Animal 15 (2021) 100124

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Combined effect of probiotics and specific immunoglobulin Y directed against *Escherichia coli* on growth performance, diarrhea incidence, and immune system in calves

A. Karamzadeh-Dehaghani [a], A. Towhidi [a,*,1], M. Zhandi [a], N. Mojgani [b], A. Fouladi-Nashta [c]

[a] Department of Animal Science, Campus of Agriculture and Natural Resources, University of Tehran, Karaj, Iran
[b] Department of Biotechnology, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
[c] Department of Comparative Biomedical Sciences, The Royal Veterinary College, London, UK

ARTICLE INFO

Article history:
Received 23 April 2020
Received in revised form 25 October 2020
Accepted 26 October 2020
Available online 23 December 2020

Keywords:
Chicken egg yolk immunoglobulin
Cytokines
E. coli
Immunoglobulins
Performance

ABSTRACT

Enterotoxigenic *Escherichia coli* (**ETEC**) K99 is one of the major pathogens associated with calf diarrhea. The induction of passive immunity in animals by immunoglobulin Y and using probiotics are inexpensive alternatives to antibiotics for the prevention and treatment of a number of bacterial infections, including diarrhea. Hence, the aim of this research was to evaluate the impact of dietary probiotics and ETEC K99-specific egg yolk antibody supplements, alone and in combination with each other, on health and growth parameters, diarrhea incidence and immune stimulation in newborn Holstein calves. One hundred and twenty neonatal calves were allocated randomly into 4 dietary groups ($n = 30$ per group) received colostrum/milk without any additives (control group), or supplemented with egg yolk powder contained *E. coli* K99-specific antibody (Ab group; 1 g/day), a commercial probiotic, Hypro-calves (Pro group; 3 g/day), and their combination (Ab + Pro group), from day (d) 1 to d28 of age. Analyses of the growth parameters, feed efficiency, fecal score, and microbiota and immune function were carried out on d0, 14, 21, and 28 of the experiment. Calves in Ab or Ab + Pro group had higher ($P < 0.05$) average daily gain compared to control and Pro groups during 0–14d. Feed efficiency of calves in Ab and Ab + Pro groups was significantly higher than that in control group during the period of 0–14d; however, no significant differences were observed in 0–28d period. Diarrhea prevalence and fecal score in Ab + Pro group were lower than control group ($P < 0.05$). Calves in Ab + Pro group had the lowest number of fecal *E. coli* in comparison to other groups on d28 ($P < 0.05$). Feeding Ab + Pro supplement increased ($P < 0.05$) concentrations of blood IgA and serum CD4 compared to the control group. Likewise, calves in Pro group had higher CD4 levels as compared to the control calves ($P < 0.05$). Serum concentration of interferon-gamma in control group was lower than other groups ($P < 0.05$). Overall, these data suggest that feeding a combination of probiotic and specific antibody against ETEC to neonate Holstein calves enhances feed efficiency, boosts immunity, and reduces diarrhea prevalence.

Implications

Diarrhea is the main factor of calf death worldwide. Calf diarrhea not only causes mortality but also causes the cost of treatment, labor force, veterinary intervention, and growth disturbance. So, reduction of calves' mortality can help to economize the dairy herds. Furthermore, due to the widespread use of antibiotics as part of the therapeutic protocols in cases of diarrhea, the issue of the resistant bacterial pathogens to these antibiotics can be critical for domestic animals and human health. Therefore, developing novel methods such as antibody or/and probiotics application could be practical to decrease calf diarrhea and antibiotics using.

Introduction

Neonatal calf diarrhea is one of the most challenging clinical syndromes that lead to significant economic losses in herds (Lorenz et al., 2011). Calf diarrhea is a disease with multifactorial etiology caused by both infectious and noninfectious factors. Many factors, including the calf's exposure to pathogens, the environment conditions, the management factors, and the nutritional and immunological condition of young calves (lack of colostrum feeding, failure to absorb colostral antibodies) impact on the occurrence of diarrhea (Hulbert and Moisá, 2016). Infectious diarrhea is the major cause of morbidity and mortality in the newborn dairy calves throughout the world. Among the diarrhea pathogenic

* Corresponding author.
E-mail address: atowhidi@ut.ac.ir (A. Towhidi).
[1] Department of Animal Science, Campus of Agriculture and Natural Resources, University of Tehran (Daneshkadeh Street, Building no.6, Karaj, Iran.

https://doi.org/10.1016/j.animal.2020.100124

agents, Enterotoxigenic *Escherichia coli* (**ETEC**) is the major cause of diarrhea during the first weeks of life. The main factors impacting ETEC pathogenicity are fimbria antigens, mainly K99 (*E. coli* K99$^+$), and heat-stable enterotoxins (Shams et al., 2012). Fimbria antigens promote adhesion of bacterial cells to small intestine (Shams et al., 2012). Bacterial enterotoxins by increasing osmolality pull water into the intestinal lumen, resulting in fluid secretion and diarrhea (Cho and Yoon, 2014). The course of the disease is rapid from decrease in the absorption of essential nutrients, weight loss, weakness, diarrhea, and severe dehydration to death in less than 24 h (Smith, 2009).

To alleviate these problems, antibiotics have been widely used in diets. However, the use of antibiotics in animal breeding is questionable because they have resulted in serious complications due to drug resistance and their residues in the animal products (Cheng et al., 2014). Moreover, in the recent years, it has been shown that antibiotics rarely affect the disease outcome, because their positive effects are observed at least three days after administration (Duse et al., 2015). Therefore, finding a suitable alternative strategy to antibiotics is required. Recently, oral passive immunization using chicken immunoglobulin Y (**IgY**) has attracted considerable attention because it has many advantages over the mammalian immunoglobulin G (**IgG**) such as cost-effectiveness, accessibility, and high yield. Oral administration of specific chicken IgY has been shown to be highly effective against a variety of intestinal pathogens especially diarrheal pathogens in different animals (Diraviyam et al., 2014).

Another feed supplement that has been developed in the recent years as an alternative for antibiotics is probiotics (Hume, 2011). Probiotics are live microbial feed additives which can confer a health benefit to the host by improving its microbial balance (Gorbach, 2000). Probiotics have numerous functions, including maintaining normal intestinal microorganisms, protecting animals against gastrointestinal disorders, increasing feed efficacy and BW gain, and improving immune system (Timmerman et al., 2005).

Researchers have shown that milk supplementation with various strains of probiotics can significantly improve growth rate and health of calves (Roodposhti and Dabiri, 2012). Based on the published literature, there is no report on the combined effects of egg yolk antibodies and probiotics in pre-weaning calves. Therefore, the study reported here aimed to assess impact of feeding a combined supplement containing IgY against to ETEC and probiotic on growth performance, diarrhea incidence, fecal microbial profile and immune system of suckling Holstein calves.

Material and methods

Antigen preparation

The enterotoxigenic *E. coli* K99 strain (O101:K99$^+$) was obtained from the Razi Type Culture Collection, Razi Vaccine and Serum Research Institute, Karaj, Iran. The strain was originally isolated from a diarrheic neonatal calve during study conducted during 2008 and 2009. This isolate was identified and serotyped by molecular and serological methods (Shams et al., 2012). The bacteria were cultured in tryptic soy broth medium at 37 °C for 24 h under aerobic condition to proliferate. The bacteria were then pelleted by centrifugation at 3000 × *g* for 15 min and then inactivated by mixing with 5% formalin overnight. Inactivation of *E. coli* was confirmed by back of growth after inoculating in blood agar and MacConkey agar. The formalin inactivated *E. coli* were washed three times with phosphate buffered saline and then set by comparing 1 McFarland index (equal to 3×10^8 CFU/ml). The suspension was stored at −20 °C until use.

Birds immunization and immunoglobulin Y purification

Leghorn laying hens ($n = 30$; 22-week-old) were kept in individual cages according to animal welfare recommendations (Janczak and Riber, 2015). Primary immunization of hens was performed by intramuscular breast injection of 1 ml of killed *E. coli* (3×10^8 CFU/ml) emulsified with 1 ml Freund's complete adjuvant. Then, two booster immunization injections carried out using equal volumes of antigen and Freund's incomplete adjuvant in two weeks interval. Freund's adjuvant without antigen was injected to the control group. Blood samples were collected for antibody titer determination on day 0, 2, 4, 6, 8, and then 10 weeks after the first antigen injection. In addition, the eggs were collected daily starting at the first immunization and stored at 4 °C. Purification of IgY from egg yolk was carried out using Polyethylene glycol 6000 as previously described (Zhang et al., 2018). The purity of IgY was 80%, and approximately 12.51 mg/ml in the yolks of eggs could be obtained from antigen immunized hens. In a preliminary research (unpublished data) using increasing concentrations of 0.1, 0.5, 1, 2, and 3 g of egg yolk powder, and carrying out agar well diffusion, minimum inhibition concentration, and minimum bactericidal concentration tests the optimal inhibitory of the egg yolk powder on *E. coli* growth was determined to be 1 g. Therefore, this concentration was used in the studies presented in this manuscript.

Probiotic strains

A commercial probiotic, Hypro-calves (Nature Biotechnology, Karaj, Iran) that contains dextrose and seven species of bacteria was used. These include *Enterococcus faecium*, *Pediococcus acidilactici*, *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum*. In order to determine the optimal inhibitory dose of Hypro-calves probiotic on *E. coli* growth, agar well diffusion, minimum inhibition concentration, and minimum bactericidal concentration methods were used in a preliminary research, and according to the procedures described previously (Karimi et al., 2018). Among the three concentrations tested (10^7, 10^8, and 10^9 CFU/ml), the best effect was obtained with 10^8 CFU/ml (unpublished data) considering 3 g per day Hypro-calves probiotic.

Animals and experimental groups

One hundred twenty newborn Holstein calves (60 male and 60 female average birth weight 35.7 kg) acquired from the same dairy farm were separated from their dams at birth prior to suckling. Calves were randomly allotted to one of the following treatments; colostrum or milk without any additives (control group); colostrum or milk supplemented with egg yolk powder contained *E. coli* K99-specific antibody (1 g/day; Ab group); colostrum or milk supplemented with probiotic (3 g/day; Pro group); and colostrum or milk supplemented with egg yolk powder contained *E. coli* K99-specific antibody and probiotic (Ab + Pro group). Treatment of the calves began on the first day of birth. This study lasted for 28 days, and the calves had free access to fresh water and starter feed (Table 1) at all times. The animals were maintained in individual pens under a strict management protocol as previously described (Vega et al., 2011).

Measuring calf performance and recording clinical observations

Starter intake was determined daily by difference between feed offered and feed refused. The BW of each calf was measured at birth and then weekly in the morning just before feeding. Structural growth measurements of body length, heart girth, withers height, hip height, and hip width were recorded weekly. Calves were observed daily to check health status by a dedicated veterinarian. Fecal consistency of each calf was scored daily before the morning milk feeding by a qualified technician according to the criteria described previously (Vega et al., 2011). In this study, the incidence of diarrhea was more in the first two weeks of calves' life and at this time, if severe diarrhea was detected, calves received a therapy protocol including oral electrolytes (ORS, 5 g/10 kg of BW, Damiabalance, Tehran, Iran) and Ceftiofur

Table 1
Ingredients and chemical composition of the calf starter.

Ingredients	Kg
Barley grain, ground	200
Corn grain, ground	350
Soybean meal, solve[1]	350
Canola meal, mech. extract[2]	13
Salt	10
Sodium bicarbonate	10
Wheat bran	36
Calcium carbonate	15
Magnesium oxide	5
Premix[3]	11
Total	1000.00
Chemical composition	
DM	902.7
CP, g/kg of DM	217.6
NDF, g/kg of DM	348
ADF, g/kg of DM	85
Ash, g/kg of DM	66

[1] Solvent-extracted soybean meal 45% CP.
[2] Extracted canola meal 37% CP.
[3] Premix provided/kg diet: vitamin A, 15 000 IU; vitamin D, 5 000 IU; vitamin E, 50 mg; Fe, 90 mg; Cu, 12.5 mg; Mn, 30 mg; Zn, 90 mg; Se, 0.3 mg; I, 1.0 mg.

(Excenel, 0.2 ml/10 kg of BW, Zoetis, US) for 3 d. The fecal samples were collected on days 0 and 28 after birth with sterile gloves and placed in sterile tubes. The samples were stored in −20 °C until examination. In the laboratory, the numbers of bacteria were enumerated using appropriate growth media and growth conditions as described previously (Dibaji et al., 2014). In brief, de Man, Rogosa and Sharpe (**MRS**) agar was used to culture lactobacillus, Violet Red Bile (**VRB**) agar was used to culture *E. coli* and Violet Red Bile Dextrose (**VRBD**) agar was used to culture coliforms. After shaking the tubes, one ml fecal samples was diluted in 9 ml of phosphate buffered saline. The suspension was prepared from 10^{-1} dilutions, and serial dilutions were made (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}). Then, 100 μl of dilutions (10^{-4}, 10^{-5}, and 10^{-6}) were poured was into the petri dishes which were prepared previously and contained the medium and distributed to all parts of the medium. Lactobacillus was incubated at 37 °C in anaerobic conditions for 72 h. An anaerobic jar was used to create anaerobic conditions. *E. coli* and coliforms were incubated at 37 °C under aerobic conditions for 48 h. Counting of bacteria in the petri dishes was done by a colony counter. Bacterial counts were reported as logarithm number of bacteria per g sample.

Immune response

Blood samples were collected from jugular veins using heparinized blood collection tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA), on d 3 and then weekly on d 7, 14, 21, and 28, approximately 4 h after the morning feeding. Plasma was separated by centrifugation at 3 500 rpm for 15 min and stored at −20 °C until analysis. The ELISA analysis was performed in duplicate per each sample for determining levels of immunoglobulins (IgG, IgG1, IgG2, and IgA), cytokines (IL6, IFNγ, CD4, and CD8), and acute phase proteins [haptoglobin and serum amyloid A (**SAA**)] using appropriate ELISA kits and following the manufacturer's protocol (Bioassay Technology Laboratory, Shanghai, China). In brief, 50 μl standard solutions for each immunoglobulin, cytokine, and acute phase proteins were added into standard wells of separate sterile micro-plates followed by the addition of 50 μl streptavidin-HRP to the same wells. Into the sample wells, plasma samples (40 μl) were dispensed and anti-IgG, IgG1, IgG2, IgA, IL6, IFNγ, CD4, CD8, haptoglobin, and SAA antibodies (10 μl) and streptavidin-HRP (50 μl) were added. Micro-plates were then incubated for 60 min at 37 °C and washed five times with wash buffer afterwards. After this, 50 μl of each substrate solutions A and B were added into the sample wells, respectively, and after incubation for 10 min at 37 °C in the dark, 50 μl stop solution was added to terminate the reaction. After changing color of the samples to yellow, the absorption was measured at a wavelength of 450 nm by a plate reader (DANA 3200, Germany).

Statistical analysis

A complete randomized study design was used was for these investigations, and the data were analyzed using the statistical model $Yijkl = \mu + Ai + Bj + \delta(ij)k + ABij + eijkl$.

Yijkl = dependent variable; μ = mean, Ai = fixed effect of treatment i, Bj = fixed effect of time j, δ(ij)k = random effect of calf j within treatment i, ABij = fixed effect of treatment by time interaction, eijkl = residual error. Integrated and unintegrated data were analyzed by MIXED and Genmod procedure of SAS (SAS Institute 2004), respectively. The differences among the least square means were determined using the PDIFF procedure of LSMEANS with SEM. Also, the turkey's test was used for pair-wise comparisons. Significance was declared when *P*-value was less than 0.05. Since, the daily gain was correlated with the starter intake, the starter intake was considered as a covariate factor in the statistical model and considered independent from the treatment.

Results

Growth performance and feed intake efficiency

The results of the BW, daily gain, and structural growth of Holstein calves fed diets containing egg yolk antibody and probiotic are presented in Table 2. There was no significant effect of the dietary treatments on BWs of calves in comparison with the control group ($P > 0.05$). Holstein calves received Ab and Ab + Pro supplements had significantly higher ($P < 0.05$) daily gain compared to control and Pro treatments in 0–14 d and 0–28 d periods. The structural growth parameters such as withers height, hip height, hip width, and heart girth were not affected ($P > 0.05$) by the treatments. Analysis of the data for the starter intake (dairy feed intake) and the feed intake efficiency are presented in Table 3. The starter intake in Ab + Pro treatment was significantly higher than the control and Pro groups in all periods ($P < 0.05$). Feed intake efficiency in calves received Ab and Ab + Pro was significantly higher than the control group during the period of 0–14 d ($P < 0.05$). No significant differences were observed among Pro and other treatments in this period ($P > 0.05$). None of the supplement influenced feed intake efficiency in Holstein calves during 14–28 d and 0–28 d periods ($P > 0.05$).

Diarrhea incidence, fecal score, and microbial population

According to the Table 4, diarrhea prevalence was significantly lower in calves received Ab + Pro supplement in diet compared to the control group ($P < 0.05$). There was no difference ($P > 0.05$) among other experimental groups. Diarrhea duration and time of its treatment (the therapy protocol) were not influenced by the supplements ($P > 0.05$). The incidence of diarrhea was more in the first two weeks of calf's life and at this time, calves received a therapy protocol (ORS and other therapy drugs) during severe diarrhea. The fecal score was lower in calves received Ab + Pro supplement than the control and Pro and Ab supplemented calves ($P < 0.05$). The result of fecal microbial count of Holstein calves is presented in Table 5. Calves in Ab + Pro group had the lowest number of *E. coli* as compared to other groups in d28 ($P < 0.05$). The lowest number of coliforms were also observed in calves of Ab + Pro group than other groups in d28 ($P < 0.05$). However, Ab and Pro supplements led to reduce the number of coliforms compared to the control group in d28. Also, a difference in fecal lactobacillus population was

Table 2
Effect of feeding Ab and Pro supplements on body weight, daily gain and structural growth of Holstein calves (mean ± SE).

Parameter	Treatments				*P* value
	Co	Ab	Pro	Ab + Pro	
Body weight					
0 d	35.65 ± 1.24	35.70 ± 1.23	35.55 ± 1.24	35.95 ± 1.23	0.89
14 d	37.65 ± 1.24	38.05 ± 1.25	38.15 ± 1.22	38.25 ± 1.25	0.89
21 d	42.30 ± 1.22	43.15 ± 1.19	43.30 ± 1.16	43.75 ± 1.20	0.76
28 d	46.15 ± 1.23	47.25 ± 1.22	46.75 ± 1.24	47.75 ± 1.19	0.86
Daily gain (kg/d)					
0–14 d	0.30^{b} ± 0.01	0.36^{a} ± 0.01	0.33^{ab} ± 0.01	0.36^{a} ± 0.05	0.02
14–28 d	0.44 ± 0.02	0.46 ± 0.02	0.46 ± 0.02	0.48 ± 0.08	0.38
0–28 d	0.37^{b} ± 0.01	0.41^{a} ± 0.009	0.40^{ab} ± 0.01	0.42^{a} ± 0.01	0.04
Withers height (cm)					
Initial	76.00 ± 0.38	75.60 ± 0.36	75.95 ± 0.38	75.95 ± 0.37	0.38
Final	80.70 ± 0.42	80.30 ± 0.30	80.62 ± 0.39	80.70 ± 0.42	0.52
Daily	0.167 ± 0.007	0.167 ± 0.01	0.167 ± 0.005	0.169 ± 0.005	0.43
Hip height (cm)					
Initial	80.90 ± 0.45	81.05 ± 0.48	80.90 ± 0.45	80.95 ± 0.40	0.81
Final	85.75 ± 0.44	85.95 ± 0.41	85.80 ± 0.46	85.90 ± 0.46	0.64
Daily	0.173 ± 0.007	0.175 ± 0.006	0.175 ± 0.008	0.176 ± 0.008	0.77
Hip width (cm)					
Initial	17.05 ± 0.19	17.10 ± 0.16	17.12 ± 0.22	17.07 ± 0.23	0.99
Final	19.35 ± 0.22	19.47 ± 0.14	19.42 ± 0.18	19.47 ± 0.25	0.66
Daily	0.082 ± 0.003	0.084 ± 0.004	0.082 ± 0.004	0.085 ± 0.004	0.44
Heart girth (cm)					
Initial	76.95 ± 0.40	76.77 ± 0.38	76.90 ± 0.42	76.97 ± 0.46	0.88
Final	84.50 ± 0.40	84.30 ± 0.33	84.40 ± 0.43	84.55 ± 0.48	0.83
Daily	0.269 ± 0.005	0.268 ± 0.009	0.267 ± 0.008	0.270 ± 0.006	0.91

Co = Control; Ab = Antibody; Pro = Probiotic; Ab + Pro = Antibody and Probiotic.
[ab]Values within a row with different superscripts differ significantly at $P < 0.05$.

Table 3
Effect of Ab and Pro fed on starter intake and feed efficiency of Holstein calves (mean ± SE).

Period (day)	Treatments				*P* value		
	Co	Ab	Pro	Ab + Pro	Treat	Time	Treat × Time
Starter intake (kg/d)							
0–14	0.09^{c} ± 0.002	0.10^{ab} ± 0.002	0.10^{b} ± 0.002	0.12^{a} ± 0.002	<0.0001	0.01	0.001
14–28	0.34^{d} ± 0.01	0.40^{b} ± 0.01	0.37^{c} ± 0.01	0.43^{a} ± 0.01	<0.0001	0.03	0.001
0–28	0.21^{b} ± 0.004	0.26^{a} ± 0.005	0.23^{b} ± 0.005	0.27^{a} ± 0.004	<0.0001	0.02	0.001
Feed efficiency							
0–14	0.37^{b} ± 0.01	0.43^{a} ± 0.01	0.40^{ab} ± 0.02	0.42^{a} ± 0.01	0.03	0.26	0.09
14–28	0.39 ± 0.01	0.41 ± 0.01	0.41 ± 0.01	0.42 ± 0.01	0.41	0.68	0.56
0–28	0.41 ± 0.02	0.41 ± 0.03	0.42 ± 0.01	0.41 ± 0.02	0.69	0.89	0.57

Co = Control; Ab = Antibody; Pro = Probiotic; Ab + Pro = Antibody and Probiotic; treat = Treatment; treat × time = interaction between treatment and time.
[a,b,c,d]Values within a row with different superscripts differ significantly at $P < 0.05$.

Table 4
Effect of feeding Ab and Pro on Holstein calves diarrhea and fecal score (mean ± SE).

Item	Treatments				*P* value		
	Co	Ab	Pro	Ab + Pro	Treat	Time	Treat × Time
Affected calves (%)	63.33^{a}	50.00^{ab}	53.33^{ab}	33.33^{b}	0.03	0.25	0.55
Duration (day)	3.50 ± 0.71	2.75 ± 0.64	3.40 ± 0.81	2.35 ± 0.72	0.15	0.12	0.23
Treatment,[1] (day)	1.35 ± 0.24	1.05 ± 0.28	1.20 ± 0.25	0.85 ± 0.28	0.36	0.56	0.25
Fecal score	1.64^{a} ± 0.14	1.42^{a} ± 0.13	1.52^{ab} ± 0.18	1.23^{c} ± 0.16	0.04	0.001	0.03

Co = Control; Ab = Antibody; Pro = Probiotic; Ab + Pro = Antibody and Probiotic; treat = Treatment; treat × time = interaction between treatment and time.
[a,b,c]Values within a row with different superscripts differ significantly at $P < 0.05$.
[1] The average days of receiving a therapy protocol during severe diarrhea.

found among groups in d28 so that calves in Ab + Pro group had higher number of lactobacillus than other groups ($P < 0.05$).

Immune response

Table 6 shows the effects of the treatments on the immune system of the Holstein calves. Experimental treatments did not result in any significant difference in the blood IgG, IgG1, and IgG2 concentrations of calves in all periods. The trend of IgG, IgG1, and IgG2 concentrations in different weeks of the experiment are shown in Fig. 1 (a), (b) and (c), respectively. Feeding calves with the Ab + Pro supplement resulted in significantly ($P < 0.05$) higher concentration of blood IgA than the control group, but it was not different from IgA concentration in calves supplemented with Ab and Pro groups. The concentration of IgA in

Table 5
Effect of feeding Ab and Pro on fecal microbial population of Holstein calves (log cfu/g of wet digesta) (mean ± SE).

Parameter	Co	Treatments			*P* value
		Ab	Pro	Ab + Pro	
E. coli					
0	9.46 ± 0.09	9.04 ± 0.09	9.25 ± 0.08	9.11 ± 0.07	0.43
28	8.26^{a} ± 0.10	7.88^{a} ± 0.13	8.09^{a} ± 0.18	6.73^{b} ± 0.13	<0.0001
Coliform					
0	9.59 ± 0.27	9.20 ± 0.29	9.44 ± 0.25	9.26 ± 0.25	0.52
28	8.78^{a} ± 0.10	8.30^{b} ± 0.17	8.40^{b} ± 0.04	7.26^{c} ± 0.15	0.0003
Lactobacillus					
0	7.07 ± 0.27	7.12 ± 0.22	7.26 ± 0.11	7.24 ± 0.12	0.90
28	7.88^{b} ± 0.13	8.04^{b} ± 0.12	7.94^{b} ± 0.11	8.73^{a} ± 0.11	0.02

Co = Control; Ab = Antibody; Pro = Probiotic; Ab + Pro = Antibody and Probiotic.
[a,b,c]Values within a row with different superscripts differ significantly at $P < 0.05$.

different weeks of the experiment are shown in Fig. 1 (d). Calves received Ab + Pro supplement had the highest serum CD4 concentration compared to control, Ab and Pro groups ($P < 0.05$). Similarly, serum CD4 concentration in calves fed Pro supplement was significantly higher than control calves ($P < 0.05$). CD4 concentration in the different week of experiment is shown in Fig. 1 (e). Table 6 and Fig. 1 (f) shown that no significant differences were observed in the serum CD8 concentration among treatment groups ($P > 0.05$). Concentration of serum IFNγ in Ab + Pro treated calves was significantly lower than Ab group ($P < 0.05$), but was significantly higher than control group and was not different from Pro treated calves. The different IFNγ concentrations in week of experiment are shown in Fig. 1 (g). There were no differences in the concentration of acute phase proteins including haptoglobin and SAA among the groups (Table 6 and Fig. 1 (i) and (j)).

Discussion

Newborn calf diarrhea is a major problem in dairy cow farming causing death of the calves within few days after birth resulting in significant economic loss to the industry. In the study presented here, we assessed impact of probiotic, egg yolk powder, and their combination as feed supplements on a range of parameters associated with health and growth in the neonatal Holstein calves and with aiming to decrease neonatal diarrhea.

The results showed that the supplementation of colostrum and milk with probiotic and egg yolk powder improves daily weight gain and feed efficacy, but do not have any effect on BW and body structural measures, which may be related to egg yolk nutrients. Egg yolk contains high-quality nutrients including high levels of proteins, trace minerals, vitamins and is a rich source of iron and phosphorous. Using egg yolk as a feed additive for animals, provided promising results (Vega et al., 2011). Some authors indicated that when egg is added at levels up to 10% of the diet, calves had better growth performance (Vega et al., 2011). Similarly, a significant increase in BW of calves fed probiotics was reported (Jatkauskas and Vrotniakiene, 2010). Some studies, however, have shown that treatment with probiotics did not have any positive effects on the BW and BW gain of the calves (Görgülü et al., 2003). Furthermore, supplementing a blend of probiotics, prebiotics, and egg protein did not improve growth performance of the calves (Ballou, 2011). It appears that the discrepancy between our results and previous reports is due to the differences in the levels and concentrations of egg yolk and probiotic used. Because most of the pathogenicity of *E. coli* K99 is in the first 14 days of calves' life, specific antibodies against *E. coli* K99 appear to be less effective in the 14–28 period. On the other hand, calves were treated in experimental treatments after the outbreak of severe diarrhea. It seems that the high prevalence of diarrhea in the control group in the first two weeks and consequently the higher number of treatments in this group has caused that on the 28d the final weight of the calves is not statistically significant.

In the present study, feeding a combination of egg yolk powder and probiotics reduced the diarrhea incidence and the fecal score. In addition, the microbial culture clarified lower quantities of *E. coli* and coliform and higher quantity of lactobacillus in the fecal of calves fed this combination. In order to determine the total number of lactobacillus, *E. coli*, and coliforms in the fecal samples, we used MRS, VRB, and VRBD media, respectively. Those media have specific compositions to inhibit the growth of other bacteria or distinguish the bacteria according to the appearance of colonies. The intestinal microbiota is very unstable (Signorini et al., 2012) and stressful conditions may reduce Lactobacilli populations and increase pathogen microorganisms in neonatal calves (Signorini et al., 2012). Hence, the use of probiotics and specific IgY can prevent proliferation of pathogenic bacteria in the gastrointestinal tract, diminish the incidence of diarrhea, and improve fecal scores, as shown by a number of investigators (Görgülü et al., 2003; Hennig-Pauka et al., 2003; Signorini et al., 2012; Li et al., 2016). It is known that the probiotics such as lactobacillus can produce some antimicrobial compounds such as hydrogen peroxide, organic acids, and bacteriocins (Sanders et al., 2010). In addition, they compete with pathogens for adhesion to intestinal epithelium (Oelschlaeger, 2010). On the other hand, egg yolk antibodies have shown to counteract pathogen activity by preventing the attachment of pathogens to the intestine, bacteria agglutination, toxin neutralization, and phagocytosis promotion (Li et al., 2016). Taken together, it seems that feeding a combination of probiotic and egg yolk antibody has synergistic effect on reducing the pathogenic agents such as *E. coli* and resulting in preventing neonatal calves' diarrhea, as shown by our results. However, it should be noted that counting

Table 6
Effects of feeding Ab and Pro on the immune system of Holstein calves (mean ± SE).

Parameter	Treatments				*P* value		
	Co	Ab	Pro	Ab + Pro	Treat	Time	Treat × Time
IgG (mg/ml)	71.57 ± 7.00	80.08 ± 6.23	73.35 ± 4.59	83.28 ± 7.46	0.09	0.20	0.13
IgG1 (mg/ml)	2.79 ± 0.46	3.33 ± 0.19	3.40 ± 0.18	3.62 ± 0.38	0.21	0.41	0.19
IgG2 (μg/ml)	1.00 ± 0.19	0.81 ± 0.13	1.06 ± 0.15	0.80 ± 0.30	0.13	0.19	0.03
IgA (μg/ml)	22.25^{b} ± 2.05	28.92^{ab} ± 1.90	29.89^{ab} ± 3.93	32.16^{a} ± 3.28	<0.0001	0.23	0.002
IL6 (ng/ml)	125.77 ± 19.69	119.24 ± 25.57	136.33 ± 17.43	146.65 ± 26.30	0.54	0.88	0.50
CD4 (ng/ml)	37.95^{c} ± 2.90	44.60^{bc} ± 3.98	48.29^{b} ± 5.82	57.87^{a} ± 5.64	0.0007	<0.0001	<0.0001
CD8 (ng/ml)	34.12 ± 4.02	34.86 ± 2.90	33.39 ± 3.23	36.76 ± 2.82	0.40	0.01	0.002
IFNγ (pg/ml)	60.80^{c} ± 5.36	74.42^{a} ± 3.77	67.65^{b} ± 5.54	67.93^{b} ± 4.72	0.001	0.10	<0.0001
Haptoglobin (μg/ml)	74.80 ± 7.47	61.27 ± 4.35	62.11 ± 6.74	56.36 ± 6.39	0.23	0.43	0.20
SAA (μg/ml)	4.15 ± 0.93	3.74 ± 0.89	3.62 ± 0.49	3.03 ± 0.26	0.52	0.78	0.49

Co = Control; Ab = Antibody; Pro = Probiotic; Ab + Pro = Antibody and Probiotic; treat = Treatment; treat × time = interaction between treatment and time.
[a,b,c]Values within a row with different superscripts differ significantly at $P < 0.05$.
IgG = immunoglobulin G; IgG1 = immunoglobulin G1; IgG2 = immunoglobulin G2; IgA = immunoglobulin A; IL6 = interleukin 6; CD4 = cluster of differentiation 4; CD8 = cluster of differentiation 8; IFNγ = Interferon gamma; SAA = Serum Amyloid A.

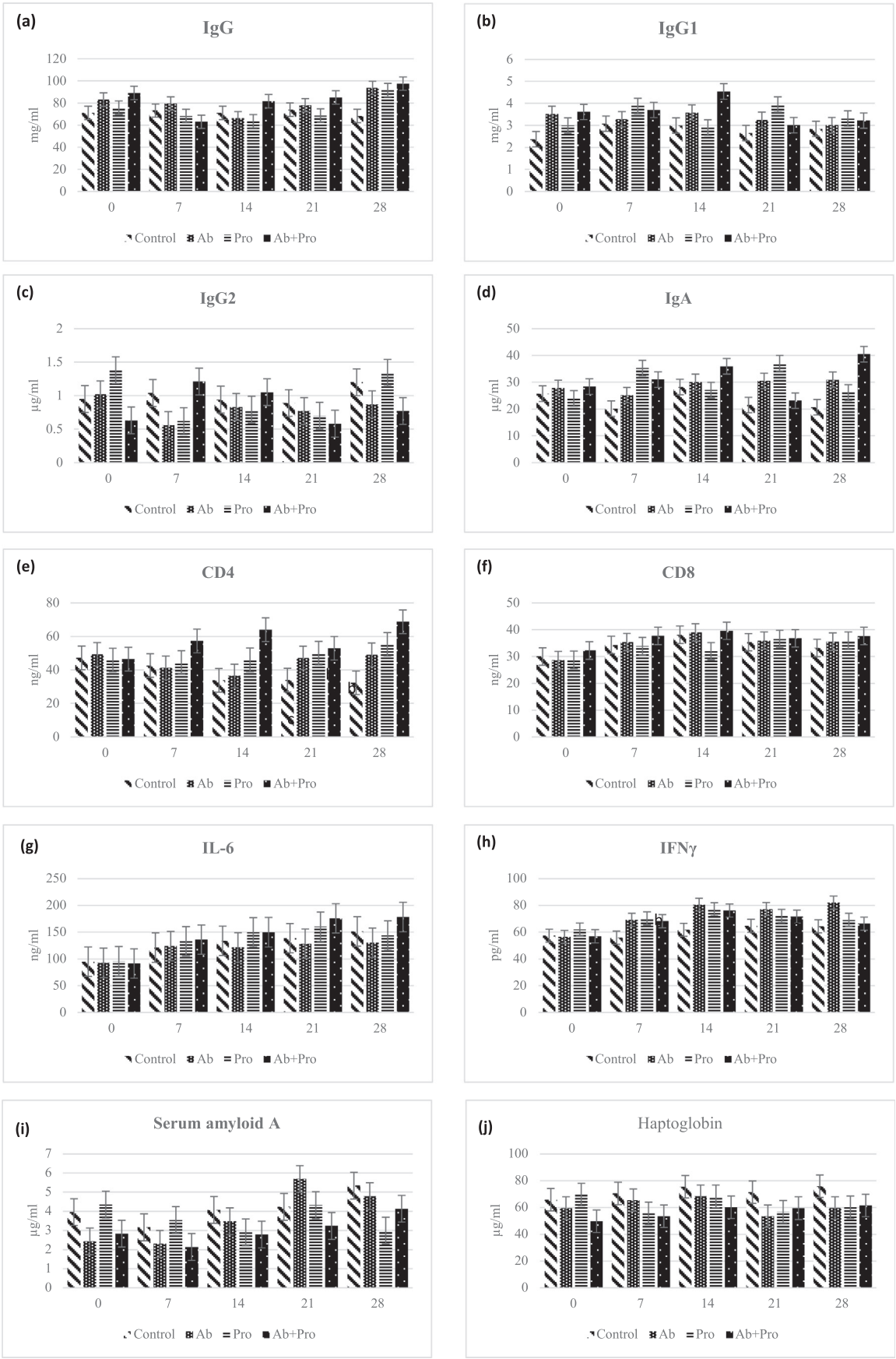

Fig. 1. Effects of feeding Ab and Pro on the immune system of Holstein calves in different week of experiment (mean ± SE). Ab = Antibody; Pro = Probiotic; Ab + Pro = Antibody and Probiotic; IgG = immunoglobulin G; IgG1 = immunoglobulin G1; IgG2 = immunoglobulin G2; IgA = immunoglobulin A; IL6 = interleukin 6; CD4 = cluster of differentiation 4; CD8 = cluster of differentiation 8; IFNγ = Interferon gamma.

the number of *E. coli* K99 in the faces sample by PCR method can better show the effect of combination of specific antibody and probiotics, which unfortunately was not possible for authors in this study.

Probiotics and IgY possess some immune stimulating properties and exert beneficial effects in balancing the host defense system. Their effects have been previously reported on enhancing mucosal barrier integrity, promoting greater antibody production and upregulating cell mediated immunity (Zhang et al., 2018). In the current study there was no statistically significant difference in the concentrations of blood IgGs among groups. It was reported that fortifying milk by the addition of probiotic increases IgG levels as an anti-spore immune response (Hong et al., 2005). In contrast, there are some reports showing that administration of probiotic to pre-ruminant calves has no positive effect on immunoglobulins (Roodposhti and Dabiri, 2012), which is in line with our results. Some investigators have indicated that feeding bovine RV-specific IgY antibody to neonatal calves enhances IgG1 concentration until day 7 after birth. These researchers have also mentioned that bovine RV-specific IgY antibody has no effect on IgG2 and its effect was only observed on 21 day of age (Vega et al., 2011).

Serum immunoglobulin A is an antibody that defends against infections at mucosal membranes and plays a role in inhibition of inflammatory reactions. Its concentration decreases during diarrhea prevalence (Dock et al., 2004). Previous studies have reported an increase IgA level with supplementing probiotic (Dock et al., 2004) and egg yolk antibody (Vega et al., 2011). However, we could not find any significant difference in IgA level by feeding egg yolk powder or probiotic alone to the calves, but by their combination, which related probably to some factors as the used levels and concentrations of egg yolk and probiotic and the type of probiotic bacteria. Based on our results, feeding a combination of the specific egg yolk antibody and probiotic seem to have a superior impact on boosting immune system in the suckling calves. It is possible that the egg yolk antibodies and probiotics exert different levels of immune-regulatory effects in a source dependent manner.

It was suggested that intestinal inflammation is accompanied by microflora imbalance. Proinflammatory cytokines, such as IL6 and IFNγ, play key roles in inflammatory processes (Muñoz-Carrillo et al., 2018). It has been reported that a decreased number of peripheral leukocytes is associated with a high risk of calf diarrhea (Wang et al., 2007). In addition, a lower quantity of CD8 and CD4 cells and decreased cytokine expression levels were reported in neonates with scouring, compared with healthy children (Wang et al., 2007). In the present study, feeding probiotic and its combination with egg yolk powder to the calves led to increase CD4 and IFNγ concentrations, but did not have any effects on IL6 and CD8 levels. Numerous studies have reported the effects of probiotics and IgY on proinflammatory and anti-inflammatory cytokines (Li et al., 2016). It was also implied that probiotic microorganisms might stimulate the intestine epithelial and associated lymphoid tissues and may activate local immune responses. Qadis and colleagues showed that probiotics enhance blood CD8 cells in scouring calves (Qadis et al., 2014). In another study, the CD4 cell numbers increased in response to probiotic treatment in cows (Kohiruimaki et al., 2008). On the other hand, it was reported that oral administration of *Salmonella typhimurium*-specific IgY antibody leads to a decrease in the CD4 level but an increase of the CD8 concentration during microbial infection (Li et al., 2016). The IFNγ concentration enhanced in calves with diarrhea and challenged with Salmonella (Li et al., 2016). Specific IgY antibody has little effect on diminution of the IFNγ level (Bergman et al., 2005).

In current study, the addition of specific IgY and probiotics alone or in combination with each other did not increase the Hp and SAA levels in calves, which is inconsistent with results of others (Balikci and Al, 2014). Such differences could be attributed to other contributing factors and require further investigation.

Conclusion

The results of the present study indicated that feeding a combination of probiotic and egg yolk powder containing *E. coli* K99-specific antibody has beneficial effects on calf health. It enhanced the daily weight gain and stimulated components of the immune system resulting in reduced diarrhea prevalence in pre-weaning Holstein calves. It is noteworthy that because of a good management in the calf barns of the farm and receiving a therapy protocol only during severe diarrhea, none of the calves died during this study.

Ethics approval

Not applicable.

Data and model availability statement

None of the data were deposited in an official repository.

Author ORCIDs

Amir Karamzadeh-Dehaghani: 0000-0003-1271-5225.
Armin Towhidi: 0000-0002-3288-1709.
Mahdi Zhandi: 0000-0003-2886-9747.
Naheed Mojgani: 0000-0002-3138-3433.
Ali Fouladi-Nashta: 0000-0003-4944-2400.

Author contributions

Amir Karamzadeh-Dehaghani: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing.

Armin Towhidi: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

Mahdi Zhandi: Methodology, Supervision.

Naheed Mojgani: Conceptualization, Methodology, Validation.

Ali Fouladi-Nashta: Writing – review & editing.

Declaration of interest

None.

Acknowledgements

None.

Financial support statement

This work was supported by University of Tehran grant number 7108017.6.40 and also sponsored by Nature Biotechnology Company (Biorun).

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