Immune status of broiler chickens experimentally infected with avian *E.coli* supplemented with amino acid mixture rich in Glycine

Ismail Raheel [1], Ahmed Orabi [2*], Ahmed Erfan[3], Hassan Shaheen[4], Shaimaa Hassan[5]

[1] Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Beni-Suief University, Egypt.

[2] Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Egypt.

[3] Head of biotechnology unit, Animal health institute, Egypt

[4] Veterinarian in Egyptian livestock field

[5] Department of microbiology, Animal Health Research Institute, Agriculture research center, Egypt.

Correspondence author; Ahmed Orabi / drorabi2012@yahoo.com / Orabivet@cu.edu.eg : tel. +201000883784

1. Abstract

Meat producing broiler chickens may needs dietary amino acids of animal origin to obtain high growth, good health, muscles yield and healthy immune response. To investigate the effects of the amino acids mixture from animal origins rich in glycine on performance and immunity of broiler chickens an in vivo experiment was conducted at which T1 andT2 groups supplemented with amino acid mixture in every 10 days for successive 3 days, 8 hr. daily interval at dose 1 ml/L in drinking water and the second (T2 group) was experimentally challenged with avian pathogenic *E.coli* (APEC) and the third group (T3) had no supplementation. The obtained results showed that amino acids mixture treated groups(T1 and T2) had a remarkable immune-modulation as noticed by significant elevation of phagocytic count and activities(lysozymes and nitric oxide production) ,expression of IL-4, IL-10 and interferon- gamma-genes using RT-PCR. Performance studies proved that there was an improvement of body weight gain and lowered mortality rate in amino acids treated group.

Key words: Glycine amino acid, Avian *E. coli*, Immunomodulation, growth enhancing, Broiler chickens.

2. Introduction

Broiler chickens average daily gain during the last decades, increased to more than 50 g/day, and the market age was reduced to six weeks due to improvement the genetic potential **[1,2]**. Availability of amino acids in feedstuffs is an important feature of dietary protein quality, as all dietary sources of protein are heterogeneous mixtures of different proteins, therefore, these proteins would be digested and different amino acids were taken up from the gut **[3]**. Digestible amino acids used in diet formulation as it makes it possible to increase the inclusion levels of different ingredients in poultry diets **[4]**. There are ten amino acids cannot be synthesized by poultry and must be supplied in the diet to cover maintenance, growth, and production requirements. The ten essential amino acids are lysine, methionine, tryptophan, arginine, threonine, isoleucine, histidine, leucine, phenylalanine, and valine, while glycine is considered to be essential for the modern broiler chickens, since the rate of its synthesis is not adequate to support growth and maximum muscles production **[5]**. Undoubtedly that immune system requires proper nutrition **[6]**,as studies have shown that random level of protein or of essential amino acids in birds even deficiencies or excesses can lead to shift of immune responses **[7]**. Among amino acids, both arginine and tryptophan have been proven to positively influence the avian systemic immune response **[8]**.Among amino acids, both arginine and tryptophan has been proven to positively influence the avian systemic immune response **[8]**. Although most *E. coli* strains belong to the normal flora of the intestines and are non-pathogenic, there are some strains that are able to establish themselves outside of the intestines and cause diseases, these falls into the extra-intestinal pathogenic *E. coli* (ExPEC) **[9]**.Colibacillosis is the extra-intestinal infections caused by APEC which lead to reduced yield, quality and reduce immune response with high mortality and morbidity rate **[10]**. Glycine act as an important compound in the synthesis of many physiologically molecules as nucleotides as purines and haem **[11]**.also, glycine itself is a potent antioxidant as has a role in, glutathione synthesis **[12]**and also improve leucocytes anti-oxidative capability. The pharmacological importance of glycine represented in glycine-gated chloride channel in leucocytes cells helping in cytokines production regulation and improves immune response **[13,14]**, so the current work studied the effect of supplementing amino acids mixture rich in glycine on performance, and immune status of broiler chickens challenged with avian pathogenic *E.coli* compared with non-challenged group.

3. Materials and Methods

Ethical approval

This investigation was performed in accordance with the recommendations in the updated Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. All procedures were approved by the Beni-sueif University Ethical Committee in compliance with the United Kingdom (UK) Animals.

Broiler chickens husbandry

According to **[15]** with little modification**,** a total of 3 hundred (-day-old) Cobb broiler chickens were obtained from a commercial hatchery. Birds had free access to pelleted feed and water. Random distribution of chicks into 3 groups of 100 birds each, the experimental groups were as follows: T1; Amino acid mixture rich in Glycine treated T2 Amino acid mixture rich in Glycine treated and avian Pathogenic *E.coli* challenged and T3; Amino acid mixture rich in Glycine untreated. Chickens were weighed weekly; also blood and spleen samples from each group were collected for evaluation of immune status of treated birds.

Dietary treatments

Amino acid mixture rich in Glycine is a plenty mixture of amino acids from animal origin with high concentration of glycine amino acids distributed by ATCO, veterinary pharmaceutical integration. It added to treated groups every 10 days for successive 3 days, 8 hr. daily interval 1 ml/L in drinking water.

Challenge strains

avian pathogenic *E.coli* serogroup O_{78} used in the challenged study was local isolate full identified and classified phenotypically and genotypically and accession number on Genbank MW699361 (SEC-EGY/ORABI/Raheel/2020). The challenged dose was $1.5x10^8$ cfu/ml through drinking water according to **[16]**.

Immune matrix of broiler chickens supplemented with amino acid mixture and experimentally infected with APEC

HI titer; Blood samples were collected and tested using HI test by using inactivated H5N1 antigen (A/chicken/Egypt/18-H/2009) was used for detection of AIV-H5 antibodies and Lasota strains (8HA units) for detection of NDV antibodies **[17]**.

Phagocytic cell assay; 96-Well Phagocytosis Assay, CytoSelect™ used according to **[18]** and Nitric oxide, Lysozyme level recorded according to **[19]**. The results of all immune parameters were presented as mean±SE. All given parameters were compared between studied groups using the one-way ANOVA with fixed effects of the factors using (Start Soft Inc).

Cytokines assay using qRT- PCR; RNA extraction from tissue samples was applied according to (Yuan et al., 2006) and the reaction prepared by using specific primes for IL-4**[20]**,IL-10**[21]**and ,IFNG **[22]**.

Re-isolation of avian *pathogenic E.coli*

According to **[23]**;10 g of solid sample in 90 ml of normal saline followed by enrichment for 24 h incubation at 37°C in non-differential broth such as nutrient broth. This procedure will allow multiplication of *E. coli* then streaked on MacConkey's and EMB agar and biochemically and serologically identified.

Performance measurements

Feed intake (FI), BWG, FCR and mortality percent were weekly determined **[24]**.

4. Results

Results of innate immunity parameters

the phagocytic cell count determined in T1, T2 and at which there were propagation in the count cells from 10^2 to 10^6 in these groups at 10 and 30 days old respectively in comparison with T3 that show slow cell count from 10^2 to 103 and also the secretory mediators of activated macrophage increased as nitric oxide and lysozyme and were determined as follow, T1;lysozyme from 1.5±0.356 to 5.5±0.556/nitric oxide from 2.5±0.215 to 18.4±0.235 and T3; lysozyme from 1.5±0.365 to 78.3±0.256/nitric oxide from 2.4±0.117to 28.5±0.525 at 10 and 30 day old respectively, while T2 showed low level of these mediator (table 2) .

Results of humoral immunity parameters

HI test used for traceability of the vaccination program reaction success, the results in table (1) for AI antibodies revealed that there were increasement in T1 from 1.94 ± 1.34 at 10 day old to 3.25± 1.65 then for 4.92± 1.73 and at 20 and 30 day old , while in T2 from 1.82 ± 1.75 to 2.66± 1.36 and 3.95± 1.43 at at10, 20 and 30 day old respectively ,in comparison with the ND antibodies titers there were also increasement in T1(from 4.82±1.22 to 5.43± 1.54), T2 (from 3.92±1.46 to 4.34± 1.52) at 20 and 30 day old. However the results of untreated control negative T3 showed little changes

Results of Avian cytokines gene expression analysis using qRT- PCR

The fold change in some avian cytokines showed in table (2), which revealed that there were fold change increasement in T1: IL4; from 5.1337 to 11.1579 / IL10; from 3.9449 to 7.5685/ IFNG ; form 7.6741 to 13.5479, T2: IL4; from 4.3772 to 8.5742/ IL10; from 2.5847 to 5.6178/ IFNG; form 6.1903 to 10.4831) at 15 and 25 day old respectively with little change in T3.

Results of re-isolation rate of avian *E. coli*

The re-isolation rate of the challenged serogroup was 12% in experimentally challenged group (T2)

Results of Performance parameters

Performance parameters recorded as feed intake(FI g/bird),BWG(kg/bird) and FCR, the results revealed that the FI in all experimented groups was 3300 g/bird, while the BWG was 2.4 ,2.2 and 2.1 kg/bird in T1,T2 and T3 respectively and the FCR was1.45, 1.5, 1.571 in T1,T2 and T3 respectively

5. Discussion

Consumer pressure for broilers raised has contributed to increased diets free of animal by-product meals being fed to broilers. Increase the use of the amino acid as feed ingredients has will continue in the coming periods, due to limited resources accompanied by excess requirements of amino acids levels in new broilers strains performance and efficiency assessments **[25,26]**. However diets for broiler chickens based mainly on cereals, oilseeds, and this raise least cost formulation on the total glycine or glycine plus serine amino acids **[27]**. In the present study Amino acid mixture rich in Glycine, which is a commercial amino acid combination rich in glycine explored as immune modulatory and performance enhancer agent in broilers chickens at which the immune status of the chicks under experiments evaluated by different parameters measurement. Humoral immune response depend on production of huge amount of antibodies against foreign bodies and vaccines which act as antigens, so in the current study the antibodies titer of Avian influenza and Newcastle used vaccine were measured using HI test at which the amino acid treated groups T1 showed best titer at different age old , the results in table (1) for AI antibodies revealed that there were increasement in T1 from 1.94 ± 1.34 at 10 day old to 3.25± 1.65 then for 4.92± 1.73 and at 20 and 30 day old , while in T2 from 1.82 ± 1.75 to 2.66± 1.36 and 3.95± 1.43 at at10, 20 and 30 day old respectively ,in comparison with the ND antibodies titers there were also increasement in T1(from 4.82±1.22 to 5.43± 1.54), T2 (from 3.92±1.46 to 4.34± 1.52) at 20 and 30 day old. However the results of untreated control negative T3 showed slowly modified titer and this give conclusion that supplementation with amino acid aid in an efficient immune response even in case of pathogens challenge as avian pathogenic *E.coli*. Although the increased use of all vegetable based diets fed to broilers has resulted in increased recent research on glycine responses in broilers.

Glycine amino acid share in many metabolic roles ranging from amino acid to non-protein nitrogen pathways and also energy metabolism **[28,29]**and in poultry, glycine plays a critical role in uric acid formation for nitrogen elimination **[30,31]**.Cytokines are mediators which are important for immune cells function and communication to evade any pathogens and control infections and this have a strength point for growth, performance and yield of broilers chickens, so the recorded results for some selected avian cytokines for determination using qRT- PCR revealed that there were fold change increasement in T1: IL4 ;from 5.1337 to 11.1579 / IL10; from 3.9449 to 7.5685/ IFNG ; form 7.6741 to 13.5479 and in T2: IL4; from 4.3772 to 8.5742/ IL10; from 2.5847 to 5.6178/ IFNG; form 6.1903 to 10.4831) at 15 and 25 day old respectively with disappointed results in T3 as there were confinement of these mediators which reflect on immune response against infectious agents as IL4 act as a potent mediators of many immune cells as mast cell, eosinophil's and Th2 cells **[32]**, while IL10 has potent anti-inflammatory actions by preventing tissue damage and maintain normal cell homeostasis **[33]**, in the other hand interferon gamma has a critical roles in modulation of innate and cellular immune response toward viral pathogens **[34]**. Glycine amino acid reduces inflammation in infected animals as low level of glycine in diets decrease immune responses in chickens treated with lipopolysaccharides**[35]**, which was followed by glycine dietary supplementation **[36]**, as it decrease TNFα levels in plasma improved survival rate and decrease morbidity. Interestingly, glycine protected animals against arthritis induced by peptidoglycan polysaccharide, help in control of stress-induced gastrointestinal mucosal injury, protect from the ischemia shock caused by endotoxin sepsis and hemorrhage **[14]**. Experimental infection with avian pathogenic E.coli have been crucial in the study of immune modulation of amino acid mixture rich in Glycine by allowing investigation of the macrophage clearance ability and shedding of the challenged strains, in the current investigation the re-isolation rate of the challenged pathogenic serogroup was 12% in T2 group. Macrophages are one of the mononuclear phagocytic systems, that it is a crucial player in both the innate and adaptive immune responses, for this reasons the present investigation studied the phagocytic count and its mediators there were augmentation in the count cells from 10^2 to 10^6 in T1 and T2 groups at 10 and 30 days old respectively in comparison with T3 that showed slow cell count from 10^2 to 10^3 and exacerbate nitric oxide and lysozyme level in T1 and T2 more than T3 (table 2) which indicated that activation of macrophages cells by action of dietary supplementation of amino acid mixture rich in glycine **[37]**. The good immune response ,the best performance criteria as broilers are among the most efficient feed converting livestock in the world, so During the present experimental study the performance parameter were determined as feed intake, body weight gain(kg/bird) and feed conversion at which the results showed that the weight gain were 2.4, 2.2 and 2.1 kg/bird in T1,T2 and T3 respectively and the FCR was1.45,1.5 and 1.571 in T1,T2 and T3 respectively which confirm that the treated group with amino acid mixture has potent muscle yield and health digestive system for feed conversion.

6. Conclusion

In conclusion for best growth, high muscle yield, alert immune response and less inflammatory reaction in broiler chickens amino acid mixture rich in glycine dietary supplement is required.

7. References

1. Silva Junior RGC, Lana GRQ, Rabello CB-V, Barboza WA and Lana SRV, 2005. Exigências de metionina+ cistina para frangos de corte machos de 1 a 21 e de 22 a 42 dias de idade, em clima tropical. *Revista Brasileira de* Zootecnia, 34(6), 2399-2407. https://doi.org/10.1590/S1516-35982005000700028.

2. Araújo LF, Junqueira OM, Araújo CS DS, Sakomura NK, Andreotti MDO, and Sugueta SM, 2002. Diferentes perfis de aminoácidos para frangos de corte no período de 43 a 56 dias de idade. *Revista Brasileira de Zootecnia*, *31*(1), 387-393.

3. Hughes RJ, Choct M, 1999. Chemical And Physical Characteristics Of Grains Related To Variability In Energy And Amino Acid Availability In Poultry. Australian Journal Of Agricultural Research, 50: 689-701.

4. Li X, Mollet P, Huang K and Bryden WL, 2002. Inclusion Level of Cottonseed Meal In Broiler Diets. In: 7th World Poultry Science Association Asian Pacific Federation Conference and 12th Australian Poultry And Feed Convention. Proceedings... S.L, P.256.

5. Friedman M, 1996. Nutritional value of proteins from different sources. A review. *Journal of Agricultural and Food Chemistry* 44, 6.29.

6. Butcher GD and Miles RD, 2002. Interrelationship of nutrition and immunity. VM139. Available online at www.edis.ifas.ufl.edu (accessed 21 April 2010).

7. Abdukalykova S and Ruiz-Feria S, 2006. Arginine and vitamin E improve the cellular and humoral immune response of broiler chickens. International Journal of Poultry Science, 5, 121-127.

8. de Jonge WJ, Kwikkers KL, te Velde AA, van Deventer SJ, Nolte MA, Mebius RE, Ruijter JM, Lamers MC and Lamers WH, 2002. Arginine deficiency affects early B cell maturation and lymphoid organ development in transgenic mice. *The*

Journal of clinical investigation, *110*(10), 15391548.https://doi.org/10.1172/JCI16143.

9. Abraham S, Gordon DM, Chin J, Brouwers HJ, Njuguna P, Groves MD, Zhang R and Chapman TA, 2012. Molecular characterization of commensal Escherichia coli adapted to different compartments of the porcine gastrointestinal tract. *Applied and environmental microbiology*, *78*(19), 6799–6803. https://doi.org/10.1128/AEM.01688-12.

10. Sadeyen J R, Kaiser P, Stevens M P, and Dziva F, 2014. Analysis of immune responses induced by avian pathogenic Escherichia coli infection in turkeys and their association with resistance to homologous re-challenge. Veterinary research, 45(1), 1-12.

11. Kim SW, Mateo RD, Yin YL and Wu G, 2007. Functional amino acids and fatty acids for enhancing production performance of sows and piglets. Asian-Aust J Anim Sci 20, 295–306.

12. Fang YZ, Yang S and Wu G, 2002. Free radicals, antioxidants, and nutrition. Nutrition, 18(10), 872–879. https://doi.org/10.1016/s0899-9007(02)00916-4.

13. Froh M, Thurman RG and Wheeler MD, 2002. Molecular evidence for a glycine-gated chloride channel in macrophages and leukocytes. *American journal of physiology. Gastrointestinal and liver physiology*, *283*(4), 856–863. https://doi.org/10.1152/ajpgi.00503.2001.

14. Zhong Z, Wheeler MD, Li XL, Froh M, Schemmer P, Yin M, Bunzendaul H, Bradford B and Lemasters JJ, 2003. Glycine: a novel anti-inflammatory, immunomodulatory, and cytoprotective agent. Curr Opin Clin Nutr Metab Care 6, 229–240.

15. Rostagno HS, Albino LFT, Donzele JL, Gomes PC, de Oliveira R F, Lopes DC, Ferreira AS, Barreto SLT and Euclides R F, 2011. Brazilian tables for poultry and swine: Feed composition and nutritional requirements. 3rd ed. UFV, Viзosa, Minas Gerais, Brazil.

16. Fernández A, Lara C, Loste A and Marca MC, 2002. Efficacy of calcium fosfomycin for the treatment of experimental infections of broiler chickens with Escherichia coli O78:K80. *Veterinary research communications*, *26*(6), 427–436. https://doi.org/10.1023/a:1020582207129

17. Office International des Épizooties (OIE), 2014. Chapter 2.3. 4 (Avian influenza) in Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.www.oie.int.

18. Yu Z, Ono C, Aiba S, Kikuchi Y, Sora I, Matsuoka H and Tomita H, 2015. Therapeutic concentration of lithium stimulates complement C3 production in dendritic cells and microglia via GSK-3 inhibition. Glia. 63(2):257-270. https://doi.org/10.1002/glia.22749

19. Raheel IAR, Orabi A and El Masry A, 2019. Natural herbs CLEANACTIV®; Immune-modulator, health activator and growth promoter in broiler chickens. *International Journal of Veterinary Science*, *8*(4), 267-270.

20. Suzuki K, Okada H, Itoh T, Tada T, Mase M, Nakamura K, Kubo M and Tsukamoto K, 2009. Association of increased pathogenicity of Asian H5N1 highly pathogenic avian influenza viruses in chickens with highly efficient viral replication accompanied by early destruction of innate immune responses. Journal of virology, 83(15), 7475–7486. https://doi.org/10.1128/JVI.01434-08.

21. Samy AA, El-Enbaawy MI, El-Sanousi AA, Abd El-Wanes SA, Ammar AM, Hikono H and Saito T, 2015. In-vitro assessment of differential cytokine gene expression in response to infections with Egyptian classic and variant strains of highly pathogenic H5N1 avian influenza virus. International Journal of Veterinary Science and Medicine *3*(1-2), 1-8.

22. Markowski-Grimsrud CJ and Schat KA, 2003. Infection with chicken anaemia virus impairs the generation of pathogen-specific cytotoxic T lymphocytes. Immunology. *Immunology*, *109*(2), 283–294. https://doi.org/10.1046/j.1365-2567.2003.01643.x

23. Irwin PL, Nguyen LHT, Paoli GC and Chen CY, 2010. Evidence for a bimodal distribution of Escherichia coli doubling times below a threshold initial cell concentration. BMC Microbiology; 10(1):207. https://doi.org/10.1186/1471-2180-10-207.

24. Coneglian JLB,Vieira SL, Berres J and Freitas DMD, 2010. Responses of fast and slow growth broilers fed all vegetable diets with variable ideal protein profiles. Revista Brasileira de Zootecnia; 39(2):327–334.

25. McGill E, Kamyab A and Firman JD, 2012. Low crude protein corn and soybean meal diets with amino acid supplementation for broilers in the starterperiod 1. Effects of feeding 15% crude protein. Int. J. Poult. Sci. 11, 161–165.

26. Kidd MT, Tillman PB, Waldroup PW and Holder W, 2013. Feed-grade amino acid use in the United States: the synergistic inclusion history with linearprogramming. Journal of Applied Poultry Research, 22(3), 583-590

27. Dean DW, Bidner TD and Southern LL, 2006. Glycine supplementation to low protein, amino acid-supplemented diets supports optimal performance of broiler chicks. Poultry science, 85(2), 288–296. https://doi.org/10.1093/ps/85.2.288

28. Akinde AO, 2014a. Amino acid efficiency with dietary glycine supplementation: part 1. World's Poult. Sci 70, 461–474.

29. Akinde AO, 2014b. Amino acid efficiency with dietary glycine supplementation: part 2. World's Poult. Sci 70, 575–584.

30. Powell S, Bidner TD and Southern LL, 2009. The interactive effects of glycine, total sulfur amino acids, and lysine supplementation to corn-soybean meal diets on growth performance and serum uric acid and urea concentrations in broilers. *Poultry science*, *88*(7), 1407–1412. https://doi.org/10.3382/ps.2008-00433.

31. Powell S, Bidner TD and Southern LL, 2011. Effects of glycine supplementation at varying levels of methionine and cystine on the growth performance of broilers fed reduced crude protein diets. *Poultry science*, *90*(5), 1023–1027. https://doi.org/10.3382/ps.2010-01247

32. Wills-Karp M, Finkelman FD, 2011. Innate lymphoid cells wield a double-edged sword. Nature immunology. ;12:1025–1027.

33. Anderson CF, Oukka M, Kuchroo VJ, Sacks D. CD4+CD25-Foxp3− 31 cells are the source of IL-10-mediated immune suppression in chronic cutaneous leishmaniasis. J Exp Med. 2007;204:285–97.

34. Tannenbaum CS, Hamilton TA,2000. Immune-inflammatory mechanisms in IFNgamma-mediated anti-tumor activity. Semin. Cancer Biol. 10, 113– 123.

35. Ikejima K, Iimuro Y, Forman DT and Thurman RG, 1996. A diet containing glycine improves survival in endotoxin shock in the rat. The American journal of physiology, 271,G97–G103. https://doi.org/10.1152/ajpgi.1996.271.1.G97

36. Konashi S, Takahashi K and Akiba Y, 2000. Effects of dietary essential amino acid deficiencies on immunological variables in broiler chickens. *The British journal of nutrition*, *83*(4), 449–456.

37. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity. (2010) 32:593–604. doi: 10.1016/j.immuni.2010.05.007

Table 1: Immune matrix of Amino acid rich in Glycine

Parameter	Age	T1	T2	T3
ND-HI	10 days	2.12±1. 35	1.84±1. 43	1.83±1. 54
	20 days	4.82±1.22	3.92±1.46	2.83±1.43
	30 days	5.43± 1.54	4.34± 1.52	3.24± 1.42
AI-HI	10 days	1.94 ± 1.34	1.82 ± 1.75	1.78 ± 1.46
	20 days	3.25± 1.65	2.66± 1.36	1.93± 1.86
	30 days	4.92± 1.73	3.95± 1.43	2.8± 1.37
Phagocytic cell count	10 days	10^3	10^3	10^2
	20 days	10^4	10^4	10^3
	30 days	10^6	10^6	10^3
Nitric oxide µmol/ml	10 days	2.5±0.215	2.4±0.117	2.4±0.235
	20 days	15.2±0.145	25.6±0.415	6.8±0.315
	30 days	18.4±0.235	28.5±0.525	9.5±0.415
Lysozyme µmol/ml	10 days	1.5±0.356	1.5±0.365	1.4±0.156
	20 days	3.5±0.422	6.5±0.345	2.2±0.132
	30 days	5.5±0.556	8.3±0.256	3.2±0.254

T1: Amino acid rich in Glycine treated, T2: Amino acid rich in Glycine treated and avian Pathogenic *E.coli* challenged, T3: Amino acid rich in Glycine untreated and unchallenged (control negative)

Table 2: Results of cytokines indices

Time point	Gp.	*IL4*		*IL10*		*IFNG*	
		CT	Fold change	CT	Fold change	CT	Fold change
15 day	T1	19.21	5.1337	20.42	3.9449	18.04	7.6741
	T2	18.17	4.3772	19.76	2.5847	17.08	6.1903
	T3	21.33	-	22.16	-	20.74	-
25 day	T1	17.59	11.1579	18.96	7.5685	17.43	13.5479
	T2	19.08	8.5742	20.50	5.6178	18.91	10.4831
	T3	21.07	-	21.88	-	21.19	-

T1: Amino acid rich in Glycine treated, T2: Amino acid rich in Glycine treated and avian Pathogenic *E. coli* challenged, T3: Amino acid rich in Glycine untreated and unchallenged (control negative)