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Lymphocytic proliferation and interleukin-2 production in chickens supplemented with growth promoters

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Abstract

The efficacy and safety of growth promoters as immunopotentiators in chickens have been extensively evaluated under laboratory conditions. In this study, 210 (day old chicks) chicks were allotted into seven groups. Group 1 served as a control, group 2 and 3 were fed Reomin. Group 4 and 5 were supplemented with Gibberellic acid, a plant hormone with androgenic features, through drinking water (single and double doses). Group 6 and 7 were fed Digestamin supplemented to basal diet at recommended single and double doses. All chicks were vaccinated against H5N1twice at three weeks interval. Blood samples as well as tissue specimens were collected for assessment of T-Lymphocyte Proliferation Assay (LPA), differential leucocyte count, serum total protein and albumin and interleukin-2 (IL-2) production. Furthermore, wattle dermal reaction was conducted. The Gibberellic acid treated groups showed highest lymphocytic count and IL-2 production. Meanwhile chickens treated with probiotics showed highest values of LPA, total serum proteins and globulin as well as wattle dermal thickness. These results indicated that the evaluated growth promoters are safe and efficacious for enhancing immune response against H5N1antigen.

Keywords: Lymphocyte; interleukin-2; chickens; growth promoters

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Introduction

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 subtype) is a major threat to poultry industry inducing devastating epidemics with dramatic economic losses (Otte et al., 2004). So, the current trial was designed to improve the immune status of chickens vaccinated against H5N1 using growth promoters through dietary supplementation. Immunosuppression resulting from suppressor factors such as mycotoxins, some diseases, stress conditions and other adverse environmental factors have important effects on the vaccination protection levels against avian influenza H5N1 (Liu et al., 2011). Alternatively, there are several factors of immunomodulatory agents that are capable of stimulating immune responsiveness of chickens to vaccines. A great attention was paid toward "probiotics" which act as growth promoters contain organisms as *Lactobacillus acidophilus* and *L. plantarum* which contribute to the intestinal microbial balance in a positive way to enhance production. The beneficial effect of the probiotics might be mediated by a direct antagonistic effect against specific pathogens improving their metabolism and feed efficiency (Goldin and Gorbach, 1984). This work was planned to study the immunostimulatory effect of some growth

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promoters on cell mediated criteria in chickens vaccinated against H5N1 represented in LPA, IL-2 production, lymphocytic count and wattle dermal reaction.

Materials and Methods

Two hundred and ten day old Sasso chicks of mixed were housed in separate floor units under similar management and hygienic conditions. Chicks were weighed and randomly allotted into seven groups of 30 chicks each. Diet and water were provided *ad libitum*. Chicks were fed on a commercial starting diet (23% crude protein and 3000 kcal energy/kg) for the first week and later changed to growing and finishing diet supplemented with experimental treatments until day 65.

The growth promoters applied in this study: Gibberillin, Roemin contains China Wax Carevet. Digestamin and horseradish (Gemeinschaft, F.U.H. Egger, GMbH, "P.G.E." Austria). H120-B1 Hitchner, Lasota and Gumboro live attenuated vaccines were obtained from Izo S.P.A., Italy. Rassortant Avian influenza virus vaccine inactivated (H5 Subtype, Re-1 strain) produced by Yebio Bioengineering Co., Ltd. Qing Dao, China.

Seven experimental groups were used in this study. The first group served as a control, in the chicks were fed on the basal diet only. The second and the third groups received Reomin at low dose of 1gm/kg of ration and high dose of 2g/kg of ration respectively. The fourth and fifth groups received Gibberellin at low dose of 0.325 mg/L water and high dose of 0.65 mg/L water respectively. The sixth and seventh groups received Digestamin in the diet at low dose of 6 gm/kg of ration and high dose of 12g/kg of ration respectively.

Blood samples containing EDTA (as anticoagulant) was added to whole blood for differential leucocytic count. Serum samples was separated by centrifugation and stored at –20ºC until analysis of some blood chemistry. Determination of total protein (g/dL) was estimated according to the method described by Cornall (1949) using commercial kits (BioMerieux, France). Determination of albumin (g/dL) was colorimetrically measured according to the method described by Pinnell and Northam (1978) using commercial kits (Bio Merieux, France). Determination of serum globulin (g/dL) was estimated by subtracting the albumin concentration from total protein.

Lymphocyte Proliferation Assay (LPA) was estimated according to the method described by Perros and weightman (1991). LPA was measured at two time-points during the study at 2 weeks after the first dose of vaccination and at 2 weeks after the booster dose of vaccination against avian influenza.

For estimation of IL-2 production, tissue specimens at two weeks after booster vaccination were obtained from three birds from each replicate. Specimens from liver were collected and stored at -70 ºC in liquid nitrogen until further extraction of RNA was done with TR Izol reagent (Life Technologies, Inc., Grand Island, NY, USA). Specimens from spleen for estimation of interleukins, (IL-2) was calculated by Reverse Transcriptase RT -PCR using the following primer sets
Primer 1:5` – CTTTGGCTGTATTTCGGTAGC- 3'
Primer 2:5` – AAGTTGGTCAGTTCATGGAGAA – 3'

Wattle dermal reaction was conducted on day 63 of age. The thickness of the right wattle was measured in all groups. Then 0.1ml avian influenza vaccine was injected intradermal into the right wattle and the thickness was measured for three successive days as a direct method of measuring cell mediated immunity Ian (1992). Statistical analyses were carried out using (ANOVA) according to Snedecor and Cochran (1967).

Results and Discussion

The T-lymphocyte proliferation reflects the change in cellular immunity in chickens the results of LPA are shown in Table 1.

Table 1: Evaluation of cellular immune response by lymphocyte 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	L.d	0.15 ± 0.01^{d}	0.33 ± 0.00^{bc}
	d.d	0.23 ± 0.01^{bc}	0.33 ± 0.01^{bc}
Gibberellin	L.d	0.32 ± 0.01^{a}	0.32 ± 0.01^{cd}
	d.d	0.25 ± 0.01^{b}	0.29 ± 0.01^{d}
Digestamin	L.d	0.31 ± 0.01^{a}	0.36 ± 0.02^{b}
	d.d	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. Ld means low dose-dd means double doses

It revealed that there was a significant increase in LPA in all the treatments. This increase may be attributed to the antigenic load resulting from lactobacillus bacteria, which induce stimulation of the immune system. Havenaar and Spanhaak (1994) reported that probiotics stimulate the immunity of the chickens 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 stimulate the immune system. The lactic acid producing bacteria present in Reomin and Digestamin could interact with M cells which activate Payer's patches lymphocyte to be liberated from the intestine and reach the circulation (Muir, 1998). Moreover, Kohler et al. (2003) recorded that the wall of lactic acid producing bacteria is mainly composed of peptidoglycans and polysaccharides stimulating

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	L.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.d	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	L.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.d	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	L.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.d	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. Ld means low dose-dd means double doses.

macrophages to release IL2, IL1 which are mainly concerned with activation of lymphocytes. Also, Bakr et al. (1999) attributed the improvement in LPA performed by the Gibberellin to the increase of lymphocytes intracellular cyclicguanosine mono-phosphatase which stimulates blast transformation.

The differential leucocytic count for chickens fed different levels of growth promoters are illustrated in Table 2. Significant increase in lymphocyte percent in all groups as compared to the control group was recorded. This may be attributed to IL2 which enhanced the cytotoxicity of the macrophages and secretion of IL1, enhanced immunoglobulin synthesis and proliferation of β lymphocyte, enhancing proliferation of T cells and natural killer cells. The lymphocytosis which appears in the differential leucocytic count is suggestive of the immunogenic stimulation as the lymphocytes play a major role in the humoral and cell mediated immunity of chicken (Sturkie, 1986).

The data of measuring IL2 indices are shown in Table 3 and Figure 1. There were significant increase in expression of mRNA IL2 in Gibberellin groups (low and high dose) and Digestamin groups (low and high dose) as compared to the control group and there was significant increase in Reomin high dose as compared to the control group. Following infection or vaccination, cytokines (proteins) 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 (Schnetzler et al., 2001). Chicken IL2 shared similar properties with mammalian IL2 by being expressed by activated T. cells (Kaiser and Mariani, 1999). 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 (Farner et al., 1997). Yurong et al. (2005) described that the use of probiotic increase the number of T-cells in the ceacal tonsil. Farnell et al. (2006) noted that the treatment of chicken with probitoics lead to significant increase in the oxidative burst and deregulation of heterophils. 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 produces H_2O_2 to the intestine. H_2O_2 is a part of peroxidase enzyme that plays a role in raising the host immunity and protects the host against infection.

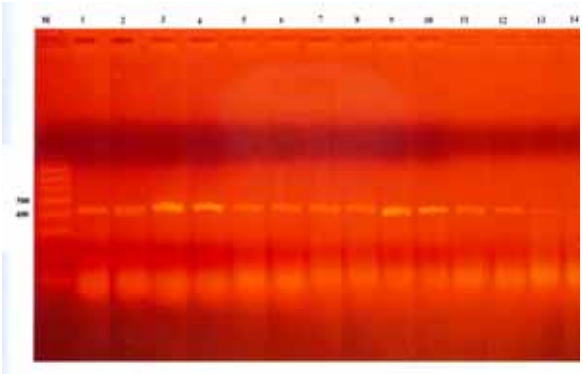

Fig. 1: Electrophoresis photo showing showing level of IL2 mRNA in T cells stimulated by growth promoters.

M = Marker DNA
The bottom two bands are 400 and 500 bp
Lane 2 and Lane 3 represent reomin double 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 and Lane 15 represents control group.

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

Plasma total protein was not significantly altered by feeding diets supplemented with different doses of the applied growth promoters at three weeks old. Thus, evaluated growth promoters had no adverse effect on liver functions. At the age of seven weeks, there was

Table 3: Values of 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	L.d	3.80 ± 0.19	3.51 ± 0.07^{cd}	5.00 ± 0.02^{a}
	d.d	3.46 ± 0.21	3.56 ± 0.06^{c}	3.91 ± 0.13^{b}
Gibberellin	L.d	3.98 ± 0.24	3.47 ± 0.09^{cd}	3.05 ± 0.12^{d}
	d.d	3.27 ± 0.05	3.80 ± 0.03^{b}	3.80 ± 0.14^{b}
Digestamin	L.d	3.65 ± 0.20	3.59 ± 0.01^{cd}	3.59 ± 0.24^{bc}
	d.d	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. Ld means low dos,e, dd means double doses.

Table 4: Values of 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	L.d	2.16±0.08^{a}	1.41±0.08^{ab}	1.89±0.04^{a}
	d.d	2.06±0.036^{ab}	1.47±0.03^{a}	1.45±0.03^{d}
Gibberellin	L.d	2.24±0.05^{a}	1.54±0.03^{a}	1.78±0.06^{ab}
	d.d	1.65±0.09^{c}	1.47±0.04^{a}	1.67±0.05^{bc}
Digestamin	L.d	1.66±0.17^{c}	1.39±0.03^{ab}	1.60±0.04^{cd}
	d.d	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. Ld means low dose-dd means double doses.

Table 5: Values of 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	L.d	1.64±0.17	2.02±0.15^{c}	3.11±0.05^{a}
	d.d	1.40±0.20	2.09±0.02^{c}	2.46±0.11^{b}
Gibberellin	L.d	1.74±0.19	1.93±0.11^{c}	1.27±0.13^{d}
	d.d	1.61±0.09	2.33±0.01^{b}	2.13±0.18^{bc}
Digestamin	L.d	1.99±0.04	2.46±0.04^{b}	1.99±0.23^{bc}
	d.d	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. Ld means low dose-dd means double doses.what about N.S see the table (N.S= non-significant variations).

Table 6: 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	L.d	0.87±8.82	0.87±8.82	1.47±0.17	1.27±3.33^{b}
	d.d	0.73±0.12	1.37±0.13	2.27±0.37	2.60±0.40^{a}
Gibberellin	L.d	1.00±0.15	1.57±0.29	1.70±0.35	1.97±0.55^{ab}
	d.d	1.23±8.82	1.40±0.21	2.00±0.50	2.93±0.58^{a}
Digestamin	L.d	1.23±0.17	1.60±0.31	2.30±0.42	2.27±0.15^{ab}
	d.d	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. Ld means low dose-dd means double doses.

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	L.d	0.87±8.82	0.87±8.82	1.47±0.17	1.27±3.33^{b}
	d.d	0.73±0.12	1.37±0.13	2.27±0.37	2.60±0.40^{a}
Gibberellin	L.d	1.00±0.15	1.57±0.29	1.70±0.35	1.97±0.55^{ab}
	d.d	1.23±8.82	1.40±0.21	2.00±0.50	2.93±0.58^{a}
Digestamin	L.d	1.23±0.17	1.60±0.31	2.30±0.42	2.27±0.15^{ab}
	d.d	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. Ld means low dose-dd means double doses.

Plasma total protein was not significantly altered by feeding diets supplemented with different doses of the applied growth promoters at three weeks old. Thus, evaluated growth promoters had no adverse effect on liver functions. At the age of seven weeks, there was significant increase in total protein concentration in the groups which received double dose of Reomin, Gibberellin and Digestamin as compared to the control group. These higher levels might be due to the stimulated hepatic activities resulting in the release of enzymes regulating the blood glucose and serum protein as recorded by Abd El-Gawad et al. (2004).

The values of serum globulin are shown in Table 6. The results revealed highly significant elevation in serum globulin values at 7 weeks age and at the end of experiment in all treated groups. This may be attributed to the hepatostimulatory and hepatoprotective effect of probiotic as recorded by Sarma et al. (2003) or enhancement of immunity parameters by microbial probiotic supplementation noticed by Sorenson (1982).

It was also observed that the chickens fed diets containing the growth promoter (high dose of Reomin, Gibberellin, and Digestamin) produced a better response to the vaccine injection by increasing wattle thickness compared to the control group. The wattle dermal reaction produced by T lymphocyte proliferation response has been well documented and has been shown to be a reliable indicator of *in vivo* cellular immunity in poultry (McCorkle et al., 1980).

When the antigen is injected into an animal sensitized by the vaccination, a delayed hypersensitivity response occurs, no change is detectable either grossly or histologically for several hours (12-24). 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 seen to be infiltrated with mononuclear cells (macrophages and lymphocytes). The inflammatory reaction reaches its greatest intensity by 24 to 72 hours before gradually fading as recorded by Thomson (1978). Eventually

supplementation of Reomin, Digestamin and Gibberellin enhance proliferative response of T cells to mitogen through the release of IL2 and enhancing the cytotoxicity of natural killer cells. Similar results were obtained by Matsuzaki et al. (1998). Mortality recorded during experimental period was about 2% with no significant difference between the groups.

In conclusion, our results confirmed that the growth promoters supplemented in chickens resulted in higher level of IL2 and LPA which ultimately resulted in improved cellular immunity.

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