

The Efficacy of Na-Butyrate Encapsulated in Palm Fat on Experimentally Induced Necrotic Enteritis and Enumeration of intestinal resident *Clostridium perfringens* in Broiler Chickens

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Abstract: Experimental induction of necrotic enteritis (NE) successfully achieved by daily oral inoculation of 4×10^8 CFU of *C. perfringens*/bird in PBS for 4 successive days to immunocompromised broiler chickens (using IBDV vaccination with a hot vaccine strain).

NE infected bird groups showed variable degrees of diarrhea, inappetance enteritis and mortalities. Average lesion scoring indicated the efficacy of Na butyrate encapsulated in palm fat (Admix 30) in lowering damage associated with NE. The recorded mortality was lower in Admix 30 treated groups vs. their non-treated ones. Enumeration of intestinal *C. perfringens* in non-infected chickens (resident *C. perfringens*) as well as in experimentally induced NE groups revealed reduction in the mean values in Admix 30 treated groups vs. non-treated infected ones. In conclusion; the present investigation proved the effectiveness of Admix 30 as a powerful environmentally friendly alternative for natural control method of NE in poultry enterprises.

Key words: Necrotic enteritis, *Clostridium perfringens*, acidifiers.

I. Introduction

C. perfringens is a spore-forming gram positive anaerobic rod which is a common inhabitant of the intestine of healthy broiler chickens belonging to the resident microbiota (Sengupta et al.; 2011) However, this microorganism along with predisposing factors causing mucosal damage (dietary ingredients or changes, severe stress, coccidiosis, or immunosuppressive affections) can collaborate to the overgrowth of *C. perfringens* and subsequent toxin production which is requisite to develop NE, (Barnes, 1997, Collier et al.; 2008 and Llancó et al.; 2012). NE is an important clinical disease produced by *C. perfringens* that affects the poultry industry worldwide causing serious economic loss, about of two dollar billions/year (Kaldhusdal and Lovland; 2000). The intestinal number of *C. perfringens* in healthy and in NE-affected birds are different. The *C. perfringens* population is found to be normally less than 10^2 to 10^4 colony-forming units (CFU) per g of the intestinal contents in the small intestine of healthy chickens compared to 10^7 - 10^9 CFU/g in diseased birds (Kondo, 1988). NE occurs in broilers aging between 2-6 weeks (Songer, 1996 and Cooper and Songer, 2010). In Europe the incidence of *C. perfringens* causing necrotic enteritis (NE) has increased since the ban on in-feed antibiotic growth promoters (AGP) (Van der Sluis, 2000 and Van Immerseel et al., 2004). Many recent studies of NE have focused on finding different ways to control this disease (Shojadoost et al., 2012). The issue of controlling NE and other enteropathogens without the use of AGP is becoming a big challenge. Accordingly; natural alternatives concepts based on natural ingredients for gastrointestinal tract (GIT) integrity and antibacterial action became highly commendable. Lückstädt (2003) mentioned under this point of view that acidifiers can be part of the feeding concept to replace AGP. Because of their pH-reducing and antimicrobial effects; acidifiers appear as one of the most feasible and functional alternative to AGP. Accordingly, experts in the poultry industry have given the use of acidifiers closer scrutiny. The auspicious effect of acidifiers over the organism is due to the better adhesion of the lactic acid bacteria to GIT epithelium in comparison with the pathogenic bacteria, and stooping the implementation of those bacteria over the mucus membranes of the intestine. Awaad et al (2011) has shown the interest of using protected organic acidifiers into the feed of broiler chickens submitted to *C. perfringens* infection and concluded that taking in consideration the facts that organic acids do not require withdrawal period, increase shelf-life of poultry products they can make a valuable contribution to flock health and safety of food that might provide a significant tool for the poultry industry in combating occurrence of intestinal diseases and in reduction of food borne pathogens.

The present trial is conducted to determine the effects of usage of Na-butyrate encapsulated in palm fat on experimentally induced NE and resident *C. perfringens* enumeration (intestinal colonization).

II. Material and Methods

Na-butyrate: Na-butyrate encapsulated in palm fat (Admix® 30 produced by NUTRI-AD International, Belgium) was used in this trial in the following dietary levels in the test groups; starter diet: 1 kg/ton, grower diet: 0.5 kg/ton and finisher diet: 0.25 kg/ton

Experimental design:

One day-old male Arbor Acres plus broiler chickens (n=800) were used in this study. Duration of the trial extended from one day of age up to slaughter (35 days). These birds were allotted into 4 equal groups (1-4) consisting of 200 birds each and assigned into 10 equal replicates of 20 birds. All groups ran contemporaneously. Chickens of groups 1 and 3 treated with Admix® 30 while groups 2 and 4 fed on a plain ration without treatment.

As immunosuppressed chickens are more likely to develop NE, so that some researchers have used methods to induce immunosuppression. These methods mostly use Infectious Bursal Disease vaccine (IBD); it has resulted in a significant increase in NE lesions (McReynolds et al. 2004). Accordingly; vaccination against infectious bursal disease (IBD) using 228-E vaccine was given at 14th day of age; birds of groups 1 and 2 were individually infected by crop gavages with 4×10^8 CFU/ml/bird of *C. perfringens* in phosphate buffered saline (PBS) for 4 successive days after Olkowski et al. (2006), Gholamiandehkordi et al. (2007) and Timbermont et al. (2009). Chickens of groups 3 and 4 were kept without infection. The used strain of *C. perfringens* was type A B2 NET B, isolated from cases of chicken NE.

All experimented birds were vaccinated against different diseases according to the vaccination programs usually adopted in Egyptian chicken broiler farms. Chicken groups 1 and 2 (*C. perfringens* challenged) were kept in a separate room and those of groups 3 and 4 (non-challenged) were kept in another room. All chickens were floor reared in separate pens and kept in environmentally controlled rooms.

Diets:

Chickens fed *ad libitum* a mash commercial starter diet (23% crude protein and 3000 kcal ME/kg diet) during the first 2 weeks of age, commercial grower diet (22% crude protein and 3150 kcal ME/kg diet) from 2-4 weeks of age, and then commercial finisher diet (19% crude protein and 3200 kcal ME/kg diet) from 4-5 weeks of age. Birds had free access to water. No chemotherapy (neither antibiotic, nor anticoccidial drug or coccidiostat) was added during the entire period of the trial.

Measured parameters:

I. Intestinal colonization of C. perfringens (Bioassay):

For intestinal colonization of *C. perfringens* 10 birds of the 4 groups were randomly sacrificed at 14, 21 and 28 days post infection (PI) (1 bird/replicate). Following euthanasia, birds necropsied and 0.2 g of intestinal contents from each bird were serially diluted in sterile PBS to 1:100, 1:1000, and 1:10000 and 0.1 ml of each dilution and poured on the surface of sheep blood agar plates and tryptose sulfite-cycloserine (TSC) agar (supplemented by D-cycloserine) with egg yolk emulsion. These were overlaid with the same medium but without egg yolk. After anaerobic incubation at 37°C for 24 hours; typical *C. perfringens* colonies (black colonies) on TSC agar or large dome-shaped colonies with a double zone of hemolysis on blood agar plates counted and reported as colony-forming units (CFU) per gram. The colonies picked and confirmed by criteria of Harmon (1984) and Carrido et al. (2004).

II- Lesion scoring of gut:

Necropsied birds at day 21 and 28 examined for gross pathological lesion scoring of the small intestine. The scoring system criteria were the six-point system of Keyburn et al. (2006) modified by Shojadoost et al. (2012) as follows:

0 = No gross lesions -

1 = Thin or friable walls, or diffuse superficial but removable fibrin

2 = Focal necrosis or ulceration, or non-removable fibrin deposit 1 to 5 foci

3 = Focal necrosis or ulceration, or non-removable fibrin deposit 6 to 15 foci

4 = Focal necrosis or ulceration, or non-removable fibrin deposit 16 or more foci

5 = Patches of necrosis 2 to 3 cm long variable

6 = Diffuse necrosis typical of field cases variable, but extensive.

III-Health status and Mortality assay:

During the evaluation; the health status of the birds checked daily.

Statistical analyses:

One-way analysis of variance adopted using SAS software general liner models procedure (SAS Institute, 2000). The main factor was Admix 30 supplementation as a mean effect. Mean values assessed for significance using Duncan's multiple range tests. The following model was used for data analysis: $Y_{ij} = \mu + \alpha_i + e_{ij}$

Where: Y_{ij} : The J^{th} observation of the i^{th} dose of Admix 30.

μ : Overall mean.

α_i : Effect of dose of Admix 30 ($i = 1, 2$).

e_{ij} : Unexplained error.

Statements of statistical significance are based upon $P \leq 0.05$.

III. Results and Discussion

In the present investigation experimental induction of NE successfully achieved by daily oral inoculation of 4×10^8 CFU of *C. perfringens*/ bird in PBS for 4 successive days to immunocompromised broiler chickens (using IBDV vaccination with a hot vaccinal strain). Experimentally infected bird groups showed variable degrees of diarrhea, inappetance enteritis and mortalities.

The recorded mortality was lower in Admix 30 treated groups vs. their non-treated ones which could be attributed to its antibacterial effect (Table 1).

The average lesion scoring reached 1.8 and 0.4 in NE infected treated group with Admix 30 (group 1) vs. 2.6 and 0.7 in NE infected non treated group (group 2) at 7th and 14th day post *C. perfringens* challenge respectively indicating the efficacy of Admix 30 in lowering damage associated with NE (Table 2 and Fig.1).

Enumeration of GIT resident *C. perfringens* at 14th day of age revealed diverse values; with higher levels found in non treated blank control birds (group 4) as compared with Admix 30 treated birds (group 3). Similar finding was also determined at 28th day of age in the same group. On the other hand; *C. perfringens* enumeration in NE infected group reduced these values at different examined intervals in Admix 30 treated birds (group 1) vs. non-treated NE infected birds (group 2) (Table 3). Aforementioned findings could be attributed to the pH-reducing and antimicrobial effects of Admix 30 as an acidifier; a conclusion which completely accords with that reported by Lückstädt (2003). Obtained bacteriological results might be explained in the view of the report of Dhawale (2005) who mentioned that organic acids in non-dissociated state (non-ionized) are more lipophilic, penetrate the semi-permeable membrane of the bacterial cell wall and enter the cytoplasm. He concluded that the antibacterial effects of organic acids work through: modification of internal pH; inhibition of fundamental metabolic functions; accumulation of toxic anions and disruption of the cellular membrane

Although the most effective method to prevent or to control NE is the use of antimicrobials mixed to feed and water, bacterial resistance to bacitracin, tetracycline, clindamycin, lincomycin and erythromycin has been reported in several countries, such as, Denmark, Switzerland, Norway, Belgium, Jordan and Brazil (Silva et al, 2009, Gharaibeh et al. 2010 and Salvic et al. 2011). In countries that have stopped of using AGP, the problems of diseases associated with *C. perfringens* in broiler chickens have increased (Grave et al.; 2004). Taking in consideration our obtained findings (*C. perfringens* enumeration, mortality rates and lesion scoring of experimentally induced NE); the role of Na butyrate encapsulated in palm fat (Admix 30) is a powerful environmentally friendly alternative that cannot be denied as a natural control method for NE in poultry enterprises. This argument confirms findings of Awaad et al. (2011) who clearly showed that using acidifiers is considered a novel and effective alternative to antibiotics that could reduce the severity of *C. perfringens* associated with NE in broiler chickens.

Food safety is probably the biggest issue facing poultry production systems today. Preventing contamination of poultry products with food borne pathogens remains a considerable challenge for producers and integrations. Ghadban et al. (1998) and Brynstad (2002) reported on *C. perfringens* as one of the food borne pathogens responsible for the severe food borne necrotic enteritis in man (enteritis necroticans or pigbel disease) which is fatal specially in young and elderly and its enterotoxin has been shown to be the virulence factor responsible for causing the symptoms of *C. perfringens* type A food poisoning which is the more common in the industrialized world. Eventually; the present investigation proves the effectiveness of Na butyrate encapsulated in palm fat (as an animal feed acidifier) in reducing enumeration of resident as well as experimentally induced *C. perfringens* and reducing the severity of NE in broilers which seems to be essential to the higher need for top quality poultry.

Table 1. Effect of Admix 30 on mortality of *C. perfringens* infected and non-infected broiler chickens.

| Group No. | Weekly Mortality Rate (%) | | | | | Cumulative Mortality Rate |
|-----------------|---------------------------|-----------|-----------|-----------|-----------|---------------------------|
| | 1 Week | 2 Weeks | 3 Weeks | 4 Weeks | 5 Weeks | |
| 1-Admix30+CPI* | 2.22±1.13 | 2.59±0.96 | 0.00±0.00 | 1.48±0.82 | 2.59±1.24 | 8.88±1.93 |
| 2-Control+CPI | 2.59±0.79 | 1.48±0.82 | 1.11±1.11 | 3.33±1.51 | 4.07±1.78 | 12.58±3.27 |
| 3-Admix30 | 2.96±0.49 | 1.48±0.60 | 0.37±0.37 | 1.48±0.82 | 1.11±0.57 | 7.40±1.23 |
| 4-Blank Control | 2.22±0.82 | 1.85±0.83 | 1.11±0.57 | 1.85±0.99 | 3.70±1.10 | 10.73±1.60 |
| Probability | 0.9104 | 0.7441 | 0.5410 | 0.5727 | 0.3493 | 0.3650 |

*CPI = *C. perfringens* infection

Table2. Results of lesion score post NE induction

| 21 day-old chickens (7 days PI) | | | | | |
|----------------------------------|----------------------|--------------------|-----------------------------|----------------------------|----------------------|
| Groups No. | Lesion score** of NE | No. of birds/score | No. of examined birds/group | Total lesion score / group | Average lesion score |
| 1-Admix30 +CPI* | 1 | 5 | 10 | 18 | 1.8 |
| | 2 | 2 | | | |
| | 3 | 3 | | | |
| 2-Control + CPI | 2 | 4 | 10 | 26 | 2.6 |
| | 3 | 6 | | | |
| 3-Admix30 | 0 | 10 | 10 | 0 | 0 |
| 4-Blank Control | 0 | 10 | 10 | 0 | 0 |
| 28 day old chickens (14 days PI) | | | | | |
| 1-Admix30 +CPI* | 0 | 6 | 10 | 4 | 0.4 |
| | 1 | 4 | | | |
| 2-Control + CPI | 0 | 5 | 10 | 7 | 0.7 |
| | 1 | 3 | | | |
| | 2 | 2 | | | |
| 3-Admix30 | 0 | 10 | 10 | 0 | 0 |
| 4-Blank Control | 0 | 10 | 10 | 0 | 0 |

*CPI = *C. perfringens* infection

**Lesion score after Keyburn et al. (2006) modified by Shojadoost et al. (2012).

Table 3. Means of enumeration of resident and experimentally induced *C. perfringens* (10³CFU/g).

| Group No. | Age of broiler chickens | | |
|------------------|----------------------------|-----------------------------|---------------|
| | 14 days | 21 days | 28 days |
| 1-Admix30 + CPI* | 0.042±0.020 ^{b**} | 11.920±8.727 ^b | 2.353±1.030 |
| 2-Control + CPI | 0.260±0.094 ^{ab} | 353.200±94.154 ^a | 55.630±19.545 |
| 3-Admix30 | 31±0.019 ^b | 0.180±0.0772 ^b | 0.430±0.398 |
| 4-Blank Control | 300±0.133 ^a | 0.080±0.0335 ^b | 69.200±59.312 |
| Probability | 0.0462 | 0.0001 | 0.2878 |

*CPI = *C. perfringens* infection

** Means with different, superscripts, within age, are significantly different (P ≤ 0.05).

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Fig.1. Different intestinal lesions recorded in experimentally induced NE in broiler chickens.