

# Efficacy of dietary supplementation of bacteriophages in treatment of concurrent infections with enterotoxigenic *Escherichia coli* K88 and K99 in postweaning pigs

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## Summary

Postweaning pigs challenged with enterotoxigenic *Escherichia coli* (ETEC) K88 and K99 and fed a diet supplemented with ETEC K88- and K99-specific bacteriophages exhibited greater weight gain, lower fecal consistency score, and less fecal shedding and intestinal adhesion of ETEC K88 than did pigs fed the unsupplemented diet.

**Keywords:** swine, bacteriophage, postweaning diet, enterotoxigenic *Escherichia coli*, feces

**Received:** September 15, 2015

**Accepted:** February 2, 2016

**Resumen - Eficacia de la suplementación dietética de bacteriófagos en el tratamiento de infecciones recurrentes con la *Escherichia coli* enterotoxigénica K88 y K99 en cerdos post-destete**

Los cerdos post-destete probados con *Escherichia coli* enterotoxigénica (ETEC) K88 and K99 y alimentados con una dieta suplementada con bacteriófagos específicos de ETEC K88 y K99 mostraron mayor ganancia de peso, puntaje menor de consistencia fecal, y menos eliminación fecal y adhesión intestinal del ETEC K88 que los cerdos alimentados con una dieta no suplementada.

**Résumé - Efficacité d'un ajout de bactériophages à l'alimentation pour le traitement d'infections concomitantes par *Escherichia coli* K88 et K99 chez des porcs en période post-sevrage**

Des porcs en période post-sevrage ont été infectés avec des souches entérotoxigènes d'*Escherichia coli* (ETEC) K88 et K99 et nourris avec une diète supplémentée avec des bactériophages spécifiques contre des ETEC K88 et K99. Ceux-ci ont montré un gain de poids supérieur, un score plus faible de la consistance fécale, et moins d'excrétion fécale et d'adhésion intestinale des ETEC K88 que les porcs nourris avec une nourriture non supplémentée.

Postweaning diarrhea or colibacillosis is a costly disease causing substantial mortality, as well as growth retardation, in swine production.<sup>1-3</sup> Colibacillosis is typically associated with avid intestinal adhesion and fecal shedding of enterotoxigenic *Escherichia coli* (ETEC). The ETEC causing diarrhea in postweaning pigs carries the F4 (K88) or F18 fimbrial antigen in most cases.<sup>4-6</sup> The F5 (K99) antigen has also been found in postweaning diarrhea in Central China<sup>7</sup> as well as in South Korea (Jeong-Hee Han, unpublished data, 2014), although piglets are less susceptible to ETEC K99 than to K88 with increasing age.<sup>8</sup>

Common therapies used for prevention and treatment of colibacillosis are antibiotics<sup>9,10</sup>

and pharmacological concentrations of zinc oxide (ZnO) ranging from 2000 to 4000 mg per kg diet in many countries,<sup>2,4,9-11</sup> including the United States.<sup>11</sup> Use of antibiotics as feed additives has been banned in the European Union since 2006, and subsequently in Korea since mid-2011,<sup>12</sup> because of increasing concerns about the emergence of antibiotic-resistant pathogens.<sup>13</sup> Moreover, the concentration of ZnO in feed is now limited to 150 mg per kg by regulation in the European Union,<sup>14</sup> which may lead non-European countries to adopt a similar regulation. It is thus necessary to find alternatives to in-feed antibiotics as well "pharmacological" ZnO.

Bacteriophages or phages have recently received re-emerging attention as alternatives to antibiotics because of several merits as feed additives, including their high stability within the feed and digestive tract as well as their high specificity of transfection.<sup>15-17</sup> However, only limited information is available as to the effects of phage therapy in the pig, although dietary phages have been shown to be effective for alleviating the severity of diarrhea in postweaning pigs challenged with a hemolytic K88-positive ETEC strain as well as in unchallenged piglets.<sup>18</sup> Thus, more studies are needed before dietary phages can be established as prophylactic or therapeutic agents against porcine colibacillosis. The present study was therefore initiated to evaluate the efficacy of dietary phages on treatment of colibacillosis induced by a concurrent oral challenge with ETEC K88 and K99 in postweaning pigs.

## Materials and methods

The experimental protocol for the present study was approved by the Institutional Animal Care and Use Committee of Kangwon National University.

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This article is available online at <http://www.aasv.org/shap.html>.

Han SJ, Oh Y, Lee CY, et al. Efficacy of dietary supplementation of bacteriophages in treatment of concurrent infections with enterotoxigenic *Escherichia coli* K88 and K99 in postweaning pigs. *J Swine Health Prod.* 2016;24(5):259-263. <https://doi.org/10.54846/jshap/943>

The phages used in the present study were prepared by iNtRON Biotechnology, Inc (Sungnam, Korea), as follows. Briefly, the ETEC K88-specific and K99-specific phages were isolated on agar plates of K88 and K99 bacterial cultures, respectively, from the feces of 30- to 70-day-old grower pigs on a commercial swine farm. The isolated phages, which were identified as *Myoviridae* and *Siphoviridae* families, respectively, were plaque-purified, diluted in a 0.2 M Tris buffer (pH 7.5) containing 0.1 M NaCl, 1 mM MgSO<sub>4</sub>, and 0.01% gelatin, and freeze-dried. The ETEC K88-specific and K99-specific phages added to a common pig diet are known to retain their titers for 12 months (Dr Sang-Hyeon Kang, iNtRON Biotechnology, Inc; oral communication, December, 2015).

Thirty candidate piglets, which had been born to Duroc-sired Landrace × Yorkshire dams on a commercial farm, were castrated on day 2 after birth. Pigs were injected intramuscularly with 4 mg ceftiofur sodium per kg body weight once a day for 3 consecutive days during the suckling period, beginning on day -7 of the experiment, to attempt to remove commensal ETEC if present. Of the 30 candidates, 18 piglets that did not excrete either ETEC K88 or K99, as determined by real-time polymerase chain reaction (PCR) on genomic DNA extracted from feces,<sup>9</sup> were selected at weaning (28 days of age). The animals were transported to a university animal experimental station and allotted arbitrarily to three pens (groups) of six animals each on the day of selection and transportation, corresponding to day 0 of the experiment. Two groups of animals were challenged orally with  $3.0 \times 10^8$  colony-forming units (cfus) of each of ETEC K88 and K99 in a total volume of 6 mL of phosphate buffered saline (pH 7.4). Animals in the remaining group were administered the same volume of vehicle. The ETEC K88 used for the challenge was identified as F4 fimbriae-positive, heat-labile, enterotoxin-positive, hemolysin serogroup O8; ETEC K99 was identified as F5 fimbriae-positive, heat-stable, enterotoxin-positive, hemolysin serogroup O8. The unchallenged group and one challenged group were provided with a basal nursery diet (Control and Chal-Basal groups, respectively) that had been used in previous studies.<sup>9,19,20</sup> The remaining challenged group received the same diet supplemented with  $1.0 \times 10^9$  plaque-forming units of each of ETEC K88- and K99-specific phages per kg (Chal-Phage group). The animals were on the feeding trial for 7 days, beginning on day 0.

Fecal consistency was scored daily beginning on day 1 according to a four-ladder whole-number scale<sup>5</sup> as described previously: 0 = normal feces; 1 = soft feces; 2 = mild diarrhea; 3 = severe diarrhea.<sup>19</sup> Rectal temperature was measured on days 0, 3, and 7 using an electronic thermometer (ThermoScan; Braun GmbH, Kronberg, Germany). Fecal samples were collected on days 1, 3, and 7, also as described previously.<sup>9,19</sup> All animals were euthanized at the end of the 7-day feeding trial. After measuring digesta pH of the stomach, jejunum, and ascending colon using litmus paper (Thomas Scientific, Swedesboro, New Jersey), mucosal tissues from the small intestinal segments and the mesenteric lymph node were collected as described.<sup>18,20</sup> The numbers of ETEC K88 and K99 shed in feces and bound to the tissue were determined by real-time PCR targeting the genes coding for fimbriae K88 and K99, respectively, also as described.<sup>9</sup>

All data were analyzed using the general linear model procedure (SAS 9.2; SAS Institute Inc, Cary, North Carolina), except for fecal shedding and intestinal adhesion of ETEC K88 and K99. The model included treatment only when there was a single observation per animal. In an analysis of repeated measurements, in which the model included treatment, day, and their interaction, the effects of treatment and day, including its interaction with treatment, were tested using animal and day × animal nested within treatment as error terms, respectively. For fecal shedding and intestinal adhesion of ETEC K88 and K99, data from unchallenged animals were excluded from statistical analysis after confirmation of the absence of either pathogen in the feces and intestinal tissues. Frequency of the appearance of pathogen-positive feces and pathogen-positive intestinal tissue were analyzed using the chi-squared test. Means were separated by *t* test;  $P < .05$  was considered statistically significant and  $P < .10$  was considered a tendency.

## Results

The rectal temperature of the Control group did not change during the 7-day experimental period (Table 1). Mean rectal temperature was lower in the Chal-Phage group than in the Chal-Basal group, but did not differ between the Chal-Phage and Control groups. Fecal consistency score increased transiently after day 1 in the challenged groups; mean score was greatest in the Chal-Basal group, followed sequentially

by the Chal-Phage and Control groups. Average daily gain, which was less in the challenged groups than in the Control group, was greater in the Chal-Phage group than in the Chal-Basal group. The digesta pH value measured at necropsy was lower in the Control group than in the Chal-Basal group for the stomach, jejunum, and colon and also in the Chal-Phage group versus the Chal-Basal for the colon, with a tendency to be lower in the Chal-Phage group than in the Chal-Basal for the jejunum ( $P = .07$ ). The ETEC K88 (Figure 1, Panel A) and K99 (Figure 1, Panel B) were detected in feces of the challenged groups, but not the Control group. The mean number of cfus of ETEC K88 transformed to base 10 logarithm (log) per gram feces was greater in the Chal-Basal group than in the Chal-Phage group, but the log number of cfus of ETEC K99 did not differ between the two groups.

The log number of cfus of ETEC K88 bound to the tissue was less in the Chal-Phage group than in the Chal-Basal group for the ileum and cecum, but did not differ between the two groups for the duodenum, jejunum, colon, or mesenteric lymph node (Table 2). Adhesion of ETEC K99, however, did not differ between the two groups in any region of the digestive tract.

## Discussion

Clinical measurements in the present study indicated that the postweaning pigs concurrently challenged with ETEC K88 and K99 developed the intended colibacillosis as manifested by the higher body temperature and fecal consistency score, as well as lower weight gain in the Chal-Basal and Chal-Phage groups versus the Control group, and severity of clinical signs was less in the Chal-Phage group than in the Chal-Basal group. These results, as a whole, were similar to the effects of the ETEC K88 challenge and dietary supplementation of antibiotics or 2500 mg ZnO per kg diet, respectively, in weaning pigs in earlier studies.<sup>9,19</sup>

The greater digesta pH value of the stomach in the Chal-Basal group, compared to the Control group, was consistent with the earlier result of ETEC K88 challenge in weaning pigs,<sup>19</sup> but not with that of the study of Wellock et al,<sup>21</sup> where digesta pH did not change in pigs challenged with ETEC O149 239/03. It thus remains to be determined why different ETEC strains exerted varying effects on gastric acidity. It has been reported that proliferation of beneficial microflora

**Table 1:** Effects of dietary supplementation of enterotoxigenic *Escherichia coli* (ETEC) K88- specific and K99-specific bacteriophages on clinical signs, growth performance, and digesta pH in postweaning pigs challenged with ETEC K88 and K99\*

	Control		Challenged		SEM	P
	Basal (n = 6)	Basal (n = 6)	+Phage (n = 6)			
Rectal temperature (°C)						
Day 0	38.72	38.68 <sup>x</sup>	38.72 <sup>x</sup>			
Day 3	38.72 <sup>a</sup>	39.87 <sup>b,y</sup>	39.05 <sup>c,y</sup>	0.097 <sup>†</sup>		NA
Day 7	38.68	38.90 <sup>x</sup>	38.83 <sup>xy</sup>			
Overall <sup>‡</sup>	38.71 <sup>a</sup>	39.15 <sup>b</sup>	38.87 <sup>a</sup>	0.057		< .01
Fecal consistency score <sup>§</sup>						
Day 1	0.17	0.33 <sup>x</sup>	0.17 <sup>x</sup>			
Day 2	0.17 <sup>a</sup>	1.33 <sup>b,y</sup>	0.50 <sup>a,xy</sup>			
Day 3	0.17 <sup>a</sup>	2.17 <sup>b,z</sup>	1.33 <sup>c,z</sup>			
Day 4	0.33 <sup>a</sup>	2.00 <sup>b,z</sup>	1.00 <sup>c,yz</sup>	0.237 <sup>†</sup>		NA
Day 5	0.17 <sup>a</sup>	1.33 <sup>b,y</sup>	0.67 <sup>a,xy</sup>			
Day 6	0.17 <sup>a</sup>	1.17 <sup>b,y</sup>	0.50 <sup>a,xy</sup>			
Day 7	0.17 <sup>a</sup>	1.00 <sup>b,y</sup>	0.50 <sup>ab,xy</sup>			
Overall <sup>¶</sup>	0.19 <sup>a</sup>	1.33 <sup>b</sup>	0.67 <sup>c</sup>	0.111		< .01
Growth performance						
Initial weight (kg)	11.4	10.1	10.6	0.46		.18
Final weight (kg)	16.4 <sup>a</sup>	11.5 <sup>b</sup>	13.2 <sup>c</sup>	0.52		< .01
ADG (kg)	0.361 <sup>a</sup>	0.098 <sup>b</sup>	0.186 <sup>c</sup>	0.021		< .01
Digesta pH						
Stomach	2.75 <sup>a</sup>	3.65 <sup>b</sup>	3.28 <sup>ab</sup>	0.188		.02
Jejunum	6.67 <sup>a</sup>	7.17 <sup>b</sup>	6.87 <sup>ab</sup>	0.111		.02
Colon	6.70 <sup>a</sup>	7.23 <sup>b</sup>	6.80 <sup>a</sup>	0.100		< .01

\* A total of eighteen 28-day-old postweaning pigs received an oral administration of  $3.0 \times 10^8$  colony-forming units (cfu) of ETEC K88 and of ETEC K89 in 3 mL phosphate buffered saline (PBS) each or 6 mL PBS (Control) on day 0 of the experiment. The animals were fed a nursery diet containing no phage (Basal) or  $1.0 \times 10^9$  plaque-forming units (pfu) of ETEC K88-specific bacteriophages and the same number of pfus of ETEC K99-specific bacteriophages per kg diet (+Phage) for 7 days and were subjected to necropsy, including measurement of digesta pH. Data are means of six animals. Overall average daily feed intakes were 0.406, 0.392, and 0.397 kg per animal for the Control-Basal, Challenged-Basal, and Challenged-Phage groups, respectively.

† Applies to all day × treatment combinations.

‡ Both for the day and day × treatment  $P < .01$  (ANOVA).

§ 0 = normal feces; 1 = soft feces; 2 = mild diarrhea; 3 = severe diarrhea.

¶ For day and day × treatment,  $P < .01$  and  $P = .09$ , respectively (ANOVA).

<sup>a,b,c</sup> Means within a row with no common superscript differ ( $P < .05$ ; t test).

<sup>x,y,z</sup> Means within a column with no common superscript differ ( $P < .05$ ; t test).

SEM = standard error of the mean; ADG = average daily gain; NA = not applicable.

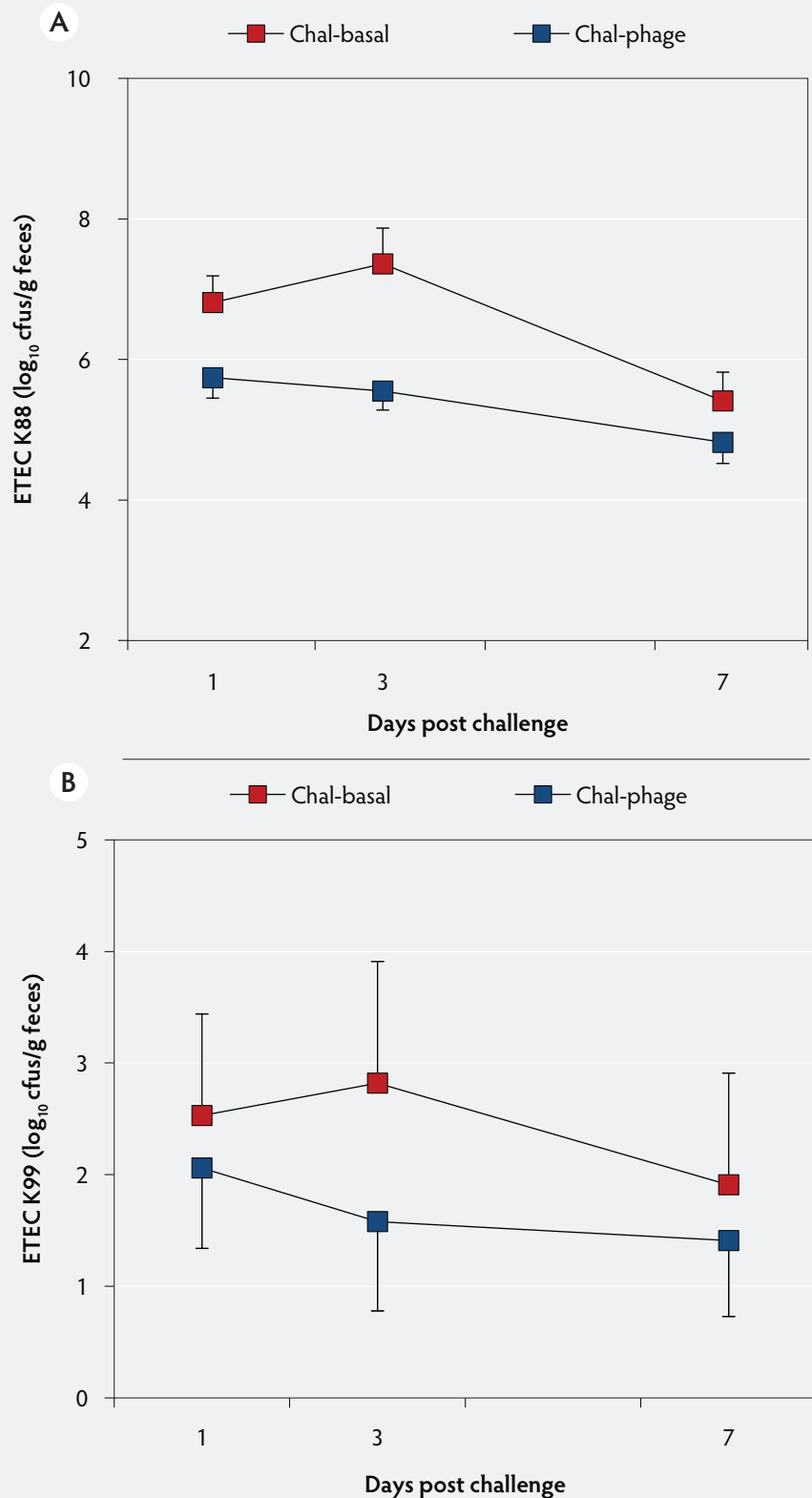
was favored at a lower pH,<sup>22</sup> whereas at higher pH, ETEC colonization was enhanced.<sup>23</sup> It is also well known that ETEC enterotoxins cause electrolyte losses and diarrhea.<sup>2,3,24</sup> Thus, the higher pH of the jejunal digesta, as well as the better fecal consistency score in the Chal-Basal group versus the Control group, which was consistent with the results of Kwon et al<sup>19</sup> and Wellock et al,<sup>21</sup> is likely to have been the result of the

ETEC infection in the Chal-Basal group. Conversely, the lower digesta pH in the colon, as well as the tendency to lower pH in the jejunum for the Chal-Phage group versus the Chal-Basal group, is presumed to have resulted from alleviated ETEC infection as a consequence of the phage therapy.

Fecal shedding and intestinal adhesion of ETEC, which were lower for the K99 strain

than for the K88 strain, were lower in the Chal-Phage group versus the Chal-Basal group for ETEC K88, but not for K99. This may reflect the lower infectivity of the K99 antigen compared with that of K88 in postweaning pigs,<sup>25</sup> although a possibility of confounding effects of ETEC K88 and K99 as well as the two phage strains could not be ruled out under the present experimental conditions.

**Figure 1:** Fecal shedding of *Escherichia coli* (ETEC) K88 (Panel A) and K99 (Panel B) of post-weaned pigs after oral administration of the ETEC K88 and K99 pathogens: effects of in-feed phages. Treatments described in Table 1. Colony forming units (cfus) were transformed to base 10 logarithms. Data are means (with standard error of the means) of six animals. The *P* values for treatment, day, and treatment × day were < .01, < .01, and .26, respectively, for ETEC K88, and .53, .76, and .89, respectively, for ETEC K99 (ANOVA). Chal = challenged.



## Implications

- Phage therapy appears to be effective for treatment of ETEC K88 infection, but not that of K99 infection, in post-weaning pigs.
- More studies on the effects of the ETEC K88-specific phage in piglets infected with ETEC K88 alone are needed.

## Acknowledgements

This work was supported by the College of Veterinary Medicine and Institute of Veterinary Science of Kangwon National University. The authors thank Dr Sang-Hyeon Kang (iNtRON Biotechnology, Inc, Seoul) for providing the ETEC K88-specific and K99-specific bacteriophages used in the present study. Dr Seung-Jae Han and Dr Yeonsu Oh contributed equally to this work.

## Conflict of interest

None reported.

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**Table 2:** Effects of dietary supplementation of enterotoxigenic *Escherichia coli* (ETEC) K88-specific and K99-specific bacteriophages on intestinal adhesion of ETEC in postweaning pigs challenged with ETEC K88 and K99\*

	Basal (n = 6)	+Phage (n = 6)	P
ETEC K88, log <sub>10</sub> cfus/g tissue (no. of ETEC-positive pigs)			
Duodenum	1.57 ± 0.996 (2)	0.74 ± 0.738 (1)	.52
Jejunum	3.03 ± 1.37 (3)	1.42 ± 0.902 (2)	.35
Ileum	7.24 ± 0.460 (6)	5.57 ± 0.263 (6)	.01
Cecum	6.32 ± 0.504 (6)	3.92 ± 0.800 (6)	.03
Colon	3.31 ± 1.510 (3)	1.84 ± 1.175 (2)	.46
Mesenteric lymph node	3.84 ± 1.233 (4)	2.19 ± 0.979 (3)	.32
ETEC K99, log <sub>10</sub> cfus/g tissue (no. of ETEC-positive pigs)			
Duodenum	0 (0)	0 (0)	NA
Jejunum	2.43 ± 1.108 (3)	1.42 ± 0.905 (2)	.50
Ileum	2.59 ± 1.184 (3)	1.52 ± 0.965 (2)	.50
Cecum	1.91 ± 1.238 (2)	0.83 ± 0.83 (1)	.49
Colon	1.79 ± 1.129 (2)	0.93 ± 0.932 (1)	.57
Mesenteric lymph node	2.80 ± 1.277 (3)	2.18 ± 0.984 (3)	.71

\* Treatments described in Table 1. The numbers of cfus of ETEC K88 and K99 were determined by real-time polymerase chain reaction targeting the respective fimbrial genes using genomic DNA extracted from the intestinal tissue as template and were transformed to base 10 logarithms. Data are means ± standard errors of the means of six animals, with the log<sub>10</sub> cfu value for the ETEC-negative animal calculated as 0. The numeral in parenthesis represents the number of the corresponding ETEC-positive animals out of six. The effect of the dietary treatment on the frequency of the corresponding ETEC-positive samples was not significant (chi-squared test) in any of the fecal and tissue samples. Data from Control animals without either ETEC K88 or K99 in fecal or intestinal tissue samples were excluded from this table. Cfus = colony-forming units; NA = not applicable.

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