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ACTIVATION OF INNATE IMMUNE SYSTEM IN RESPONSE TO A HYDRATED NUTRITIONAL SUPPLEMENT TREATMENT

By

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ABSTRACT

The aim of this study was to conduct an investigation into the effect of palm dates on innate immunity of broiler. One hundred twenty broiler chicks were portioned out in 6 experimental groups for 5 weeks. The experimented chicks received palm date in diet and 10 birds from each group were oral administration with challenging bacteria *E.coli*, *S.* Typhimurium*, C. perfringens, P. multocida* and *A. paragallinarum.* 5 randomly serum, spleen and intestinal samples were collected from each group weekly before and after the bacterial challenge for evaluation of innate and acquired immunity of the challenged birds. IL-6, IFN-γ and IgA in serum of in control group were the lowest in all weeks compared to other groups except in week two IgA value was similar to *C. perfringens* and higher than *S.* Typhimurium group in week four. *E.coli* group was lower than the other challenged groups in IL-6, IFN-γ and IgA in all weeks except IL-6 in week five and six it was higher than *S.* Typhimurium group and *A. paragallinarum* group, IFN-γ was similar to *C. perfringens* group and *P. multocida* group in first and second week and IgA value was higher than *S.* Typhimurium group in week three, four and five. *C. perfringens* group was the highest in IL-6 in week three, four and five and the highest of IFN-γ in week three. IgA values of *A.paragallinarum* group were higher than other groups in week six. *P. multocida* group was the highest IgA in all weeks except in week one and six; it was the highest value of IFN-γ in week four and six.

INTRODUCTION

For the pathogens that enter the body, the first line of defense is provided by innate immune mechanisms such as phagocytic cells that include heterophils and macrophages **(Qureshi *et al.,* 2000)**, complement **(Koppenheffer and T.L., 1998)**, and natural killer (NK) cells **(Gobel *et al.,* 2001, Sharma and schat., 1991)**. Innate responses do have some specificity in

recognizing pathogens **(Janeway and Medzhitov., 2002, Lemaitre *et al.,* 1996)**. Previous studies have shown that proinflammatory cytokines such as IL-1β, interferon-γ, IL-6, chemokine IL-8, and anti-inflammatory cytokines (IL-10 and transforming growth factor-β4) expression are regulated in the intestinal tissues during Salmonella challenge **(Withanage *et al.*, 2004, 2005; Fasina *et al.,* 2008)**. Cytokines are essential effector molecules of innate and acquired immunity and are crucial signaling molecules in cellular communication **(Kumar *et al.,* 2011)**. Toll-like receptors (TLRs) are pathogen recognition receptors, which initiate the pathways controlling expression of cytokines and chemokines and represent a link between innate and acquired immunity **(Werling and Jungi, 2003)**. Toll-like receptors are key primary innate immune receptors for sensing microorganisms through recognition of PAMP by immune cells, thus facilitating the initiation of a primary response against invading pathogens and recruitment of adaptive immune responses **(Tipping, 2006; Avlas *et al*., 2011)**. The chicken has been found to have 11 known TLR (TLR1 (Types 1, 2), TLR2 (Types 1, 3), TLR3, TLR4, TLR5, and TLR7, as well as chicken-specific TLR 15, TLR16, & TLR21) **(Keestra *et al.,* 2007, Brownlie and Allan, 2011)**. Likewise, recognition of microbial associated molecular patterns by avian TLR activates the basic signaling pathways, as seen in mammals **(Kogut *et al.,* 2012)**.

IgA complexes with a secretory component present on the surface of mucosal epithelial cells to form sIgA **(Wieland *et al.,* 2004).** Production of IgA, produced by intestinal B cells, provides an additional protective layer from luminal microbiota **(Vaishnava *et al.,* 2011, Macpherson and Uhr, 2004)**. A minor source of IgA in secretions is derived from blood via the hepatobiliary IgA transport system. In contrast to the transepithelial IgA pathway, hepatocytes express a specific receptor for blood IgA **(Allen *et al.,* 1987)**.

A recent report showed that aqueous extracts of dates have antioxidant, antimicrobial and anti-mutagenic activity **(Mohamed and Al-Okbi., 2005, Bilgari *et al.,* 2008, Saddiq and Bawazir, 2010)**.An important finding showed that dates constitutes good source of antioxidant **(Zineb *et al*.,2012)** and another study has shown that dates have the highest concentration of polyphenols among the dried fruits **(Vinson *et al.,*2005)**. The antioxidant activity of phenolic compounds is a result of their redox properties, which can play an important role in absorbing and neutralizing free radicals **(Garcia *et al.,*1994)**. Phytochemical show significant antioxidant capacities and antioxidant capability in lowering the prevalence

and lower mortality rates of cancer **(Velioglu *et al*.,1998)**. Another finding in the support of dates as antioxidant reported that dates are a good source of antioxidants due to the carotenoids and phenolics with quantity 3942 mg/100 g and antioxidants constituents 80400 μmol/100 g **(Bilgari *et al.,* 2008)**. Phenolic compounds present in dates including p-coumaric, ferulic,and sinapic acids, flavonoids,and procyanidins **(GU*et al.,*2003, Mansouri *et al.,* 2005).**

So the aims of the present study were to investigate the effect of palm dates on innate and humoral immunity of broiler**.**

MATERIAL AND METHODS

Sample:

One hundred twenty broiler chicks were portioned out in 6 experimental groups for 5 weeks. The experimented chicks received palm date in diet and 10 birds from each group were oral administration with challenging bacteria *E.coli*, *S.* Typhimurium*, C. perfringens*, *P. multocida* and *A. paragallinarum*. 5 randomly serum, spleen and intestinal samples were collected from each group weekly before and after the bacterial challenge for evaluation of innate and acquired immunity of the challenged birds.

ELISA kits for determination of IL-6 and IFN-γ (according to Kaiser *et al*., 2000 and Karakolev *et al.*, 2015).

IL6 an IFN gamma concentration were analyzed by using Sandwich-ELISA as follow. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Chicken IL-6. Standards or samples were added to the appropriate micro ELISA plate wells and bound by the specific antibody. Then a biotinylated detection antibody specific for Chicken IL-6 and Avidin-Horseradish Peroxidase (HRP) conjugate was added to each micro plate well successively and incubated. Free components were washed away. The substrate solution was added to each well. Only those wells that contain Chicken IL-6, biotinylated detection antibody and Avidin-HRP conjugate would appear blue in color.

The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color turns yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm +/- 2 nm. The OD value was proportional to the concentration of Chicken IL-6. You can calculate the concentration of Chicken IL-6 in the samples by comparing the OD of the samples to the standard curve.

ELISA kits for determination of IgA (According to Guillermo Tellez *et al.,* **2017).** An indirect ELISA was performed to quantify IgA. The commercial chicken IgA ELISA quantitation set (Catalog No. E30-103, Bethyl Laboratories Inc., Montgomery, TX 77356) was used according to the manufacturer's instructions. In brief, 96-well plates (Catalog No. 439454, Nunc MaxiSorp, Thermo Fisher Scientific, and Rochester, NY) were coated with 1 μg/100 μL of goat polyclonal anti-chicken IgA diluted in 0.05 mol/L carbonate-bicarbonate, pH 9.6. The plates were covered with a lid and allowed to incubate overnight at 4 °C. Then the contents of the plates were emptied, and the plates were tapped on a dry paper towel, and rinsed 5 times with washing solution (50 mmol/L Tris, 0.14 mol/L NaCl, 0.05% Tween 20, pH 8.0) of 350 μL/well. Individual wells were then blocked (125 μL/well) with 20% Super Block (Pierce Inc., Rockford, IL) in phosphate buffered saline for 60 min at room temperature. The plates were again emptied, tapped to dry and stored desiccated without further washing step. Samples were thawed to room temperature and diluted in sample/conjugate diluent (50 mmol/L Tris, 0.14 mol/L NaCl, 1% bovine serum albumin, 0.05% Tween 20) and 100 μL was added to the respective wells. A standard curve was used in order to quantify the total IgA in the samples, and chicken reference serum IgA from the quantitation kit was serially diluted in sample/conjugate diluent to get concentrations of 1 000, 500, 250, 125, 62.5, 31.25 and 15.625 ng/mL; sample/conjugate diluent alone was used as the zero standard (blank). Standard dilutions were added to the respective wells (100 μL/well). Plates were then incubated for 1 h at room temperature and rinsed 5 times with washing solution. Goat anti-chicken IgA-horseradish peroxidase (HRP) conjugated detection antibody from the IgA quantitation set was diluted (1:40 000) in sample/conjugate diluent and 100 μL were transferred to each well. The plate was incubated for 60 min at room temperature. After incubation, HRP detection antibody was removed and the plate washed again 5 times as previously described. After washing, 100 μL of tetrame thy lbenzi dine substrate (Catalog No. TMBS-1000-01, TMB Super Sensitive One Component HRP Microwell Substrate, SurModics IVD, Eden Prairie, USA) was added to each well and incubated for 15 min at room temperature, protected from light. The reaction was stopped with 100 μL of 3% - 7% maleic acid solution (Catalog No. LSTP-1000-01, BioFx® 450 nm liquid stop solution for TMB Microwell Substrates, SurModics IVD, Eden Prairie, USA), and absorbance was measured at 450 nm using an ELISA plate reader (Synergy HT, multi-mode

microplate reader, BioTek Instruments, Inc., Winooski, VT, USA). The value of absorbance at 450 nm minus the blank value for each standard concentration was plotted on the vertical (Y) axis versus the corresponding chicken IgA concentration on the horizontal (X) axis using the Gen5™software(BioTek Instruments,Inc.,Winooski,VT,USA).Chicken IgA concentration obtained was multiplied by the dilution factor to determine the amount of chicken IgA in the undiluted samples.

Methods of Histopathology for intestinal tissues of birds:

Intestinal specimens were collected from bird in each group then were fixed in 10% buffered formalin **(Bancroft and Layton, 2013).**

RESULTS

Results of IL-6 in serum of SPF broilers chickens:

Mean±SD of IL-6 in serum of SPF broilers chickens at six rearing weeks which revealed that in control group (Gp 1) Mean±SD of IL-6 were 16± 0.15, 34± 0.13, 58± 0.12, 125± 0.22, 245± 0.14, 356± 0.11 while in *E.coli* group (Gp 2) were 17±0.12,44±0.17,68 ± 0.11, 180 ±0.15 ,290 ±0.14 , 400 ± 0.18 *,S.* Typhimurium group 3(Gp3) were 20 ±0.33, 55 ± 0.17 , 88 ± 0.6 , 190 ± 0.25 , 220± 0.25 , 390 ± 014 , *C. perfringens* group 4 (Gp4) were 18 ± .013 , 66 ± 0.18 , 90 ± 0.16 ,220 ± 0.2 , 310 ± 0.11 , 420 ± .013 , *A. paragallinarum* group 5 (Gp 5) were 22 ± 0.3 ,60 ± 0.4 , 85 ± 0.32 ,200 ± 0.11 , 280 ± 0.14 , 390 ± 0.2 , *P .multocida* group 6 were 20 ± 0.32 , 75 ± 0.14 , 88 ± 0.16 , 210 ± 0.15 , 300 ± 0.3 , 450 ± 0.16 .

Results of IFN-γ in serum of SPF broilers chickens:

Mean±SD of IFN-γ in serum of SPF broilers chickens at six rearing weeks which revealed that in control group (Gp 1) Mean±SD of IFN-γ were 140 ±0.330 ,170 ± 0.280 , 190 ± 0.450 , 250 ± 0.360 , 300 ± 0.440 ,350 ± 0.350 while in *E.coli* group (Gp 2) were 150 ± 0.282 , 180 ±0.344 , 200 ± 0.380 , 300 ± 0.385 , 420 ± 0.240 , 560 ± 0.484, *S.* Typhimurium group 3(Gp3) were 145 ± 0.485 , 175 ± 0.682 , 210 ± 0.484 , 320 ± 0.285 , 450 ± 0.444, 610 ± 0.285, *C. perfringens* group 4 (Gp4) were 150± 0.252, 180 ±0.355, 220 ± 0.445, 340 ±0.355, 460 ± 590, *A .paragallinarum* group 5 (Gp 5) were 160 ± 0.234, 190 ± 0.453, 210 ±0.380, 360 ± 0.524, 490 ± 0.475, 600 ± 0.245 , *P. multocida* group 6 were 150 ± 0.380 , 180 ± 0.467, 220 ± 0.256, 380 ± 0.564, 490 ± 0.246, 650 ± 0.562.

Results of IgA in serum of SPF broilers chickens:

Mean±SD of IgA in serum of SPF broilers chickens at six rearing weeks which revealed that in control group (Gp 1) Mean±SD of IgA were 15 ± 0.114, 25 ± 0.123 , 34 ± 0.142, 65 ± 0.133, 80 ± 0.115, 110± 0.122 while in *E.coli* group (Gp 2) were 16 ± 0.106, 22 ± 0.124, 35 ± 0.134, 67 ± 0.115, 85 ± 0.118, 122 ± 0.113 ,*S.* Typhimurium group 3(Gp3) were 18 ± 0.133, 24 ± 0.124, 34± 0.116, 62 ± 0.135 , 84 ± 0.118, 125 ± 0.165, *C. perfringens* group 4 (Gp4) were 16 ± 0.114, 25± 0.144, 35 ± 0.135, 70 ± 0.153, 90± 0.156, 125 ± 0.165, *A .paragallinarum* group 5 (Gp 5) were 18 ± 0.134, 26 ±0.135, 36 ± 0.187, 66 ± 0.157, 88 ± 0.186, 135 ± 0.175 , *P. multocida* group 6 were 16 ± 0.189, 28 ± 0.156, 38 ± 0.167, 72 ± 0.147, 92 ±0.167, 124 ± 0.176.

Results of Histopathology for intestinal tissues of SPF broilers chickens:

Revealed that at *E.coli* group (Gp 2) duodenum showed degenerated sloughed epithelium, congested blood vessels in lamina propria with edema and congestion of muscular layer, at *S.* Typhimurium group (Gp3) duodenum showed hyperplasia of lining epithelium with mononuclear cells infiltration in lamina propria, at *C. perfringens* group (Gp3) duodenum showed hyperplasia of lining epithelium with degeneration of crypts mononuclear cells infiltration in lamina propria and congestion of muscular layer, *A. paragallinarum* group (Gp4) duodenum showed mucosal edema and mononuclear cells infiltration, at *P. multocida* group (Gp6) duodenum showed mononuclear cells infiltration in lamina propria.

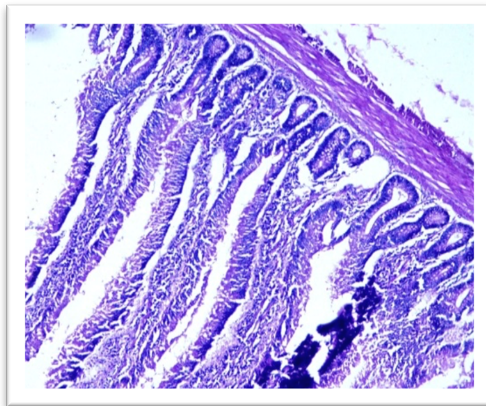

Fig (1): Controlduodenumshowed apparently normal architecture.

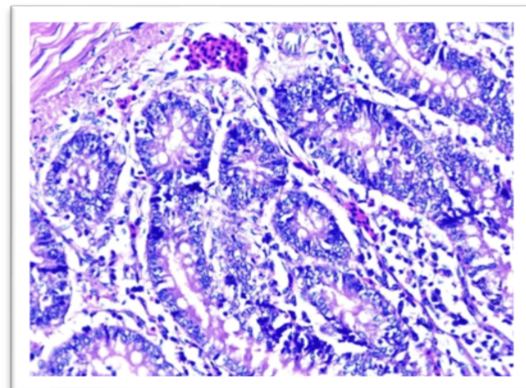

Fig.(2): Duodenum of infected group with *E.coli* showed degenerated sloughed epithelium, congested blood vessels in lamina propria with edema and congestion of muscular layer.

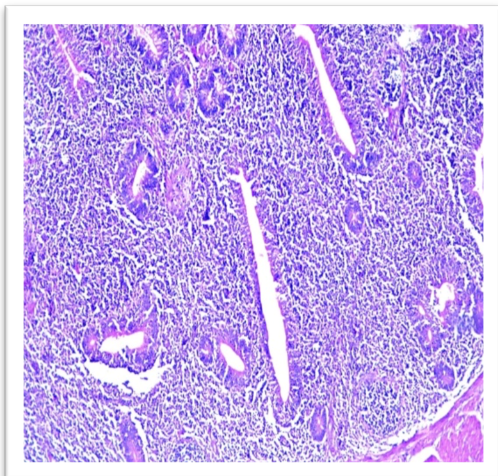

Fig. (3): Duodenum of infected group with *S.* Typhimurium showed hyperplasia of lining epithelium with mononuclear cells infiltration in lamina propria.

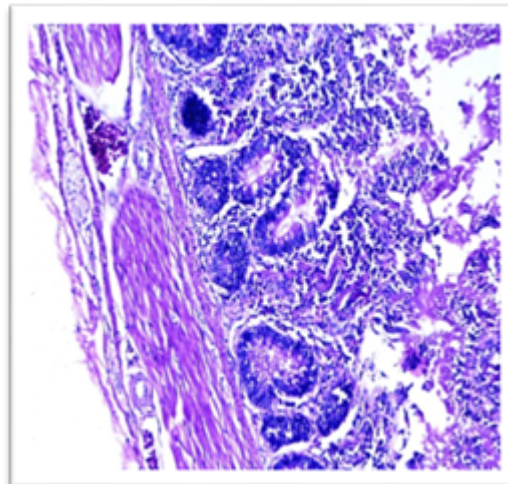

Fig. (4): Duodenum of infected group with *C .perfringens* showed hyperplasia of lining epithelium with degeneration ofcryptsmononuclear cells infiltration in lamina propria and congestion of muscular layer.

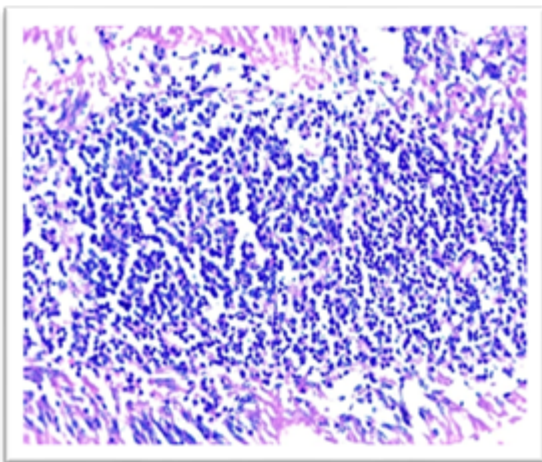

Fig. (5): Duodenum of infected group with *A. paragallinarum* showed mucosal edemaandmononuclearcells infiltration**.**

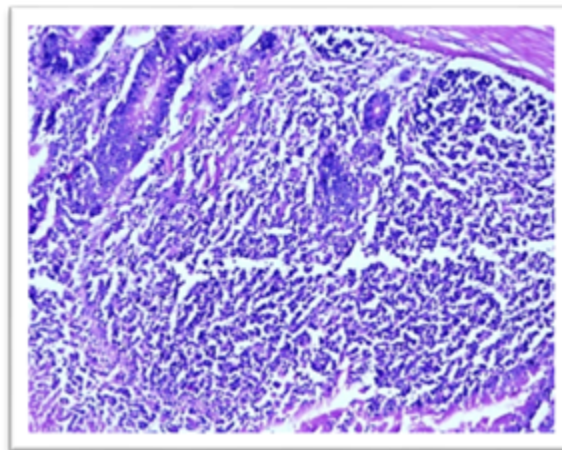

Fig. (6): Duodenum of infected group with *P. multocida* showed mononuclear cells infiltration in lamina propria .

DISCUSSION

A recent study examined the antioxidant activities in different type of dates such as Fard, Khasab and Khalas and showed that Khalas is measured to be best quality, had higher antioxidant activity, total carotenoids, and bound phenolic acids than other types of dates **(Al-Farsi *et al*., 2005)**. Other study showed that palm date fruits constitutes thirteen flavonoid glycosides of luteolin, quercetin, and apigenin at different stages of maturity **(Hong *et al.,* 2006, Bilgari *et al.,* 2008)**. Ajwa, types of dates that is only cultivated in Saudi Arabia/ Al-Madinah Al-Munawara and have significant value in several types of diseases cure and also show protective role in hepatic toxicity **(Abdu, 2011)**. An important study based on special type of dates; has shown significant antioxidant activity and caused a significant reversal of the lead induced changes in the oxidative biomarkers in serum and also Ajwa dated extract has a tissue protective effect via a free radical scavenging and antioxidant properties **(Ragab *et al* .,2013)**. Constituents of medicinal plants such as flavanoid and phenol play a significant role in cancer control through the regulation of genetic pathways without any side effect **(Gali-Muhtasib *et al*., 2006- Khan *et al*., 2011)**. Earlier studies reported that beta D-glucan from dates has shown antitumour activity **(Ishurd *et al.,* 2002)**. Study on animal model showed that glucans, constituents of date fruits exhibited a dose dependant anticancer activity with an optimum activity at a dose of 1 mg/kg in tumour **(Ishurda and John, 2005)**. Another important study has also shown that anti-tumor activity for date glucan **(Ishurd *et al.,* 2004)**. Study on Ajwa showed a protective effect and ameliorated the lesions of Ochratoxin nephero toxicity which might lead to kidney failure **(Ali and Abdu, 2011)**. An important study showed that, the effect of methanol and acetone extracts of leaves and pits Phoenix dactylifera inhibited the growth of *F. oxysporum, Fusarium sp*., *F. solani, A. alternata, Alternaria* sp **(Bokhari and Perveen, 2012)**. Some other important finding showed that methanol and acetone extracts of the P. dactylifera pits reasonably inhibited the growth of Gram positive and Gram-negative bacteria **(Ammar *et al.,* 2009, Jassim and Naji, 2010)**. Another recent study in the support of *p. dactilifera* effect as antimicrobial on *Klebsiella pneumonia* and *Escherichia coli* and also showed a role in reducing the side effects due to the use of drugs as methylprednisolone **(Aamir *et al*., 2013)**. Also, another study also

showed that Phoenix dactylifera extract has antibacterial effect against *E. fecalis*, indicating that this extract can be used for treating enteric diseases **(Aamir *et al.,* 2013)**.

Earlier studies have shown that constituents of plants such as phenolics and flavonoids act as excellent anti-inflammatory agents **(Talhouk *et al.,* 2007)**. A study in animal model showed that Phoenix dactylifera pollen has potential protective effect via modulation of cytokines expressions **(Elberry *et al.,* 2011)**. Another important finding in the support of date's fruits reported that the methanolic extract of edible portion of the fruit showed a vital role in reducing foot swelling and plasma fibrinogen **(Mohamed and Al-Okbi., 2004)**. A study in the support of dates as anti-inflammatory showed that the leaves of dates can be considered as a good source of natural antioxidant and anti-inflammation drugs **(Eddine, 2013)**.

Tissue macrophages are seeded by progenitor cells from the fetal yolk sac **(Ginhoux *et al.,* 2010, Schulz *et al*., 2012)**, can locally self-renew **(Jenkins *et al*., 2011)** and are also replenished by bone marrow-derived monocytes **(Varol *et al*., 2009)**. Peritoneal macrophages may also be induced in birds by intraperitoneal injections of inflammatory stimulants such as Sephadex beads. Some of the assays used to assess macrophage functions include a) phagocytosis, b) cytokine production upon stimulation with mitogens (lipopolysaccharide), c) ability to lyse tumor cells, and d) production of nitric oxide (NO) upon activation by T cell-produced cytokines, most notably IFN-gamma. Some of the functional characteristics of avian macrophages have been described **(Sharma., 1983, Qureshi *et al.,* 2000, Mellata *et al.,* 2003, Khatri *et al.,* 2005, Khatri *et al.,* 2006, Palmquist *et al.,* 2006).** During an infection, these microorganisms are phagocytosed by the macrophage and are contained within a membrane bounded vesicle called the phagosome. Under normal circumstances the phagosome fuses with enzyme - and toxin-containing lysosomes forming a phagolysosome where these exogenous antigens are killed and digested **(Abbas *et al.,* 2000)**. Free radicals, including reactive oxygen species and nitric oxide (NO), are products of activated macrophages and other phagocytic leukocytes and are known to be toxic to bacteria and some parasites **(Rosen *et al.,* 1995)**.

During this recall stimulation, cytokines such as IFN-g are produced by activated responder T cells. The applicability of this system to chickens has been investigated in coccidiosis, where the functional activity of chicken Peripheral Blood Lymphocytes (PBL) upon recall stimulation with *Eimeria tenella* sporozoites was demonstrated by determining both their

potential to proliferate and to produce ChIFN-g, as measured by bioassay **(Breed *et al*., 1997, 1999)**. IFN-γ increases expression of MHC Class I and Class II molecules that modulate immune response **(Kuby, 1997)**. IL-2 induces the proliferation and differentiation of T, B, and NK cells. IFN-γ has been implicated in immune response to various avian diseases **(Kaiser, 1996)**. Correlation between an IFN-γ promoter polymorphism and resistance to *E.coli* infection in chickens has been reported **(Kaiser *et al,* 1998).**

CONCLUSION

A palm date has a potential immunomodulation. Both innate and adaptive components of immune system are stimulated with palm dates after challenging infection.

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