456.50	421.30	382.13	375.92	22.01	NS	NS	*	NS	NS	NS	NS
Weight gain (g/day)												
First month	374.30	386.20	313.30	337.80	35.43	NS	NS	*	NS	NS	NS	NS
Second month	697.60	828.60	718.10	717.10	68.85	NS	NS	*	NS	NS	NS	NS
Total period	536.01	607.40	514.80	527.01	43.03	NS	NS	*	NS	*	NS	NS

1) Crude protein (CP)= 17% and amount of milk fed= 7% of body weight; 2) CP= 17% and amount of milk fed= 10% of body weight; 3) CP= 20% and amount of milk fed= 7% of body weight and 4) CP= 20% and amount of milk fed= 7% of body weight.
* (P<0.05).
SE: standard error.
NS: non significant.

Table 5 Effect of amounts of milk fed and starter protein levels on bacteria and protozoa numbers (Mean±SE) in the rumen fluid

Item	Protein level (%)		P-value	Milk fed (% body weight)		P-value
	17	20		7	10	
Bacteria numbers ($\times 10^9$ mL^{-1})	3.90±0.51	3.36±0.51	0.328	3.98±0.51	3.28±0.51	0.265
Protozoa numbers ($\times 10^6$ mL^{-1})	2.62±2.41	3.20±2.41	0.073	2.84±2.41	2.99±2.41	0.061

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SE: standard error.

Table 6 Interaction between milk fed and starter protein levels on bacteria and protozoa numbers in the rumen

Item	Experimental treatments				SE	P-value
	1	2	3	4		
Bacteria numbers ($\times 10^9$ mL^{-1})	4.23^{a}	3.58^{b}	3.73^{ab}	2.99^{b}	0.51	0.045
Protozoa numbers ($\times 10^6$ mL^{-1})	2.68	2.59	3.01	3.40	2.41	0.256

1) Crude protein (CP)= 17% and amount of milk fed= 7% of body weight; 2) CP= 17% and amount of milk fed= 10% of body weight; 3) CP= 20% and amount of milk fed= 7% of body weight and 4) CP= 20% and amount of milk fed= 7% of body weight.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SE: standard error.

Belanche *et al.* (2012) examined the effect of diet, presence or absence of protozoa on bacteria population and reported that protozoa inhibited the effect of diet on the bacteria population.

The amounts of dietary energy affects the density of protozoa in the rumen. So that, by reducing the amounts of dietary energy, protozoa population in the rumen reduce. There is direct correlation between protozoa numbers in the rumen and glucose fermentation and ammonia (Soares *et al.* 2008). In this study the diets were iso-energetic. Therefore the energy of diet did not have effect on microbiota numbers in the rumen fluid. But, protein to energy ratio should be investigated. There is negative relationship between the number of bacteria and protozoa (Teather *et al.* 1984) that our results also showed.

Fecal score and general health score

Results showed that main factors (the amounts of milk fed and starter protein levels) and their interactions did not affect fecal consistency (Tables 7 and 8). Also, amounts of milk fed (P=0.062) and starter protein levels (P=0.072) did not affect fecal fluidity, but interaction between them was significant difference (P=0.046).

Fecal fluidity in treatment 2 (CP=17% and milk intake=10% of BW) and treatment 1 (CP=17% and milk intake=7% of BW) was recorded 2.161 and 1.735 respectively and difference was significant (Table 8).

During the study, there were no significant differences between protein levels of starter (17 and 20% CP) and amounts of milk fed (7 and 10% of BW) and their interactions on number days with diarrhea (Tables 7 and 8).

Number of days with diarrhea were 2.66, 2.16, 2.25 and 2.34 for treatments 1 to 4, respectively. No significant differences were observed in general health score among treatments (P=0.178) (Table 8).

Conneely *et al.* (2014) reported that amounts of milk fed did not have a negative impact on health status of calves as indicated by fecal fluidity and days with diarrhea. Results of the present study is in contrast with Quigley *et al.* (2006) and Diaz *et al.* (2001) who stated that fecal scores, days with scours, proportion of calves treated with antibiotics, and number of days treated with antibiotics were greater when additional milk replacer was fed.

These contrasting results could be due to differences in management and climatic conditions across studies (Conneely *et al.* 2014).

Table 7 Effect of amounts of milk fed and starter protein levels on fecal score and general heath score

Items	Protein level (%)		SE	Milk fed (% body weight)		SE
	17	20		7	10	
Consistency[1]	1.296	1.189	0.176	1.139	1.346	0.152
Fluidity[2]	1.948	1.928	0.073	1.775	2.100	0.062
GHS[3]	18.655	18.370	0.087	18.375	18.655	0.072
Number days with diarrhea incidence	2.411	2.290	0.237	2.451	2.250	0.157

1: 1-normal, 2-foamy, 3-mucus, 4- sticky; 2: 1-normal, 2- soft, 3-runny, 4- watery and 3: GHS= general health score.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SE: standard error.

Table 8 Interaction between milk fed and starter protein levels on fecal score and general heath score

Items	Experimental treatments*				SE	P-value
	1	2	3	4		
Consistency[1]	1.180	1.412	1.098	1.280	0.387	0.076
Fluidity[2]	1.735^{b}	2.161^{a}	1.816^{b}	2.041^{ab}	0.421	0.046
GHS[3]	18.78	18.32	17.96	18.99	1.460	0.178
Number days with diarrhea incidence	2.66	2.16	2.25	2.34	0.720	0.841

* 1) Crude protein (CP)= 17% and amount of milk fed= 7% of body weight; 2) CP= 17% and amount of milk fed= 10% of body weight; 3) CP= 20% and amount of milk fed= 7% of body weight and 4) CP= 20% and amount of milk fed= 7% of body weight.
1: 1-normal, 2-foamy, 3-mucus, 4- sticky; 2: 1-normal, 2- soft, 3-runny, 4- watery and 3: GHS= general health score.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SE: standard error.

Table 9 Effect of experimental treatments on skin fold to injection of phytohaemagglutinin (PHA) (mm)

Hours after PHA injection	Experimental treatments				SE	P-value		
	1	2	3	4		Protein	Milk	Protein × milk
2	3.75	3.60	3.39	3.24	0.560	NS	NS	NS
4	3.09	2.90	2.87	2.96	0.470	NS	NS	NS
6	2.67	2.36	2.40	2.58	0.431	NS	NS	NS
8	2.38	2.06	2.10	2.29	0.390	NS	NS	NS
12	1.97	1.89	1.84	1.80	0.211	NS	NS	NS
24	1.43	1.32	1.39	1.65	0.300	NS	NS	NS

1) Crude protein (CP)= 17% and amount of milk fed= 7% of body weight; 2) CP= 17% and amount of milk fed= 10% of body weight; 3) CP= 20% and amount of milk fed= 7% of body weight and 4) CP= 20% and amount of milk fed= 7% of body weight.
SE: standard error.
NS: non significant.

Previous researchers reported no significant change in scour score with starters varying in CP concentrations from 19 to 25%, which is consistent to our observation (Akayezu *et al*. 1994; Stamey *et al*. 2012). Fecal scores and diarrhea incidence are influenced by physiological, environmental and management factors and are less affected by the type and composition of starters (Lesmeister *et al*. 2004). Hill *et al*. (2010) reported that fecal score increased by increasing level of milk intake. The results of this experiment are in agreement with those were reported by Hill *el at*. (2010). Ozkaya and Toker (2012) reported that amounts of milk fed, starter protein level and interaction between them did not affect scour and body temperatures of calves. In this experiment, amounts of milk fed had no significant differences on health of calves. Also, Yavuz *et al*. (2015) reported level of milk feeding between three groups (low milk: 172 L to weaning on 49 days of age, moderate milk: 315 L milk to 56 days of age and high milk: 416 L to weaning at 56 days) did not affect health status of calves pre- and post-weaning and fecal scores tended to be low (softer) in high milk (HM) group.

There was tendency for firmer feces and fewer sums of scouring days in calves receiving more milk per day. But, some researchers reported a higher occurrence of diarrhea in calves supplied higher levels of milk or milk replacers, compared with restricted fed calves (Diaz *et al*. 2001; Quigley *et al*. 2006), but others reported no difference (Jasper and Weary, 2002; Khan *et al*. 2007).

A high incidence of diarrhea is rather related to poor sanitary, management, and housing conditions than to level of milk intake (Hammon *et al*. 2002; Jasper and Weary, 2002).

But Khan *et al*. (2007) observed that milk or milk replacement restrictions affected the growth and health of calves (due to nutrient deficiencies). The amounts, composition and method of feeding are effective on the performance, health behavior and other calf traits (Browe *et al*. 2005).

Immune response

The skin test provides a measure of the proliferative response potential of circulating T lymphocytes to an injected mitogen such as phytohemagglutinin (Smits *et al*. 1999).

Results showed that starter protein levels, amounts of milk fed and their interaction had no effect in cell-mediated immune response of calves (Table 9).

CONCLUSION

Variations in different experiments about the impact of levels of milk intake and starter protein on health and microbial population of calves probably depend on the level and quality of dry feed consumption. It is possible that milk feeding interactions and source of proteins in rations might explain different responses in the present study. Protozoa population was more when the starter CP content was higher compared to the lower CP starter. Level of milk feeding and starter protein did not affect health status of calves. Perhaps, probiotic supplementation had positive effect on feed intake and performance of calves and cause these results. Under the circumstances, low levels of starter protein and milk intake did not negative effect on health status. Further studies are needed to evaluate effects of ratio of protein to energy in diet on performance and rumen conditions.

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REFERENCES

Akayezu J.M., Linn J.G., Otterby D.E. and Hansen W.P. (1994). Evaluation of calf starters containing different amounts of crude protein for growth of Holstein calves. *J. Dairy Sci.* **77,** 1882-1889.

Belanche A., Fuente G., Pinloche E., Newbold C.J. and Balcells J. (2012). Effect of diet and absence of protozoa on the rumen microbial community and on the representativeness of bacterial fractions used in the determination of microbial protein synthesis. *J. Anim. Sci*. **90,** 3924-3936.

Browe E.G., Vandehaar M.J., Daniels K.M., Liesman J.S., Chapin L.T., Heisler D.H. and Weber Nielsen M.S. (2005). Effects of increasing energy and protein intake on body growth and carcass composition of heifer calves. *J. Dairy Sci*. **88,** 585-594.

Chiquette J., Allison M.J. and Rasmussen M. (2012). Use of *Prevotella bryantii* 25A and a commercial probiotic during subacute acidosis challenge in mid lactation dairy cows. *J. Dairy Sci.* **95,** 5985-5995.

Conneely M., Berry D.P., Murphy J.P., Lorenz I., Doherty M.L. and Kennedy E. (2014). Effects of milk feeding volume and frequency on body weight and health of dairy heifer calves. *J. Lives. Sci*. **161,** 90-94.

Cray W.C., Casey T.A., Bosworth B.T. and Rasmussen M.A. (1998). Effect of dietary stress on fecal shedding of *Escherichia coli* O157:H7 in calves. *Appl. Environ. Microbiol*. **64,** 1975-1979.

Dehority B.A. (2003). Rumen Microbiology. Publication Data. Nottingham, United Kingdom.

Diaz M.C., Van Amburgh M.E., Smith J.M., Kelsey J.M. and Hutten E.L. (2001). Composition of growth of Holstein calves fed milk replacer from birth to 105 kilogram body weight. *J. Dairy Sci*. **84,** 830-842.

Drackley J. K. (2008). Calf nutrition from birth to breeding. *Vet. Clin. North. Am. Food Anim. Pract.* **24,** 55-86.

Drackley J. K., Bartlett K.S. and Blome R.M. (2002). Protein content of milk replacers and calf starters for replacement calves. Available at: www.livestocktrail.uiuc.edu/dairynet/paperDisplay.cfm?ContentID=339.

Hammon H. M., Schiessler G., Nussbaum A. and Blum J.W. (2002). Feed intake patterns, growth performance, and metabolic and endocrine traits in calves fed unlimited amounts of colostrum and milk by automate, starting in the neonatal period. *J. Dairy Sci*. **85,** 3352-3362.

Hill T.M., Aldrich J.M. and Schlotterbeck R.L. (2005). Nutrient sources for solid feeds and factors affecting their intake by calves. Pp. 113-133 in Calf and Heifer Rearing: Principles of Rearing the Modern Dairy Heifer from Calf to Calving. P. Garnsworthy, Ed. Nottingham University Press, Nottingham, United Kingdom.

Hill T.M., Bateman H.G., Aldrich J.M. and Schlotterbeck R.L. (2010). Effect of milk replacer program on digestion of nutrients in dairy calves. *J. Dairy Sci.* **93(3),** 1105-1115.

Hill T.M., Bateman H.G., Quigley J.D., Aldrich J.M., Schlotterbeck R.I. and Heinrichs A.J. (2013). Review: New information on protein requirements and diet formulation for dairy calves and heifers since the Dairy NRC 2001. *Prof. Anim. Sci*. **29,** 199-207.

Ivan M., Neill L., Alimon R. and Jalaludin S. (2001). Effects of bentonite on rumen fermentation and duodenal flow of dietary components in sheep fed palm kernel cake by-product. *Anim. Feed Sci. Technol*. **92(1),** 127-135.

Jasper J. and Weary D.M. (2002). Effects of ad libitum milk intake on dairy calves. *J. Dairy Sci*. **85,** 3054-3058.

Khan M.A., Lee H.J., Lee W.S., Kim H.S., Ki K.S., Hur T.Y., Suh G.H., Kang S.J. and Choi Y.J. (2007). Structural growth, rumen development, and metabolic and immune responses of Holstein male calves fed milk through step-down and conventional methods. *J. Dairy Sci*. **90,** 3376-3387.

Khan M.A., Weary D.M. and Von Keyserling M.A.G. (2011). Hay intake improves performance and rumen development of calves fed higher quantities of milk. *J. Daily Sci*. **94,** 3547-3553.

Labussiere E., Dubois S., van Milgen J., Bertrand G. and Noblet J. (2008). Effects of dietary crude protein on protein and fat deposition in milk-fed veal calves. *J. Dairy Sci*. **91,** 4741-4754.

Lesmeister K.E., Heinrichs A.J. and Gabler M.T. (2004). Effects of supplemental yeast (*Saccharomyces cerevisiae*) culture on rumen development, growth characteristics, and blood parameters in neonatal dairy calves. *J. Dairy Sci*. **87,** 1832-1839.

Loerch S.C. and Fluharty F.L. (1999). Physiological changes and digestive capabilities of newly received feedlot cattle. *J. Anim. Sci*. **77,** 1113-1119.

Magalhaes V.J.A., Susca F., Lima F., Branco A.F., Yoon I. and Santos J.E.P. (2008). Effect of feeding yeast culture on performance, health, and immune competence of dairy calves. *J. Dairy Sci*. **91,** 1497-1509.

McLeod K.R., Harmon D.L. and Riddell J.B. (2010). Addition of a *Bacillus* based probiotic to the diet of pre ruminant calves: Influence on growth, health, and blood parameters. *Int. J. Appl. Res. Vet. Med.* **8,** 78-85.

Newbold C.J., Wallace R.J., Chen X.B. and McIntosh F.M. (1995). Different strains of *Saccharomyces cerevisiae* differ in their effects on ruminal bacterial numbers *in vitro* and in sheep. *J. Anim. Sci.* **73,** 1811-1818.

NRC. (1989). Nutrient Requirements of Dairy Cattle. 6th Ed. National Academy Press, Washington, DC, USA.

NRC. (2001). Nutrient Requirements of Dairy Cattle. 7th Ed. National Academy Press, Washington, DC, USA.

Ozkaya S. and Toker M.T. (2012). Effect of amounts of milk fed, weaning age and starter protein level on growth performance in Holstein calves. *Arch. Tierz.* **55,** 234-244.

Quigley J.D., Wolfe T.A. and Elsasser T.H. (2006). Effect of additional milk replacer feeding on calf health, growth, and selected blood metabolites in calves. *J. Dairy Sci*. **89,** 207-216.

Roodposhti P. and Dabiri N. (2012). Effects of probiotic and prebiotic on average daily gain, fecal shedding of escherichia coli, and immune system status in newborn female calves. *J. Anim. Sci*. **25,** 1255-1261.

Russell J.B., Strovel H.J. and Chen G. (1988). Enrichment and isolation of a ruminal bacterium with a very high specific activity of ammonia production. *Appl. Environ. Microbiol*. **54,** 872-877.

SAS Institute. (2002). SAS®/STAT Software, Release 9.1. SAS Institute, Inc., Cary, NC. USA.

Sekine J., Kamel H.E.M., Hisinuma M., Nasir A., Fadel El-Seed M.A., Morita S., Hanada M. and Kondo S. (2004). Effect of protein sources with different rumen degradability in a diet on the efficiency of utilization of metabolizable energy by calves. *Arch. Tierz*. **47,** 325-335.

Sheridan J.F., Dobbs C., Brown D. and Zwilling B. (1994). Sychoneuroimmunology: Stress effects on pathogenesis and immunity during infection. *Clin. Microbiol. Rev*. **7,** 200-212.

Smits J.E., Bortolotti G.R. and Tella J.L. (1999). Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immune competence. *Funct. Ecol*. **13,** 567-572.

Soares P.C., Martinele I., D'Agosto M., Maruta C.A., Sucupira M.C.A., Antonelli A.C., Mori C.S. and Ortolani E.L. (2008). Effect of an energy deficient diet on populations of ciliate protozoans in bovine rumen. *Arq. Bras. Med. Vet. Zootec.* **60(1),** 148-155.

Soderholm J.D. and Perdue M.H. (2001). Stress and the gastrointestinal tract II. Stress and intestinal barrier function. *American. J. Physiol. Gastrointest. Liver Physiol*. **280,** 7-13.

Stamey J.A., Janovick N.A., Kertz A.F. and Drackley J.K. (2012). Influence of starter protein content on growth of dairy calves in an enhanced early nutrition program. *J. Dairy Sci*. **95,** 3327-3336.

Teather R.M., Mahadevan S., Erfle J.D. and Sauer F.D. (1984). Negative correlation between protozoal and bacterial Levels in rumen samples and its relation to the determination of dietary affects on the rumen microbial population. *Appl. Environ. Microbiol*. **47(3),** 566-570.

Timmerman H.M., Mulder L., Everts H., Van Espen D.C., Vander Wal E., Klaassen G., Rouwers S.M.G., Hartemink R., Rombou ts F.M. and Beynen A.C. (2005). Health and growth of veal calves fed milk replacers with or without probiotics. *J. Dairy Sci*. **88,** 2154-2165.

Van Soest P.J. (1994). Nutritional Ecology of the Ruminant. Cornell University Press, Ithaca, New York.

Wanapat M. and Pimpa O. (1999). Effect of ruminal NH_3-N levels on ruminal fermentation purina derivatives, digestibility and rice straw intake in swamp buffaloes. *Asian-Australasian J. Anim. Sci*. **12,** 904-907.

Warner A.C.I. (1961). Some factors influencing the rumen microbial population. *J. Gen. Microbiol*. **28,** 129-146.

Yavuz E., Todorov N., Ganchev G. and Nedelkov K. (2015). The effect of feeding different milk program on dairy calf growth, health and development. *Bulgarian J. Agric. Sci*. **21,** 384-393.