Using Micro Algea As Food Additive For Reducing Viral Infection In Poultry

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Abstract

The present study was conducted to investigate the effect of *Spirulina platensis* and *Chlorella vulgaris* separately and a mixture of both on reduction of viral infection in poultry. In two farms in Monofia Gavernrate where chicks were infected with avain influenza virus (H5N1) . The infected chicks lossed ability of having feed and drink in the two farms also affect the quality and quantity of eggs (eggs not normal in size ,have bad quality , egg shells was very fragile and cracked) this was in the second farm . The experiment started by adding 1ml/L from a mixture of *Spirulina platensis* and *Chlorella vulgaris* (50:50) in the drinking water dially for one week .At the end of the experiment the performance of the infected chicks flock was improved at the first day of application of treatment and all over the treatment course, where the mortality rate was sharply , the feed and water consumption were increased and the vitality of the chickens was also increased . Also ,the boiler chicks in the second farm started laying eggs with a good quality and quantity . In the laboratory an experiment was done to investigate the effect of *Spirulina platensis* extract on 200 one day old Cubb broiler infected chicks (obtained from El-Ahram Poultry Company) . Chicks were fed on commercial ration. The boiler chicks were divided into 4 groups 50 chicks each in a separate battery. Feed and water are available ad libitum. First group has *Spirulina platensis* extract and vaccinated with Egy Flu vaccine at 14 day old .Second group has vaccination only ,third group has *Spirulina platensis* extract only. Fourth group neither having treatment with *Spirulina platensis* extract nor vaccinated with Egy Flu vaccine (at 14 day old).All the chicks were vaccinated gainst broiler viral diseases using IB Primer,clone vaccine (against ND),Inactivated vaccine against Newcastle disease (HIPRAVIAR-BPL2) and Gumboro 228 E strain .All groups Challenge by inoculating each chicken via intra nasal rout with 0.1 ml of the Highly Pathogenic Avian Influenza (HPAI) H5N1) virus containing 106 EID50. The first group of broiler chicks that received *Spirulina* extract (3 times per week)and vaccination with (Egy Flu vaccine) were significantly higher immune response (haemagglutination inhibition) (HI antibody titer) against Egy Flu vaccine than the second group that received Egy Flu vaccine only. Both G1 and G2 showed significantly higher HI antibody titer than G3 and G4 .

Also, the results revealed that the treated boiler chicks with *Spirulina* extract and vaccinated with S/C at 14 day old with Egy Flu vaccine chicks (all over 10 days post challenge with HPAI H5N1 virus) gave high resistance to Avian Influenza (HPAI) H5N1) virus and became as normal in having feed and drinking water and their was one dead chicken only during the period of the experiment .

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The broiler chicks that neither treated with *Spirulina* extract nor vaccinated with Egy Flu vaccine were unable to be feed but they have to drink water only and the number of dead chicks increased day by day during the period of the experiment .

Key wards : *Spirulina platensis* , *Chlorella vulgaris* , performance , immune system , characteristic , laying eggs of broiler chicks .

1. Introduction

Cultivation of *Spirulina* opened a new phase in the field of micro algae production ,because of a lot of additional advantages with respect to previously cultured microalgae among which:-a) less risk of external contamination due to its ability of growing under alkaline and salty condition. b) Easier digestibility , it has a soft cell wall made of complex sugar and protein. c) Higher protein content d) Lower nucleic acid content . e) Presence of value cell component like vitamin A , chlorophyll, essential fatty acids , etc . These advantages high make *Spirullina platensis* an optimal source of high value nutritional components **Pelizer *et al.(* 2002**). A new high molecular weight polysaccharide , with immunostimulatory activity has been isolated form *Spirulina* and called "Immulina " . This highly water – soluble polysaccharide represents between 0.5 % and 2.0% (w/w) of the dry microalgae **Pugh *et al* . (2001)**. In both *S. platensis* and *S. maxima*, total nucleic acid levels of 4.2-6% of dry matter have been reported **Santillan,(1974).**The photosynthetic pigment phycocyanin has a part to play in modulating the immune system. It has been seen to have an inhibitory effect on the release of histamine from mast cells during an allergic inflammatory response **Subhashini *et al*.(2004)**. An interesting study was carried out by preparing a hot water extract of *spirulina* and subjecting it to fractionation. A part of the fractionated product was found to inhibit the replication of several viruses, especially those with an envelope such as the measles virus, and the HIV-1 virus, in human T cells, peripheral blood mononuclear cells and Langerhans cells. This component was found to be a sulfated polysaccharide, calcium spirulan **Mishima *et al*. (1998) and Akao *et al*. (2009)**. Also calcium was seen to play an essential role in a dose-dependent manner for inhibiting the cytopathic role of such viruses **Grawish *et al* . (2009)** . The orally administered *Spirulina* or phycocyanin (the *Spirulina* holoprotein that contains PCB) can exert a wide range of anti-inflammatory effects. A heaping tablespoon (about 15 g) of *Spirulina* can be expected to provide about 100 mg of PCB. Also intaking 2 heaping tablespoons daily would be likely to have important antioxidant activity in humans **McCarty, (2007)**. *Spirulina* supplementation improved the activity of the phagocytic cells, namely monocyt/macrophages, heterophils and thrombocytes in chickens. They also proposed that 1000-10000mg kg- 1 (0.1-1.0%) range of dietary *Spirulina* supplementation in chickens would be safe to use in terms of improved immunocompetence without compromising performance characteristics of chickens .Chicken macrophages exposed to a water-soluble *Spirulina* extract show enhanced phagocytic activity in vitro suggesting activation of mononuclear phagocytic system function. Furthermore, dietary supplementation of *Spirulina* (1,000 ppm to 10,000 ppm) improved thymic weights, enhanced CBH (Cutaneous Basophilic Hypersensitivity Response response), increased tumor cell killing by NK (natural killer) cells and doubled the macrophage phagocytic potential over chickens fed a basal diet. Chicks on 1,000 ppm *Spirulina* diet cleared significantly more E. Coli from circulation at 30 and 40 minutes post i.v. inoculation. Similiar reduction was seen in clearing bacteria from spleen after 80 minutes post i.v. inoculation of Staph. Aureus in chicks fed 10,000 ppm over controls. Chicks in groups from 1,000 ppm to 16,000 ppm *Spirulina* treatments showed enhanced CBH response. Chicks in 1,000 ppm group exhibited enhanced E. Coli clearance between 30 to 60 minutes and decreased splenic bacterial counts

at 80 minutes post inoculation. These studies imply *Spirulina* enhances several immunological end points in chickens both during in vitro and in in vivo exposures **Qureshi *et al*.(1995)** . **James *et al*. (2006)** found that improved lymphoid organ development. Splenic, bursal and thymic weights were higher in the *Spirulina-fed* poults compared in those fed the control diet. *Spirulina* improves the intestinal flora in fish by breaking down indigestible feed components to extract more nutrients from the feed. *Spirulina* stimulates the production of enzymes that transport fats within the fish for growth instead of storage. *Chlorella* is an attractive potential food source because it is high in protein and other essential nutrients; when dried, it is about 45% protein, 20% fat, 20% carbohydrate, 5% fibre, and 10% minerals and vitamins due to this it has the ability to : • improve the immune system. • detoxify and heal the body.• improve digestion and elimination. • enhance growth and tissue repair, and maintenance. • protect against degenerative and chronic health problems. • slow down the ageing process **Belasco, (1997) .** *Chlorella* is an attractive food source because it is high in protein and other essential nutrients; when dried, it is about 45% protein, 20% fat, 20% carbohydrate, 5% fiber, and 10% minerals and vitamins **(Zelitch, 1971)** . **Aminot and Rey (2001)** found that *chlorella* is known to have the highest amount of chlorophyll compared to all other green algae and plants. The chlorophyll content in *Chlorella* can reach as high as 7% of its total weight and it contains 5 – 10 times more chlorophyll than *spirulina*, and 10 times more than alfafa. Due to its high chlorophyll content, it is also known as the "Supreme Whole Food Concentrate" .

Chlorella's rich source of beta-carotene, carotenoids, zinc, selenium, amino acids, calcium and nucleic sugars such as mannose, rhamnose, arabinose, galactose, and xylose also helps in the building of a strong and healthy immune system **Lee *et al* ., (2003).**Supplementation of *Chlorella* tablets may give a beneficial immunostimulatory effect to normal (uninfected) people by enhancing the *NK* cell activity and producing INF-γ and IL-12 as well as IL-1β, the Th-1 cell-induced cytokines. Helper T lymphocytes (Th) are divided into two functional subclasses, Th-1 and Th-2 cells based upon the cytokines that they produce and their effects on cell mediated and humoral immunity **Nair *et al*. (2002)** .*Chlorella* stimulates the activity of immune cells and macrophages by increasing interferon levels, thus enhancing the ability of the immune system to combat pathogens and foreign proteins. It has been shown that dietary *Chlorella* supplementation enhances the immune system in humans and animals **Queiroz *et al* . (2002) , Guzmán *et al* . (2003) and Halperin *et al* .(2003). Anonym, (1998)** in the Institute for Cereal Processing Ltd. (IGV, Nuthetal- Rehbruecke, Germany) showed an increase in the number of eggs laid by hens fed on food enriched by 1% of *Spirulina platensis*. The eggs were also bigger and of better quality as the ones from control hens .*Spirulina* has been shown to be an effective means of altering chicken product quality to meet consumer preferences. For instance, the total cholesterol content of eggs can be lowered by including *Spirulina* into layer hen rations **Sujatha and Narahari(2011).** This is mainly due to *Spirulina's* high antioxidant and omega-3 polyunsaturated fatty acids (PUFA) content that enriches the nutritional value of eggs at the expense of cholesterol content **Rajesha *et al*. (2011) and Sujatha and Narahari(2011)**. Egg yolk colour has also been found to intensify linearly with increased dietary *Spirulina* levels **Ross and Dominy (1990) , Sujatha and Narahari (2011).** In white Leghorn layer hens, dietary *Spirulina* levels of 3-9% of the total ration was found to result in egg yolk colours best representative of consumer preferences **Saxena *et al.* (1983)**. *Spirulina's* effect on yolk colour results from its high level content of zeaxanthin, xanthophylls and other carotenoid pigments, particularly β-carotene, which accumulate within the yolk **Anderson *et al*. (1991) and Takashi (2003).** These same compounds have been found to also accumulate within the muscle tissue of chickens. Both **Toyomizu *et al.* (2001) and Venkataraman *et al*. (1994)** have reported this outcome with muscle tissue increasing in yellowness and redness with increasing levels of dietary *Spirulina*.

Dietary *Spirulina* levels at 1% of the total ration in the week prior to slaughter has been found to result in broiler muscle tissue pigmentation at levels best representing consumer preferences **Dismukes *et al.* (2008).**In studies on laying hens, **Al-Batshan *et al*** . (**2001)** who found on their study that laying hens, the introduction of *Chlorella* into the diet in amount of 10% of the feed lead to laying more eggs, which were bigger and had more intensive yolk, and richer in vitamins and pigments (carotene and xanthophylls). **Rajesha *et al*. (2011**) showed that egg yolk colour score was higher in layers fed flaxseed diets with 5% *Spirulina* (w/w) compared to those on a flaxseed diet (20% w/w) .

The aim of this work is to study the effect of *Spirulina platensis* and *Chlorella vulgaris* extracts separately and a mixture of both on reduction of viral infection in poultry at Menoufia governorate. The viral infection which infect the chicks causing decrease in food consumption ,drinking water , vitality of the chickens and the quantity and quality of eggs for boiler chicks(shell eggs were very fragile and cracked and not normal in size)

2. Materials and Methods

Culture medium used for *Spirulina Platensis*

The medium used for the *Spirulina* is Zarrouk's medium **(Zarrouk,1966)** , The strain of *Spirulina platensis* was kindly supplied from the algal culture collection of Mansoura University.

Determination of dry weight :-

Was done according to **Yoshida *et al* ., 1996)** .

Culture medium used for *Chlorella vulgaris* :

The medium used for the *Chlorella* is Kuhl medium (**Kuhl and Lorenzen 1964)**.

The strain of *Chlorella vulgaris* was kindly supplied from the algal culture collection of Tanta University.

Determination of dry weight :-

Was done according to (**Yoshida *et al* ., 1996)** .

Chemical analysis

Algal biochemical composition for *Spirulina platensis* and *Chlorella vulgaris* :-

Proteins:-
The total proteins have been measured according to **(Bradford method 1976)**

Determination of total lipids:

Total Lipid was determined according to the standard method of Association of Official Analytical Chemists **(A.O.A.C. ,1984).**

Determination of total carbohydrates :

Total carbohydrates were determinate according to the method described in **A.O.A.C. (1975)**

Experimental Chickens :

200 one day old Cubb broiler chicks were obtained from El-Ahram Poultry Company . Chicks were fed on commercial ration. The chicks were divided into 4 groups each of 50 in a separate battery. Feed and water are available ad libitum.

Inactivated avian influenza vaccine:

Egy-flu is inactivated oil adjuvant treassorted avian influenza vaccine (H5N1 subtype, Egy/PR8-1 strain), produced by Harbin Veterinary Research Institute (HVRI). Batch No (2013001) , manufacture date 4/5/2013, Expiry date 3/5/2014. . The vaccine is inoculated via subcutaneous rout(S/C) at a dose of 0.5 ml /chicken

Other vaccines:-

a)Variant IB vaccine:-

IB Primer vaccine is live vaccine against IB,manufactured by Fort Dodge Animal Health.Batch NO. 1084293A. Manufacture date :4-2012.Expiry date:4-2014

b) IBD vaccine:-

Gumboro 228 Eis live vaccine against Gumboro disease,produced by Intervet international. Batch No (A080A3J01) , Manufacture date :7-2013, Expiry date 7/2015.

c)ND vaccine:-

Clone is live vaccine (freeze dried) for poultry against Newcastle Disease. Manufactured by MSD Animal Health CO . Batch No (1085419A), Manufacture date :7-2012,Expiry date 7/2014.

d) Inactivated vaccine against Newcastle disease:-

HIPRAVIAR-BPL2 wasproduced by laboratories HIPRA, S.A. Spain. Batch No (28VP-1) , Manufacture date 10/2013,Expiry date 10/2014.

Highly Pathogenic Avian Influenza (HPAI) H5N1 virus:

A locally isolated and identified HPAI H5N1 isolate of 2013 was kindly obtained from Prof. Dr. Samir Nasif CLEVB, and used as a challenge virus at a dose of 106 EID50/chicken via intra nasal rout.

Materials for Hemagglutinatin and hemagglutination inhibition tests:

a- Antigens and antisera For potency test:

The homologous antigen and its corresponding antisera were obtained from AI vaccine manufacture co. and used in HA test and HI test.The used antigen was:
Inactivated H5N1 Antigen (A/chicken/Egypt/18-H/2009) and its antisera.

b-Physiological saline:

It was prepared according to Cruickshank et al., (1975) by adding 8.5 g of Sodium Chloride in distilled water up to 1000 ml; PH was adjusted to be 7.2 and sterilized by autoclaving at 121°C for 20 minutes.

c-Sodium Citrate (3.8%):

Sodium Citrate ……...3.8 g:
Distilled water……up to ……....100 ml.
It was used as anticoagulant for preparation of chicken erythrocyte suspension

d- Chicken erythrocyte suspension:

Chicken blood taken in 3.8 % sodium citrate from adult (more than 3 week old), apparently health and susceptible chickens at ratio 0f 4:1 and subjected to three successive washing cycles by centrifugation at 3000 rpm for 5 minutes. It was used as 0.5 % concentration in physiological-buffered saline (PH 7.2) for plate haemagglutination (HA) and hemagglutination inhibition (HI) tests.

e- Serum sample:

Blood samples for serum collection were taken in dry, sterile tubes. The tubes were stoppard and left in a slope position at 37 °C for 1 hour. Serum samples were separated by centrifugation at 3000 rpm and kept in small vials at -20 °C till usage. Sera were inactivated at 56 °C for 20 minutes before testing.

2. Methods:

2-1: . Potency test: (OIE, 2012)

14 day old Broiler chickens, were vaccinated S/C with a field dose recommended by the manufacturer company. Blood samples were drawn weekly and the serum samples were separated, inactivated at 56ºC/30 and kept at -20 ºC till used. The serological analysis of AI antibody level against H5 was determined by HI test using the homologous AI antigen

2.1.1: The serological tests:.

2.1.1.1:Micro-plate haemagglutination (HA) test: (OIE, 2012)

0.025 ml of PS was dispensed into each well of a plastic V-bottomed microtiter plate.
0.025 ml of antigen was placed in the first well.
Make two-fold dilutions of 0.025 ml volumes of antigen across the plate.
Further 0.025 ml of PS was placed to each well.
0.025 ml of 1% (v/v) chicken RBCs was dispensed to each well.
Mix by tapping the plate gently and then allow the RBCs to settle for about 40 minutes at room temperature.
HA determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA (no streaming); this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

2.1.1.2 haemagglutination inhibition (HI) test: (OIE, 2012)

0.025 ml of PS was dispensed into each well of a plastic V-bottomed microtiter plate.
0.025 ml of serum was placed into the first well of the plate.
Two fold dilutions of 0.025 ml was made as volumes of the serum across the plate.
Add 4 HAU of antigen in 0.025 ml to each well and leave for a minimum of 30 minutes at room temperature (i.e. about 20 ºC) or 60 minutes at 4 ºC.
0.025 ml of 1% (v/v) chicken RBCs was added to each well. And after gentle mixing, allow the RBCs to settle for about 40 minutes at room temperature.
The HI titers the highest dilution of the serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (containing 0.025 ml RBCs and 0.05 ml PBS only) should be considered to show inhibition.
The validity of the results should be assessed against a negative control serum, which should not give a titer > ¼ (> 22 or > log2 when expressed as the reciprocal), and appositive control serum for which the titer should be within one dilution of the known titer.

2.2 Efficacy test:

The vaccinated and non vaccinated chickens were challenged via intra nasal rout with 0.1 ml of the HPAI H5N1 virus containing 10^6 EID_{50}/Chicken at the 28^{th} day PV. All the challenged chickens were observed for 10 days post challenge and all the dead bird were recorded.The protection percentage were calculated as number of dead chickens/number of servived chickens. The test will be valid if the negative non treated and non vaccinated chichens died withen 4 days post challenge.

2.3.Experimental design in laboratory

All the chicks in the experimente were vaccinated against broiler viral diseases using IB Primer (against IB) at 1^{st} day of age,clone vaccine (against ND) at 7^{th}days of age ,Inactivated

vaccine against Newcastle disease (HIPRAVIAR-BPL2) at 9th day of age , Gumboro 228 E strain at 13th day of age. **And treated as the following table:**

Table (1)

Treatment , vaccination for broiler chicks at 14 day old and its challenge

No of the group	Treatment with* *Spirulina* extract	Vaccination** At 14 day old	Challenge ***
G1	Yes	Yes	Yes
G2	No	Yes	Yes
G3	Yes	No	Yes
G4	No	No	Yes

G1 (group 1) , G2 (group 2) , G3 (group3), G4(group4)

***Spirulina* extract was added to the drinking water at a rate of 3 times/week and a dose of ml/Liter drinking water.

** Vaccination via S/C rout at a dose of 0.5 ml / chicken at 14 day old.

*** Challenge by inculating each chicken via intra nasal rout with 0.1 ml of the Highly Pathogenic Avian Influenza (HPAI) H5N1) virus containing 10^6 EID_{50} at the 28th day post vaccination

2-4 : Experimental design of farm 1:

1-The first farm in Menoufia Governorate has about more than one thousand chicks were infected by avain influenza vaccine (H5N1) plates (1,2 and 3) .

2- The experiment started by using 1ml/L from mixture of *Spirulina platensis* and *Chlorella vulgaris* (50:50) in the drinking water dially for one week .

3- Observation was done every day to follow up mortality , vitality , food consumption and drinking water .

2-5: Experimental design of farm 2 :

1- The second farm in Menoufia Governorate has about 5,000 layer chicks this farm was infected by (H5N1) plates (1,2,3 and 4) .

2- The experiment started by using 1ml/L from mixture of *Spirulina platensis* and *Chlorella vulgaris* (50:50) in the drinking water dially for one week .

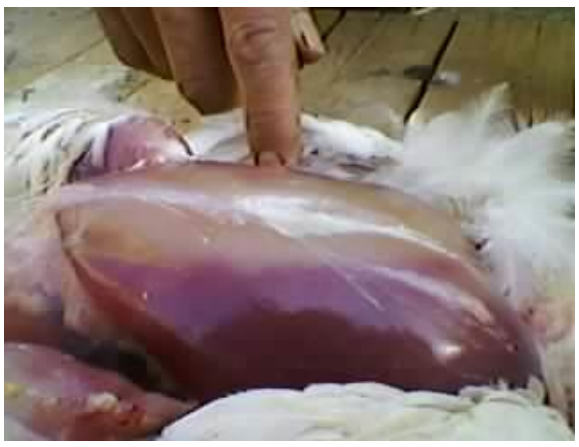
3- Observation was done every day to follow up mortality , vitality , food consumption , drinking water and egg production .

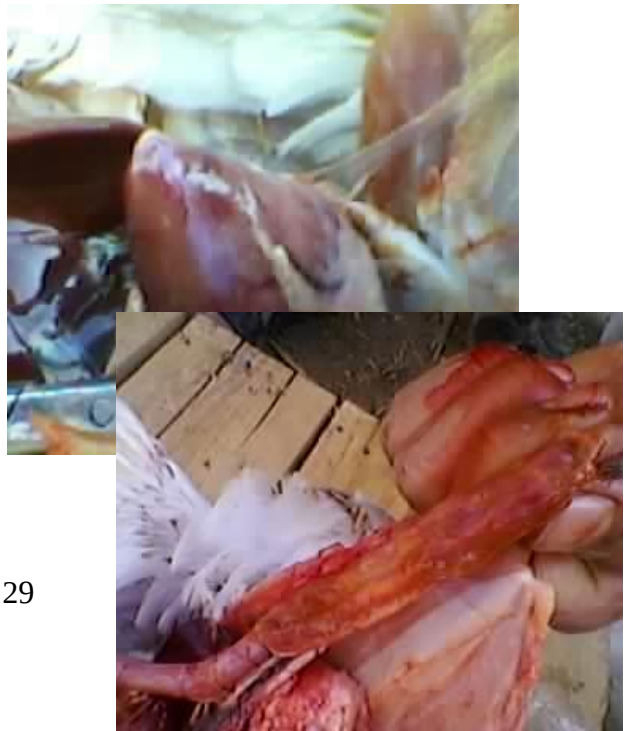
Before treatment:

Plate (1): As infected broiler chickens 25 day old depression dullness , anorexia and unable to move .

Plate (2) : Al infected broiler chickens showing high mortalities

Plate (3): Swelling of the liver, heart and blood in the intestines and diarrhea





Plate(4): Egg shells was very fragile and cracked

3. Results and Discussion

3.1 Results

Table (2):The biochemical analysis of *S. platensis* biomass grown in Zarrouks basal medium (1966)

Component	Concentration (%w/w)
Crude protein	62.5
Lipids	8.5
Total carbohydrate	11
Fiber	8
Minerals	10

Table (3):The biochemical analysis of *Chlorella vulgaris* biomass grown in kull medium(1962)

Component	Concentration (%w/w)
Crude protein	52
Lipids	17
Total carbohydrate	14
Fiber	7.5
Minerals	9.5

Table (4) : Efficacy of *Spirulina* extract on the immune response of chickens against ai inactivated avian influenza vaccine

Table (4) showed that the group (1) of broiler chickens that received *Spirulina* extract 3 times per week and vaccinated with Egy Flu vaccine were significantly higher immune response (haemagglutination inhibition) (HI antibody titer) against Egy Flu vaccine than group (2) that received Egy Flu vaccine only. Both G1 and G2 showed significantly higher HI antibody titer than G3 and G4 as in Figure (1).

	HI antibody titers against Homologous antigen				**Protection %**
Chicken groups	1st week PV	2nd week PV	3rd week PV	4Th week PV	
G1	38	148	410	550	95 %
G2	30	141	362	522	90 %
G3	10	10	10	10	15 %
G4	0	0	0	0	0

Table (5): Mortality pattern and protection % of the challenged Chicken groups all over 10 days post challenge with HPAI H5N1 virus:

Group	Challenge	No. of bird	Deaths at day post challenge										Total of deaths	protection %
			1	2	3	4	5	6	7	8	9	10		
1	28th DPV	20						1					1	95
2	28th DPV	20				1	1						2	90
3	42nd DO	20			3	5	3	2	2				17	15
4	42nd DO	20		3	12	5							20	0

G1 :Broiler chickens treated with *Spirulina* extract and vaccinated S/C at 14 day old with Egy Flu vaccine ; G2; vaccinated S/C at 14 day old with Egy Flu vaccine only ; G3: Broiler chickens treated with *Spirulina* extract only; G4 : Broiler chickens did not treated with *Spirulina* extract nor vaccinated with Egy Flu vaccine (Negative Control group). All groups were challenged at the $th week PV with the highly pathogenic avian influenza virus H5N1 that isolated during 2014 as in Figures (2,3 and 4). S/C (Subcutaneous rout) , DPV(Day Post vaccination) ,DO (Day old)

Table (5) : showed that 20 chickens from each group were challenged with the Highly Pathogenic Avian Influenza Virus at a dose of 10^6 EID_{50} /chicken and observed for 10 days post challenge , Table (5) Showed the mortality pattern that clearly demonstrate that *Spirulina* treatment significantly increased the protection % in the vaccinated and treated group (G1,95%) compared with the only vaccinated group (G2,90%), Besides increasing the protection % and changing the mortality pattern in the treated non vaccinated group (G3, 15% and take longer time) compared with the non treated non vaccinated group (G4, 0% and all the chickens died withen 84 hours).

The observation on farm (1)

Table (6) The chicks mortality , vitality , food consumption and water drinking at the first farm in Monoufia Governorate and during one week of the experiment.

Table (6) revealed that the performance of the infected chicks flock was improved at the first day of application of treatment and allover the treatment course, as the mortality rate was sharply decreased and the feed and water consumption were increased also the vitality of the chicks was also increased after they became better plates (5,6,7 and 8) in the last of week they re-infected again by E- Coli because of the farm conditions became poor in ventilation and hygiene as in Figures (5,6 and7) .

Farm	Natural		During infection		During treatment		
In Menoufia Governorate has about more than one thousand chick	Feeding	Drinking water	Feeding	Drinking water	Feeding	Drinking water	Dead on the first day of the infection was 60-70 then almost about 50 and then 12 on the last day of treatment then increased as a result of infection with E-coli
	700-800 kg/24h	800-900/ 8h	500-550kg/24h	500/11h	550kg	500-550 /9h	
			400kg	400/11h	550kg	600/9h	
			300kg	300/10h	600kg	650/8h	

The observation on farm (2) :-

Table (7): The chicks mortality , vitality , food consumption , water drinking and quantity and quality of eggs at second farm in Monoufia Governorate during one week of the experiment .

Farm	Natural		During infection		During treatment		
Farm in Menoufia Governorate has about 5,000 chicks and eggs were produced per day	Feeding	Drinking water	Feeding	Drinking water	Feeding	Drinking water	Dead on the first day was more than 800 and then began gradually reduced to about 600 and then 200 and then increased again due to infection with E- coli .Infection have also been reported in the quantity and quality of eggs and egg shells was very fragile and cracked
	2000 kg/24 h	1000/ 8h	900-1000 kg	800/10 h	850kg	850/9h	
			800kg	700/10 h	900-950kg	900/9h	
			700kg	600/10 h	950-1000kg	900/8h	

Table (7) revealed that the performance of the infected flock was improved at the first day of age application .The improved parameters are the mortality rate which decreased sharply from 50 chicken per day to 12 per day allover 3 days, the feed and water consumption and vitality of the chickens were also increased also,the chicks in this farm were put eggs dially .Because of the infection the eggs were not normal in size ,have bad quality , egg shells was very fragile and cracked after they became better in the last week they re-infected again by *E- Coli* because of the farm conditions became poor in ventilation and hygiene as in Figures (8,9 and10) plates (5,6,7 and 8).

After treatment

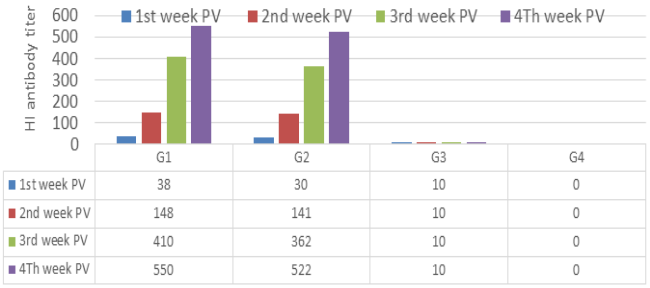
Plate(5): The chickens to be alert and the mortality rate begins to be decreased one day post treatment with *Spirulina platensis*

Plate(6): At the second day post treatment the chickens showed more albert and the mortality slightly decreased.

Plate(7): Fifth day post treatment the majority of most the chickens showed normal and healthy status with significant reduction in mortality rate .

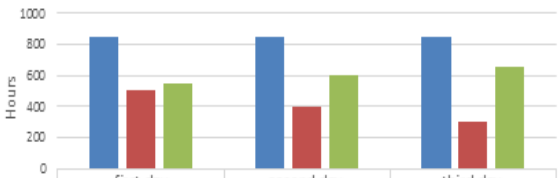
Plate(8): Fifth day post treatment the majority of most of the chickens showed normal and healthy status with significant reduction in mortality rate .

Figures



	G1	G2	G3	G4
1st week PV	38	30	10	0
2nd week PV	148	141	10	0
3rd week PV	410	362	10	0
4Th week PV	550	522	10	0

Figure (1):- HI antibody titers against Homologous antigen



	first day	second day	third day
control	850	850	850
during infection	500	400	300
during treatment	550	600	650

Figure (6):-Water drinking at first farm in Monoufia governorate and during one week of the experiment.

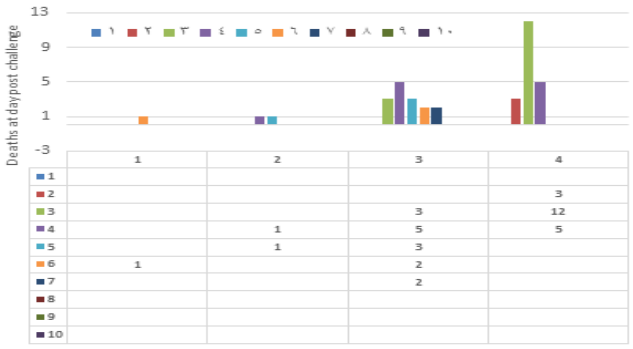


Figure (2):Mortality pattern and protection % of the challenged Chicken groups all over 10 days post challenge with HPAI H5N1 virus

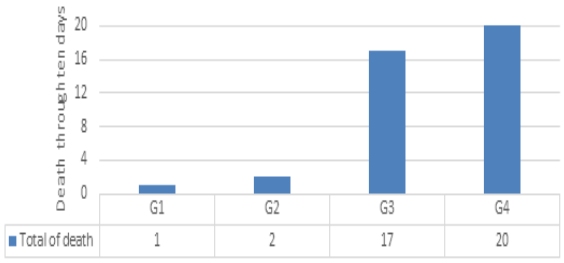


Figure (3);Mortality pattern and protection % of the challenged Chicken groups all over 10 days post challenge with HPAI H5N1 virus

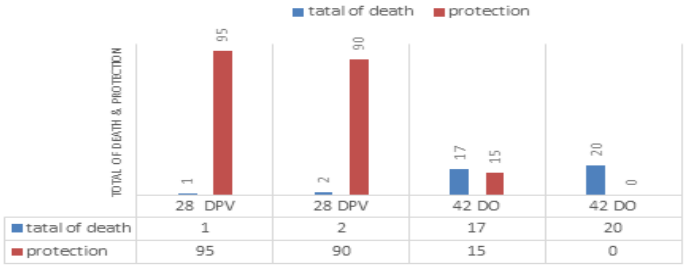


Figure (4):Mortality pattern and protection % of the challenged Chicken groups all over 10 days post challenge with HPAI H5N1 virus

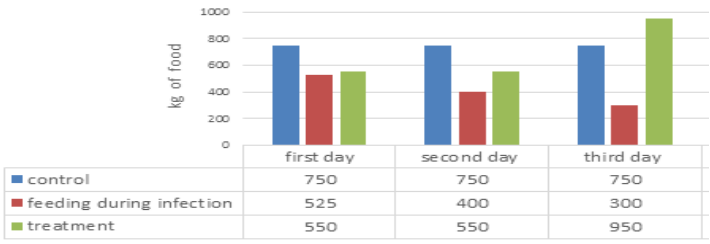


Figure (5) :-The mortality , vitality and food consumption at the first farm in Monoufia governorate and during one week of the experiment

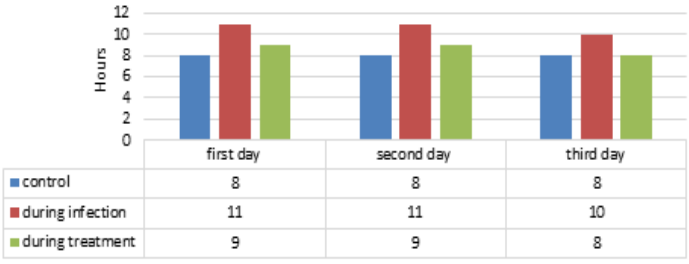


Figure (7) :-Consumption of water in first farm during infection and during treatment.

Figure (8):-The mortality, vitality and food consumption at the second farm in Monoufia governorate and during one week of the experiment.

Figure (9):-Water drinking at second farm in Monoufia governorate and during one week of the experiment.

Figure (10):-Consumption of water in second farm during infection and during treatment

3.2 Discussion:

Amha (2002) noted that *Spirulina platensis* rich content of protein, vitamins, essential amino acids, minerals, and essential fatty acids. *Spirulina* is 60-70% protein by weight and contains a rich source of vitamins, especially vitamin B12 and provitamin A (β-carotene), and minerals. One of the few sources of dietary γ-linolenic acid (GLA), it also contains a host of other phytochemicals that have potential health benefits this match with the present results that showed *Spirulina platensis* contain 62.5 % from protein so it considerd an excellent source of protein , 8.5 % lipids , 11% from carbohydrate , 8% fiber so it is a good source in digestion and 10% minerals as in Table (2) . **Halle *et al* .(2009)** noted that *Chlorella* is a green microalgae that contain plentiful nutrients including essential amino acids and

vitamins.This match with the present results which revealed that *Chlorella vulgaris* contain 52 % from protein so it considered an excellent source of protein , 17 % from lipids so it considered a good source for biodiesel , 14% carbohydrate , 7.5% fiber so it is a good source in digestion and 9.5% minerals as in Table (3) .

Jensen and Drapeau (2001) found that the improved response on microbial community of the intestine might be attributed to the high nutrient concentration of cell wall of fresh liquid *Chlorella* (FLC). In addition, a large amount of chlorophyll and fibrous cell walls in *Chlorella* is an important factor to increase the beneficial lactic acid bacteria in the gut of the broiler chicks. In this study, FLC was provided to the experimental birds without applying any processing techniques. Naturally, FLC contains all nutrients including vitamins, minerals, fiber, chlorophylls, and chlorellin. Chlorellin fights only against pathogenic organisms without destroying beneficial microflora in the intestine, which in turn has the ability to improve the health and productivity of broiler chickens.This match with the present results where revealed that *Chlorella* contains a large amount of fibers and this fibers are useful in digestion and also using cells of *Chlorella* as it is without any processes gave the best results Table(3).

The present study revealed that *Chlorella* rich with minerals which improve the immune system (Table 3) this match with **Halle *et al* . (2009)** who found that *Chlorella* is reach in selenium . Results indicate numerical improvement in cell immune in this study so it could be related to high amounts of selenium in *Chlorella*. Each kind of improvement in immune system could cause increase bioenergetic efficiency in body so increase body weight and decrease feed conversion ratio. Therefore it appears logical that performance increase in *Chlorella* and commercial treatments.

In the present study a mixtre of *Spiulina* and *Chlorella* extracts enhanced the immune system and vitality of chicks when 1 ml was added to the drinking water (Table 4) while adding 1 ml/l in drinking water of *Spiulina* extract only for four groups . The results (Table 2) showed that the group that received *Spirulina* extract 3 times per week and vaccinated with Egy flu vaccine were significantly higher immune respose (HI) antibody titer against Egg flu vaccine (95%) than group 2 (90%) that received Egy flu vaccine only. Both group 1 and 2 showed significantly higher HI antibody than group 3 and 4 (Table 4 and Figure 1) . Also, the results of the effect of *Spirulina* on challenged chicks all over 10 days post challenge with HPAI H5N1 virus revealed that broiler chickens treated with *Spirulina* extract and vaccinated with S/C at 14 day old with Egy Flu vaccine (first group) gave high resistance to Avian Influenza (HPAI) H5N1) virus and become as normal in having feed and drinking water and their was one dead chicken only during the period of the experiment while the broiler chickens neither treated with *Spirulina* extract nor vaccinated with Egy Flu vaccine were unable to be feed but they have to drink water only and the number of dead chicks were increased day by day during the period of the experiment (Table 5 and Figures 2,3 and 4) These results agree with **Qureshi *et al* .(1996)** who noted that Chicks fed *Spirulina* levels of 10 g/kg of diet had increased NK-cell activity compared to the control group, showing an enhanced disease resistance potential .

Also **Mason (2001)** reported that many microalgae, particularly *Spirulina* and *Chlorella*, are good sources of β carotene, vitamin B12, and β-glucan, an immunoregulatory polysaccharide component that plays a vital role in the body immune functions and inflammatory processes . Gamma-interferon is a protein produced by immune cells and protects the body from infections. *Chlorella* stimulates the activity of immune cells and macrophages by increasing

interferon levels, thus enhancing the ability of the immune system to combat pathogens and foreign proteins.

Al-Batshan *et al.*(2001) achived that chicken phagocytic activity had an incremental linear increase with increasing dietary *Spirulina* levels of 0.5, 1 and 2% of diet .

Willis *et al* . (2007) noted that therefore, using nutritional methods to support immune system could be a useful tool. Utilization of feed additives is the one of the most important methods for instance using prebiotic is profitable in broilers because prebiotics by adhesion to intestine lymphoid tissue could stimulate mucosal immune system.

Kang *et al*. (2013) reported that the number of white blood cells (WBC) was significantly higher ($P < 0.05$) in broilers fed fresh liquid *chlorella* (FLC) compared with dried *chlorella* powder (DCP), and the number of lymphocytes was also significantly higher ($P < 0.05$) in FLC compared with virginiamycin as antibiotic growth promoters A(AGP) and DCP. The (WBC) indicate the response of immune system these results matche with the present results which indicate that by adding 1ml/l from a mixture of *Spiulina* and *Chlorella* enhance immune system also, when adding1 ml/l from *Spiulina* only (Table 4) .Tables (6 and 7) and Figures (5,6,7,8,9 and10) revealed that the performance of infected chicks was improved at the first day of application of treatment (with a mixture of *Spiulina* and *Chlorella)* in the drinking water and allover the treatment course as the mortality rate was sharply decreased , the feed and water consumption were increased and the vitality of the chicken was also increased. These results match with that of **Sharma (1991)** who noted nowadays hypersensitivity to diseases is the most important problem in broilers farms. In this way annually, different kinds of diseases are occurred in flocks that are caused lots of mortality and losses. Digestive system completes immune system in poultry thus each alteration in intestine environment which occurred by feed could affects mucosal immune system in poultry. The mixture from *Chlorella* and *Spirulina* inhance immune system and mortality . **Gružauskas *et al.* (2004)** reported that *Spirulina* improve absorption of minerals, protect from diarrhea, and optimize nutrient digestion processes. Feeding *Spirulina platensis* containing diets may increase the lactobacillus population and enhance the absorbability of dietary vitamins .

Kaoud (2012) and Kharde *et al.* (2012), who reported that feed conversion ratio significantly ($P<0.05$) improved by dietary inclusion of *Spirulina platensis* as compared with the control broilers. They also reported that *Spirulina platensis* supplementation significantly decreased mortality rate of broilers. The present results revealed that the amount of natural feeding of 700-800 and 2000 kg/24 h for the two farms respectively , also the drinking water of 800-900 and 1000/8 h for two farms respectively decreased day by day during the infection with H5N1 virus (500-550 kg / 24 h)(500/11h) but after drinking water with a mixture of *Chlorella* and *Spirulina* extract they became better having feed and drinking water also (Tables 6 and 7) and (Figure 5,6,7,8,9 and10) , plates (5,6,7 and 8). These results match with that of the **Mariey *et al*. (2014)** who found that total feed intake for the whole experimental period was significantly ($P<0.05$) higher for birds fed 0.3 and 0.2 g *Spirulina*/kg diet (3591.16 and 3568.87 g), followed by birds fed 0.1 g and 0.0 g *Spirulina*/kg diet (3554.82 and 3551.67 g), respectively.Also chicks fed dietary *spirulina platensis* had significantly ($P<0.05$) obvious improvement of viability rate, being 99.46% for birds fed 0.2 or 0.3 g *Spirulina*/kg diet and 98.63% for birds fed 0.1 g *spirulina*/kg diet versus 96.33% for the control birds. A significant improvement in feed conversion ratio was achieved by birds fed *spirulina* diets may due to at least in part, an improvement in live body weight or the improvement of viability percentage.In the present study as in Tables (6 and 7) and Figures

(5-10) 1 ml/l of the two species *Chlorella* and *Spirulina* was added to the drinking water during the infection of chicks with H5N1 because during the infection chicks unable to be feed but have to drink water only so adding the mixture in the drinking water at the end of the experiment enhance their feeding and leed to improvement in quality and quantity of eggs as eggs shell and eggs yolk. These results matches with that of **Anonym, (1998)** who reported that an increase in the number of eggs laid by hens fed on food enriched by 1% of *Spirulina platensis*. The eggs were also bigger and of better quality as the ones from control hens. **Rajesha *et al.* (2011)** they showed that egg yolk colour score was higher in layers fed flaxseed diets with 5% *Spirulina* (w/w) compared to those on a flaxseed diet (20% w/w).

Al-Batshan *et al* . (2001) found on their study on laying hens that the introduction of *Chlorella* into the diet in amount of 10% of the feed lead to laying more eggs, which were bigger and had more intensive yolk, and richer in vitamins and pigments (carotene and xanthophylls).

Sakaida (2003) and Nikodémusz *et al*. (2010) observed that feeding *Spirulina*-containing diets gave a beneficial effect on productive performance of laying hens. **Sujatha and Narahari (2011)** noted that egg yolk carotenoids pigment and omega-3 fatty acid levels increase when White Leghorn hens fed 150 g flaxseeds + 200 mg vitamin E + 3 g *Spirulina* per kg diet .

4.Conclusion:

Avian Influenza (HPAI) H5N1) virus is the most common cause of losses, not only in large commercial flocks, but also in backyard chickens. Mostly, they do not respond to drug therapy. Prevention and control relies on vaccination where this is effective but the vaccination is very expensive .We conclud that using *Spirulina platensis* and *Chlorella vulgaris* as it is with vaccine give high immune response for the infected chicks and they became healthy. Also,it is easy to obtain them and cheap in price.

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الملخص باللغة العربية

استخدام بعض الطحالب الدقيقة كأضافات غذاء للدواجن لأنخفاض معدلات الاصابة االفيروسية

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٤)المعمل المركزى للرقابة على المستحضرات الحيوية البيطريه

أجريت هذه الدراسة الحاليه لمعرفة تأثير *Spirulina platensis* و *Chlorella vulgaris* على حده او مزيج منهما معا للحد من الاصابه الفيروسيه فى مزرعتين للدواجن بمحافظه المنوفية حيث يوجد دواجن مصابه بأنفلونزا الطيور (HSN1) حيث فقدت الدواجن المصابه قابليتها للشرب والأكل وفى احدى المزرعتين كان لهذه الاصابة تأثير فى إنتاجية البيض للفراخ البياضه من حيث الجوده و الكميه (حجم البيض – الكميه – قشر البيضه كان هشا وسهل الكسر)

بدأت التجربة المعملية بإضافه ١ مللى/ لتر من خليط ا*Spirulina platensis* و*Chlorella vulgaris* بنسبه ٥٠% الى ٥٠% فى ماء الشرب يوميا ولمده اسبوع واحد – فى نهاية التجربة بدأ تقدم ملحوظ فى أداء الدواجن المصابه من أول يوم من المعامله وخلال فتره التجربه أيضا حيث أنخفض معدل الوفيات بشده وزاد إستهلاك الماء والطعام وذادت الحيويه أيضا وبدات الدواجن البياضه فى المزرعه الثانيه بوضع البيض بصوره جيده كميا ونوعيا.

أجريت تجربة معملية أخرى لدراسه تأثير *Spirulina platensis* على مجموعة فراخ بياضه مصابه عددها ٢٠٠ دجاجه عمر يوم واحد (تم الحصول عليها من الأهرام للدواجن) تتغذى على غذاء تجارى – تم تقسيم هذه المجموعه الى اربع مجموعات تحتوى كل مجموعه على ٥٠ دجاجه كل مجموعه فى بطاريه على حدة حيث يعطى لها الماء والطعام حسب الرغبه وجميع الدواجن مطعمه ضد أمراض فيروسيه تمهيديه (IB) عند اليوم الأول من العمر ومصل (ND) عند اليوم السابع من العمر ومصل نيوكاسل (HIPR AVIAR BP12) عند اليوم التاسع من العمر ومصل (جمبرو E ٨ ٢٢) عند اليوم الثالث عشر من العمر.

بالنسبه للمجموعة الاولى التى تناولت *Spirulina platensis* وتطعيمها بمصل (Egy Flue) عند اليوم الرابع عشر والمجموعه الثانيه اخذت المصل فقط والمجموعه الثالثه تناولت الطحلب فقط والمجموعه الرابعه لم تتناول لا المصل ولا الطحلب .

تم فصل من كل مجموعة ٢٠ فرخ على حدة وتم اعطائهم عن طريق الأنف / ٥٠ مللم من خليط (HSN1)(HPA1) تطعيم قوى لمصل أنفلونزا الطيور تحتوى ١٠٦ E1D-50 عند ٢٨ يوم.

اثبتت النتائج أنه بالنسبه للمجموعة الاولى التى تناولت الطحلب (ثلاث مرات بالاسبوع) ومصل ال Egy Flue أثبتت مناعه قويه ضد المرض عن المجموعه الثانيه المطعمه فقط بالمصل.

اما المجموعتان الاولى والثانيه فقد اثبت عيارا مميزا للأجسام المضادة (HI) ضد مصل Egy Fluعن المجموعتان الثالثه والرابعه كما أثبتت النتائج أن الفراخ التى تناولت طحلب الاسبيرولينا والتى تم اعطائها عند اليوم ١٤ من العمر مصل Egy Flue أظهرت مفارقه كبيره للانفلونزا (HSN1)(HPA1) وأصبحت طبيعيه فى تناول الاكل والشرب ولم يحدث سوى حالة وفاة واحده خلال فتره التجربه أما بالنسبه للفراخ التى لم يتم تطعيمها بمصل ولم تتناول طحلب الاسبيرولينا كانت غير قادرة على تناول الاكل لكن كانت تتناول الماء بشراهه وكان معدل الوفيات يزداد يوم بعد يوم خلال فترة التجربة.