Diarrhea Duration and Performance Outcomes of Pre-Weaned Dairy Calves Supplemented with Bacteriophage

Evandro Schmoeller
Federal University of Pelotas: Universidade Federal de Pelotas
Adriane D. C. de Matos
Federal University of Pelotas: Universidade Federal de Pelotas
NATALIA RAHAL
Federal University of Pelotas: Universidade Federal de Pelotas https://orcid.org/0000-0002-1633-2355
Josiane O. Feijo
Federal University of Pelotas: Universidade Federal de Pelotas
Cassio C. Brauner
Federal University of Pelotas: Universidade Federal de Pelotas
Francisco Augusto B. Del Pino
Federal University of Pelotas: Universidade Federal de Pelotas
Marcio N. Correa
Federal University of Pelotas: Universidade Federal de Pelotas
Viviane R. Rabassa (vivianerabassa@gmail.com)
Federal University of Pelotas: Universidade Federal de Pelotas

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Abstract

This study aimed to evaluate lytic bacteriophage supplementation in pre-weaned dairy calves over neonatal calf diarrhea and respiratory diseases occurrence, performance and biochemical parameters. Also, to determine bacterial agents causing NCD. Two hundred Holstein×Gyr crossbred female calves were divided into two groups: Control (CON, n = 100), no supplementation; and Bacteriophage (PHAGE, n = 100) bacteriophage supplementation (1 g/day) from d 3 until d 70 of life. Calves were monitored daily for respiratory disease and diarrhea, as for age at the first diarrheic episode and its duration. Fecal samples were cultured for isolation of *Escherichia coli* and *Salmonella* spp. colonies and PCR was performed to identify *E. coli* virulence genes and to confirm *Salmonella* spp. Performance outcomes were evaluated up to 80 d of age. Blood samples were collected to determine serum levels of total proteins, albumin, cholesterol, γ-glutamyl transferase and urea. PHAGE group had fewer days in diarrhea and duration of the first episode was lower, compared to CON group. Fecal samples of three animals in PHAGE and nine in CON were positive for *E. coli* in PCR. Thoracic perimeter tended to be higher in supplemented animals. Average daily gain mean of PHAGE was higher in the first 30 d of life, at the beginning of step-down weaning (up to 42 d) and after weaning (up to 80 d). PHAGE mean was lower for albumin and higher for urea. Therefore, phage therapy during the pre-weaned period reduced the duration of neonatal diarrhea, providing greater weight gain for calves.

Introduction

Appropriate calf management is fundamental for dairy cattle production, because the neonatal period presents several challenges to these animals. Metabolic instability, associated with the beginning of the immune response development makes these animals susceptible to diseases and increased mortality (Volpato et al., 2017).

Among diseases of this period, neonatal calf diarrhea (NCD) is associated with significant economic losses due to high morbidity and mortality, cost of treatment and impaired development (Coura et al., 2015; Foster, 2009). NCD can be infectious (caused by viruses, bacteria and protozoa); or related to nutrition, influenced by temperature, quantity and quality of milk or milk replacer; and climate conditions (Smith, 2009). Regarding the primary cause, with imbalance of intestinal microbiota due to diarrhea, bacterial enteritis ends up occurring as an opportunistic infection in most clinical conditions (Cho et al., 2013).

Escherichia coli and *Salmonella* spp. are the main etiologic agents of bacterial NCD and are commonly treated with metaphylactic antibiotic treatment. Antibiotic therapy has been questioned for stimulating factors of antimicrobial resistance and death of microorganisms that act symbiotically to the intestine, participating in the development of intestinal tissue and the immune system (Macpherson et al., 2004). This imbalance can impair inflammatory response modulation (Lei et.al, 2015), causing a competition between symbiotic agents and pathogenic microorganisms for nutrients and intestinal epithelial surface, prolonging or aggravating NCD (Backhed et al, 2005; Rakoff-Nahoum et. al, 2004).

In order to decrease the indiscriminate use of antibiotics, phage therapy has emerged as an alternative in prophylactic control of bacterial diarrhea. Bacteriophages are mandatory intracellular viral particles that parasitize bacteria with high specificity (Santos et al., 2014). These phages can have different life cycles, known as lytic or lysogenic. In the lytic cycle, they become excellent tools in controlling pathogenic bacteria, as they use bacterial metabolism to replicate and produce endolysins, causing cell wall destruction and bacterial death (Harper and Enright, 2011). Studies have shown positive effects on maintaining ruminants' intestinal microbiota balance (Callaway et al., 2008; Sheng et al., 2006) and controlling septicemia in calves and birds (Barrow et al., 1998).

Therefore, we aimed to evaluate lytic bacteriophage supplementation in pre-weaned dairy calves over NCD and respiratory diseases occurrence, performance and biochemical parameters of dairy calves. Also, to determine bacterial

agents causing NCD.

Material And Methods

Animals and experimental conditions

The study was conducted on a commercial dairy farm in Passos, Minas Gerais, Brazil, under the protocols approved by Ethics Committee on Animal Experimentation - CEEA of the Federal University of Pelotas (number 14807). Sample size was established using G*Power software 3.9.1.2 version. Two hundred Holstein × Gyr crossbred female calves with different breed admixture (1/2 H + 1/2 G; 3/4 H + 1/4 G, 5/8 H + 3/8 G) were maintained at an outdoor system known as “tropical housing”. Briefly, calves were neck-tied to a chain fixed to a wire stretched on the ground (tied to wood posts) and had access to shade provided by a shading screen. Immediately after birth, all calves were bottle-fed colostrum until the first 8 hours of life and until the first 24 hours 10% BW of colostrum was given to calves. Colostrum quality was measured using Brix refractometry, considering good quality when values were ≥ 20% (Quigley et al., 2013). Until 3 d of life calves were kept in individual pens and were bucket or bottle fed 4 L of transition milk (milk from cows from days 2 to 5 post-partum), divided into two meals. From the third day, calves were bucket fed with a 3 L of milk replacer Nurture Prime, Nutron, Brazil (22% CP, 18% fat DM basis, 38% lactose DM basis), twice a day, up to 42 d of life. After d 42, 3 L of milk replacer was given only in the afternoon beginning the step-down weaning process. In addition, from the 3 d of life animals had free access to starter (12.59% CP and 15.2% starch DM basis) and water and at 60 d corn silage was offered (NRC, 2000). Weaning was performed at 70 d of life, with calves remaining in this housing system until 80 d.

To verify transfer passive of immunity (TPI), blood sample was collected within 48 h of life via jugular venipuncture using vacuum tube with EDTA (5mL). These were centrifuged at 2183 x g to obtain plasma and total plasma protein (TPP) was analyzed in an optical refractometer. Failure of TPI (FTPI) was determined in calves with TPP below 5.5 g/dL (Tyler et al., 1996), and calves with FTPI were homogeneously distributed between groups.

This study had a completely randomized design. Animals were homogeneously divided into two groups, considering birth weight, breed admixture, birth order and FTPI. Bacteriophage group (PHAGE), n = 100 (50 ½, 48 ¾, 2 $^{5}/_{8}$ of Holstein), was fed 1g of a product containing bacteriophage (FAGOLAC, Bayer Animal Health, Brazil) added to milk replacer from d 3 d until weaning, and had initial weight mean of 35.48 ± 0.75 kg. Control Group (CON), n = 100 (51 ½, 48 ¾, 1 5/8 of Holstein blood), was fed milk replacer without addition of the product, and had initial weight mean of 35.31 ± 0.71 kg.

Calves were monitored daily up to 80 d of life for occurrence of diarrhea and respiratory disease. For diarrhea, animals were considered sick when fecal score was ≥ 2 and for respiratory disease when greyish to yellowish nasal discharge was presented accompanied by fever and abnormal respiratory sounds. From these records, the following rates were determined: morbidity (number of sick calves/total number of calves enrolled in the experiment); mortality (number dead calves/ total number of calves enrolled in the experiment); lethality (number of calves that died from diarrhea or respiratory disease/ total number of calves that presented diarrhea or respiratory disease); and relapse (number of animals that got sick twice or more).

Clinical diagnostics were based on evaluation of dehydration, respiratory and heart rates and rectal temperature (Smith, 2009). Sick calves were treated according to farm’s protocol similar for both groups. For diarrhea, treatment with trimethoprim-sulfamethoxazole (20mg/kg B.W., once a day, intramuscular injection) was used for 3 d or more (according to clinical condition) associated with oral fluids. Penicillin (procaine benzylpenicillin 7,500UI/kg; benzathyne benzylpenicillin 5,000UI/kg; dihydrostreptomycin 17.06mg/kg, once a day, intramuscular injection) associated with meloxicam (0.5 mg/kg B.W., once a day, intramuscular injection) were used for 3–5 days to treat respiratory disease.

Fecal score (FS) and duration of diarrhea

FS was determined on a scale of 0 to 4, where 0 = normal consistency, 1 = pasty, 2 = loose or watery feces, 3 = profuse diarrhea with liquefied stools, and 4 = profuse diarrhea with liquefied and bloody feces (adapted from Kertz and Chester-Jones, 2004). Onset of diarrheic episodes occurred when calves presented FS ≥ 2 and ended when FS ≤ 1. The total number of days calves remained with diarrheic feces (FS ≥ 2), considering all diarrheic episodes of each calf, and duration of the first diarrheic episode (days) were determined throughout the first 30 d of life.

Fecal samples and bacterial characterization

Sixty-six fecal samples (PHAGE = 31; CON = 35) on the first day of the first diarrheic episode were collected directly from the rectum, packed in a sterile cup and frozen at -20ºC until microbiological analysis. Subsequently, these samples were cultured in two types of medium, for *Salmonella spp.* and *E. coli* growth.

For *Salmonella* spp. identification, samples were initially grown in tetrathionate broth. The broth was kept in a bacteriological oven at 35–37°C for 72 hours. Every 24 hours of incubation, an aliquot was removed from the broth and added to MacConkey agar, incubated again under the same conditions for 24 hours. Biochemical tests were performed with colonies with *Salmonella* spp. visual characteristics, following the technique described by Quinn et al. (1993).

For *E. coli*, lactose fermenting colonies found in MacConkey agar medium were submitted to biochemical characterization (Quinn et. Al., 1993). The PCR was performed for colonies characterized as *E. coli* and *Salmonella spp*.

Molecular methods

The genomic DNA extraction of *E. coli* and *Salmonella* spp. colonies was performed according to the protocol described by Green and Sambrook (2012). The DNA concentration was of samples was 5 ng/µL, with purity ratio ≥ 1.8 quantified by spectrophotometry using NanoVue Plus (GE Healthcare Life Sciences, Buckinghamshire, UK).

Amplification procedure followed the protocols described in the literature. For *E. coli*, the *USPA* gene was PCR amplified and sequenced to confirm genus and species designation, generating a fragment of 884 base pairs (bp) (Chen; Griffiths, 1998). After confirmation, the PCR amplification of the following virulence genes were attempted: (i) *EAE* (384 bp), intimin, present in the enteropathogenic E. coli (EPEC); (ii) *STX1* (180 bp), which encode for Shiga-toxin-producing *E. coli* (STEC); (iii) *HLYA* (534 bp), encoding for hemolysin, present in the enterohaemorrhagic *E. coli* (EHEC); and (iv) *ESTIA* (157 bp), encoding for thermostable toxin A. For genus confirmation of *Salmonella* spp., the *HILA* gene was PCR amplified and sequenced, generating a 413 bp fragment (Craciunas et al., 2012).

The conventional PCR was performed as follows: 12.5µL of Taq polymerase master mix 2x (Cellco), 8.5µL of water for PCR (Ludwig Biotec), 10 pmol (1 µL) of each forward and reverse primers (Extend), and 10ng (2 µL) of template DNA, for a final volume of 25µL. Amplification was performed on MJ Research® PTC 100 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) and PCR products were subjected to electrophoresis on an agarose gel 1.5% (for fragments larger than 200 bp) and 2% (for fragments smaller than 200 bp) with a standard molecular weight of 1kb (Invitrogen). Then, the amplified products were visualized on a transilluminator (Loccus, L-Pix Touch).

Performance outcomes

The assessment of calves' body weight, average daily gain (ADG), thoracic perimeter (chest circumference), withers height and rump width were performed at birth and weekly until 30 d of life, and subsequently evaluated at days 42, 60 and 80. The body weight was determined through the circumferential measurement of thoracic perimeter using a graduated tape in kg. Average daily gain was determined by dividing weight gain in the period for the number of days in the same period. Wither's height (from the ground to scapular junction) and croup width (between the ischial tuberosities) were measured using a graduated (cm) tape. A total of 175 calves (PHAGE = 83; CON = 92) were included in these evaluations, calves that died before the end of experimental period were excluded.

Blood collection and biochemical analyzes

For biochemical analyses a sampling of both groups was used (PHAGE = 43; CON = 41). Blood samples were collected through jugular venipuncture at d 0 (up to 24 hours after birth) and at d 7, 14, 21, 30 and 60 using a vacuum system and a tube without anticoagulant. Samples were centrifuged at 2183 x g for 15 min, serum was stored in 1.5 ml microtubes and frozen at -20ºC until the time of analysis. The Labmax Plenno Automatic Biochemical Analyzer, standard model (Labtest, Brazil), was used and analyzes were performed following manufacturer's protocols and the reagent kits specifically (Labtest, Brazil) for each parameter: total proteins, albumin, cholesterol, γ-glutamyl transferase (GGT) and urea.

Statistical analysis

The performance and biochemical data were analyzed using the PROC MIXED method, considering animal as random effect, and group and age, as well as their interactions, as a fixed effect. Each animal was considered an experimental unit. Normality was verified by PROC UNIVARIATE of residues. Diarrhea duration was analyzed using One-way ANOVA, while the categorical variables TPP, morbidity, mortality, lethality, disease recurrence and positive PCR isolates for *E. coli* and *Salmonella* spp. were assessed by the Chi-square and Fischer's exact test. All analyses were performed on SAS University Edition (SAS Institute Inc., Cary, USA), and mean difference were considered significant when $P < 0.05$ and tendency when $0.05 < P > 0.1$.

Results

Morbidity, mortality, lethality and disease relapse rates did not differ between groups (Table 2). First diarrheic episode duration and diarrheic feces permanence (all days with diarrhea during the first month of life) were shorter in PHAGE ($P < 0.001$ and $P = 0.03$, respectively) during the neonatal period (Fig. 1). On days 3, 5 to 13 and 25 of life, PHAGE had fewer diarrheic calves ($P \leq 0.05$; Table 3; Fig. 2).

Regarding age at first diarrhea, there was no difference between the groups (PHAGE = 6.15 and CON = 5.57; $P = 0.99$). Sixty-six feces' samples were tested in microbiological analysis and 27 of these had colonies with biochemical characteristics of *E. Coli* and 2 samples with colonies of *Salmonella* spp. Positive samples for *USPA* gene tended to be higher in CON group (9; PHAGE: 3; $P = 0.09$). In one *E. coli* positive sample from CON, *EAE* gene (intimin virulence factor) characteristic of EPEC was expressed. None of the samples was positive for *Salmonella* spp. in the molecular analysis.

The PHAGE group had higher ADG mean in the neonatal period (first 30 d of life; $P = 0.004$), at the beginning of step-down weaning (up to 42 days of life; $P = 0.004$) and until after weaning (80 d of life; $P = 0.009$) (Fig. 3). The supplemented group had greater weight at the end of the experiment ($P < 0.001$) and tended to have a greater thoracic perimeter ($P = 0.06$), while withers height and croup width did not differ ($P > 0.05$) between groups (Table 3).

Albumin and urea levels were different between groups, the PHAGE mean was lower for albumin (PHAGE = 2.65 g/dL; CON = 2.76g/dL) ($P = 0.004$) and higher for urea (PHAGE = 21.4 mg/dL; CON = 17.82mg/dL) ($P < 0.0001$). Other parameters, GGT (PHAGE = 297.51 U/L; CON = 295.08 U/L), total proteins (PHAGE = 6.33 g/dL; CON = 6.29 g/dL) and cholesterol (PHAGE = 87.85 mg/dL; CON = 86.63 mg/dL), did not differ ($P > 0.1$). All biochemical variables differed considering calves' age ($P < 0.0001$), and for albumin and total proteins interaction between experimental groups and age was observed ($P < 0.0001$) (Fig. 4).

Discussion

Enteric diseases are common on the first weeks of calves' lives, negatively affecting ADG, and consequently increasing female heifers' age at first conception (Svensson et al, 2003; Botteon et al., 2008). Additionally, diseases occurrence in

neonatal period may cause future additional health problems, decreasing quality and quantity of milk production (Kozasa et al., 2005; Laureyns et al., 2009). Therefore, reduction in morbidity and duration of diarrhea can determine higher ADG and, consequently, improvements in animal health and production. In this study, we observed a shorter duration of diarrhea in the PHAGE group in the neonatal period and, consequently, a greater weight gain during the pre-weaned period.

Reduction in the diarrhea period and fewer calves with diarrhea up to 13 d of life in PHAGE may be associated with the control of bacterial proliferation provided by bacteriophage use. Co-infection is commonly seen in diarrheic calves (Cho and Yoon, 2014), and an intestinal dysbiosis and immune suppression may facilitate the proliferation of opportunistic bacteria increasing the duration and severity of the disease (Cho et al., 2013). Such fact was reported by Smith et al (1987) and Sulakvelidze et al. (2001), that confirmed the phage effect on reducing the magnitude of infectious processes. The less aggravated diarrheic episodes possibly justify serum protein and albumin levels increased significantly in the PHAGE group when compared to the CON after the 21st d of life. Also, calves supplemented with phage presented faster clinical recovery, less loss of protein, and possibly greater food intake, with better absorption of nutrients due to smaller extent of intestinal damage (Allison, 2017).

Loss of fluids and less nutrient absorption, associated with inflammatory process caused during diarrhea has a negative correlation with animals' weight gain (Barrington et al., 2002; Gifford et al., 2012). Thus, a shorter diarrhea duration may explain the higher ADG of calves that received milk replacer added with phages. It is noted that this difference is even greater considering only the neonatal period, which is the moment of the highest incidence of diarrhea (García et al., 2000).

In a study conducted with 122 dairy herds in Sweden, calves with neonatal diarrhea were more likely to develop respiratory infections up to six months of age (Hultgren et al., 2008). However, this association was not found in some herds in the USA and in Canada (Windeyer et al., 2014). In the present study, the use of phages was not able to reduce the incidence of respiratory disease, possibly because even reducing the duration of diarrhea, there was still a load of pathogens being eliminated in the environment.

Phage therapy probably acted on bacterial co-infection and secondary bacterial enteritis (Cho et al., 2013), however neonatal diarrhea may be caused by other factors or agents not controlled by phage use, such as nutrition, environment and virus (Cho and Yoon, 2014). Regarding causes of neonatal diarrhea, Santin et al. (2014) observed that 50.3% of fecal samples of calves between 5 days to 2 months of age were positive for *Cryptosporidium* spp., increasing to 66.7% when considering only animals up to 2 weeks of age. Thus, diarrhea prevalence was not reduced in this study probably due to primary causing factors or agents other than bacteria. However, phage therapy was able to decrease diarrhea duration by controlling opportunistic bacterial infections.

In vitro studies have shown satisfactory effects on the reduction of *E. coli* O: 157:H7 occurrence (Niu et al., 2009; Coffey et al., 2011; Carter et al., 2012), however, in vivo experiments show contradictory results. The phage therapy was effective in controlling *E. coli* intestinal population in cattle (Sheng et al., 2006; Niu et al., 2008) and sheep (Callaway, 2008). Nonetheless, other studies did not find the same effect (Bach et al., 2003; Raya et al., 2006; Rivas et al. 2010). Bacteria quantification was not possible in this study, but we believe that the reduction in the diarrheic episode duration in PHAGE was promoted by some decrease in the pathogenic bacteria burden.

Still, the success of the phage therapy may be associated with administration route. Ronzema et al. (2009) demonstrated that oral route is superior to intra-rectal. Sheng et al. (2006) observed that the rectal application (4 doses of 25 ml with $1x10^{10}$ CFU) was sufficient to control bacterial population, although in this study continuous administration of phages in lower concentrations in the water was maintained ($1.8x10^{6}$ CFU). Also, Anand et.al (2015) found that broad-spectrum phage was able kill 47.3% of *E. coli* isolates from diarrheic calves.

In this study, lytic bacteriophage added to milk replacer managed to control diarrhea, as observed by the decrease in the permanence of diarrheic feces during neonatal period. Additionally, greater weight gain in the first 80 d of life was obtained for calves supplemented with bacteriophage.

Declarations

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Conflicts of interest/Competing interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and material

Data will be made available on request

Code availability

Not applicable

Authors' contributions

VR, MC, FP and CB conceived and designed research. ES and VR conducted experiments. JF, AM and NR conducted hematological analysis. AM conducted molecular analysis. JF, VR and NR analyzed data. ES, NR and AM wrote the manuscript. VR and FP reviewed and edited. All authors read and approved the manuscript.

Ethics approval

The research protocol was approved by Ethics Committee on Animal Experimentation - CEEA of the Federal University of Pelotas (Protocol No. 14807)

Consent to participate

Not applicable

Consent for publication

Not applicable

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Tables

Table 1
Sequence of primers and conditions used in PCR for genes of *Escherichia coli* and *Salmonella* spp. in feces samples of Holstein × Gyr crossbred female calves with diarrhea.

Gene	Protein	*Primer sequences*	*amplicon size* (pb)	No. of cycles	annealying temperature (°C)	Reference
hilA	Hyper Invasibility	F- GCGAGATTGTGAGTAAAAACACC	413	35	63	Craciunas et al. (2012)
		R- CTGCCCGGAGATATAATAATCG				
uspA	Universal stress protein	F-CCGATACGCTGCCAATCAGT	884	30	70	Chen; Griffiths (1998)
		R-ACGCAGACCGTAGGCCAGAT				
eae	Intimin	F-GACCCGGCACAAGCATAAGC	384	35	60	Paton; Paton (1998)
		R-CCACCTGCAGCAACAAGAGG				
stx1	Shiga toxin 1	F-ATAAATCGCCATTCGTTGACTAC	180	35	60	Paton; Paton (1998)
		R-AGAACGCCCACTGAGATCATC				
estla	Thermoestable toxin A	F-CCTCTTTTAGCAGACACTGAATCATTG	157	30	63	Mueller et al. (2007)
		R-CAGGCAGGATTACAACAAAGTTCACAG				
hlyA	Hemolysin	F- GCATCATCAAGCGTACGTTCC	534	35	60	Paton; Paton (1998)
		R- AATGAGCCAAGCTGGTTAAGCT				

Table 2
Mortality, morbidity, lethality and relapse rates of Holstein × Gyr crossbred female calves with (PHAGE; n = 100) or without (CON; n = 100) the addition of bacteriophage in milk replacer during pre-weaning (70 days).

Parameter	Group		*P* value
	CON	PHAGE	
Morbidity[1]	70% (70/100)	63% (63/100)	0.29
Diarrhea morbidity (%)			
Respiratory disease morbidity (%)	29% (29/100)	26% (26/100)	0.66
Mortality[2]	2% (2/100)	1% (1/100)	0.57
Diarrhea mortality (%)			
Respiratory disease mortality (%)	3% (3/100)	5% (5/100)	0.47
Lethality[3]	2.9% (2/70)	1.6% (1/63)	0.62
Diarrhea lethality (%)			
Respiratory disease lethality (%)	10.3% (3/29)	13% (5/26)	0.57
Relapse[4]	24.3% (17/70)	25.4% (16/63)	0.88
Diarrhea relapse (%)			
Respiratory disease relapse (%)	13.8% (4/29)	11.53% (3/26)	0.80

1 - Morbidity: number of ill calves/total number of calves; **2 - Mortality**: number of dead calves/ total number of calves; **3 - Lethality**: number of calves that died from diarrhea or respiratory disease/ total number of calves that presented diarrhea or respiratory disease; **4 - Relapse**: number of animals that got sick twice or more.

Table 3
Number of calves Holstein × Gyr crossbred female calves with diarrhea receiving (PHAGE; n = 100) or not (CON; n = 100) the addition of bacteriophage in milk replacer during pre-weaning (70 days).

AGE	Number of calves with diarrhea		P value
	CON	PHAGE	
1	2/48	2/47	*0.9828*
2	4/48	8/47	*0.2025*
3	9/48	2/47	*0.0273**
4	9/48	8/47	*0.826*
5	16/48	5/47	*0.0077**
6	15/48	6/47	*0.03**
7	16/48	9/47	*0.1165*
8	25/48	12/47	*0.008**
9	24/48	11/46	*0.0089**
10	22/48	11/45	*0.0312**
11	27/48	9/45	*0.0003**
12	18/48	4/45	*0.0012**
13	11/48	3/45	*0.0285**
14	2/48	3/45	*0.5932*
15	1/48	1/44	*0.9504*
16	1/48	1/44	*0.9504*
17	1/48	2/44	*0.5066*
18	2/48	0/44	*0.171*
19	5/48	1/44	*0.114*
20	4/48	2/44	*0.4623*
21	2/48	2/44	*0.9291*
22	5/48	2/44	*0.2887*
23	7/48	3/44	*0.232*
24	5/48	0/44	*0.0277**
25	3/48	5/44	*0.3846*
26	8/48	5/44	*0.4657*
27	3/48	4/44	*0.6077*
28	2/48	2/44	*0.9291*

*Statistical difference detected by Fisher's exact test (P < 0.05)

AGE	Number of calves with diarrhea		P value
	CON	PHAGE	
29	6/48	2/44	*0.1762*
30	2/48	2/44	*0.9291*
*Statistical difference detected by Fisher's exact test (P < 0.05)			

Table 4

Performance parameters of Holstein × Gyr crossbred female calves up to 80 days of life with (PHAGE; n = 83) or without (CON; n = 92) the addition of bacteriophage in milk replacer during pre-weaning (70 days).

Parameter	Group				*P* value		
	CON		PHAGE		Group	Age	Group*Age[2]
	Mean	SEM[1]	Mean	SEM[1]			
Thoracic perimeter (cm)	77.67	0.21	78.30	0.26	0.06[†]	<0.001[*]	0.26
Withers height (cm)	77.29	0.18	77.44	0.13	0.51	<0.001[*]	0.81
Croup width (cm)	18.26	0.29	18.53	0.15	0.42	<0.001[*]	0.32
Weight (kg)	47.34	0.25	49.51	0.26	<0.001[*]	<0.001[*]	0.09[†]
ADG (kg)	0.432	0.016	0.491	0.017	0.011	<0.001[*]	0.47

[1]Standard error of the mean

[2]Interaction between group and age (days) and group

[*]Statistical difference identified by Tukey-Kramer test (*P*<0.05)

[†]Tendency identified by Tukey-Kramer test (0.05 < *P* >0.1)

Figures

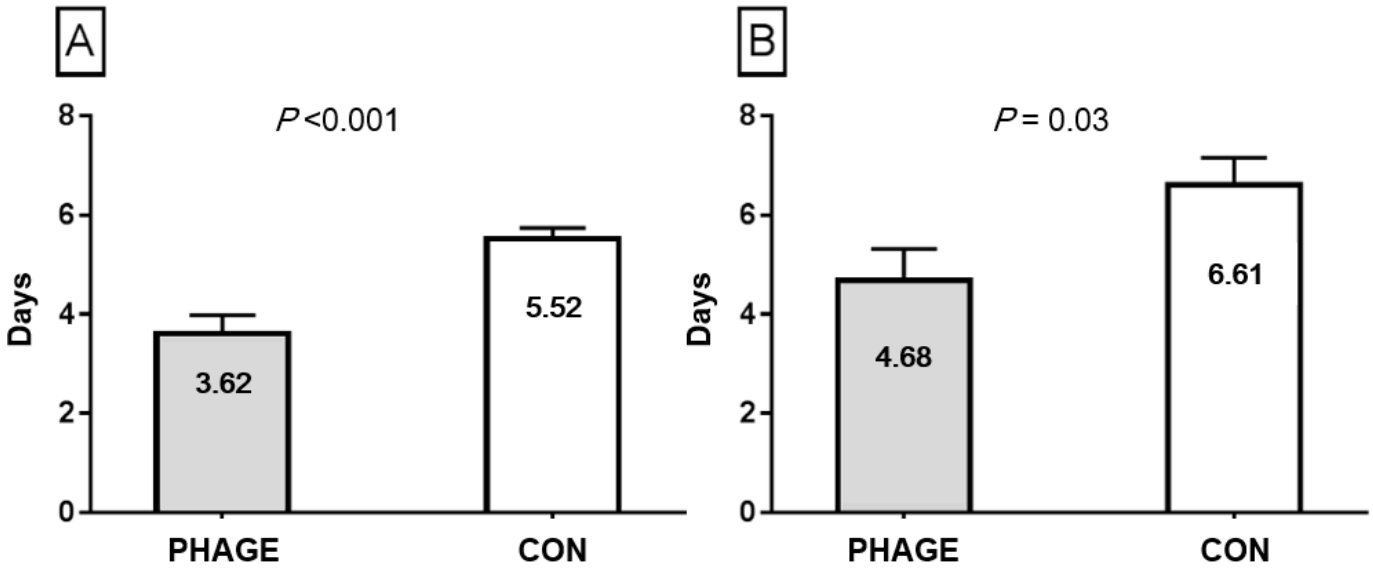

Figure 1

Period (days) of duration of the first diarrheic episode (A) and duration of diarrheic feces (B) in the neonatal period of Holstein × Gyr crossbred female calves with (PHAGE; n = 42) or without (CON; n = 50) the addition of bacteriophage in milk replacer during pre-weaning (70 days) Difference between groups by One-way ANOVA (P<0.05) Bars indicate standard error of the means

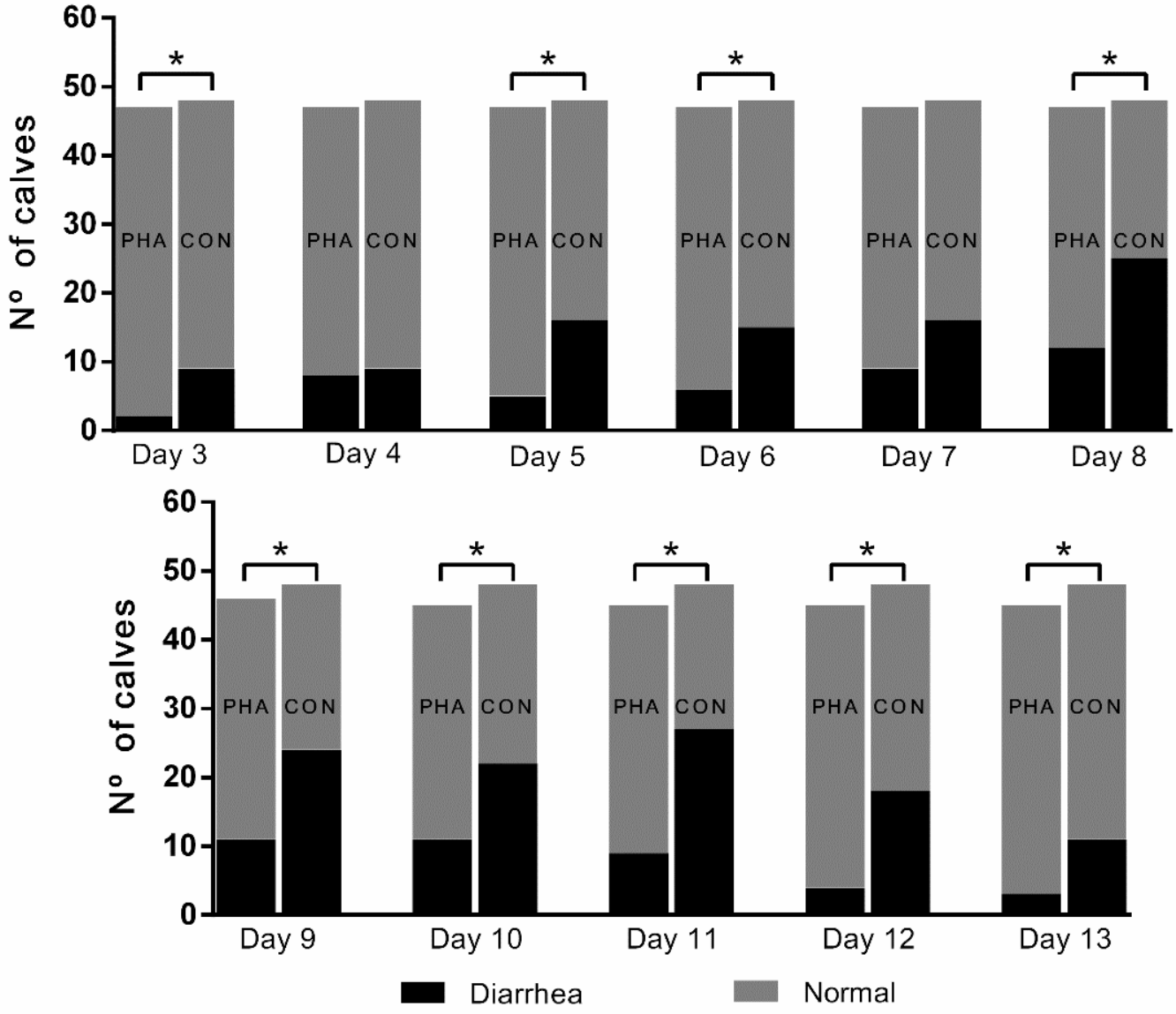

Figure 2

Number of Holstein × Gyr crossbred female calves with diarrheic and normal feces from days 3 to 13 of life with (PHAGE) or without (CON) the addition of bacteriophage in milk replacer during pre-weaning (70 days) *Comparison between groups by Fisher's exact test (P=0.05)

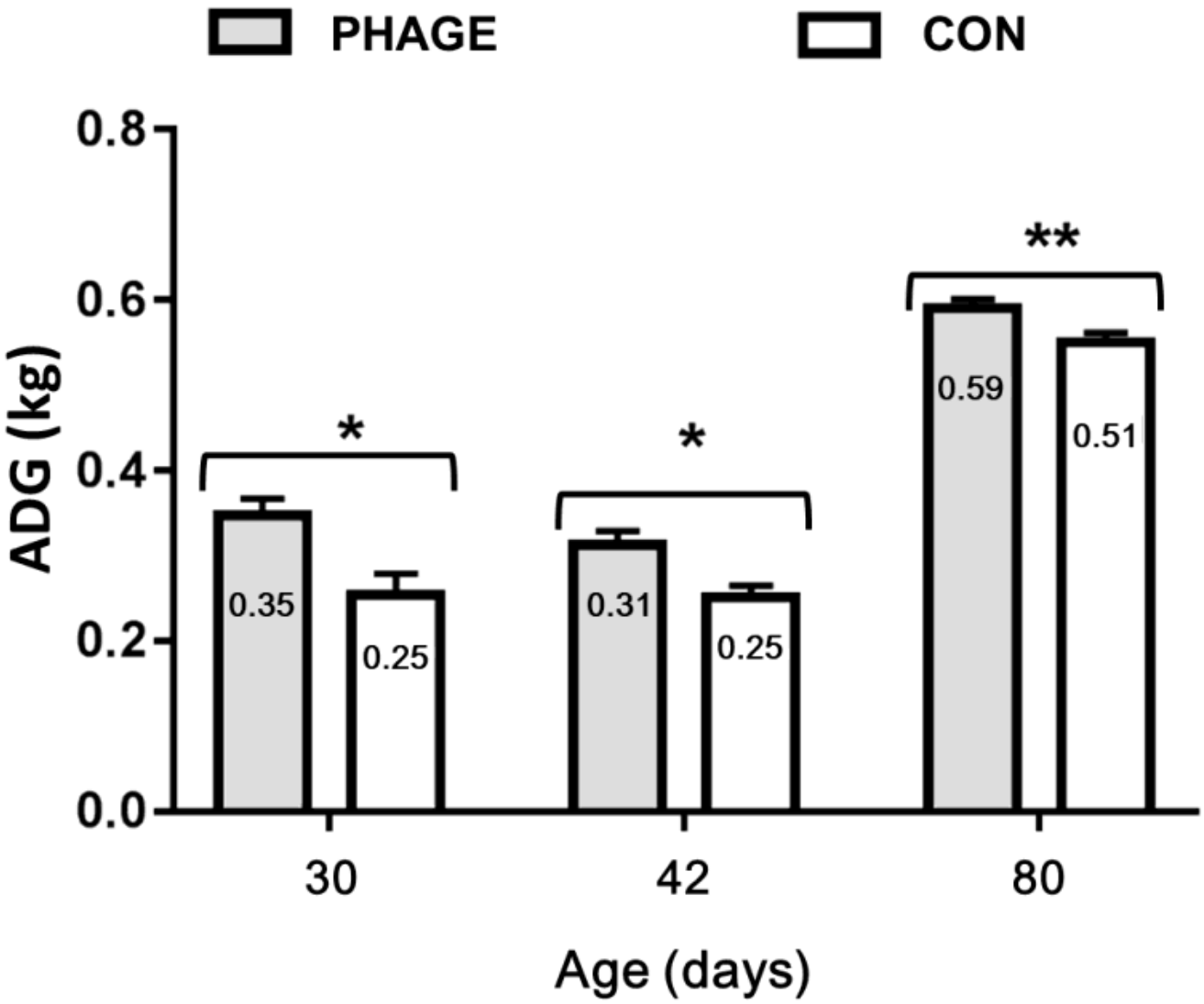

Figure 3

Average daily gain (ADG) at the end of the neonatal period (30 days), at the beginning of step-down weaning (42 days) and after weaning (80 days) of calves with (PHAGE; n = 83) or without (CON; = 92) the addition of bacteriophage in milk replacer during pre-weaning (70 days) Comparison between groups by Tukey-Kramer test (*P<0.01 ** P=0.01)

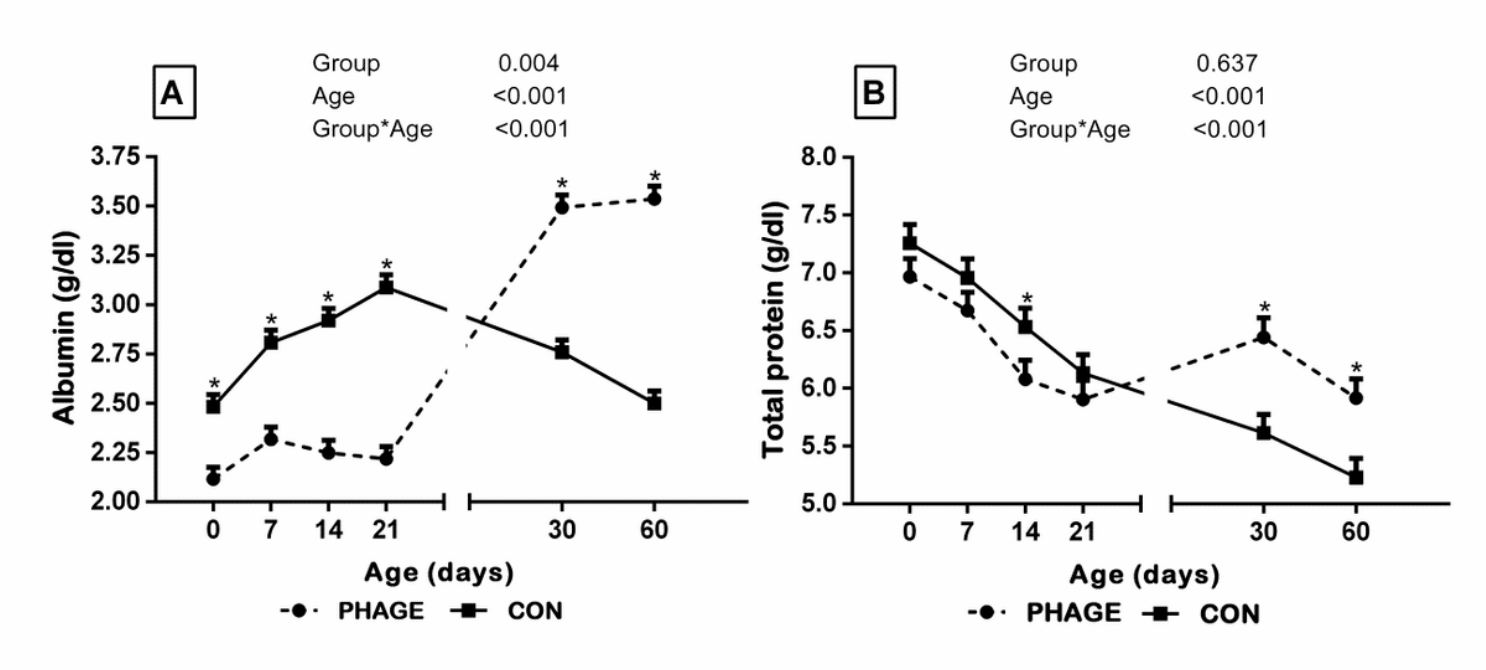

Figure 4

Mean value of albumin (A) and total protein (B) of calves rotating with (PHAGE; n = 43) or without (CON; n = 41) the addition of bacteriophage in milk replacer during pre-weaning (70 days) *Indicates statistical difference by the Tukey-Kramer test ($p < 0.05$) Interaction between age (days) group and Tukey-Kramer test ($p < 0.05$)