Lymphocytic Proliferation And Interleukin-2 Production In Chicken Immunized With Avian Influenza (H5N1) Vaccine Under Growth Promoters Supplementation

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ABSTRACT

The efficacy and safety of growth promoters as immunopotentiators in chicken vaccinated with avian influenza (H5N1) have been extensively evaluated under laboratory conditions. In this study, 210 one day old chick were allotted into seven groups, group 1 as a control, group 2, 3, 6 and 7 were fed on basal diet supplemented with probiotics Reomin and Digestamin at recommended dose and double the recommended doses. Group 4 and 5 were supplemented with Gibberellic acid through drinking water (single and double doses) which is classified as plant hormone with androgenic features. All chicken were vaccinated twice three weeks interval against H5N1. Blood samples and serum samples as well as tissue specimens were collected for assessment: T-Lmphocyte Proliferation Assay (LPA), differential leucocytic count, serum total protein, albumin and Interleukin (IL-2) production. In addition wattle dermal reaction.

The Gibberellic acid treated groups showed the highest lymphocytic count and IL-2 production. Meanwhile chicken treated with probiotics showed highest values of LPA, serum total proteins and globulins as well as wattle dermal thickness .These results indicated that the evaluated growth promoters are safe and efficacious for enhancing immune response to H5N1vaccine, and reduce economic losses that H5N1 virus outbreaks can produce in poultry flocks.

INTRODUCTION

Highly pathogenic avian influenza H5N1 is a subtype of genus A of the Orthomyxoviridae family, a deadly threat to the world poultry production, may result in flock mortalities as high as 100%. Nowadays, the main control strategic measures available in Egypt are effective disease surveillance, enhanced biosecurity of the poultry farms and the proper use of effective and potent vaccines.

Immunosuppression resulting from mycotoxins, Gumboro disease, Marek's disease, chicken anemia virus and other adverse environmental factors has important effect on the protection levels achieved by vaccination program against H5N1. Alternatively, there are several immunomodulatory agents that are capable of stimulating immune responsiveness of chicken to vaccines.

So a great attention was paid toward "probiotics" which act as growth promoters that keep intestinal microbial balance in a positive way, in order to enhance life performance. The beneficial effect of probiotics might be mediated by a direct antagonistic effect against specific pathogens *(1)*, improving their metabolism and feed efficiency *(2)* and/or stimulation of immunity *(3)*.

This work was planned to study the immunomodulatory effect of some growth promoters on cell mediated criteria in chicken vaccinated against H5N1 as judged by LPA,IL-2 production, Lymphocytic count and wattle dermal reaction.

MATERIAL AND METHODS

A –Experimental birds

Two hundred and ten (210) one day old Sasso chick (50% males and 50% females), were housed in separate units (floor reared), under similar managmental and hygienic conditions. Chicks were weighed and randomly allotted into 7 groups, (30 chicks each). Feed and water were provided *ad-libitum*.

Chicken were fed on a commercial starter diet (23% crude protein and 3000 k cal metabolisable energy/kg feed) for the first week and then switched to grower and finisher diet

supplemented with experimental treatments until 65 days old.

B- The growth promoters

1-Gibberillin (Brelex®, valent distributor, each tablet contains 0.92gm Gibberillic acid GA3).

2-Roemin contains active Lactobacills bacteria 10^8 cfu/gm (China Wax Carevet).

3-Digestamin contains fermented soybean meal, fermented grass (lactic acid bacteria 10^5 cfu/gm) and horseradish. (Gemeinschaft, F.U.H. Egger, GMbH, "P.G.E." Austria).

C-Vaccines

H120-B1 Hitchner, Lasota and Gumboro live attenuated vaccines (Izo S.P.A.) Italy, were used.

Rassortant Avian influenza virus inactivated vaccine (H5 Subtype, Re-1 strain). Produced by Yebio Bioengineering Co., Ltd. f Qing Dao, China. Imported by Kemit. Company. Batch. No. 2008045. Vaccines were used according to recommended protocol.

D-The experimental design

Seven experimental groups were used in this study. The 1st group was the control one; the chicks were fed on basal diet only. The 2nd and the 3rd groups received Reomin in the diet at low (1gm/ kg of ration) and double dose (2g / kg) respectively. The 4th and 5th groups received Gibberellin at low (0.325 mg/L water) and double dose (0.65 mg/L water) respectively. The 6th and 7th groups received Digestamin, in the diet at low (6 gm/kg) and double dose (12g/kg) respectively.

E- Haematological examination

1-Blood samples

Blood samples were used for differential leucocytic count.

2-Serum samples

Serum samples for analysis of some blood chemistry parameters. Total serum protein *(4)* and serum albumin *(5)* were colorimetrically measured using commercial kits (bioMerieux, France)

Globulin was estimated by subtracting the albumin concentration from total Protein.

F-Evaluation of cell mediated immunity

1-Lymphocyte Proliferation Assay (LPA)

LPA measures the ability of lymphocytes placed in short- term tissue culture to undergo colonel proliferation when stimulated *in vitro* by a foreign antigen *(6).*

LPA was measured at two point- time during the study: at 2 weeks after the first dose of vaccination and at 2 weeks after the second dose of vaccination against avian influenza.

Chemicals and reagents were obtained from JRH Bioscience Sera-Lab. Ltd. Co. and Sigma chemical Co. USA.

2-Assay of IL-2 production

Tissue specimens

Two weeks post revaccination, 3 birds from each replicate were sacrificed. Specimens from liver were collected and stored in liquid nitrogen at-196 ℃ until further extraction of RNA by TRIzol reagent (Life Technologies, Inc., Grand Island, NY, USA) and estimation of interleukin (IL-2) by Reverse Transcriptase RT -PCR.

Primer sequences for RT-PCR.

Primer sets for chicken IL2:-

Forward Primer 1:5` – CTTTGGCTGTATTTCGGTAGC- 3'

Reverse Primer 2:5` – AAGTTGGTCAGTTCATGGAGAA – 3'

3 - Wattle dermal reaction

At the 63rd day of age 0.1ml from avian influenza vaccine was injected intradermally into the right wattle and the thickness was measured for three successive days as a direct method for measuring cell mediated immunity *(7).*

Data obtained in this study representing the different variables were statistically analyzed *(8).*

RESULTS AND DISCUSSION

The poultry industry is facing a ban on the use of antibiotics as feed additives in many parts of the world. Consequently, there is a growing interest in finding viable alternatives

for disease prevention and growth enhancing supplements. The effect of probiotics as natural additives have gained remarkable public interest and importance by proving their efficiency and obvious positive effects on animal health, which improve the balance of microflora involved in digestion and enhance the general immune system of chicken.

In many tropical areas including Egypt, highly pathogenic avian influenza (H5N1) is a common major threat to poultry industry inducing devastating epidemics with dramatic economic losses. So, the current trial was designed for improving the immune status of chickens vaccinated against H5N1 using growth promoters through dietary supplementation.

The criteria for evaluation of immunomodulatory features of studied growth promoters

Lymphocytic Proliferation Assay (LPA)

The results of the lymphocyte proliferation assay are shown in Table 1. The T-lymphocyte proliferation reflects the change in cellular immunity in chicken .The results revealed that there was a highly significant increase in LPA in all the treatments which received the growth promoters at 3 and 7 weeks of age in low and double doses.

This increase may be attributed to the antigenic load resulting from lactobacillus bacteria, which induce stimulation of the immune system. Probiotics stimulate the immunity of the chicken in two ways: flora from probiotic migrate throughout the gut wall and multiply to a limited extent or antigen released by the dead organisms are absorbed and this stimulates the immune system *(9)*. The lactic acid producing bacteria present in Reomin and Digestamin could interact with microfold cells which activate Payer's patches lymphocyte to be librated from the intestine and reach the circulation *(10)*. Moreover, the wall of lactic acid producing bacteria is mainly composed of peptidoglycans and polysaccharides stimulating macrophages to release IL2 and 1L1 which are mainly concerned with activation of lymphocytes *(11)* . Also, the improvement in LPA performed by the Gibberellin to the increase of lymphocytes intracellular cyclicguanosine mono-phosphatase which stimulates blast transformation *(12, 13)*.

Table 1. Evaluation of cellular immune response by lymphocytic proliferation assay:

Groups		At 3 weeks of age	At 7 weeks of age
Control		0.15 ± 0.01 (d)	0.20 ± 0.01 (e)
Reomin	R.d	0.15 ± 0.01 (d)	0.33 ± 0.00 (bc)
	D.rd	0.23 ± 0.01 (bc)	0.33 ± 0.01 (bc)
Gibberellin	R.d	0.32 ± 0.01 (a)	0.32 ± 0.01 (cd)
	D.rd	0.25 ± 0.01 (b)	0.29 ± 0.01 (d)
Digestamin	R.d	0.31 ± 0.01 (a)	0.36 ± 0.02 (b)
	D.rd	0.21 ± 0.01 (c)	0.41 ± 0.00 (a)

In the same column means followed by the same letter are not significantly different at 0.01 level of significance. R.d= recommended dose-D.rd= double recommended doses

During the primary influenza infection or vaccination, the viral clearance depends on CD8 T lymphocyte *(14)*. Meanwhile, T. helper cells can be further subdivided into Th1 and Th2 according to the cytokines produced, influenza infection induces strong Th1 response (1L2 and INF). Some evidence indicates that protective immunity is mediated by Th1 like responses *(15)*.

Differential leucocytic count

The differential leucocytic count for chicken fed different levels of growth promoters are illustrated in Table (2). The results revealed that there was highly significant increase in lymphocyte present in all groups as compared to the control group. This may be attributed to IL2 which might enhance the cytotoxicity of the macrophages and secret IL1; enhance immunoglobulin synthesis and proliferation of B lymphocyte, enhancing proliferation of T. cells and natural killer cells. The lymphocytosis which appears in the differential leucocytic count is the suggestive of the immunogenic stimulation as the lymphocytes play a major role in the humoral and cell mediated immunity of chicken *(16)*.

Table 2. Lymphocyte and heterophil percent in differential leucocyte count of broilers

Groups		3rd week of age (primary immune response)		7th week of age secondary (secondary immune response)		End of Experimental	
		Lymphocyte	Heterophil	Lymphocyte	Heterophil	Lymphocyte	Heterophil
Control		60.33±0.88 (c)	29.67±0.88 (a)	61.67±1.20 (d)	28.33±1.20 (a)	62.67±1.20 (c)	27.33±1.20 (a)
Reomin	R.d	64.67±0.88 (ab)	25.33±0.88 (bc)	64.33±1.20 (cd)	25.67±1.20 (ab)	71.67±0.88 (ab)	18.33±0.88 (bc)
	D.rd	63.33±1.45 (bc)	26.67±1.45 (ab)	65.33±1.45 (bcd)	24.67±1.5 (abc)	69.67±1.45 (b)	20.33±1.45 (b)
Gibberellin	R.d	68.00±1.53 (a)	22.00±1.53 (c)	73.33±1.45 (a)	16.67±1.45 (d)	74.67±1.76 (a)	15.33±1.76 (c)
	D.rd	66.67±1.20 (ab)	23.33±1.20 (bc)	74.67±2.03 (a)	15.33±2.03 (d)	75.00±1.53 (a)	15.00±1.53 (c)
Digestamin	R.d	66.00±1.15 (ab)	24.00±1.15 (bc)	67.00±0.58 (bc)	23.00±0.58 (bc)	69.67±0.88 (b)	20.33±0.88 (b)
	D.rd	67.00±0.58 (ab)	22.67±0.33 (c)	69.00±0.58 (b)	21.00±0.58 (c)	72.33±1.45 (ab)	17.67±1.45 (bc)

In the same column means followed by the same letter are not significantly different at 0.01 level of significance.

IL-2 Production

The data of measuring IL2 indices are shown in Table 3 and Fig. 1. The results revealed that there was highly significant increase in expression of mRNA IL2 in Gibberellin groups (low and double doses) and Digestamin groups (low and double doses) as compared to the control group and there was significant increase in Reomin high dose as compared to the control group.

Table 3. Data of measuring IL2 indices which lies between 400 and 500 bp on the marker DNA

	Molecular weight	Amount
Lane 2	450	1.5615
Lane 3	450	1.6213
Lane 4	450	3.1736
Lane 5	450	2.6013
Lane 6	450	1.4034
Lane 7	450	1.3021
Lane 8	450	1.4231
Lane 9	450	2.8310
Lane 10	450	3.2134
Lane 11	450	1.4233
Lane 12	450	1.6221
Lane 13	450	1.2012
Lane 14	450	1.0000

IL2 indices was measured at two weeks after booster vaccination.

Following infection or vaccination, cytokines are produced by the immune system to regulate its responses by mediating a multitude effects ranging from activation and differentiation of immune cells to enhance the immune function and production of other cytokines *(17)*. Chicken IL2 shared similar properties with mammalian IL2 by being expressed by activated T. cells *(18)*. Mammalian IL2 is an essential cytokine for many types of immune responses including T cells differentiation and activation, B cell development, and NK cell stimulation, *(19, 20)*. Probiotics modulate the expression of various pro and anti-inflammatory cytokines *(21)*. The use of probiotic increase the number of T-cells in the ceacal tonsil *(22)*.Treatment of chicken with probiotics led to a significant increase in the oxidative burst and degranulation of heterophils *(23)*. Consequently, the elevated levels of lymphocyte assay and IL2 indices in avian influenza vaccinated groups supplemented by different levels of growth promoters is attributed to the number of immunoregulatory functions of lactic acid producing bacteria in Reomin and Digestamin. Digestamin, also contains horse radish peroxidase which produce H_2O_2 to the intestine.H_2O_2 is part of peroxidase enzyme that play a role in rising the host immunity and protect the host against infection.

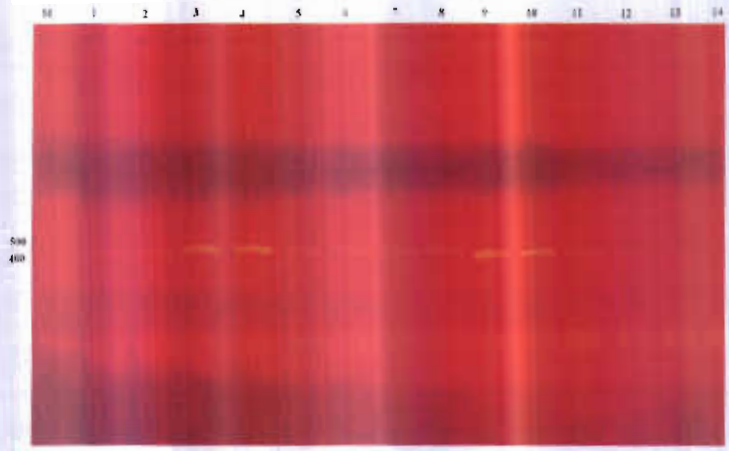

Fig. 1. Electrophoresis photo level of IL2 mRNA in T cells stimulated by growth promoters.
M = Marker DNA
The bottom two bands are 400 and 500 bp
Lane 2 and Lane 3 represent reomin double recommended dose group.
Lane 4 and Lane 5 represent digestamin double dose group.
Lane 6 represents digestamin group
Lane 7 represents reomin group
Lane 8 represents gibberellin group
Lane 9 and Land 10 represent gibberellin double dose group
Lane 11 represents gibberellin group
Lane 12 represents digestamin group
Lane 13 represents reomin group
Lane 14 represents control group.

Serum total protein

The effect of different experimental growth promoters on total protein is illustrated in Table 4.

Serum total protein was not significantly altered by feeding diets supplemented with different doses of the applied growth promoters at three weeks of age. So, evaluated growth promoters had no adverse effect on liver functions .Similar data were recorded as a result of inclusion yeast culture in broiler diet *(24-26)*. At the age of seven weeks and at the end of the experiment ,there was a significant increase in total protein values in the groups received double dose of Reomin, Gibberellin and Digestamin as compared to the control group. These higher levels might be due to stimulated hepatic activities resulting in the release of enzymes regulating the blood glucose and serum protein levels. Supplementing Primalac® (probiotic) to broiler diets significantly increased the average values of blood total protein *(27)*. Lasalocid (growth promoter) supplementation resulted in an improvement of nitrogen utilization and relatively elevated the concentration of total protein *(28)* .

Table 4. Serum total protein (g/dL) of different experimental groups (means + SE).

Groups		At 3rd week of age	At 7th week of age	At end of experiment
Control		3.88 ± 0.18	3.37 ± 0.01 (d)	3.17± 0.29 (cd)
Reomin	R.d	3.80 ± 0.19	3.51 ± 0.07 (cd)	5.00 ± 0.02 (a)
	D.rd	3.46 ± 0.21	3.56 ± 0.06 (c)	3.91 ± 0.13 (b)
Gibberellin	R.d	3.98 ± 0.24	3.47 ± 0.09 (cd)	3.05 ± 0.12 (d)
	D.rd	3.27 ± 0.05	3.80 ± 0.03 (b)	3.80 ± 0.14 (b)
Digestami n	R.d	3.65 ± 0.20	3.59 ± 0.01 (cd)	3.59± 0.24 (bc)
	D.rd	3.62 ± 0.07	4.26 ± 0.01 (a)	2.29 ± 0.06 (e)

In the same column means followed by the same letter are not significantly different at 0.01 level of significance.

Serum albumin

Serum albumin are illustrated in Table 5, all treatments showed no significant differences in albumin level as compared with control group. So, growth promoters had no adverse effect on liver functions and blood components.

Table 5. Serum albumin (g/dL) of different experimental groups (means±SE).

Groups		At 3rd week of age	At 7th week of age	At 9th of experiment
Control		1.86 ±0.08 (bc)	1.44 ±0.04 (ab)	1.53 ±0.08 (cd)
Reomin	R.d	2.16 ± 0.08 (a)	1.41± 0.08 (ab)	1.89 ± 0.04 (a)
	D.rd	2.06 ±0.036 (ab)	1.47 ± 0.03 (a)	1.45 ± 0.03 (d)
Gibberellin	R.d	2.24 ± 0.05 (a)	1.54 ± 0.03 (a)	1.78 ±0.06 (ab)
	D.rd	1.65 ± 0.09 (c)	1.47 ±0.04 (a)	1.67 ±0.05 (bc)
Digestamin	R.d	1.66 ± 0.17 (c)	1.39 ± 0.03 (ab)	1.60 ±0.04 (cd)
	D.rd	1.62 ± 1.46 (c)	1.30 ±0.05 (bc)	1.63 ±0.05 (bc)

In the same column means followed by the same letter are not significantly different at 0.01 level of significance.

Serum globulin

The values of serum globulin are shown in Table 6, the results revealed highly significant elevated values at 7 weeks of age and at the end of experimental period in all treated groups. This may be attributed to the hepatostimulatory and hepatoprotective effects of probiotics *(29)* or enhancement of immunity parameters by microbial probiotics supplementation *(30).*

Wattle dermal reaction

The wattle dermal reaction are summarized in Table 7. In the present study, it could be noticed that the chicken fed diets containing the growth promoter (high dose of Reomin, Gibberellin and Digestamin) had significantly higher response to vaccine injection by increasing wattle thickness compared to the control groups.

Table 6. Serum globulin (g/dL) of different experimental growth promoters (means±SE).

Groups		At 3rd week of age	At 7th week of age	At end of experiment
Control		2.02 ± 0.15	1.93 ± 0.06 (c)	1.64±0.30 (cd)
Reomin	R.d	1.64 ± 0.17	2.02 ± 0.15 (c)	3.11 ± 0.05 (a)
	D.rd	1.40 ± 0.20	2.09 ± 0.02 (c)	2.46 ±0.11 (b)
Gibberellin	R.d	1.74 ± 0.19	1.93 ± 0.11 (c)	1.27 ±0.13 (d)
	D.rd	1.61 ± 0.09	2.33 ± 0.01 (b)	2.13±0.18 (bc)
Digestamin	R.d	1.99 ± 0.04	2.46 ±0.04 (b)	1.99±0.23 (bc)
	D.rd	2.00 ± 009 N.S	2.87 ± 0.04 (a)	2.61±0.11 (ab)

In the same column means followed by the same letter are not significantly different at 0.01 level of significance.

Table 7. Evaluation of cellular immune response by measuring wattle thickness (mm).

Groups		Before injection	First day post Injection	Second day post Injection	Third day post Injection
Control		0.80 ± 5.77	1.13 ± 0.13	1.10 ± 0.00	1.10 ± 0.00 (b)
Reomin	R.d	0.87 ± 8.82	0.87 ± 8.82	1.47 ± 0.17	1.27± 3.33 (b)
	D.rd	0.73 ± 0.12	1.37 ± 0.13	2.27 ± 0.37	2.60± 0.40 (a)
Gibberellin	R.d	1.00 ± 0.15	1.57 ± 0.29	1.70 ± 0.35	1.97± 0.55 (ab)
	D.rd	1.23 ± 8.82	1.40 ± 0.21	2.00 ± 0.50	2.93± 0.58 (a)
Digestamin	R.d	1.23 ± 0.17	1.60 ± 0.31	2.30 ± 0.42	2.27± 0.15 (ab)
	D.rd	0.93 ± 0.20	1.53 ± 0.39	2.20 ± 0.40	2.73± 0.54 (a)

In the same column means followed by the same letter are not significantly different at 0.01 level of significance.

The wattle dermal reaction T lymphocyte proliferation response has been studied and has been shown to be a reliable indicator of *in vivo* cellular immunity in poultry *(31, 32).* The skin response reflects a complex series of physiological events such as mitogen receptor

and lymphocyte macrophage interaction, release of chemical mediators, cellular proliferation and changes in vascularity *(33).*

The skin is one of the largest organ of the body and the most common site for manifestation of immune reaction *(34, 35).* Collectively these responses characterized the immune response of the skin subsequent to natural antigen exposure or vaccination. The immune response in the skin involves mast cell, macrophages, T. cells and dendritic cells which include langerhan's cells which secrete cytokines that attract lymphocytes from nearby circulation and play a role as killers and scavengers of opsonized antigen *(34,36).*

When the antigen is injected into an animal sensitized by the vaccination, a delayed hypersensitivity response occurs; no changes are detectable either grossly or histologically for several hours (12-24hours). Vasodilatation and increased vascular permeability occur at the site of injection as a result, erythema and an indurate (hard) swelling eventually developed, on histological examination the lesion is infiltrated with mononuclear cells (macrophages and lymphocytes). The inflammatory reaction reaches its greatest intensity by 24 to 72 hrs before gradually fading *(37).*

Mortality: 2% mortality was recorded during experimental period, with no difference between groups.

Eventually, supplementation of Reomin, Digestamin and Gibberellin enhanced proliferative response of T. cells to mitogen through the release of IL2 and enhanced the cytotoxicity of natural killer cells. Similar results were obtained *(38, 39).* In summary, our results clearly confirmed that the growth promoters supplementation in chickens immunized with H5N1 vaccines might act as effective immunologic adjuvant resulted in higher stimulation indices for IL2 production and LPA, subsequently improving the cellular immunity .These results are consistent with higher lymphocytic count and increasing wattle thickness. On the other hand higher values of protein and globulin in the groups supplemented with growth promoters were recorded.

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الملخص العربي

الاستثارة الخلوية الليمفاوية و إنترليوكين-2 في الدجاج المحصن بلقاح أنفلونزا الطيور (H5N1) تحت المعاملة بمنشطات النمو

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في هذا البحث تم دراسة كفاءة وسلامه منشطات النمو كمحفز للمناعة في دجاج اللحم المحصن ضد انفلوانزاالطيور(H5N1) وقد تم استخدام عدد 210 كتكوت عمر يوم وزعت في 7 مجموعات متساوية ومتجانسة .ألمجموعه 1الضابطه والمجموعات 2و3و6و7 تم أضافه البروبيوتيك(روميين ودايجستامين) بالجرعة المعتادة والمضاعفة إلى العلف المجموعات 4و5تم أضافه مادة جابر يلين بالجرعة المعتادة والمضاعفة في مياه الشرب وهى عبارة عن هرمون نباتي منظم للنمو .

تم تحصين جميع الطيور بجرعتين من لقاح أنفلونزا الطيور الميت عند عمر أسبوع وكذلك عمرا ربعه أسابيع .تم تجميع عينات دم وسيرم وأنسجة لعمل الفحوص الاتيه:معامل الاستثارة للخلايا الليمفاوية ,العدد النوعي لخلايا الدم البيضاء,قياس البروتين والألبومين,IL-2انترلوكين 2عن طريق اختبار البلمرة المتسلسل المتضاد كذلك تم اجراءاختبار حساسية لجلد الدلايات.

وقد لوحظ في المجموعات المعالجة بمادة جابر يلين زيادة معنوية في عدد الخلايا الليمفاوية ومادة IL-2 في نفس السياق مجموعات الدجاج المغذى على علائق مضاف إليها البروبيوتيك أظهرت زيادة معنوية في معامل الاستثارة بالخلايا الليمفاوية والبروتين الكلى والجلوبيولين كذلك زيادة سمك جلد الدلايات.

مما سبق يتضح أن منشطات النمو التي قيمت في هذه الدراسة أمنه وفعاله في تحفيز الاستجابة المناعية لتحصين أنفلونزا الطيور H5N1 بغرض تقليل الخسائر ألاقتصاديه الناجمة عن وباء الأنفلونزا في قطعان الدواجن.