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Effect of Fatty Acids in Feed on Levels of Antibody Titers and CD4 and CD8 T-Lymphocyte against Newcastle Disease Virus of Vaccinated Broiler Chicken

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***Abstract*—**400 one-day-old male broiler chicks (Ross-308) randomly divided to 2 main groups, 1st main group (GA) was feeding basal diet with medium chain fatty acid (MCFA) at rate of 0.15% and divided to four subgroups, 3 subgroups vaccinated with different routes with Newcastle Disease Virus (NDV) and non-vaccinated group. The 2nd main group (GB) feeding basal diet without MCFA and divided the same as 1st main group. The parameters used in this study included: ND antibody titers at 1, 10, 21, 28, 35 and 42 days of age and values of CD4 and CD8 at 1, 20, 30 and 42 days of age. This experiment detected increase in ND antibodies titers in (G1, G2, G3) groups were fed on basal diet MCFA comparing to groups were fed without adding MCFA (G5, G6, G7) and control groups (G4, G8). The results of cellular immune response (CD4 and CD8) T-cells in broiler chicks indicated that there was obviously significant relationship between dietary Fatty Acid (FA) versus the diet without FA on the level of CD4 parameter, for the entire experimental period. The effect of different ages was statistically significant in creating different values of CD4 level, whereas the CD4 level decreases markedly with age. However, analyzing the data of different vaccination methods, oculonasal method of vaccination led to the highest value of CD4 compared with the oral, S/C and control groups. There were statistical differences in CD8 values due to supplementation of FA versus the basal diet and due to the effect of different age periods. As for the age effect, the CD8 value at 20 days of age was significantly higher than at 42 and 30 days.

***Keywords*—**Broiler, CD4 and CD8, fatty acids, Newcastle disease.

I. Introduction

POULTRY industry represents an important sector in animal production, especially in the developing countries. Poultry products, specially chickens, are considered one of the most important sources of food continues to increase globally [1]. Newcastle disease (ND) is one of the pathogenic viral diseases of avian species. It is economically significant because of the highly mortality and morbidity associated with it [2]. Various approaches have been used for identifying the specific components of the immune response involved in protection [3]. Vaccination is the practice of using modified (attenuated or killed) microorganisms, or portions to induce immune response to a particular disease without actually causing the disease. This may be through protecting birds from infection or by limiting the effects of infection [4]. As in mammals, cell-mediated immunity plays an important role in active protective immunity in birds [5]. Many nutrients are capable of modulating the immune system [6]. The aim of using immunomodulators in the diet is to enhance immune performance and increase prevention against diseases in chickens and domestic animals [7]. Enhancing the characteristics of diet, feed acceptability and bird health due to using of feed additives [8]. Dietary fats play an important role in maintaining the health and growth of poultry as significant sources of energy. Their other essential role in poultry feed is to reduce dustiness, increase palatability and serve as a lubricant in feed preparation [9]. Essential oils enhance production of digestive secretions, stimulate blood circulation, exert antioxidant properties, reduce levels of pathogenic bacteria and may enhance immune status [10]. Using these medicinal plant oils in the diet showed significant effects on performance, carcass quality, feed conversion ratio Feed Conversion Ratio (FCR) and body weight gain of treated chicks [11]. Many nutrients are capable of modulating the immune system as stated by [6]. Enhance broiler performance and immunological performance is due to using aromatic herbal extract. Addition of aroma biotic lead to significantly increase of HI titer of NDV, increase weight of lymphoid organs (thymus, Bursa of Fabricius) and improvement of leukocytes were noticed by [12].

II. Material and Methods

A. Experimental Birds and Housing

400 one day-old male broiler chicks (Ross-308) were obtained from a commercial (VANO) hatchery in Erbil city. The chicks represented a very homogenized sample in the initial where it ranged from 43-44 gm. This was accomplished by weighting 400 birds of the male broiler chicks individually. Thereafter the 400 chicks were divided into two groups named GA and GB, where the GA (200 birds) group of chicks reared on FA supplemented 0.15% starter, grower, and finisher pellet diet (Table I). The added FA is characterized by being MCFA (Aroma biotic) produced by Vitamix Belgium Company. The other GB (200 birds) group of chicks were reared on the same basal diet without FA added. Each of GA and GB chicks where subdivided to 4 groups, 50 birds each, and was subjected to

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different methods of vaccination against ND, orally, oculonasal, S/C, and control (non-vaccinated). Vaccination was applied when birds were 10 days-old. According to the three vaccination methods and control, the sub groups were symbolically named G1, G2, G3 and G4 for birds of GA, respectively. By the same talking G5, G6, G7 and G8 were referred to the birds of GB. The treatment chicks were reared in floor pen (2.5×1m) on chicken paper liter allowed the access of water and subjected to 24hour light. The electrically heated house was furnishing the birds with a temperature schedule consist of an initial temp of 34ċ was reached on day of the experiment. For all birds, feed was given ad-libitum with feed through space held constant. During the experiment, extra care was taken to secure biosecurity.

TABLE I
COMPOSITION AND CHEMICAL ANALYSIS OF THE BASAL DIET FEED TO THE EXPERIMENTAL BIRDS

N	Ingredients	Starter % (1-2wks)	Grower % (3-4wks)	Finisher % (5-6wks)
1	Corn	380	390	450
2	wheat	160	200	200
3	bran	85	80	70
4	Soybean	324	270	218
5	Oil	10	19	23
6	Lysine	1	1.5	1.5
7	Methionine	1	1.25	1.25
8	Colin	1	1	1
9	Calcium	15	14	13
10	Di-calcium phosphate	15	14	14
11	Vitamin	3	3	3
12	minerals	0.2	0.2	0.2
13	Anticoccidia	0.5	0.5	0.5
14	Enzyme	0.75	0.75	0.75
15	Antifungal	1	2	1
16	Salt	2.55	2.8	2.8
	Chemical analysis			
1	Crude protein	22.06%	20.12%	18.04%
2	Energy	2817.4	2916.45	3011.97
3	Methionine	0.45	0.45	0.42
4	Methionine and cysteine	0.74	0.72	0.68
5	Lysine	1.28	1.18	1.04
6	Calcium	0.99	0.92	0.87
7	Available phosphate	0.43	0.41	0.40
8	Sodium	0.16	0.16	0.16
9	Crude fiber	2.96	2.87	2.73
10	Crude fat	3.26	4.18	4.69

Supplied per Kg of diet: Vit. A, 10 000 IU; Vit. D3, 2 000 IU; Vit. E,10 mg; Vit. K3,2 mg; Vit. B1, 2mg; Vit. B2, 6 mg; Vit. B6, 2 mg; Vit. B12, 10 mcg; Niacin, 30mg; Pantothenic acid, 10mg; Folic acid,0.75mg; Biotin, 50mcg; Choline,300mg; Copper, 4 mg; Iron, 40mg; Manganese, 70mg; Zinc,40mg; Iodine,1.2mg; Selenium, 0.1mg; Cobalt, 0.2mg.

B. Blood Sampling

1. Serum for Enzyme Linked Immunosorbent Assay (ELISA)

Wing vein is most commonly used for collecting blood from birds. At days 1, 10, 21, 28, 35, and 42 of experiment blood samples were taken randomly from each group (N=6 per group). From birds, between 1 and 2 ml of blood per bird was collected aseptically (using 2, 3 and 5 ml disposable syringes) in standard test tubes from the wing vein for evaluating of the maternal and post-vaccination antibody titer to NDV and for cellular immune response (CD4 and CD8 T-lymphocytes) at 1, 20, 30, 42 days of age too. These samples were kept undisturbed for 2-4 hours at room temperature for clotting and then kept at 4 Cº over-night. By centrifugation, the sera from these samples were separated, collected in Eppendorf tube, and stored at −20 Cº until further use.

Indirect ELISA for NDV Antibodies titer measurement: used for detection of humoral Immune Response.

NDV specific ELISA plates from Synbiotics Co., San Diego, CA, USA with an automated microplate reader (ELx800, BIO-TEK Instruments Inc, Winooski, Vermont, USA) to test the serum samples. The software provided by the manufacturer quantified the amount of antibody titer in each individual sample and calculated the mean arithmetic titer for the group of serum samples from each group.

For Cellular Response: use Sandwich ELISA for measurement of chicken (CD4 and CD8) T-lymphocytes titers in serum:

The serum samples were tested with CD specific ELISA kit (Mybiosource) USA, according to the manufacturer's protocol using an automated microplate reader (ELx800, BIO-TEK Instruments Inc., Winooski, Vermont, USA) under (450) nm wavelength. The software provided by the manufacturer quantified the amount of CDs titer in each individual sample and calculated the mean arithmetic titer for the group of serum samples from each group which are estimated by Nanogram (ng)/L unit (Figs. 1 and 2).

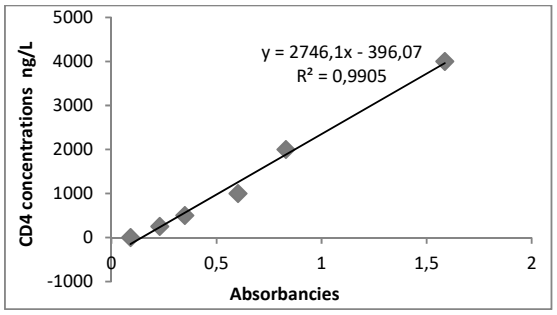

Fig. 1 Standard curve for CD4 concentration

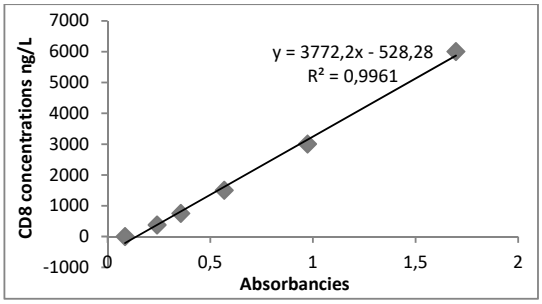

Fig. 2 Standard curve for CD8 concentrations

C. Statistical Analysis

Data were analyzed using statistic program [13] for analyzing the effect of aroma biotic in broiler on ND antibodies

titers, CD4 T-lymphocytes and CD8 T-lymphocytes depending on general linear model analysis of variance according to the equation (Yij = U + Ai + b1(X1-Xi) + b2(X2-Xi) + eij) followed by determination of regression factor between random samples, then we using Duncan multiple range test for detection the significant variations between the means [14].

III. Results

A. ND Antibody Titer

The effect of dietary FA, vaccination methods and interaction on ND titer at 1 and10 days of age was not significantly different, whereas the effect of dietary FA on ND titer was only obvious in birds fed diet with FA after vaccination (Table II), where the ND titer in birds at 3rd week significantly over dominated those fed diet without FA supplemented diet treatments at those particular ages the G1and G5 were similar in their effect had higher ND titers and significantly different from other groups , while the G4 and G8 were lest ND titers detected. On 4th week of age the G1, G2, G5, and G6 were similar in their effects on ND titers and significantly higher than the other groups. When birds were 5 weeks of age, the G1and G5 were similar in their effect had higher ND titers and significantly different from other groups, while the G4 and G8 were lest ND titers detected. 6th week-old birds showed significant differences in the ND titer value with the different methods of vaccination as illustrated in (Table II) (Fig. 3), in which the G3 and G7 were the highest ND titers values and significantly different the other groups. While the G4 and G8 the least values.

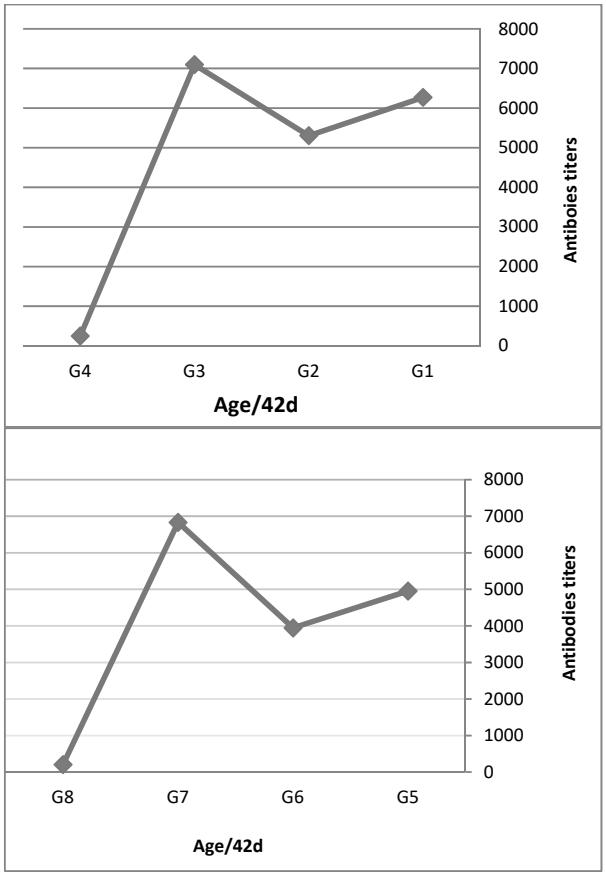

Fig. 3 The means ND titers according to the different vaccination methods and type of diet at 42 days of ag

TABLE II
ND Antibody Titer of Broiler Chicks as Affected by Dietary Fatty Acids and Vaccination Methods at Different Ages

		10 day	21 day	28 day	Treatment 35 day	Age (day) 42 day
Interaction (GA)	G1	3975.6±184ba	6709±223.7a	7668±320a	7147±459.9a	6269±342b
	G2	4322±632.7ba	5291±254.9b	6741±373a	5551±355bc	5304±436.3b
	G3	3414±208.2ba	3093±240.8d	4594±475b	5461±531bc	7095±250.3a
	G4	4025±587.9ba	2033.8±292e	976.8±67c	737.6±85.3d	245.83±41d
Interaction (GB)	G5	38976±476ba	6188.5±229a	7048±536a	6229±546ba	4953.1±327b
	G6	3956±336ba	4390.8±189c	6540±471a	4649±345.7c	3946±422.7c
	G7	3017.8±412b	2446±188ed	4056±441b	5073±549bc	6833±233.8a
	G8	4690.8±485.9a	1887±243.9e	891±205.8c	687.8±87.5d	203.8±39.5d

B. Cluster of Differentiations (CDs) T-Lymphocyte

The effect of three factors interaction (type of diet, vaccination methods and age) and their combination on CD4 and CD8 was studied as stated in (Tables III and IV).

C. Cluster of Differentiation 4 (CD4) T-Lymphocyte

Means for CD4 measured in Nanogram/L for each of the main treatment and their interactions are presented in (Tables IV-XIII). The birds at 10 days of age**,** the results confirmed that there was a higher value of CD4 observed in G7 and significantly different from the other groups, whereas the G4 and G8 similar in their effect and had the least value between groups. As for data at 20 days of age the G1and G6 higher values statistically different when compared with G4, G5 and G7. On the other hand when birds at 30 days of age the G6 showed higher value had similar influence with G2, G4, G5, on the hand significantly different from the G1, G7, G3 and G8. However, each of the G2, G8 at 42 days of age had higher values and significantly different from the groups except the G6 in which similar effect.

D. Cluster of Differentiation 8 (CD8) T-Lymphocyte

Data analyzing for CD8 T-lymphocytes in broiler chickens in (Table IV). There were statistical differences in CD8 values due to supplementation of FA versus the basal diet as stated in

(Table IV). When bird at 10 days of age the G1 and G7 had higher values of C8 detected and significantly different when compared with other groups. Whereas birds at 20 days of age the G1, G6 and G8 had similar effect on CD8 T-lymphocytes and significantly different from the other groups.

TABLE III
CLUSTER OF DIFFERENTIATION4 (CD4) AS EFFECTED BY DIETARY FATTY ACID, AGE OF BIRDS, VACCINATION METHODS AND THEIR INTERACTIONS IN BROILER CHICKS

Treatment		Age (day)			
		10 day	20 day	30 day	42 day
Interaction (GA)	G1	1152.7±191bac	1714±29.9a	692.8±20d	776.5±29.8b
	G2	968±8.4bc	1573±180ba	1093±98cb	868.5±48.7a
	G3	1303.8±25.6ba	1326±121bac	890±23.5cd	691.4±22.7b
	G4	1146±126.7bac	963.3±139c	1064±27cb	779.9±76b
Interaction (GB)	G5	777±92c	1154±66.7bc	1068±36cb	723.6±15.8b
	G6	927±23.5bc	1752±336.7a	1136±61.5b	736.7±17b
	G7	1570.8±323.8a	900.8±36c	879±22cd	800±71ba
	G8	824.6±19.8c	1359±47bac	965±60cb	849±139.8a

On day 35, the G3, G6, G7 and G8 were similar in their effect on CD8 T-lymphocytes and significantly higher compared with other groups, in general the CD8 T-lymphocytes values in this period decreased compared with 10 and **20** days of experiment. While birds at 42 days of age the CD8 T-lymphocyte values were dramatically diminished when compared with other period of age, on the other hand in this particular age the G7 had higher CD8 values, while the G1 was the lowest values of CD8 T-lymphocytes.

TABLE IV
CLUSTER OF DIFFERENTIATION8 (CD8) AS AFFECTED BY DIETARY FATTY ACID, AGE OF BIRDS, VACCINATION METHODS AND THEIR INTERACTIONS IN BROILER CHICKS

Treatment		Age (day)			
		10 day	20 day	30 day	42 day
Interaction (GA)	G1	2571±108ba	3206±239.8a	1313±261.6c	545.9±65d
	G2	1564±114d	2056±195cb	1396.5±33.7c	1194.7±118bac
	G3	2016±220dc	2496±39b	1819±110ba	781.6±74.7dc
	G4	2357±174bc	2260±79.4b	1322.9±100c	1360±229.8ba
Interaction (GB)	G5	1910±129dc	2034±117cb	1551±74bc	871±56.4bdc
	G6	1837.8±42d	3483±379.6a	1911±64ba	1446±248.9a
	G7	3017.5±261a	1532±106.4c	2171.6±172a	1175.8±98bac
	G8	1562±32.9d	3134±75.9a	2228±119.6a	1197.5±187bac

IV. DISCUSSION

The effect of dietary FA on ND titer was only obvious in birds fed diet with FA at 21 and 42 days of age periods. This in accordance with findings reported by [6]; nutrients are capable of modulating the immune system, so agreement with those of [12] by using Aromatic herbal extract increase. HI titer of NDV was significantly higher with addition aroma biotic.

Studying the effect of vaccination regardless of the type of diet revealed that the ND titer was statistically different with different methods of vaccination on day 21 and 28 days of age, where the oral way of vaccinating birds achieved the highest mean of the ND titer followed by the second highest value by oculonasal vaccination method which intern followed by the S/C. The control birds showed the least of ND titer. Reference [15] represented that chicks vaccinated with NDV vaccine either alone or in combination had significantly higher antibody titers than chicks not vaccinated with NDV vaccine. As observed in the study of [16], the host innate immune response to virus infection is an immediate reaction designed to retard virus replication and aid the host in developing specific protection from the adaptive immune responses.

The positive effect of CD4 (helper) T-cells on the different aspects of immune system may be due to the increase number of cells as well as their innate functional ability, such as production of IL-4 and IL-6.Secretion of the interleukin are crucial for activity, proliferation and terminal differentiation of rusty B-cells specific antibody secretion plasma cells [17].

Analyzing 42 days of age, FA supplemented diet groups caused significantly higher level of ND titer than the diet without FA groups. Different types of dietary fatty acids have been shown to have variable effects on bacterial clearance and disease outcome through suppression or activation of immune responses [18].

As to vaccination methods at 42 days of age, the three ways of vaccination significantly affected the ND titer where the oral and oculonasal were decreased. Reference [19] stated that antibodies titer in bird's blood against ND decrease whilst birds aged, whereas the S/C treatment showed the highest level. Interaction analysis revealed that the S/C method significantly improved the level of the ND titer compared with other three treatments. It is due to the oil and adjuvant that vaccine slowly absorbed and induced production of antibody. Reference [20]

stated that antibody level became higher after 30 days by S/C route.

In the results of current study, increasing the level of CD4 as in chick fed FA may be due to the improving effect of FA in the diet, on general health status and physiological performance of chicks, including significant immune enhancing properties. This result agrees with [21]-[23] who reported that the development of lymphoid organs have demonstrated to be influenced by the nutrition of the bird. Nutrients are required to provide the building blocks for the immune cells and tissues including such as cells like T and B lymphocytes, macrophages and natural killer cells [24].

Cell functioning, however, depends on the efficient activation of the metabolic pathways in order to obtain ATP and structural molecules such as nucleotides, phospholipids and macromolecular synthesis [25]. On the other hand, FAs are associated with a series of metabolic pathways, being synthesized from amino acid and glucose, esterified to glycerol from phospholipids and triacylglycerol or broken down acetyl co A or CO2, generating energy. Probably due to this key position in cell metabolic processes, FAs can control their own synthesis and breakdown, as long, interfere with neighboring metabolic processes [26]. However, the development of immune system in poultry is a dynamic process initiated during embryogenesis but not complete until weeks after hatch. Therefore, early nutrition is expected to play important roles in the development and function of the immune system [27].

On the other hand, CD4 levels declined during age proceeding regardless type of diet or vaccination. This result agrees with [28], who's reported an age associated with a decrease of CD4- cell in white leghorn layer. Reference [29] documented that aging is associated with a reduction in T-lymphocyte proliferation, and impaired immune functions. All previous findings refer in different ways to the same target that age related defects as oxidative stress, cell-tissue aging phenomenon, and leads to decline T-cell (and most body cells) function and activities.

A standard evaluation of vaccination efficiency is measuring specific antibody titers to vaccine [30]. It is also well known that the cellular immunity system is a key factor in vaccine-induced antiviral immunity [31]. The high level of CD4 in oculonasal vaccination method group in results of the current study refers to fast inducing action of this method. Also, it has been shown that cell-mediated immune response plays a key role in viral infections as well as vaccine-induced protective immunity [32]. CD8 defined T-cell are crucial in specific cytotoxicity against viral infected target cells. The cells presently CD8 molecule response antigen restricted by MHC-I on target cell and play a major role in the specific cell mediated immune responses [33].

Cluster of differentiation8 (CD8) T-lymphocyte, the tissue distribution of the avian CD8 molecule is very similar to the mammalian CD8 [34]. The findings of the study show that CD8 level was higher in FA group than no FA group regardless of other factors. This may be due to the improving effect of FA on body cells as a whole, which is similar to the beneficial effect of FA in CD4 group. Many previous studies have shown that FA has diver roles in all cells. They are important as a source of energy, as structural components of cell membranes (including immune cell), as signaling molecule, and as precursors for the synthesis of eicosanoids [35]. Thus, they have considerable effect on the membrane structural and function of immune cells [36].

However, CD8 shows a highly level in 20 days group, and there was decline in the CD8 level with age progressing. This expected finding may be due to aging- inducing manner in diminishing the population and activity of most body cells including immune cells [37]. Also, senescence of T-lymphocytes is characterized by phonotypical and functional changes including the loss of characteristic T-cell surface markers [38]. However, the high level of CD8, in general, in vaccinated birds compared to non-vaccinated group, agreed with most of previous studies such as [39] who reported that ND virus specify cell-mediated immunity can already be detected within the first week after the vaccination.

In general, commercial ND vaccines are known to induce protective immunity with T-cell playing a major role in clearance of the virus [40], [41] documented that 2 week old chickens were vaccinated by live type-1 NDV, after that the number of CD8 T-cells increased dramatically and peaked at day 5. Their interpretation to that is the number changes for CD8 T-cells could be the self-protection mechanism of immune system to face plenty of viruses.

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