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The impact of exogenous dietary nucleotides in ameliorating *Clostridium perfringens* infection and improving intestinal barriers gene expression in broiler chicken

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ABSTRACT

The current study aimed to evaluate the efficiency of dietary nucleotides-supplementation on broiler chickens to alleviate the intestinal *Clostridium perfringens* (*C. perfringens*) levels and its adverse effect on gut and growth performance parameters. In this study, a total of 270 one-day-old mixed broiler chicks (Cobb 500) were randomly divided into six treatment groups with three replicates of 15 chicks/ replicate. Treatment 1 (CX), a negative control group was fed corn-soybean basal diet without added nucleotides. Treatment 2 (CN 0.05) and treatment 3 (CN 0.1), consisted of chicks were fed the basal diet with the addition of nucleotides on top at two levels (0.05 and 0.1%) respectively. Treatment 4 (PX), treatment 5 (PN 0.05), and treatment 6 (PN 0.1) consisted of chicks that were challenged with *C. perfringens* inoculum (~4×10^8 CFU/ml) on day 14, 15, 16 and 17of the experiment and were fed diets similar to treatments 1, 2, and 3 respectively. The trial continued for 35 days. At the end of the experiment, the intestinal *C. perfringens* counts, microscopic lesion scores, intestinal histomorphology, intestinal barriers (occludin and mucin mRNA expression) and growth parameters were determined. The results showed that the pathogen challenge significantly ($P^{<}0.05$) increased both *C. perfringens* levels and intestinal lesion scores. Which adversely affects intestinal barriers and intestinal histomorphology resulting in a significant decrease ($P^{<}0.05$) in body weight gain (BWG) with an increase in feed conversion ratio (FCR). Whereas, nucleotides-supplementation, at 0.1%, significantly decreased both *C. perfringens* levels and intestinal lesion scores, and significantly improved intestinal barriers and intestinal histomorphology which consequently resulted in improved growth performance parameters to be nearly the same as that of the control un-supplemented group. In conclusion, nucleotides markedly ameliorated the negative effects of *C. perfringens* challenge by improving the intestinal barrier function and intestinal histomorphology which positively reflected on the growth performance of challenged birds.

1. Introduction

Undoubtedly, gut health has been considered as a crucial factor to achieve a high and cost-effective growth rate, particularly with the massive use of an intensive broiler production system (high stocking density). Gastrointestinal tract (GIT), particularly the small intestine, is the key organ that governs the basic metabolism through regulation of nutrient absorption and metabolism. Several factors could negatively affect intestinal health status such as infectious agents, environmental and management conditions, which consequently impairs nutrient uptake and absorption, and alters bird productive performance parameters (Yegani & Korver, 2008).

The bacterial infection is the most critical factor that impairs both poultry immune and productive status. So, antibiotic growth promoters (AGPs) were added to the poultry diet in sub-therapeutic doses to improve intestinal health and reduce bacterial infections. However, there is an increase in public concerns with regards to drug residue in poultry products and the emergence of antibiotic – bacterial resistance. So, the inclusion of AGPs in the animal diet has been banned in many countries. On the other hand, as a result of using an AGP-free diet, the birds became more susceptible to enteric diseases, particularly necrotic enteritis (NE), which is caused by *C. perfringens* type-A (McDevitt, Brooker, Acamovic & Sparks, 2006). NE is considered as the most cost-effective disease that confronts the broiler industry (Young & Craig, 2001). NE results in a great economic loss in terms of reducing bird growth performance parameters and increase mortality percentage (Beltran-Alcrudo, Cardona, McLellan, Reimers & Charlton, 2008), which consequently increase demands to find alternatives to antibiotics

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(Swiatkiewicz, Arczewska-Wlosek & Jozefiak, 2014).

So that, the nutritionists are trying to develop several natural alternatives to AGPs to prevent performance losses in poultry production. The nutritional strategy argues intestinal health directly or indirectly. One of these natural alternatives is supplementing the young animal diet with bioactive substances such as nucleotides (Sauer, Bauer & Mosenthin, 2009).

Nucleotides are the building block of protein and nucleic acids of low relative molecular mass and regarded as "semi-essential nutrients" (Carver, 2007; Yu, 1998). They are needed for the multiplication of all living cells (Cosgrove, 1998) and physiological activities of animals (Superchi et al., 2012). Also, they are crucial for the body as a cellular energy source (ATP) (Carver & Walker, 1995). Besides, nucleotides act as a fundamental component in carbohydrate, protein, fat and nucleic acid metabolism (Carver & Walker, 1995; Cosgrove, 1998). Therefore, they play a critical role in Protein synthesis (Yamauchi et al., 1998), Cell mitosis (Carver & Walker, 1995), Lipid metabolism (Gil, Corral, Martinez & Molina, 1986), hematopoiesis (Yamamoto, Wang, Adjei & Ameho, 1997), immunity (Hung, 2015) and gut health (Martinez-Puig et al., 2007; Sauer et al., 2012a).

The nucleotides are naturally present in feed ingredients of the diet with a high amount (such as animal protein-soluble, fish meal and fish by-product, legumes, yeast extracts) (Deveresse, 2000; Ingledew, 1999). Whereas, they exist in low levels in Soybean meal and corn, oil, oilseeds (Deveresse, 2000).

In the past, nucleotides were not believed to be an essential nutrient in animals and humans' diets. It was thought that animals were able to produce sufficient nucleotides that meet their physiological needs via a de-novo synthesis or a salvage pathway (Hoffmann, 2007).

However, LeLeiko, Bronstein, Baliga and Munro (1983) stated that de-novo synthesis could not provide enough nucleotides under certain circumstances, as in the case of rapid enterocyte turnover induced by rapid growth, reproduction and environmental stressors. Therefore, nucleotides are often added to animals' diets in the form of pure substance or yeast extracts (Alizadeh et al., 2016; Sauer et al., 2012a).

This study was carried out to evaluate the impact of exogenous dietary supplementation of nucleotides (0.05 and 0.1% of diet) on broiler performance, gut health parameters (Histopathology, total colony count of *C. perfringens* type-A and gene expression for mucin and occludin genes).

2. Materials and methods

All procedures and protocols follow the rules of the Cairo University Institute of Animal Care and Use Committee (CU- IACUC), Veterinary Medical and Agricultural Sciences Sector under the number of CU/II/F/29/18.

2.1. Nucleotide characteristics

Nucleoforce Poultry® (Bioibérica, S.A., Spain) is a balanced concentrate of free nucleotides and active precursors obtained from dried yeasts (*saccharomyces cerevisiae*). Nutrient compositions of Nucleoforce poultry® (NP) are shown in Table 1a.

Table 1a
Nutrient compositions of Nucleoforce poultry (NP) (Bioibérica, S.A., Spain).

Item	Chemical analysis (as fed basis)
Crude protein	20.34%
Protein nitrogen	3.25%
Non-protein nitrogen (from nucleotides)	12.09%
Crude ash	3.38%
Crude fiber	0.10%

2.2. Experimental design

The trial was conducted at Animal Health Research Institute – Egypt – Doki, Reference Laboratory for Veterinary Quality Control on Poultry Production.

A total of 270 one-day-old mixed broiler chicks (Cobb 500) were obtained from Pyramids poultry Company, Egypt. Chicks with mean body weight (BW) of 40.23 ± 0.09 g randomly divided into six treatment groups with three replicates of 15 chicks/ replicate. Birds were housed in an open house system on wood shavings with a fixed lighting program (23 h on — 1 h off) during the entire experimental period (5 weeks). Clean drinking water was regularly available for birds. Standard hygienic conditions and prophylactic vaccination program against viral diseases were applied for all groups.

A 3 × 2 factorial arrangement of treatments was applied in this trial. Three dietary treatments consisted of treatment 1 (CX), a negative control group which was fed the basal diet with no NP added. Two positive control groups, treatment 2 (CN 0.05) and treatment 3 (CN 0.1), were fed the basal diet with the addition of exogenous dietary nucleotides (NP) on top, at two levels (0.05 and 0.1%) of the diet, respectively. The other three treatments, Treatment 4 (PX), treatment 5 (PN 0.05) and treatment 6 (PN 0.1), consisted of chicks that were challenged with ~4 × 108 CFU/ml/bird of *C. Perfringens* type-A in phosphate-buffered saline (PBS) for four successive days (day 14, 15, 16 and 17) during the experiment (Timbermont et al., 2009). Diets fed to chicks in (PX, PN 0.05, and PN 0.1) were the same as diets fed to chicks in (CX, CN 0.05, and CN 0.1) treatments, respectively. Experimental diets were formulated to fulfill the recommendations of Cobb 500 (Cobb, 2018) breed nutrition specifications and were offered to the birds for starter (0–14 days), grower (15–28 days) and finisher (29–35 days) periods. Calculation and chemical analysis of various diets were performed according to AOAC International (2000). Diet composition and chemical analysis are shown in Table 1b.

2.3. Challenge model and preparation of C. perfringens inoculum

The challenge model applied in this trial was a coccidia-free model destined as a modification of the models described by McReynolds et al. (2004). On day 14 of the trial, the experimental birds were vaccinated against infectious bursal disease (IBD) with the 228-E vaccine to boost the immune-suppressive impact. That increased the incidence of necrotic enteritis (NE) (McReynolds et al., 2004).

The inoculum of *C. perferingens* type-A was prepared, according to Du et al. (2016), from an isolated field strain for cases of chicken suffered from NE and did not carry the NetB gene as determined by polymerase chain reaction (PCR). The organism was cultured in anaerobic condition on tryptose-sulphite-cycloserine for 18 h at 37 °C. Then, inoculated aseptically into cooked meat media and incubated anaerobically overnight at 37 °C.

On day 14, 15, 16, and 17 of the experiment, chicks of groups (PX, PN 0.05, and PN 0.1) were challenged with a fresh preparation of *C. perfringens* type-A (Timbermont et al., 2009). Birds of challenged groups were individually infected by utilizing a crop gavage, a round-tipped animal feeding needle (15-gage, 78 mm; Solomon Scientific, Plymouth Meeting, PA) attached to a repeating syringe (Popper & Sons Inc., New Hyde Park, NY), to deliver 3 ml of the inoculum into the crop of every chick according to Thanissery et al. (2010). The Concentration of *C. perfringens* within the inoculum for every day was 4×10^8 CFU/ml/bird with freshly prepared sterile phosphate-buffered saline (PBS). At the same time, birds of unchallenged groups (CX, CN 0.05, and CN 0.1) obtained a similar volume of meat medium.

2.4. Sampling for intestinal contents, tissue samples, and lesion score

At the end of the experiment (day 35), three chicks from each replicate were slaughtered and sacrificed. The small intestine was

Table 1b
Diet composition and chemical analysis.

Item	Starter 0 - 14 d	Grower 15–28 d	Finisher 29–35 d
Ingredient (g/100 g)			
Yellow corn	57.59	61.69	63.80
SBM 46%	30.10	27.80	25.10
Corn Gluten meal	6.00	3.20	3.00
Meth.	0.30	0.25	0.22
Lys.	0.45	0.30	0.15
Thr.	0.06	0.06	0.03
Oil	1.20	2.80	3.90
MCP	1.70	1.50	1.40
*Broiler premix	0.30	0.30	0.30
Choline chloride	0.05	0.05	0.05
Lime stone	1.80	1.60	1.55
Sod. Chloride	0.30	0.30	0.35
Sod. Bicarbonate	0.15	0.15	0.15
Total	100	100	100
Calculated analysis (%)			
ME, kcal/kg	3008	3086	3167
CP	22.00	19.80	18.60
EE	2.60	2.71	2.78
CF	3.03	2.95	2.81
Lysine	1.32	1.21	1.09
Methionine	0.50	0.48	0.45
Ca.	1.00	0.95	0.88
AP	0.45	0.42	0.40
Chemical analysis (%) (as fed basis)			
CP	22.03	19.92	18.74
CF	3.06	3.01	2.97
Ca	1.02	0.97	0.90
Total P	0.47	0.43	0.41

*__broiler premix:__ vitamin A 16.000 IU, vitamin D3 1.600 IU, vitamin E 20 mg, vitamin K3 6 mg, vitamin B1 4 mg, vitamin B2 7 mg, niacin 26 mg, vitamin B6 6 mg, vitamin B12 0.05 mg, folic acid 2 mg, D-biotin 0.06 mg., Ca-D pantothenate 13 mg, carophyll-yellow 26 mg, and cholinechloride410 mg.**Trace mineral premix (per kg of diet): Mn 85 mg, Fe 65 mg, Zn 65 mg, Cu 6 mg, Co 0.3 mg, I 2 mg, and Se 0.16 mg.

Table 2
Experimental design.

Treatments	Diet	NP% of diet	*C. perfringens* type-A challenge
T1 (CX)	Basal diet	0.00	–
T2 (CN 0.05)	Basal diet	0.05	–
T3 (CN 0.1)	Basal diet	0.10	–
T4 (PX)	Basal diet	0.00	+
T5 (PN 0.05)	Basal diet	0.05	+
T6 (PN 0.1)	Basal diet	0.10	+

aseptically-excised and sectioned into three segments. The First segment is the duodenum (from gizzard till the entry of bile and pancreatic ducts). The second part is jejunum (from the entry of the ducts till Meckel's diverticulum). The third section is ileum (from Meckel's diverticulum till the ileocecal junction). Then according to Thanissery et al. (2010), approximately 7.5 cm length of both upper ileum (anterior to yolk stalk) and lower ileum (anterior to the ileocecal valve) were removed and kept on ice for subsequent enumeration of *C. perfringens*. Next, according to Liu, Guo and Guo (2012), duodenal samples (~3 cm) were collected, preserved in 2 ml screw-capped tubes, and frozen in liquid nitrogen for mRNA determination. Last, according to Thanissery et al. (2010), the duodenum was excised, washed twice with phosphate-buffered saline (PBS) and fixed in 10% neutral buffered formalin solution for morphologic examination and microscopic lesion scores.

2.5. Determination of intestinal C. perfringens concentration

Total colony count of ileal *C. perfingens* type-A was done according to Thanissery et al. (2010). Approximately 5 g of every ileum sample was weighed, conveyed to 10 ml of anaerobic FTG broth, and stomached for 30 s (Stomacher 400 Circulator, Seward Ltd., London, UK). The stomached digesta (1 ml) was directly transferred to 9 ml of anaerobic FTG broth and subjected to 10-fold serial dilution. The dilutions were then plated on TSC agar and incubated anaerobically at 37 °C for twenty-four h. Then the distinguished black colonies' number was counted. The concentration of *C. perfringens* was finally expressed as $\log^{10}$ colony forming units (cfu) per gram of intestinal content.

Table 3
Microscopic lesion scoring system.

Microscopic lesions in gut section	Score
Villus fusion	
Occasional fusion of two villi in a section	1
Occasional fusion of more than two villi or several fusions of two	2
Multiple areas where more than two villi were fused	3
Large clusters of fused villi throughout	4
Dilation of capillaries	
A few mildly dilated	1
Mildly dilated throughout	2
Moderately dilated throughout	3
Severely dilated throughout	4
Capillary hemorrhage	
A few red blood cells outside capillaries in some villi	1
A few red blood cells outside capillaries in most villi	2
Many red blood cells outside capillaries in parts of section	3
Severe haemorrhages throughout	4
Epithelial cell defects	
Flattening of epithelial cells in a few villus tips	1
Defect or micro-erosion at tips of a few villi	2
Defect or micro-erosion at tips of multiple villi	3
Severe erosions, large epithelial cell defects	4
Red blood cells gut lumen	
A few	1
Some aggregates	2
Multiple aggregates	3
Whole lumen filled with aggregates	4
Proteinaceous material gut lumen	
Some spots of material	1
Multiple spots of material	2
Very large clumps of material	3
Lumen full of material	4

2.6. Microscopic lesion score of C.perfringens

Microscopically intestinal lesion scoring was performed according to Gholamiandehkordi et al. (2007). Moreover, the further quantification of necrotic enteritis histological observations was carried out using a scoring system described in Table 3.

2.7. RNA isolation and quantitative real-time polymerase chain reaction for occludin and mucin mRNA expression

RNA extraction from the duodenal mucosa sample was applied using the RNeasy Mini 234 Kit when 30 mg of the tissue sample was added to 600 µl of RLT buffer containing 10 µl of β-mercaptoethanol per 1 ml. Samples homogenization were performed through placing tubes into the adaptor sets, which are fixed into the clamps of the tissue Lyser. Disruption performed in 2 min with a high-speed (30 Hz) shaking step. One volume of 70% ethanol was added to the cleared lysate, and the steps were completed according to RNeasy Mini 234 Kit instructions (Catalogue no. 74,104). On column DNase, digestion was done to get rid of residual DNA. The oligonucleotide primer utilized in the study were listed in Table (4a).

The PCR master mix was prepared with a total of 25 µl. Like the following, 2x QuantiTect SYBR Green PCR Master Mix (12.5 µl), reverse transcriptase 0.25 µl, Forward primer (20 pmol) (0.5 µl), reverse primer (20 pmol) (0.5 µl), RNase Free Water 8.25 µl and template RNA 3 µl. The cycle condition was mentioned in the Table (4b).

Table 4a
Oligonucleotide primers and probes used in SYBR Green real time PCR Source: Metabion (Germany).

Gene	Primer sequence (5′ – 3′)	Reference
Muc2	GCCTGCCCAGGAAATCAAG CGACAAGTTTGCTGGCACAT	Chen, Tellez, Richards & Escobar, 2015
Occludin	GAGCCCAGACTACCAAAGCAA GCTTGATGTGGAAGAGCTTGTTG	Chen et al., 2015
ß. actin	CCACCGCAAATGCTTCTAAAC AAGACTGCTGCTGACACCTTC	Yuan, Guo, Yang & Wang, 2007

The reaction was carried out in a Stratagene MX3005P real-time PCR machine. The Stratagene MX3005P software was used for determining the amplification curves and Ct values. For estimation of the variation in gene expression with the RNA from the different samples, The CT of every sample was compared with that of the control group according to the "ΔΔCt" method explained by Yuan, Reed, Chen and Stewart (2006).

2.8. Intestinal histomorphology and goblet cell histochemistry

The fixed duodenal samples were embedded in paraffin blocks. Consequently, sections of 4 μm thickness were stained with the haematoxylin-eosin method. The histomorphometric analysis assessed in a total of 30 low microscopic fields (4X) per group (five fields for every bird). The analyzed morphometric variables comprised: villus length (from the tip of the villus till muscularis mucosa), villus height (from the tip of the villus till the villus-crypt junction) with using the lamina propria as the base, villus width at half height, crypt depth as the depth of the integration between adjacent villi, and the muscularis thickness (the external muscle layer) and muscularis mucosa. Villus area was estimated from the villus height and width at half height. Values are the means from 24 different villi with measuring only vertically oriented villi and crypts (Uni, 1999). Then the villus height/crypt depth ratio was calculated.

Mucin-type identification was performed according to Liu et al. (2012) using differential staining techniques for goblet cell count. Duodenal Sections were stained with the periodic acid Schiff reaction for neutral mucin, with Alcian blue 8 GX at pH 2.5 for acid mucin, and with high iron diamine reaction without prior oxidation for sulfomucin (with a small modification: 40% rather than 10% FeCl3). Goblet cells containing mucin were counted along the villi with measuring the associated villus surface area in 10 full-length villi. The density of goblet cells was counted as the number of goblet cells per unit surface area (mm^2). All examinations and measurements were carried out using an Olympus optical microscope and Prog Res® CapturePro software (version 2.7; Jenoptik, Jena, Germany).

2.9. Performance parameters

Birds of various experimental groups were weighed individually at the start of the trial. Bodyweight (BW) and feed intake (FI) were recorded weekly on a pen basis for the calculation of body weight gain (BWG) and feed conversion ratio (FCR) during the whole experimental period. Mortality was recorded daily.

2.10. Statistical analysis

Statistical analysis was performed using the GLM procedure of the SAS Institute (2004). The differences between the treatment means were analyzed using Tukey's HSD test with a significance level of 5% (Tukey, 1991).

3. Results

3.1. Intestinal C. perfringens type-A concentration and lesion scores

The results of bacterial enumeration and lesion scores are illustrated in table 5. Overall, both *C. perfringens* counts and intestinal lesion scores were significantly affected by both pathogen challenge and NP supplementation.

The results revealed that the *C. perfringens* type-A concentrations of all unchallenged treatments (CX, CN-0.05, and CN-0.10) were significantly ($P < 0.05$) lower than those of challenged groups (PX, PN0.05, and PN0.1). The level of *C. perfringens* in the challenged un-supplemented group (PX) (5.83 Log^{10} CFU / g) was comparable to the level recorded by other literature for necrotic enteritis (NE) infected chicks. For instance, Pedersen, Bjerrum, Heuer, Lo Fo Wong and Nauerby (2008)) recorded a *C. perfringens* level of 4.98 Log^{10} CFU / g in chicks challenged with *C. perfringens* and maintained on an antibiotic-free diet. Moreover, all challenged groups (PX, PN-0.05, and PN-0.1) showed a higher intestinal lesion score ($P<0.05$) compared with those of birds in unchallenged groups (CX, CN-0.05, and CN-0.10).

The average bacterial counts of challenged groups supplemented with NP, at 0.05 and 0.1%, significantly ($P<0.05$) decreased (3.767 and 2.600 Log^{10} cfu / g, respectively) in comparing with that (5.833 Log^{10} cfu / g) of the challenged non-supplemented group (PX). Subsequently, the intestinal lesion scores of birds in the PN-0.05 and PN-0.1 groups were significantly ($P<0.05$) lower than that of the PX group. Furthermore, both bacterial count and intestinal lesion scores of birds in PN-0.1 group, supplemented with 0.1% NuPro and challenged by *C. perfringens,* were markedly reduced compared with other challenged groups (PX and PN-0.05), it almost identical to that of unchallenged groups (CX, CN-0.05, and CN-0.10).

3.2. Occludin and mucin (MUC2) mRNA expression

Table 6 represents the effect of NP supplementation on mRNA expression of occludin and MUC2 of broiler chickens challenged or/and unchallenged with *C. perfringens*. Generally speaking, both MUC2 and occludin mRNA expression was significantly ($P<0.05$) increased by NP supplementation, at 0.05 and 0.1%.

The data indicated a significant ($P<0.05$) increase in MUC2 and occludin mRNA expression of NP unchallenged groups (CN-0.05 and CN-0.1) compared with those of the unchallenged control group (CX).

Table 4b
The cycle condition according to Quantitect SYBR green PCR kit.

Gene	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)		
			Secondary denaturation	Annealing (Optics on)	Extension	Secondary denaturation	Annealing	Final denaturation
Muc2	50°C	94°C	94°C	60°C	72°C	94°C	60°C	94°C
	30 min.	5 min.	15 s.	30 s.	30 s.	1 min.	1 min.	1 min.
Occludin	50°C	94°C	94°C	60°C	72°C	94°C	60°C	94°C
	30 min.	5 min.	15 s.	30 s.	30 s.	1 min.	1 min.	1 min.
ß. actin	50°C	94°C	94°C	51°C	72°C	94°C	51°C	94°C
	30 min.	5 min.	15 s.	30 s.	30 s.	1 min.	1 min.	1 min.

Table 5
Effect of NP supplementation on intestinal *clostridium perfringens* concentration and microscopic lesion scores.

Items			*Clostridium* Log [10] cfu / g	Average intestinal lesion scores				
				Villus fusion	Capillaries dilatation	Capillaries Hemorrhages	Epithelial Cell defect	Proteinaceous Material in duodenal lumen
Treatments	[1]NP%	Challenge *C. perfringens* type-A						
CX	0.00	–	1.833^{c}	1.033^{c}	1.177^{b}	1.320^{cd}	1.453^{d}	1.033^{c}
CN-0.05	0.05	–	1.933^{c}	1.100^{c}	1.323^{b}	1.183^{de}	1.327^{d}	1.060^{bc}
CN-0.10	0.10	–	1.633^{c}	0.997^{c}	0.930^{b}	0.930^{e}	1.107^{d}	0.970^{c}
PX	0.00	+	5.833^{a}	3.767^{a}	3.133^{a}	3.103^{a}	3.933^{a}	3.783^{a}
PN-0.05	0.05	+	3.767^{b}	2.133^{b}	2.417^{b}	2.423^{b}	2.683^{b}	1.467^{b}
PN-0.10	0.10	+	2.600^{bc}	1.410^{c}	1.500^{c}	1.503^{c}	1.953^{c}	1.233^{bc}
[2]SEM			0.570	0.181	0.262	0.090	0.133	0.128
Main effects								
NP								
0%			3.850^{a}	2.400^{a}	2.155^{a}	2.212^{a}	2.693^{a}	2.408^{a}
0.05%			2.800^{ab}	1.617^{b}	1.870^{b}	1.803^{b}	2.005^{b}	1.263^{b}
0.1%			2.117^{b}	1.203^{c}	1.215^{c}	1.217^{c}	1.530^{c}	1.102^{b}
SEM			0.389	0.128	0.185	0.063	0.094	0.090
C. perfringens type-A challenge								
–			1.778^{b}	1.043^{b}	1.143^{b}	1.144^{b}	1.296^{b}	1.021^{b}
+			4.067^{a}	2.437^{a}	2.350^{a}	2.343^{a}	2.857^{a}	2.161^{a}
SEM			0.329	0.105	0.151	0.052	0.077	0.074
P-value								
NP			0.026	$^{<}$0.001	0.011	$^{<}$0.001	$^{<}$0.001	$^{<}$0.001
C. perfringens			0.003	$^{<}$0.001	$^{<}$0.001	$^{<}$0.001	$^{<}$0.001	$^{<}$0.001
NP × *C. perfringens*			0.041	$^{<}$0.001	$^{<}$0.001	$^{<}$0.001	$^{<}$0.001	$^{<}$0.001

[a,b,c]Means within a column with different superscripts are significantly different ($P^{<}$0.05).
[1]Nucleoforce Poultry, a balanced concentrate of free nucleotides commercial product obtained from dried yeasts (saccharomyces cerevisiae).
[2]SEM: standard error of mean.

Table 6
The effect of NP supplementation on mRNA expression of occludin and MUC2.

Items			Mucin (MUC2)	Occludin
Treatments	[1]NP%	*C. perfringens* type-A challenge		
CX	0.00	–	1.000^{c}	0.953^{c}
CN-0.05	0.05	–	1.133^{b}	1.063^{b}
CN-0.1	0.10	–	1.243^{a}	1.163^{a}
PX	0.00	+	0.587^{e}	0.347^{e}
PN-0.05	0.05	+	0.840^{d}	0.583^{d}
PN-0.1	0.10	+	1.103^{bc}	0.833^{bc}
[2]SEM			0.022	0.031
Main effects				
NP				
0%			0.793^{c}	0.650^{c}
0.05%			0.987^{b}	0.823^{b}
0.1%			1.173^{a}	1.068^{a}
SEM			0.015	0.022
C. perfringens type-A challenge				
–			1.126^{a}	1.060^{a}
+			0.843^{b}	0.634^{b}
SEM			0.013	0.018
P-value				
NP			0.015	0.037
C. perfringens			0.007	0.027
NP × *C. perfringens*			$^{<}$0.001	0.003

[a,b,c]Means within a column with different superscripts are significantly different ($P^{<}$0.05).
[1]Nucleoforce Poultry, a balanced concentrate of free nucleotides commercial product obtained from dried yeasts (saccharomyces cerevisiae).
[2]SEM: standard error of mean.

Regarding *C. perfringens* challenged groups, the results demonstrated that *C. perfringens* challenge adversely affected MUC2 and occludin mRNA expression. However, NP supplementation, at 0.05 and 0.1%, to the challenged groups (PN-0.05 and PN-0.1) resulted in a significant ($P^{<}$0.05) improvement in both MUC2 and occludin mRNA expression compared with that of a challenged non-supplemented group (PX).

Furthermore, both MUC2 and occludin mRNA expression of birds in PN-0.1 group, 0.1% NP supplemented group challenged with *C. perfringens*, were significantly ($P^{<}$0.05) improved to be nearly the same as that of the unchallenged control group (CX) and unchallenged group supplemented with 0.05% NP (CN-0.05).

3.3. Intestinal histomorphology and goblet cell number

The results of intestinal histomorphology and goblet cell number are demonstrated in Table 7. Overall, intestinal histomorphology and goblet cell histochemistry was significantly affected by both pathogen challenge and NP supplementation.

With regards to intestinal histomorphology, the data showed a significant ($P^{<}$0.05) increase in intestinal villus height of NP unchallenged groups (CN-0.05 and CN-0.1), by 4.62 and 12.29% respectively, and a significant ($P^{<}$0.05) decrease in crypt depth compared with that of the unchallenged control group (CX). The intestinal villus height and crypt depth of NP unchallenged groups (CN-0.05 and CN-0.1) directly reflected on villus length/crypt depth (V/C) ratio, especially V/C ratio of the CN-0.1 group which significantly increased in comparing with the CX group. Moreover, NP unchallenged groups (CN-0.05 and CN-0.1) recorded a significant ($P^{<}$0.05) increase in intestinal mucosal thickness compared with that of the unchallenged control group (CX).

On the other hand, the results of *C. perfringens* challenge demonstrated that the pathogen challenge negatively affected the villus height, crypt depth, and V/C ratio of challenged groups (PX, PN-0.05, and PN0.1) compared with that of unchallenged groups (CX, CN-0.05, and CN-0.10). However, NP supplementation, at 0.05 and 0.1%, to the challenged groups (PN-0.05 and PN-0.1) resulted in a significant ($P^{<}$0.05) increase in both villus height and intestinal mucosal thickness in comparing with that of a challenged non-supplemented group (PX).

In the case of goblet cell count, the data revealed a significant ($P^{<}$0.05) increase in goblet cell count of NP unchallenged groups (CN-0.05 and CN-0.1) compared with that of the unchallenged control group (CX).

Table 7
The effect of NP supplementation on intestinal histomorphology and goblet cell number.

Items			Villus height (μm)	Crypt depth (μm)	Villus: crypt ratio	Thickness of mucosa (μm)	Goblet cells number
Treatments	[1]NP%	*C. perfringens* type-A challenge					
CX	0.00	–	64.170[c]	16.200[a]	4.433[c]	82.220[c]	24.133[c]
CN-0.05	0.05	–	67.137[b]	13.633[b]	4.924[cb]	97.153[b]	25.693[b]
CN-0.10	0.10	–	72.060[a]	12.850[b]	6.143[a]	112.283[a]	30.027[a]
PX	0.00	+	45.127[f]	10.367[c]	4.300[c]	54.700[f]	10.683[f]
PN-0.05	0.05	+	57.160[e]	11.110[c]	4.367[c]	69.317[e]	14.950[e]
PN-0.1	0.10	+	60.680[d]	11.200[c]	5.303[b]	75.243[d]	22.027[d]
[2]SEM			0.497	0.310	0.262	0.603	0.365
Main effects							
Diet							
0%			54.648[c]	13.283[a]	4.367[b]	68.460[c]	17.408[c]
0.05%			62.148[b]	13.372[a]	4.598[b]	83.235[b]	20.322[b]
0.1%			66.370[a]	12.025[b]	5.723[a]	93.763[a]	26.027[a]
SEM			0.351	0.219	0.185	0.426	0.258
C. perfringens type-A challenge							
–			67.789[a]	14.894[a]	5.136[a]	97.219[a]	26.618[a]
+			54.322[b]	10.892[b]	4.657[a]	66.420[b]	15.887[b]
SEM			0.287	0.179	0.151	0.348	0.211
P-value							
NP			0.045	0.046	0.046	0.043	0.008
C. perfringens			0.041	0.044	0.144	0.010	0.021
NP × *C. perfringens*			<0.001	<0.001	0.429	<0.001	<0.001

[a,b,c]Means within a column with different superscripts are significantly different ($P<0.05$).
[1]Nucleoforce Poultry, a balanced concentrate of free nucleotides commercial product obtained from dried yeasts (saccharomyces cerevisiae).
[2]SEM: standard error of mean.

By far, the results illustrated that pathogen challenge passively affected the number of goblet cell of challenged groups (PX, PN-0.05 and PN0.1) comparing with that of unchallenged groups (CX, CN-0.05 and CN-0.10). However, NP supplementation, at 0.05 and 0.1%, to the challenged groups (PN-0.05 and PN-0.1) resulted in a significant ($P<0.05$) increase in the goblet cell count compared with a challenged non-supplemented group (PX).

3.4. Performance parameters

Table 8 depicts the effect of NP supplementation on the growth performance parameters of broiler chickens challenged or/and unchallenged with *C. perfringens*. At first glance, the results revealed that both body weight gain (BWG) and feed intake (FI) were significantly ($P<0.05$) increased by NP supplementation, at 0.05 and 0.1%, which reflected on the FCR ratio that numerically improved.

The data indicated considerable improvement in BWG of NP unchallenged groups (CN-0.05 and CN-0.1), by 5.08 and 7.05% respectively, and a noticeable increase ($P<0.05$) in FI comparing with that of the unchallenged control group (CX). The BWG and FI reflected on FCR, which numerically decreased in comparison with that of the CX group.

Regarding *C. perfringens* challenged groups, the results demonstrated that *C. perfringens* challenge adversely affected BWG, FI, and FCR. However, NP supplementation to the challenged groups (PN-0.05

Table 8
The effect of NP supplementation on growth performance parameters of broiler chickens.

Items			Initial BW (g)	Final BW (g)	Total weight gain (g)	Total F. I per chick (g)	FCR (g/g)
Treatments	[1]NP%	*C. perfringens* type-A challenge					
CX 1	0.00	–	40.23[a]	2001.42[b]	1961.19[b]	3166.22[b]	1.61[cd]
CN-0.05	0.05	–	40.17[a]	2100.94[a]	2060.77[a]	3276.40[a]	1.59[cd]
CN-0.10	0.10	–	40.30[a]	2139.60[a]	2099.43[a]	3304.05[a]	1.57[d]
PX	0.00	+	40.17[a]	1754.89[d]	1714.62[d]	2939.67[d]	1.72[a]
PN-0.05	0.05	+	40.27[a]	1875.58[c]	1835.28[c]	3039.98[c]	1.66[b]
PN-0.10	0.10	+	40.23[a]	1958.53[b]	1918.30[b]	3132.67[b]	1.63[bc]
[2]SEM			0.09	14.900	14.938	14.927	0.014
Main effects							
NP							
0%			40.25[a]	1878.16[c]	1837.91[c]	3052.94[c]	1.67[a]
0.05%			40.23[a]	1988.26[b]	1948.02[b]	3158.19[b]	1.62[ab]
0.1%			40.20[a]	2049.07[a]	2008.87[a]	3218.36[a]	1.60[b]
SEM			0.032	10.536	10.563	10.555	0.010
C. perfringens type-A challenge							
–			40.19[a]	2080.65[a]	2040.46[a]	3248.89[a]	1.59[b]
+			40.27[a]	1863.00[b]	1822.73[b]	3037.44[b]	1.67[a]
SEM			0.03	8.603	8.625	8.618	0.008
P-value							
NP			0.854	0.036	0.036	0.042	0.116
C. perfringens			0.296	0.008	0.008	0.009	0.027
NP × *C. perfringens*			0.854	0.123	0.124	0.103	0.296

[a,b,c]Means within a column with different superscripts are significantly different ($P<0.05$).
[1]Nucleoforce Poultry, a balanced concentrate of free nucleotides commercial product obtained from dried yeasts (saccharomyces cerevisiae).
[2]SEM: standard error of mean.

and PN-0.1) resulted in a significant ($P^{<}0.05$) improvement in both BWG and FCR in comparing with that of a challenged non-supplemented group (PX). Moreover, the performance parameters of PN-0.1 group, 0.1% NP supplemented group and challenged with *C. perfringens*, nearly similar to that of the unchallenged control group (CX).

4. Discussion

The reduced *C. perfringens* infection in NP challenged groups (PN-0.05 and PN-0.1) attributed the effect of NP supplementation to improve gut health parameters like villus height, v/c ratio, mucosal thickness, and goblet cell number. Besides, increasing the goblet cell's number, which is a secretory epithelial cell which exists within the intestine and is in charge of the mucus production, forms a protective gel-like layer covering the surface epithelium and defends against bacterial invasion (Forstner & Forstner, 1994). Additionally, nucleotides could enhance probiotic predominance (*lactobacilli and bifidobacteria*) and reduce the concentration of enterobacteria. As found out by Gil et al. (1986) and Tanaka and Mutai. (1980) who recorded a marked reduction in the concentration of enterobacteria in stool samples from infants with exogenous dietary nucleotides-supplementation. A similar result was stated by Mateo, Peters and Stein (2004) in weanling pigs. Moreover, nucleotides could even have stimulated the intestinal immune system to raise intestinal IgA production as found in dogs (Swanson et al., 2002), rats (Kudoh et al., 1999) and chickens (Gao et al., 2008) fed yeast supplements. Immunoglobulin A binds to antigens (like *C. perfringens* α-toxin) and hinders them from passing through the mucosal membrane and establishing infection and lesions (Kulkarni, Parreira, Jiang & Prescott, 2010). The obtained results are convenient with that of Thanissery et al. (2010) who found a numerical reduction in *C. perfingens* levels in challenged and NP treated groups. Our results prove that nucleotide supplementation can positively influence the microflora within the alimentary canal. That hinders the proliferation of pathogenic bacteria such as *C. perfingens* type-A.

Furthermore, the reduced intestinal lesion scores of birds supplemented with NP indicated that NP markedly alleviated the negative effects of *C. perfringens* challenge through improving the intestinal barrier (occludin and MUC2) function which resulted in improving the growth performance of birds challenged with *C. perfringens*. The suggested mechanism by which NP ameliorates the intestinal barrier is that the genetic code of the genes that used for direct protein synthesis and production of cell structures constituted mainly of a nucleotide sequence. Thus, the nucleotides-supplementation will provide genetic codes for gene expression, RNA synthesis, and messenger RNA production (Carver & Walker, 1995).

The MUC2 is a fundamental component of the protective mucus layer which can protect the intestine against pathogen and promote the procedure of restitution (Hernandez et al., 2009; Zeinali, Ghazanfari & Ebrahimi, 2017) and has been established as an indicator for gut health in poultry and other species (Dkhil, Delic & Al-Quraishy, 2013 and Li et al., 2015; Forder, Nattrass, Geier, Hughes & Hynd, 2012). Deplancke et al. (2001) demonstrated that C. perfringens is mucolytic bacteria capable of degrading the mucin of the mucous coat, which enables bacteria to spread on the epithelial surface.

Occludin and junctional adhesion molecule (JAM), claudins, and zonula occludens (ZO) are unique proteins that form the extracellular barrier of the intestine (Schneeberger & Lynch, 2004). This barrier is well-known as tight junctions (TJs), which work as a fence against macromolecular transmission (Ballard, Hunter & Taylor, 1995). The disruption of intestinal tight junction proteins may lead to an increase in the permeability of luminal antigens and bacterial translocation (Ulluwishewa et al., 2011). Little data are available on tight junction protein expression in *C. perfringens* infected birds; among them, Liu et al. (2012) reported decreased occludin gene expression within the small intestine of the *C. perfringens* infected birds. Dietary nucleotides are reported to play a role in the growth and differentiation of the alimentary canal (Carver & Walker, 1995), by enhances the function of intestinal epithelial cells (Seifert & Schultz, 1989). Moreover, there are some shreds of evidence that dietary nucleotides could enhance innate immune responses and host resistance against the damaging effect of the toxin in chickens (Frankič, Pajk, Rezar, Levart & Salobir, 2006).

The results of the improved intestinal morphological picture by dietary nucleotide supplementation agreed with Wu et al. (2018). Enterocytes have a limited capacity of de-novo synthesizing nucleotides (Yamamoto et al., 1997). So they are dependent on exogenous and endogenous nucleotides in addition to the absorption of most dietary nucleotides within the small intestine, which is over 90% (Uauy, 1994). Consequently, parenteral supplementation of nucleic acids supports mucosal cell proliferation and function by increasing the formation of mucosal protein and increasing concentration of DNA (Carver, 1994; Uauy, Birch, Birch & Uauy, 1990). As demonstrated by Carver and Walker (1995); Sanderson and Youping (1994) who reported increased mucosal weight and stimulated enterocyte differentiation by dietary nucleotides-supplementation. Generally, villus crypt ratio is a parameter for measuring the maturity and functional capacity of enterocytes (Hampson, 1986). Whereas, deeper crypts signify rapid cellular turnover to permit the villus renewal as a direct response to normal sloughing or inflammation caused by pathogens or their toxins (Yason, Summers & Schat, 1987).

The adverse effect of pathogen challenge on villus height, crypt depth, and C/V ratio also recorded by Olkowski, Wojnarowicz, Chirino-Trejo and Drew (2006). As, *C. perfringens* bacteria are releasing toxins, like Necrotic Enteritis Toxin (NetB) (Keyburn et al., 2008). The effect of *C. perfringens* toxins can be ameliorated by dietary nucleotide supplementation as recorded by Ortega, Nunez, Gil and Sanchez-Pozo (1995) who reported that nucleotide supplementation promoted rapid recovery of small intestine atrophy after feed deprivation (Ortega et al., 1995), and diarrhea in rats (Bueno et al., 1994). Moreover, several studies (Maldonado, Navarro, Narbona & Gil, 2001; Frankic et al., 2006; Hess & Greenberg, 2012) reported that dietary nucleotides-supplementation has the capability for increasing cell-mediated immunity, and improving the resistance against bacterial infections.

The role of NP in stimulating proliferation and maturation of the gastrointestinal tract (Carver & Walker, 1995), which is the main site for absorption and utilization of diet from the gut (Boleli, Maiorka & Macari, 2002), positively reflected on the growth performance parameters. Additionally, nucleotides act as the main component in carbohydrate, fat, protein and nucleic acid metabolism (Carver & Walker, 1995; Cosgrove, 1998) which are essential for maintenance and repair of muscle tissue (Grimble & Westwood, 2000). Our results of NP on the performance parameters compatible with that of Madrigal et al. (1993), who recorded that the yeast and yeast cell components significantly improved feed utilization and BWG in broiler chickens. A similar was found by Bradley, Savage and Timm (1994) in turkey poults. On the contrary, Pelícia et al. (2010) detected non-significant differences among broilers fed diets supplemented with nucleotides. Furthermore, Jung and Batal (2012) stated that nucleotides are not a fundamental nutrient; thus, it is not recommended to use them within the diets of broilers reared under normal conditions. However, exogenous dietary nucleotides-supplementation for broiler chicken could be essential to keep up maximum growth performance when birds are exposed to stress conditions (Esteve-Garcia, Martinez-Puig, Borda & Chetrit, 2007; Rutz et al., 2004).

The adverse effect of *C. perfringens* challenge on performance parameters attributed to the fact that the damage of the intestinal mucosa by pathogen challenge leads to a concomitant loss of brush-border enzymes (such as alkaline phosphatase and maltase) and transport proteins that chargeable for the ultimate stages of digestion of macromolecules and nutrient transport (Fasina et al., 2007; Jeurissen et al., 2002).

NP significantly attenuated the adverse effects of *C. perfringens* challenge by improving the intestinal barrier function, which positively

reflected on the growth performance of birds challenged with *C. perfringens*. Our results are compatible with M'Sadeq, Wu, Choct, Forder and Swick (2015) who found that dietary supplementation of the yeast cell wall was effective in restraining the decline of bird performance; which is induced by *C. perfringens* and *Eimeria* challenge. In contrary, Alizadeh et al. (2016) showed that dietary supplementation of yeast-derived products did not affect the growth performance of broiler chickens challenged with *C. perfringens*.

5. Conclusion

From the results described above, it could be concluded that dietary supplementation of nucleotides in broiler diets, at 0.1%, noticeably attenuated the negative effects of *C. perfringens* challenge by improving the intestinal histomorphology and intestinal barrier function. Which consequently resulted in a significant reduction in *C. perfringens* counts and intestinal lesion scores that positively reflected on the growth performance of birds challenged with *C. perfringens*.

Declaration of Competing Interest

None.

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Ethical statement

All procedures and protocols follow the guidelines of Cairo University Institutional Animal Care and Use Committee (CU- IACUC), Veterinary Medical and Agricultural Sciences Sector under number of CU/II/F/29/18.

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