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In ovo Inoculation of cLF36 on Post-hatch Performance, Intestinal Histo-morphometry and Microflora of Broiler Chickens Challenged with *Clostridium perfringens*

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Abstract

In ovo injection of camel lactoferrin (cLF36) as an antimicrobial peptide was applied in Ross 308 fertile eggs and tested in 320 post-hatched chickens challenged with *Clostridium perfringens* (Cp). In 8 treatments and five replicates of 8 birds each, performance, jejunum morphometry and ileal microbial counts of chickens were assayed. Feed intake and feed conversion ratio of the chickens affected by treatments. Together with the positive control group under the Cp (10^8 cfu/g) challenge and the negative control group under the antibiotic (AB) challenge, the highest villi length was observed. The highest crypt depth was related to the treatment with the Cp challenge and the lowest value was related to the *in ovo* injection of cLF36 group and combined Cp and AB challenges. The number of *Clostridium spp.* in the ileal contents increased in the chickens challenged with Cp ($P < 0.05$). The greatest change was observed in the treatment with injection of cLF36 during the embryonic period and challenge with Cp and the lowest value was related to negative control treatment. In addition, the difference between treatments with cLF36 *in ovo* injection during the embryonic period and challenge with or without Cp challenge was significantly increased. In the groups under the Cp challenge, the population of *E. coli* was numerically increased. Based on the obtained results, cLF36, derived from camel milk, could change some of the indices in performance. It caused morphological changes in the villi of ilium and caused a decrease the microbial counts of *Clostridium spp*., similar to the AB group in the chickens challenged with Cp. Our research attempts to create a new window for *in ovo* administration of cLF36, according to its beneficial effects in the present study, can be introduced as a candidate for growth-promoting antibiotics.

Introduction

Clostridium perfringens (Cp) is a gram-positive, anaerobic, spore-forming bacillus that is found in the environment as well as in the gastrointestinal tract of humans and animals and is one of the most common causes of foodborne diseases, which is an important threat. In fact, Cp is responsible for severe infections such as enterotoxaemia, gangrene dermatitis, and necrotizing enteritis (NE), especially in poultry (Songer, 1996; Van Immerseel *et al*., 2004; Popoff, 2013; Timbermont *et al*., 2011., Caly *et al*., 2015).

Animals are often infected through bacteria or spores in their food, from contaminated beddings, or through proximity to sick animals in the early stages of life. Young animals have an immature immune system and do not have a developed beneficial microflora; therefore, they are at risk. Infected animals with Cp show degraded mucosal cells in the small intestine and severe lesions in the jejunum and ileum. Infection with Cp includes depression, less mobility and diarrhea in the animals are the most obvious symptoms (Wu *et al*., 2018).

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The rapid death of chickens with NE (within 24 hours) often inhibits treatment of the disease. Antibiotics in feed or water were routinely used worldwide as growth promoters and for the prevention of NE caused by Cp in poultry (Caly *et al*., 2015). Currently, antibiotic residues in poultry products and pathogen emergence antibiotic resistance have caused concern to consumers (Daneshmand *et al*., 2019).

With the ban on the use of antibiotics in Europe (Food Additives Regulation 1831/2003/EC), which came into force in January 2006. The importance of research on antibiotic alternatives to prevent NE in the poultry industry seems to be crucial. Researchers recently focused on some predisposing factors such as coccidiosis, and diet modification, along with Cp challenges. They are trying to find out the most applied method regarding faster relief of NE in poultry, especially in fast-growing chicken in commercial strains.

Antimicrobial peptides (AMP) have recently been introduced as potential options as antibiotic growth promoters (Wang *et al*., 2016; Daneshmand *et al*., 2019). In general, AMPs are small biological molecules (<10 KDa) containing 12-50 amino acids that have antimicrobial activity against a wide variety of bacteria, viruses, and fungi (Javadmanesh *et al*., 2021). The beneficial effects of AMPs on growth performance, intestinal morphology, nutrient digestibility, intestinal microflora, and immune system function of farm animals have been previously demonstrated (Choi *et al*., 2013; Yoon *et al*., 2013; Daneshmand *et al*., 2019). Recently, a chimeric peptide was extracted from camel milk, named camel lactoferrin (cLF36, containing 36 amino acids), which has shown antibacterial and anticancer properties in previous *in vitro* studies (Tanhaeian *et al*., 2018a; Tanhaeian *et al*., 2018b).

The effects of AMP on various health characteristics of animal models under normal conditions are available in recently published papers. However, few data are available to confirm the effect of AMP on Cp challenged birds. Accordingly, the aim of our investigation is to evaluate the effect of cLF36 as a candidate for growth-stimulating antibiotics on growth performance, intestinal morphology and microbial population in broiler chickens challenged with Cp.

Materials and methods

The ethics approved all experimental protocols related to the animals in the present study, necessary care and use of animals' committee of Ferdowsi University of Mashhad (Protocol No. 53695.3) in order to minimize pain, abuse of animals, following relevant instructions and regulations were carried out.

Birds, treatments and experimental design. Three hundred and twenty day-old chicks (Ross 308) hatched after *in ovo* injection on the 15th day of incubation and fed the experimental diets (Table 1) were randomly placed in floor pens (1.1 m × 1.3 m) covered with wood shavings. The birds were assigned to 8 treatments with five replications (8 chicks per replication) as follows:

Negative controls including three treatments as T1, without punch and challenge; T2, without punch by challenge 1 ml (10^8 cfu/ml) of Cp and diluent at 14 days post-hatch for 7 days; T3, without punch and use of preventive dose 30 gram/100 liter of oral oxytetracycline 20% antibiotic (AB) at 14 days post-hatch for 5 days ; T4, positive control group with egg punch and challenged with Cp and diluent at 14 days post-hatch for 7 days; T5, sham control group with egg punch and 0.5 ml of 0.1% serum saline injection at 15 days of egg incubation and challenged with Cp and diluent at 14 days post-hatch for 7 days; T6, with punch and 0.5 ml of cLF36 (160 µg/ml) injection at 15 days of egg incubation without Cp challenge; T7; with punch and 0.5 ml of cLF36 (160 µg/ml) injection at 15 days of egg incubation and challenge of Cp and diluent at 14 days post-hatch by gavage; T8, with punch and 0.5 ml of cLF36 (160 µg/ml) injection at 15 days of egg incubation and challenge of Cp (10^8 cfu/ml) +AB and at 14 days post-hatch in drinking water.

All diets were in the mash-form and were prepared according to the starter (1-10 days) and grower (11-24 days) period to meet or exceed the minimum requirements of commercial strain of Ross 308 (Table 1). During the experiment, the birds had free access to food and water. The air temperature was set at 33°C for the first three days and then gradually decreased to 21°C until the 24th day and remained constant until the end of the experiment (day 24). The lighting program consisted of 23 hours of light in the first five days and then gradually changed to 22 hours of light and 2 hours of darkness on the 10th day and remained constant until the end of the experiment.

Camel lactoferrin and challenges

The Antimicrobial peptide used in the present study was a modified form of camel lactoferrin (cLF) consisting of 36 amino acids plus six histidine residues. Preparation of the recombinant plasmid vector was made by transforming the synthetic cLF chimera into DH5α13-15 bacteria (Tahmoorespur *et al*., 2019; Tanhaeian *et al*., 2018a; Tanhaeian *et al.*, 2018c). In the present study, 0.5 ml of saline serum solution containing 160 µg of cLF36/ml was injected into the amniotic cavity of experimental eggs on the 15th day of incubation. The experimental hatched birds were individually challenged by Cp gavage at 14-21 days of age once a day (1×10^8 cfu/ml). In unchallenged groups (not contaminated with Cp), an oral dose of sterile phosphate-buffered saline (PBS) was used as a sham control for seven consecutive

days. Birds were slaughtered at 24 days of age and gut samples were taken and stored at their appropriate media (Olkowski *et al*., 2005).

Table 1. Composition of experimental diets fed to Ross 308 broiler chickens

Ingredients (%)	Starter stages (1-10 days)	Grower stages (11-24 days)
Corn	49.17	52.15
Soybean meal (44%)	42.15	38.7
Soybean oil	4.20	5.19
Di-calcium phosphate	1.56	1.34
Limestone	1.41	1.31
Salt	0.19	0.23
Sodium bicarbonate	0.15	0.09
DL-methionine	0.35	0.29
L-lysine hydrochloride	0.20	0.13
L-threonine	0.12	0.07
Vitamin premix[1]	0.25	0.25
Mineral premix	0.25	0.25
Nutrient analysis (%)		
Metabolizable energy (Kcal/Kg)	3000	3100
Crude protein	23.00	21.50
Calcium	0.96	0.87
Available phosphorus	0.48	0.43
Sodium	0.16	0.16
Methionine	0.65	0.58
Lysine	1.28	1.15
Methionine + Cystine	0.95	0.87

[1] provided vitamins and minerals per kilogram of diet: vitamin A, 9000 IU; cholecalciferol, 2000 IU; Vitamin E, 18 IU; Vitamin K3, 2 mg; Vitamin B12, 0.015 mg; Thiamine, 1.8 mg; Riboflavin, 6.6 mg; Niacin, 10 mg; Folic acid, 0.1 mg; Biotin, 0.15 mg; Pyridoxine, 3 mg; Pantothenic acid, 30 mg; Choline chloride, 0.50 mg; Zinc, 84.7 mg; Manganese, 100 mg; Selenium, 0.2 mg; Iodine, 1 mg; Copper; 10 mg; Iron, 50 mg.

Performance data

On days 14 and 24, body weight (BW), average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) were measured. Mortality, if any, was recorded twice a day, and their data for performance traits were adjusted.

Sample collection and intestinal histo-morphometry

One bird from each experimental unit (4 birds/treatment) was randomly selected on days 14 and 24, euthanized by cervical dislocation. The intestine was carefully separated from the entire viscera, and excess adhering materials were precisely removed. Ileal contents were collected individually in sterile tubes for microbiological analysis. A section (about 5cm) was separated from the tissue of the middle - jejunum for morphological analysis. The jejunum samples were kept in 10% formaldehyde phosphate buffer for 48 hours. Then the samples were trimmed and processed on a tissue processor (Excelsior ™ AS, Thermo Fisher Scientific, Loughborough, UK), fixed and embedded in paraffin (Thermo Fisher Histo Star Embedder, Loughborough, UK) and cut with a microtome (Leica HI1210, Leica Microsystems Ltd., Wetzlar, Germany) to a slice of 3 µm, placed on slides and dehydrated on a hotplate (Leica ASP300S, Leica Microsystems Ltd., Wetzlar, Germany). The Prepared samples were dyed with hematoxylin and eosin and observed under a microscope (Olympus BX41, Olympus Corporation, Tokyo, Japan). Villi width (VW) at the base, middle part, and the distal third of each villus; villus height (VH) from the top of the villus to the villi-crypt junction; crypt depth (CD) from the base of the adjacent villus to the submucosa; VH/CD ratio, and villus surface were measured (10 villi/bird). The average slide measurement per sample was stated as a mean for each bird (Kermanshahi *et al*., 2017; Daneshmand *et al*., 2019). To count the microbial population of *E. coli*, *Clostridium spp*., *Lactobacillus spp*. and *Bifidobacterium spp*. in the collected ileal contents, briefly, the ileal contents of samples were thoroughly mixed, serially diluted from 10^{-1} to 10^{-7} with sterile PBS and homogenized for 3 min and then, dilutions were plated on different agar media. Regarding the enumeration of bacteria, *Lactobacillus spp*. and *Clostridium spp*., dilutions were plated on MRS agar (Difco, Laboratories, Detroit, MI) and SPS agar (Sigma, Germany) and anaerobically cultured at 37°C for 48 hours. Black colonies in the SPS agar medium were recorded as the count of *Clostridium Spp.* Eosin Methylene Blue (EMB) agar (Merck, Darmstadt, Germany) and BSM agar (Sigma-Aldrich, Germany) were used for *E. coli* and *Bifidobacterium spp*., respectively and incubated at 37°C for 24 hours.

Table 2. The effect of experimental treatments on the performance of broiler chickens from 1-24 days of age with or without cLF36 challenge at incubation period

Performance		Average Daily Feed Intake (g)			Average Daily Gain (g)			Feed Conversion Ratio		
Periods (day)		Starter	Challenge	Total	Starter	Challenge	Total	Starter	Challenge	Total
		1-14	14 -24	1- 24	1-14	14 -24	1-24	1-14	14 -24	1- 24
Treatments						Challenge				
NC (no punch) (T1)	- Cp	40.85^{bc}	92.13^{a}	68.32^{b}	30.55	56.72	42.99	1.34	1.63^{abc}	1.59^{ab}
NC (no punch) (T2)	+ Cp	40.81^{bc}	79.84^{b}	60.78^{c}	30.75	73.55	69.42	1.33	1.45^{bc}	1.44^{c}
NC (no punch) (T3)	+ AB	40.64^{bc}	82.58^{b}	62.44^{c}	31.13	55.42	43.27	1.31	1.50^{abc}	1.44^{bc}
PC (with punch) (T4)	+ Cp	41.14^{c}	77.80^{b}	60.54^{c}	31.80	55.98	44.47	1.29	1.40^{c}	1.36^{c}
Shame control, IOI NaCl 0.1% (T5)	+ Cp	41.53^{a}	94.91 ^{a}	71.60^{ab}	31.09	56.72	44.07	1.34	1.67^{ab}	1.63^{b}
IOI cLF36 (T6)	- Cp	41.30^{ab}	91.67^{a}	70.34^{ab}	30.96	52.94	42.99	1.33	1.74^{a}	1.64^{b}
IOI cLF36 (T7)	+ Cp	41.31^{ab}	81.24^{b}	61.9^{c}	29.97	56.54	43.06	1.38	1.45^{bc}	1.44^{bc}
IOI cLF36 (T8)	+Cp + AB	41.00^{abc}	95.83^{a}	72.01^{a}	30.73	57.50	44.15	1.34	1.67^{ab}	1.63^{a}
P values		0.0006	0.0001	0.0001	0.4852	0.9087	0.5912	0.3549	0.0008	0.0001
±*SEM*		0.06	1.17	0.79	0.19	0.75	0.26	0.01	0.03	0.01

[a-c] In each column, means with different superscripts are significantly different (P< 0.05). ±SEM, standard error of means. Negative controls (NC) including three treatments as T1, without punch and challenge; T2, without punch by challenge 1 ml (10^8 cfu/ml) of *Clostridium perfringens*, Cp and diluent at 14 days post-hatch for 7 days; T3, without punch and use of preventive dose 30 gram/100 liter of oral oxytetracycline 20% antibiotic (AB) at 14 days post-hatch for 5 days ; T4, positive control (PC) group with egg punch and challenged with Cp and diluent at 14 days post-hatch for 7 days; T5, sham control group with egg punch and 0.5 ml of 0.1% serum saline injection at 15 days of egg incubation and challenged with Cp and diluent at 14 days post-hatch for 7 days; T6, with punch and 0.5 ml of *in ovo* cLF36 (IOI, 160 µg/ml) injection at 15 days of egg incubation without Cp challenge; T7; with punch and 0.5 ml of cLF36 (160 µg/ml) injection at 15 days of egg incubation and challenge of Cp and diluent at 14 days post-hatch by gavage; T8, with punch and 0.5 ml of cLF36 (160 µg/ml) injection at 15 days of egg incubation and challenge of Cp (10^8 cfu/ml) +AB at 14 days post-hatch in drinking water.

All microbiological assays were performed in triplicate, the mean values were used for statistical analysis and the results were expressed in colony-forming units (Kermanshahi *et al*., 2017; Daneshmand *et al*., 2019).

Statistical analysis

The data were statistically analyzed using the analysis of variance (ANOVA) method and by the general linear model procedure in balanced completely randomized design (GLM) of SAS software (SAS Inst., Inc., Cary, NC). Tukey's test was used to compare the difference between the means treatments and P values <0.05 were considered significant ($P < 0.05$).

Results

Growth performance

The effect of treatments on growth performance attributes after hatching is shown in Table 2. Analysis of the average body weight of hatched chicks at 14 and 24 days showed that *in ovo* injection during embryonic period on the weight of chicks before and after challenge with Cp had no significant effect ($P > 0.05$). The effect of experimental treatments on ADG was not significant in any period ($P > 0.05$). Average daily feed intake in the pre-challenge period among the treatments (1-14 days of age) was significantly different ($P < 0.05$). *In ovo* injection of electrolyte (0.1% saline serum) showed the highest amount of ADFI among the treatments ($P < 0.05$).

The effect of treatments in the post-challenge period (14-24 days of age) on ADFI was significant ($P < 0.05$). The treatment under *in ovo* injection of cLF36 and the combined challenge of antibiotics and Cp (Cp + AB) showed the highest amount of ADFI, and the lowest amount was related to the positive control (punched eggs without any substance injection) and under the Cp challenge. The effect of treatments on the amount of ADFI during the whole experimental period (1-24 days of age) was significant ($P < 0.05$). The highest amount of ADFI was seen in *in ovo* injection of cLF36 and Cp +AB challenge and the lowest ADFI was seen in the positive control.

The effect of the treatments in the post-challenge period (14-24 days) on the FCR was significant ($P < 0.05$). The treatment under cLF36 injection and Cp challenge (positive control) showed the lowest FCR and the highest FCR value was related to the treatment under cLF36 injection and Cp + AB challenge. The effect of experimental treatments on FCR for the whole experimental period (1-24 days) was also significant ($P < 0.05$). The highest value of the FCR was seen in the *in ovo* injection of cLF36 challenged with Cp + AB, and the lowest FCR was seen in the positive control.

Jejunum Histo-morphometry

The effect of *in ovo* injection of cLF36 into the amnion sac on day 15 of embryonic development on the morphology of the jejunum at 24 days of post-hatched chickens is shown in Table 3.

Table 3. The post-hatched effect of *in ovo* injection of cLF36 on jejunal histo-morphometry of the chickens at 24 days of age

Histo-morphometry parameters		Villi length (µm)	Villi Width (µm)	Crypt Depth (µm)	VL/CD (µm)	Villi Surface area mm^2
Treatments		Challenge				
NC (no punch) (T1)	-Cp	1279^{a}	175^{ab}	178^{b}	7.20^{a}	0.702^{a}
NC (no punch) (T2)	+Cp	1121^{ab}	168^{b}	187^{b}	6.50^{ab}	0.592^{ab}
NC (no punch) (T3)	+AB	1240^{a}	178^{ab}	192^{ab}	6.48^{ab}	0.695^{ab}
PC (with punch) (T4)	+Cp	1181^{a}	174^{ab}	186^{b}	6.36^{ab}	0.649^{ab}
Shame control, IOI NaCl 0.1% (T5)	+Cp	1303^{a}	170^{ab}	222^{a}	5.86^{b}	0.695^{ab}
IOI cLF36 (T6)	-Cp	1176^{a}	178^{ab}	178^{b}	6.50^{ab}	0.658^{ab}
IOI cLF36 (T7)	+Cp	1127^{ab}	202^{a}	202^{ab}	5.67^{b}	0.717^{a}
IOI cLF36 (T8)	+Cp +AB	942^{b}	173^{ab}	170^{b}	5.59^{b}	0.509^{b}
P values		0.0001	0.0571	0.0011	0.0013	0.0195
±SEM		23.06	2.85	3.61	0.12	0.02

[a-b.] In each column, means with different superscripts are significantly different (P< 0.05). ±SEM, standard error of means. Negative controls (NC) including three treatments as T1, without punch and challenge; T2, without punch by challenge 1 ml (10^8 cfu/ml) of *Clostridium perfringens*, Cp and diluent at 14 days post-hatch for 7 days; T3, without punch and use of preventive dose 30 gram/100 liter of oral oxytetracycline 20% antibiotic (AB) at 14 days post-hatch for 5 days ; T4, positive control (PC) group with egg punch and challenged with Cp and diluent at 14 days post-hatch for 7 days; T5, sham control group with egg punch and 0.5 ml of 0.1% serum saline injection at 15 days of egg incubation and challenged with Cp and diluent at 14 days post-hatch for 7 days; T6, with punch and 0.5 ml of *in ovo* cLF36 (IOI, 160 µg/ml) injection at 15 days of egg incubation without Cp challenge; T7; with punch and 0.5 ml of cLF36 (160 µg/ml) injection at 15 days of egg incubation and challenge of Cp and diluent at 14 days post-hatch by gavage; T8, with punch and 0.5 ml of cLF36 (160 µg/ml) injection at 15 days of egg incubation and challenge of Cp (10^8 cfu/ml) +AB at 14 days post-hatch in drinking water.

The evaluation of the morphological parameters of the jejunum in broiler chickens at 24 days of age showed that cLF36 feeding can affect the histological indices of the jejunum ($P < 0.05$). No significant difference between the negative control group and the cLF36 *in ovo* feeding group in the embryonic period without Cp challenge was observed, and together with the positive control group under Cp challenge and the negative control group with AB challenge and saline injection, chickens had the highest villi length. The highest crypt depth was in the treatment with saline serum injection and Cp challenge (T5), and the lowest value was related to *in ovo* injection cLF36 group with Cp + AB challenge. The maximum value of villi width (202 µm) corresponded to the treatment group with cLF36 *in ovo* injection and Cp challenge groups (T7) and the minimum value (168 µm) was related to the negative control treatment with Cp challenge (T2), ($P < 0.05$). The highest villi surface area was concerned to the negative control group without Cp challenge (T1) and cLF36 *in ovo* injection group with Cp challenge (T7) and the lowest villi surface area was related to *in ovo* injection of cLF36 treatment under both Cp + AB challenges ($P < 0.05$).

Bacterial population

The effect of experimental treatments on the ileal bacterial population is shown in Table 4. The population of harmful bacteria (*Clostridium spp.*) increased in chickens challenged with Cp ($P < 0.05$), which can indicate the success of the challenge, and the greatest change was observed in the T7 group with cLF36 *in ovo* injection during the embryonic period and post-challenge with Cp ($10^{2.35}$ cfu/g) and the lowest value was seen in NC treatment. The colonization of beneficial bacteria (*Lactobacillus spp*. and *Bifidobacterium spp.*) showed an increase in the challenged groups, but the observed difference was not significant.

Despite the challenge with Cp and antibiotics, no significant changes were observed in the population of *Lactobacillus spp*. and *Bifidobacterium spp*., and the largest microbial population was observed in the treatment with cLF36 injection during the embryonic period and challenge with Cp and antibiotics (T8). In the treatments under Cp challenge, the *E. coli* population also increased, although the changes in the microbial population between the treatments were not significant. The highest *E. coli* value was related to the treatment with cLF36 *in ovo* injection during the embryonic period and the challenge with Cp and antibiotics (T8).

Table 4. The post-hatched effect of *in ovo* injection of cLF36 on the ileal microbial population of broiler chickens (log_{10} cfu/g) at 24 days of age

Bacterial population		*Clostridium spp.*	*Bifidobacterium spp.*	*Lactobacillus spp.*	*E. coli*
Treatments			Challenge		
NC (no punch) (T1)	-Cp	1.43^{c}	6.12	7.87	4.65
NC (no punch) (T2)	+Cp	1.88^{bc}	5.91	7.38	4.94
NC (no punch) (T3)	+AB	1.80^{bc}	6.25	7.61	4.75
PC (with punch) (T4)	+Cp	1.90^{ab}	6.50	7.55	4.83
Shame control, IOI NaCl 0.1% (T5)	+Cp	1.75^{bc}	6.23	7.64	4.77
IOI cLF36 (T6)	-Cp	1.85^{bc}	6.24	7.08	4.77
IOI cLF36 (T7)	+Cp	2.35^{a}	6.51	7.47	4.75
IOI cLF36 (T8)	+Cp +AB	2.13^{ab}	6.75	8.02	5.36
P values		0.0001	0.7869	0.7081	0.7328
±SEM		0.06	0.12	0.12	0.10

[a-c.] In each column, means with different superscripts are significantly different (P< 0.05). ±SEM, standard error of means. Negative controls (NC) including three treatments as T1, without punch and challenge; T2, without punch by challenge 1 ml (10^8 cfu/ml) of *Clostridium perfringens*, Cp and diluent at 14 days post-hatch for 7 days; T3, without punch and use of preventive dose 30 gram/100 liter of oral oxytetracycline 20% antibiotic (AB) at 14 days post-hatch for 5 days ; T4, positive control (PC) group with egg punch and challenged with Cp and diluent at 14 days post-hatch for 7 days; T5, sham control group with egg punch and 0.5 ml of 0.1% serum saline injection at 15 days of egg incubation and challenged with Cp and diluent at 14 days post-hatch for 7 days; T6, with punch and 0.5 ml of *in ovo* cLF36 (IOI, 160 µg/ml) injection at 15 days of egg incubation without Cp challenge; T7; with punch and 0.5 ml of cLF36 (160 µg/ml) injection at 15 days of egg incubation and challenge of Cp and diluent at 14 days post-hatch by gavage; T8, with punch and 0.5 ml of cLF36 (160 µg/ml) injection at 15 days of egg incubation and challenge of Cp (10^8 cfu/ml) +AB at 14 days post-hatch in drinking water.

Discussion

The aggregation of antibiotic resistance prompts scientists to investigate for antibiotic substitutes with constructive effects on growth performance and health parameters while excessive resistance in the microbiota, such as the properties observed in AMPs.

According to the results of previous studies (Khodambashi *et al*., 2017; Matsuda *et al*., 2010), in the present study, significant change was not observed in the performance and growth of chickens under Cp challenge, while diet supplementation with AMP controlled the negative effects of Cp and FCR similar to the birds fed with antibiotics. The results of

previous research, that have reported the beneficial effect of AMP on the growth performance of broiler chickens under normal conditions (Bao *et al*., 2009; Choi *et al*., 2013) and under stress (Hu *et al*., 2017) are not in agreement with the present study.

The morphological characteristics of villi in the small intestine are known as an indicator of intestinal health (Choi *et al*., 2013; Daneshmand *et al*., 2019). In addition, the intestinal lumen is the main site of nutrient absorption, which directly depends on the morphology of the villi and their surface (Lai and Gallo, 2009; Daneshmand *et al*., 2019). In our investigation, morphological analysis showed that the *in ovo* injection of AMP and oral administration of antibiotics in birds with Cp challenge had the same effect as the negative control group without any challenge for VH and VSA traits, which is consistent with the previous results (Daneshmand *et al*., 2020) under *E.coli* challenge that had shorter VH and lower VSA compared to the control group.

The results of histo-morphometry were different from the research (Daneshmand *et al*., 2019) in which the oral use of cLF36 and Cp challenge showed no significant effect of AMP of the intestinal villi in the jejunum and ileum of broiler chickens. According to the results of previous reports (Bao *et al*., 2009; Liu *et al*., 2008; Reicher *et al*., 2021), completing the diet with AMPs extracted from pig and rabbit *Sacculus rotundus*, practicing cLF36 also renovated the jejunum characteristics of broiler chickens. Generally, increasing the length of VH leads to greater VSA, which increases nutrient absorption from the intestinal lumen (Caspary, 1992) and thus increases growth performance in chickens. In the present study, despite changes in morphological characteristics of the intestine in treatment groups, supplementation of birds with cLF36 and Cp challenge along with AB did not lead to significant improvement in the performance of the chickens.

In the newest study, stimulation of the cellular basis of small intestine maturation by in ovo feeding indicated that 0.4% NaCl solution increased the total number of cells in the embryonic day of 19, 48 hours after in ovo feeding by 19% and the number of multipotent cells increased by 38%. NaCl stimulates the maturation of the small intestine and causes more expansion of epithelial villi in the first week after hatching ($P < 0.05$), which clarifies the relationship between stimulation of primary nutrition and cell maturation in the small intestine epithelium on the day of hatch, 3 and 7 (Reicher *et al*., 2022).

The beneficial effects of *in ovo* feeding-NaCl on small intestine epithelial maturation may be attributed to the increased activity of nutrient transporters by increasing the levels of Na^+ and Cl^- ions in the intestinal lumen following the consumption of NaCl-enriched amniotic fluid. *In ovo* feeding of NaCl induces small intestine epithelial proliferation through increased amniotic nutrient absorption (Kiela PR and Ghishan FK., 2016; Reicher *et al*., 2022). These claims may confirm the increase in villus length and crypt depth in the positive control group with the injection of 0.1% saline solution.

The intestinal microbiome can have a significant effect on host intestinal health through various mechanisms, including nutrient absorption, villi morphology, intestinal pH, mucosal immunity, and gene expression of transporters (Apajalahti *et al*., 2004; Castanys *et al*., 2016; Daneshmand *et al*., 2019). Antimicrobial peptide (cLF36) decreased the microbial population of the Clostridium family in chickens challenged with Cp, similar to that of the antibiotic effect, and no difference was observed in the bacterial population of the *Lactobacillus spp*., *Bifidobacterium spp*., and *E. coli* families among the experimental treatments, which is agreement with those of previous studies (Ohh *et al*., 2010; Tang *et al*., 2019).

Tetracycline may exert its antibacterial activity on the bacterial ribosomal subunits S30 and S50, which leads to the inhibition of protein synthesis (Proctor and Phillips, 2019). It reduces the population of bacteria and microbial injury in the intestine because this antibiotic has a broad spectrum of antibacterial activities on gram-negative and positive aerobic and anaerobic bacteria, including Cp and *E. coli*. The mechanism by which AMPs can affect the microbiota in the intestinal tract might be due to the antimicrobial function of peptides concerned with different surface charges of peptides and pathogens.

Antimicrobial peptides have a net positive charge that helps them to electrostatically bind to negatively charged bacterial membranes and disrupt the membranes through contact disruption and/or enzymatic digestion or crossing lipid bilayers without causing damage. Destruction of membranes may interfere with intracellular functions, such as inhibiting enzyme activity or protein and nucleic acid synthesis (Tanhaeian *et al*., 2018a; Tanhaeian *et al*., 2018c; Daneshmand *et al*., 2019).

In agreement with a previous study (Ocana *et al*., 1999), recent results indicated that cLF36 can selectively inhibit bacterial growth in the gut, similar to antibiotics, which may represent a competitive advantage of cLF36 replacement over antibiotics. Antimicrobial peptide cLF36, derived from camel milk, could not improve growth performance indicators, but in Cp-challenged chickens, similar to the antibiotic, it reduced the microbial population of Clostridium and changed the intestinal morphology.

Under the conditions of this study, it was concluded that cLF36 might be introduced as an alternative to growth-promoting antibiotics. More research is needed to clarify whether or not cLF36 might alleviate the negative effects of stressful

conditions when chickens are facing with *in ovo* injection, gavage, and incubation conditions.

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References

Apajalahti J, Kettunen A & Graham H. 2004 Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. World's Poultry Science, 60: 223–232. DOI: 10.1079/WPS200415

Bao H, She R, Liu T, Zhang Y, Peng KS, Luo D, Yue Z, Ding Y, Hu Y, Liu W& Zhai L. 2009. Effects of pig antibacterial peptides on growth performance and intestine mucosal immune of broiler chickens. Poultry Science, 88: 291–297. DOI:10.3382/ps.2008-00330

Caly DL, D'Inca Romain, Auclair E & Drider D. 2015. Alternatives to antibiotics to prevent necrotic enteritis in broiler chickens: a microbiologist's perspective. Frontiers in Microbiology, 6: 1-12. DOI: 10.3389/fmicb.2015.01336

Castanys-Muñoz E, Martin MJ & Vazquez E. 2016. Building a beneficial microbiome from birth. Advances in Nutrition, 7: 323–30. DOI:10.3945/an.115.010694

Caspary WF. 1992. Physiology and pathophysiology of intestinal absorption. American Journal of Clinical Nutrition, 55: 299–308. DOI:10.1093/ajcn/55.1.299s

Choi SC, Ingale SL, Kim JS, Park YK, Kwon IK & Chae BJ. 2013. Effects of dietary supplementation with an antimicrobial peptide-P5 on growth performance, nutrient retention, excreta and intestinal microflora and intestinal morphology of broilers. Animal Feed Science and Technology, 185(1-2): 78–84. DOI: 10.1016/j.anifeedsci.2013.07.005

Daneshmand A, Kermanshahi H, Sekhavati MH, Javadmanesh A & Ahmadian M. 2019. Antimicrobial peptide, cLF36, affects performance and intestinal morphology, microflora, junctional proteins, and immune cells in broilers challenged with E. coli. Scientific Reports, 9 (1) DOI: 10.1038/s41598-019-50511-7

Daneshmand A, Kermanshahi H, Sekhavati MH, Javadmanesh A, Ahmadian M, Alizadeh M & Aldavoodi A. 2020. Effects of cLFchimera, a recombinant antimicrobial peptide, on intestinal morphology, microbiota, and gene expression of immune cells and tight junctions in broiler chickens challenged with C. perfringens. Scientific Reports, 10: 17704. DOI: 10.1038/s41598-020-74754-x

Hu F, Gao X, She R, Chen J, Mao J, Xiao P & Shi R. 2017. Effects of antimicrobial peptides on growth performance and small intestinal function in broilers under chronic heat stress. Poultry Science, 96: 798–806. DOI: 10.3382/ps/pew379

Javadmanesh A, Mohammadi E, Mousavi Z, Azghandi M & Tanhaeian A .2021. Antibacterial effects assessment on some livestock pathogens, thermal stability and proposing a probable reason for different levels of activity of thanatin. Scientific Reports, 11(1): 10890. DOI: 10.1038/s41598-021-90313-4.

Kermanshahi H, Heravi RM, Attar A, Abbasi Pour AR, Bayat E, Hossein Zadeh M, Daneshmand A & Ibrahim SA. 2017. Effects of acidified yeast and whey powder on performance, organ weights, intestinal microflora, and gut morphology of male broilers. Brazilian Journal of Poultry Science, 19: 309 –316. DOI: 10.1590/1806-9061-2016-0351

Khodambashi Emami N, Daneshmand A, Naeini SZ, Graystone EN & Broom LJ. 2017. Effects of commercial organic acid blends on male broilers challenged with E. coli K88: performance, microbiology, intestinal morphology, and immune response. Poultry Science, 96, 3254–3263. DOI: 10.3382/ps/pex106

Kiela. Powel R and Ghishan. Fayez K.2016. Physiology of intestinal absorption and secretion. Best practice & research Clinical gastroenterology, 30(2): 145–159. DOI:10.1016/j.bpg.2016.02.007

Lai Y & Gallo LR. 2009. AMPed up immunity: How antimicrobial peptides have multiple roles in immune defense. Trends in Immunology, 30(3): 131–141. DOI:10.1016/j.it.2008.12.003

Liu T, She R, Wang K, Bao H, Zhang Y, Luo D, Hu Y, Ding Y, Wang D & Peng K. 2008. Effects of rabbit Sacculus rotundus antimicrobial peptides on the intestinal mucosal immunity in chickens. Poultry Science, 87: 250–254. DOI: 10.3382/ps.2007-00353

Matsuda K, Chaudhari A & Lee HJ. 2010. Avian colibacillosis caused by an intestinal pathogenic Escherichia coli isolate from calf diarrhea. Research in Veterinary Science, 89: 150–152. DOI: 10.1016/j.rvsc.2010.02.008

Ocana VS, De Ruiz Holgado AAP & Nader-Macias ME. 1999. Growth inhibition of Staphylococcus aureus by H2O2-producing Lactobacillus paracasei subsp. paracasei isolated from the human vagina. FEMS Immunology and Medical Microbiology, 23: 87–92. DOI: 10.1007/pl00006802

Ohh SH, Shinde PL, Choi JY, Jin Z , Hahn TW , Lim HT , Kim GY, Park YK , Hahm KS & Chae BJ. 2010. Effects of potato (Solanum tuberosum L. cv. golden valley) protein on performance,

nutrient metabolizability, and cecal microflora in broilers. European Poultry Science (EPS), 74: 30–35. DOI:10.3382/ps.2008-00491

Olkowski AA, Wojnarowicz C, Chirino-Trejo M & Drew MD. 2005. Responses of broiler chickens orally challenged with Clostridium perfringens isolated from field cases of necrotic enteritis. Research in Veterinary Science, 81: 99–108. DOI: 10.1016/j.rvsc.2005.10.006

Popoff MR. 2013. Clostridium, in Sécurité Sanitaire des Aliments: Epidémiologie et lutte Contre les Contaminants Zoonotiques, eds D. Drider and G. Salvat (Paris: Economica), 165–189.

Proctor A & Phillips GJ. 2019. Differential effects of bacitracin methylene disalicylate (BMD) on the distal colon and cecal microbiota of young broiler chickens. Frontiers in Veterinary Science, 6: 114. DOI: 10.3389/fvets.2019.00114.

Reicher N, Melkman-Zehavi T, Dayan JA. Wong E & Uni Z. 2022. Nutritional Stimulation by In-ovo Feeding Modulates Cellular Proliferation and Differentiation in the Small Intestinal Epithelium of Chicks. Animal Nutrition, 8: 91-101. DOI: 10.1016/j.aninu.2021.06.010

Songer JG. 1996. Clostridial enteric diseases of domestic animals. Clinical Microbiology Reviews, 9: 216–234. DOI: 10.1128/cmr.9.2.216

Tahmoorespur M, Azghandi M, Javadmanesh A, Meshkat Z & Sekhavati MH. 2019. A Novel chimeric anti-HCV peptide derived from camel lactoferrin and molecular level insight on its interaction with E2. International Journal of Peptide Research and Therapeutics, 3: 1593-1605. DOI: 10.1007/s10989-019-09972-7

Tang J, Ghazali FM, AbdulAziz S, Nishibuchi M & Radu S. 2009. Comparison of thermophilic Campylobacter spp. occurrence in two types of retail chicken samples. International Food Research Journal, 16: 277–288.

Tanhaeian A, Shahriari Ahmadi F, Sekhavati MH & Mamarabadi M. 2018a. Expression and purification of the main component contained in camel milk and its antimicrobial activities against bacterial plant pathogens. Probiotics and Antimicrobial Proteins, 10(4): 787-793. DOI: 10.1007/s12602-018-9416-9

Tanhaeian A, Azghandi M, Razmyar J, Mohammadi E & Sekhavati MH. 2018b. Recombinant production of a chimeric antimicrobial peptide in E. coli and assessment of its activity against some avian clinically isolated pathogens. Microbial Pathogenesis, 122: 73–78. DOI:10.1016/j.micpath.2018.06.012

Tanhaeian A, Jaafari MR, Shahriari Ahmadi F, Vakili-Ghartavol R & Sekhavati MH. 2018c. Secretory expression of a chimeric peptide in Lactococcus lactis: assessment of its cytotoxic activity and a deep view on its interaction with cell-surface glycosaminoglycans by molecular modeling. Probiotics and Antimicrobial Proteins. 11(3): 1034-1041. DOI: 10.1007/s12602-018-9496-6

Timbermont L, Haesebrouck F, Ducatelle R & Van Immerseel F. 2011. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathology. 40: 341–347. DOI: 10.1080/03079457.2011.590967

Van Immerseel F, De Buck J, Pasmans F, Huyghebaert G, Haesebrouck F & Ducatelle R. 2004. Clostridium perfringens in poultry: an emerging threat for animal and public health. Avian Pathology, 33: 537–549. DOI: 10.1080/03079450400013162

Wang S, Xiangfang Z, Qing Y & Shiyan Q. 2016. Antimicrobial peptides as potential alternatives to antibiotics in food animal industry. International Journal of Molecular Sciences, 17: 603. DOI: 10.3390/ijms17050603

Wu Y, Shao Y, Song B, Zhen W, Wang Z, Guo Y, Shahid MS & Nie W. 2018. Effects of Bacillus coagulans supplementation on the growth performance and gut health of broiler chickens with Clostridium perfringens induced necrotic enteritis. Poultry Science, 97: 2654–2666. DOI: 10.3382/ps/pey119

Yoon JH, Ingale SL , Kim JS , Kim KH, Lohakare J, Kyung Park Y, Cheol Park J, Kwon IK & Chae BJ. 2013. Effects of dietary supplementation with antimicrobial peptide-P5 on growth performance, apparent total tract digestibility, faecal and intestinal microflora and intestinal morphology of weanling pigs. Journal of the Science of Food Agriculture, 93: 587–592. DOI: 10.1002/jsfa.5840