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Short communication

Evaluation of oral administration of bacteriophages to neonatal calves: Phage survival and impact on fecal *Escherichia coli*

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ABSTRACT

Diarrhea is an important cause of morbidity and mortality in neonatal calves. Several enteropathogens are associated with diarrhea in young calves, with Enterotoxigenic *Escherichia coli* (ETEC) infection being the most common type of colibacillosis. The rise of antibiotic resistance in a number of medically important bacterial pathogens has revived interest in the use of bacteriophages as anti-bacterial therapeutic agents. Here we describe the results of a randomized, placebo-controlled, double-blinded study designed to evaluate the effect of an oral bacteriophage cocktail on fecal *E. coli* colony-forming units (CFU). Ten calves were enrolled in the study to either the bacteriophage group or the placebo group for 22 feedings. Calves in the bacteriophage treatment group (n = 6) received a total of 10^6 plaque-forming units (PFU) (volume = 5 ml) of each of four bacteriophages while the placebo group (n = 4) received only phosphate-buffered saline (5 ml). Fresh fecal samples and blood samples were collected daily from each calf and analyzed for bacterial count and presence of bacteriophage. *E. coli*-infecting phages were recovered from all phage-treated calves at concentrations of 10^2 to 10^3 PFU per gram of rectal contents, but none was detected in serum. Phage treatment caused a reduction in fecal *E. coli* when compared to the control group: the mean log CFU for the placebo-treated group was 9.25 (SE = 0.42) versus 9.11 (SE = 0.34) for the phage-treated group, but the difference was not statistically significant.

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1. Introduction

Diarrhea remains an important cause of morbidity and mortality in neonatal calves (Constable, 2004). The economic losses associated with this disease are due not only to the resulting mortality, but also to the retarded growth of the animals, the cost of both the veterinary care and the drugs used to treat the infection, and the increased labor involved (de Graaf et al., 1999). Several enteropathogens are associated with diarrhea in neonatal calves, the most prevalent being *Escherichia coli*, *Clostridium perfringens*, *Salmonella* spp., *Cryptosporidium* spp., and rotavirus and coronavirus, with their relative importance varying by geographic region (Snodgrass et al., 1986; Younis et al., 2009).

Escherichia coli is a gram-negative bacterium normally found in the intestines of most animals. Although most *E. coli* are nonpathogenic, some are able to cause intestinal and extra intestinal infections. Large numbers of *E. coli* are present in the farm environment as a result of fecal contamination. Initial exposure to pathogenic *E. coli* may occur in contaminated calving pens, but systemic infection usually requires predisposing environmental factors, inadequate transfer of passive immunity or compromised immune system by other infection. The most common type of colibacillosis in young animals is caused by the non-invasive Enterotoxigenic *E. coli* (ETEC) strains, which are also the leading cause of diarrhea among travelers and children in the developing world (Nagy and Fekete, 2005).

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The rise in antibiotic resistance in a number of medically important bacterial pathogens (Denou et al., 2009) has revived interest in the use of bacteriophages to treat bacterial infections (Merril et al., 2003). Phage therapy is considered a potentially low-cost treatment modality and was first implemented by Felix d'Herelle more than 90 years ago. However, the development of antibiotics in the 1940s put a halt to commercial production of therapeutic phages in most Western countries whereas in the Soviet Union, enteric diseases, nosocomial infections, burn and wound infections continued to be treated with phage preparations on a very large scale and apparently with some success (Sulakvelidze, 2005). Therefore, our objective was to evaluate the effect of oral administration of bacteriophages on fecal *E. coli* CFU. Additionally, it was also our objective to assess the survivability of the phages and the impact of oral phage administration on calf health. Our one-sided hypothesis was that oral administration of *E. coli* bacteriophages would cause a reduction in fecal *E. coli* CFU.

2. Materials and methods

A randomized placebo-controlled, double-blinded study was performed at the College of Veterinary Medicine, Cornell University (Ithaca, NY). From May 28, 2009 through June 7, 2009, 4 bull calves were purchased at birth from a local dairy farm plus 6 more at 1 week of age and enrolled in the study at the same time. The calves were randomly chosen and submitted to a physical examination by a veterinarian at the farm. They were commingled on the transport trailer and randomly off-loaded at Cornell University, at which time an identification tag was placed in the right ear and the animals were weighed. The calves were assigned to treatment groups and housing in the order they were removed from the trailer. On the first day of treatment a technical mistake was made and one calf received a different coded bucket from the one he had been assigned to receive. Exclusion of this calf was not necessary because the management for both groups was kept the same and no prior treatment had been given. The calf was moved from his initial group and assigned to the other group.

The calves were individually housed in permanent 0.91×1.83 m stalls with high tile walls and concrete flooring within a closed barn with an active ventilation system. This prevented contact between calves. Calves were fed commercial 22% protein/20% fat non-medicated milk replacer (Nursing Formula NT Calf Milk Replacer, Land O'Lakes Inc.) at 0.68 kg of dry matter (DM) per day, split into 2 feedings, for the duration of the study. Any milk replacer not consumed within 15 min was fed to the animal via an esophageal tube feeder. Free-choice water was available at all times. Stalls were cleaned and bedded daily with pine shavings and steam cleaned with 93 °C (200 °F) pressurized water. Gross manure contamination on the stall walls was also removed daily. Pull-over boots, water buckets, milk buckets, milk bottles, and thermometers were labeled for each treatment group and not shared between calves receiving the different treatments. Calves enrolled in the study received 22 feedings; following the farm management, where each calf receives two feedings of milk per day. Study personnel associated with calf management and sample collection were blinded to the treatments; all treatment vials were color-coded in the laboratory by a study member who did not participate in data collection, and the codes were not broken until the end of the study.

A pre-study period on days 1 and 2 was used to collect baseline data on fecal *E. coli* CFU, phage cocktail PFU, and calf health. During these two days, blood and manure samples were collected for CFU and PFU quantification as well as for obtaining calf health data. Calves started being monitored for feed intake at all feedings, and received a recorded visual health appraisal twice daily by a veterinarian (blinded to treatment allocation). At each feeding, the following data were recorded: health score, fecal score, weight, and temperature. Calf health was assessed visually by using objective criteria of appetite, fecal consistency, hydration status, respiratory effort, and attitude (Berge et al., 2005). Calves would have been removed and censored from the study if their clinical status appeared critical at any point during the study period; fortunately, this was not necessary for any calf. A single dose of Flunixin meglumine (Banamine, 50 mg/ml, 2.2 mg/kg, Schering Plough) was used to treat one calf with a depressed attitude. No antibiotic treatment was used during the study period.

On study day 3 the treatment began: each of the 6 calves in the bacteriophage treatment group received a total of 10^6 PFU (volume = 5 ml) of each of 4 genetically distinct bacteriophages while the placebo group (4 calves) received the placebo (5 ml PBS) treatment. Treatments (phage or placebo) were administered in the morning and afternoon and were added to the reconstituted milk replacer immediately before feeding. Animals in the treatment group each received a bacteriophage cocktail compounded of four bacteriophages previously isolated from the manure lagoons of two large commercial dairy farms located near Ithaca, NY (Bicalho et al., 2010). Bacteriophage high-titer stocks were propagated and purified as follows: 0.2% inoculum of an overnight culture of the propagating host strain (*E. coli* 137 previously isolated from a uterine secretion of a metritic cow) was added to 250 ml of LB broth (Difco™) and incubated at 37 °C for 5 h with shaking. Approximately 1×10^9 PFU of each isolated bacteriophage was added to the culture and, after incubation at 37 °C for 20 min without shaking, the samples were incubated for a further 18–20 h at 37 °C with vigorous shaking.

Concentrated phage preparations were obtained by precipitation of the LB lysates with NaCl/polyethylene glycol (PEG) 8000 according to protocols described by Sambrook and Russel (2001). Solid NaCl (final concentration 1.0 M) was added to each sample and, after stirring, the samples were kept on ice for 1 h. The samples were then centrifuged at $11{,}000 \times g$ at 4 °C for 10 min to remove debris and the supernatants (LB lysates) were transferred to sterile flasks. Solid PEG 8000 (final concentration 10% w/v) was added to the lysates and the samples were kept on ice for at least 1 h to allow the bacteriophage particles to precipitate. The precipitated bacteriophage particles were recovered by centrifugation at $11{,}000 \times g$ at 4 °C for 10 min and resuspended in SM buffer. These large-scale high-titer stocks were titrated using standard overlay techniques and stored at 4 °C.

Fresh fecal samples were collected daily per rectum with a latex examination glove and stored in a sterile container. The samples were kept chilled before and during transport to the

laboratory. Bacteria enumeration of fecal samples was conducted by vortexing 1 g of fecal sample with 9 ml of PBS in a 50-ml Falcon tube. Each fecal sample was diluted in PBS and aerobically cultured on MacConkey agar (Difco™) plates at 37 °C for 18 h. Each aliquot was spiral plated in triplicate.

To assess the presence and quantity of bacteriophages in the blood, blood samples from each calf were collected in a vacuum container and refrigerated until processing. After centrifugation at 20,000×*g* for 20 min, 1 ml of plasma was transferred to an Eppendorf tube and serial dilutions from 10^{-1} to 10^{-4} were prepared by adding 1 ml of the phage solution to sterile broth (SM buffer). Each phage serial dilution of each calf was quantified in 10-μl microdrops in triplicate. The plates were examined after 48 h and the number of PFU determined.

2.1. Quantification of fecal bacteriophages

To assess the presence and quantity of fecal bacteriophages, daily fecal samples were collected. In the laboratory, stool samples (1 g) were homogenized in PBS (8.5 g of NaCl and 1 g tryptone per liter) to a final volume of 10 ml. Stool samples were centrifuged for 15 min at 14,500×*g* in 50-ml Falcon tubes and filtered through a Millex AP20 prefilter followed by a 0.45-μm Minisart filter. Subsequently, the samples were stored at 4 °C and serial dilutions from 10^{-1} to 10^{-4} were prepared. Spot testing was done on LB agar Petri dishes (15 g/l agar) overlaid with 3.5 ml of LB top agar (7.5 g/l). Each Petri dish enumerated for each calf was divided in 5 quadrants (dilution 0, dilution 1, dilution, 2, dilution 3, dilution 4). Ten microliters of each dilution were applied as three spots in a clockwise distribution to each quadrant of the plate after the top agar with plating *E. coli* bacteria had solidified.

The phages were propagated and counted on an indicator strain (*E. coli* 137). The amount of phage was estimated by considering that the last dilution of the diluted phage that caused visible bacterial lysis is equivalent to 1 virion in 1 μL. In general, amplified phage plaques did not differ in appearance. Dilutions with well-separated plaques were chosen for counting from each positive stool sample.

To evaluate the effect of pH on bacteriophage viability, samples of the isolated phages were mixed in a series of tubes containing SM buffer with pH values ranging from 1.0 to 11.0, incubated for 1 h at 37 °C, and then titrated by the double-layer agar plate method.

To evaluate the effect of bacteriophage therapy on the quantity of lactose-negative fecal bacteria and also on the quantity of fecal bacteriophages, two repeated measures ANOVA analyses were performed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The outcome variables were the log CFU of fecal lactose-negative bacteria and the log PFU of fecal bacteriophages. The outcome variables were longitudinally collected (repeated measures); the error term was modeled by imposing a first-order autoregressive covariance structure to appropriately account for the within-calf correlation of the repeated measures.

3. Results

Phage treatment caused a numerical reduction of fecal *E. coli* when compared to the controls; the mean log CFU for the placebo-treated group was 9.25 (SE = 0.42) versus 9.11 (SE = 0.34) for the phage-treated group, but the difference is not statistically significant ($p = 0.15$; Fig. 1). Additionally, the log CFU of the phage-treated group decreased quickly after the first phage treatment was administered, indicating a potential treatment effect. Nevertheless, despite the known *in vitro* antimicrobial efficacy of the phage cocktail against *E. coli* strains (Bicalho et al., 2010), repeated oral application of high titers of phage cocktail did not significantly reduce fecal *E. coli* CFU. However, the statistical power of our study was limited, given that only 10 calves were enrolled. To evaluate the susceptibility of phages to acidity, an *in vitro* experiment was carried out in which the phages were exposed to a range of pH values. Dilutions of 10 and 10 showed higher lytic action among all the serial dilutions made in our laboratory. Relative viability was estimated by comparing the phage titer in acidic SM buffer with that in neutral SM buffer, pH 7.5. Phages were nonviable at pH 1 and 2. The effects of higher pH (pH 3–11) on the viability of all 4 phages used in this study were not evident (Fig. 2).

In the present study, fecal phage titers were estimated by spotting 10 μl of each dilution tube onto a bacterial lawn of *E. coli* 137. The presence of phage in each diluted sample was determined by the occurrence of plaques (clearing zones) in the bacterial lawn. Bacteriophages were recovered only from the treated group, and only after study day 3, 24 h after the first phage treatment (Fig. 3). *E. coli*-infecting phages were recovered from all phage-treated calves at concentrations of 10^2–10^3 PFU/g of fecal matter on study days 4 and 5, but no bacteriophages were detected in the feces of control calves. On study day 6, mean phage titers reached approximately 10 PFU/g, and on study days 7–10 the mean phage titers decreased to 10 PFU/g. On study day 11, the final day of the study, the mean phage titer was 10 PFU.

4. Discussion

Rozema et al. (2009) reported a significant decrease in *E. coli* O157:H7 in orally bacteriophage-treated adult cattle compared to steers who received the treatment by rectal application. They concluded that the longer retention time experienced by the orally administered phages within the digestive tract, with the digesta being present in both liquid and solid phases, increased the opportunity for the phages to interact with their target *E. coli* cells and replicate, thereby increasing both the phage population size and the likelihood of infection of additional *E. coli* cells.

Factors that can interfere with phage–bacterium interactions include inoculum size, timing and manner of administration, presence of an anti-phage immune response, and appearance of phage-resistant mutants (Smith et al., 1987). Another relevant factor, elucidated by the same authors, is the acidity of the stomach contents at the time of phage administration, which is especially important with oral phage treatments.

Our results when evaluating the susceptibility of phages to acidity are equivalent to those of Smith et al. (1987), who

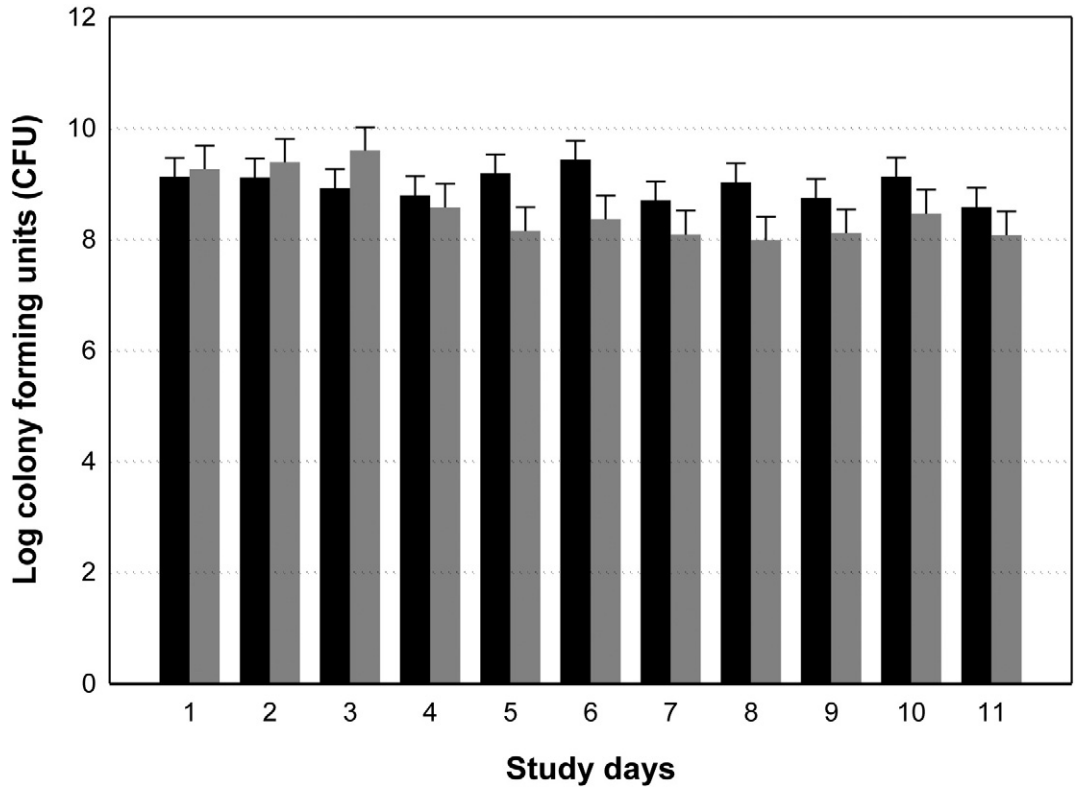

Fig. 1. Least square means (LSM) of the log-CFU by treatment groups and by study days. The placebo group (black bars) had an LSM of 9.25 (SE = 0.42) and the phage-treated group (gray bars) had an LSM of 9.11 (SE = 0.34).

reported that the 'mild' acidity (3<pH<4.3) present in the abomasum contents of calves shortly after milk feed had little harmful effect on orally administered phages, whereas the high acidity (2.3<pH<3.0) predominant in the partially empty stomach was lethal to the phages. Denou et al.(2009), using mice, found lower phage titers in the cecum and large intestine compared to the initial amount of phages added to the drinking water. Those authors mentioned the acidity of the mouse

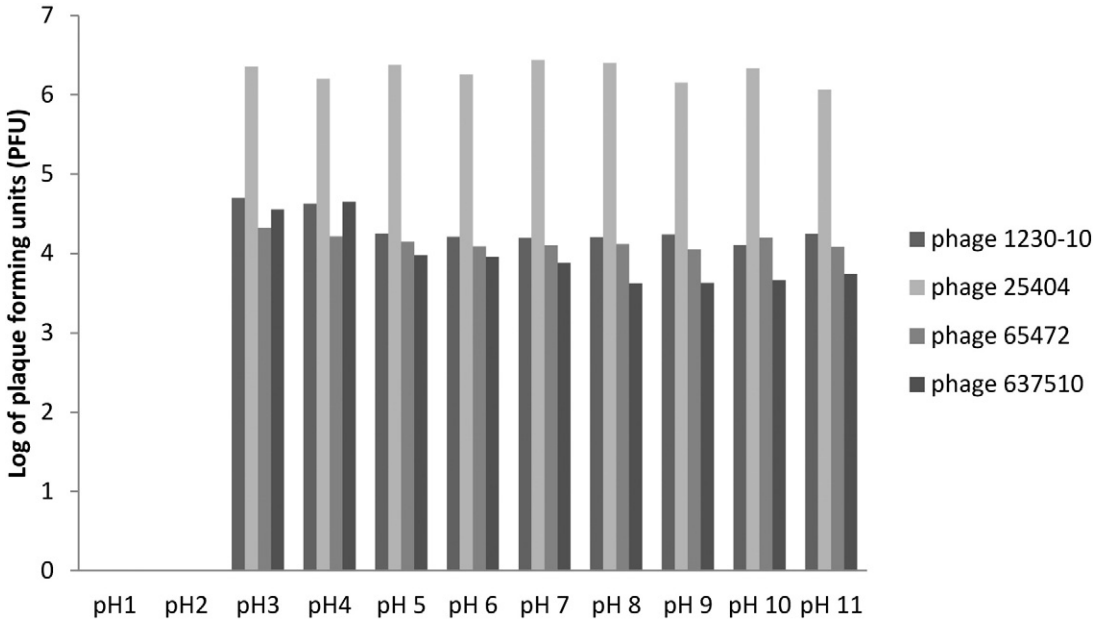

Fig. 2. Effect of pH on bacteriophage viability. Higher pH (pH 3–11) did not have an adverse effect on the viability of phages. Phages were nonviable at pH 1 and 2.

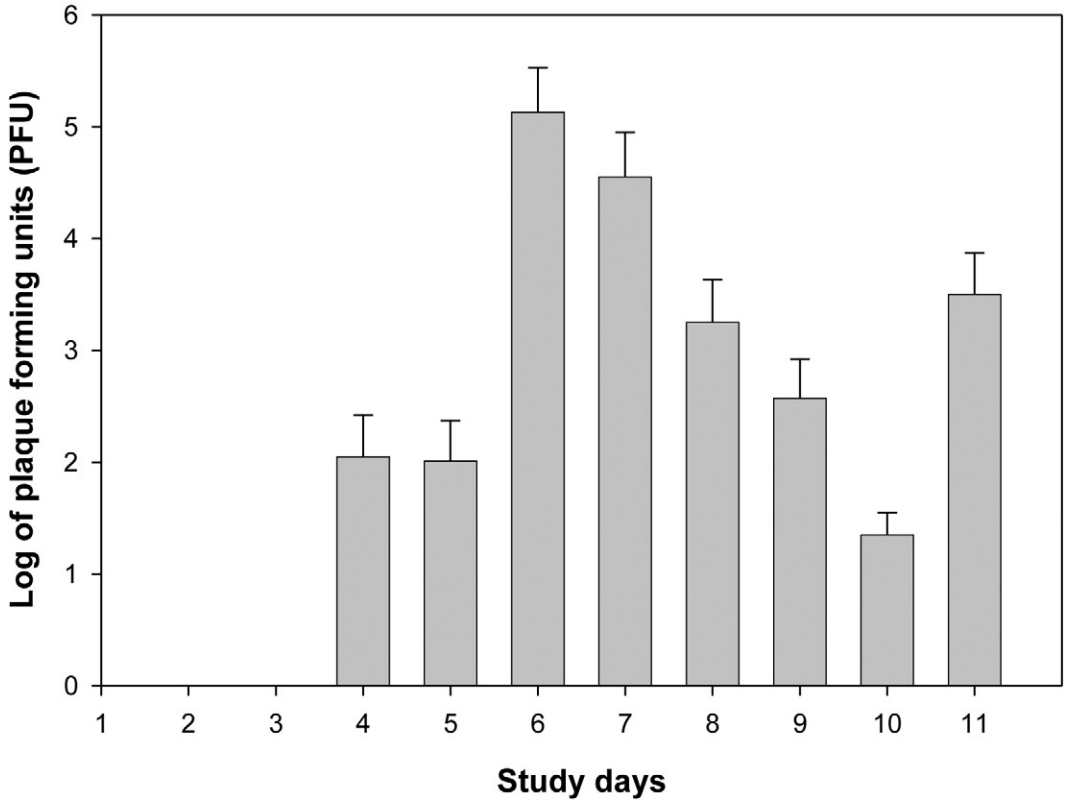

Fig. 3. Least square means (LSM) of the log-PFU by treatment groups and by study days. The data were analyzed by generalized linear mixed models using log-PFU as the outcome variable and treatment, study day and the interaction of treatment and study day as independent variables. The variable 'calf identification number' was added to the model as a random effect. Bacteriophages were identified only in the treated group and only after study day 3.

stomach (pH = 3) as a possible explanation for the phage loss. However, the findings of Bruttin and Brüssow (2005) suggest that there is considerable phage stability during gastrointestinal transit in humans, as the authors were able to detect phages in all fecal samples of volunteers who had ingested phages in drinking water at a concentration of 10 pfu/ml.

Because we constituted our phage cocktail using *E. coli* strains isolated from bovine uteri as hosts (Bicalho et al., 2010), one could argue that better results might have been obtained if fecal *E. coli* isolates had been used as hosts. The predator–prey relationship between phages and their targeted bacteria is exemplified in the work of Tanji et al., 2003. In that study, when the populations of *E. coli* O157:H7 in waste-water streams increased so did the populations of bacteriophage that were active against *E. coli* O157:H7. This is because the host specificity of each phage depends on its ability to recognize specific cell-surface receptors (Tanji et al., 2003). Such strain specificity could be an explanation for the observation that the stool samples of the control group did not present any phages (environmental in origin), because the host used for phage enumeration was an *E. coli* strain isolated from the uterus of a metritic dairy cow.

5. Conclusion

In summary, bacteriophages administered orally were recovered in high numbers from fecal samples collected 24 h after administration, thereby indicating that they had survived the low pH of the abomasum. Additionally, a numerical decrease in the CFU of lactose-negative fecal bacteria was observed in the phage-treated group; however, the difference was not statistically significant. Bacteriophage survived *in vitro* exposure to pH levels as low as 3 and as high as 11 and a complete absence of phage was observed at pH ≤ 2. No side effects of oral phage therapy were observed on the health of the study calves throughout the study period. Further study of oral bacteriophage therapy is warranted in field trials, using larger number of animals, as a potential non-antibiotic therapy against newborn colibacillosis.

Conflict of Interest

Have no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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