Evaluation of Performance and Immunomodulation in *Eimeria* spp and *Clostridium perfringens*-Challenged Broilers Administered Intermittently an *Echinacea*-Based Preparation

Taha Kumosani[1]

Elie K. Barbour[2]*

[1]*Biochemistry Department, Faculty of Science, Production of Bioproducts for Industrial Applications Research Group and Experimental Biochemistry Unit, King Abdulaziz University (KAU), Jeddah, Saudi Arabia*

[2]*Department of Agriculture, Faculty of Agricultural and Food Sciences, American University of Beirut, Beirut, Lebanon; Adjunct to Biochemistry Department, Faculty of Science and Production of Bioproducts for Industrial Applications Research Group, King Abdulaziz University, Jeddah, Saudi Arabia.*

**Corresponding author: Elie K. Barbour, Department of Agriculture, Faculty of Agricultural and Food Sciences, American University of Beirut, P.O. Box 11-0236, Beirut, Lebanon. Tel. 961-1-350000, Extension 4460; Fax: 961-1-744460; E-mail: eb01@aub.edu.lb.*

KEY WORDS: Broilers, Clostridium, Eimeria, Immunomodulation, Performance

ABSTRACT

Aim

To study the impact of intermittent administration of an Echinacea-based preparation (EBP) in broilers on performance and immunomodulation against a multivalent challenge by four species of Eimeria and C Perfringens.

Methods and Results

A total of 60 day-old Cobb birds were divided into three treatments, with 20 birds per each. Birds of Treatment 1 were administered the EBP intermittently at the ages of 1-3, 8-10, 15-17, and 22-24 days, standardized at 0.44 mg alkamides/liter of drinking water, while deprived of challenge. Birds of Treatment 2 were similarly treated with EBP, followed by an intra-esophageal challenge at 28 d of age with four Eimeria spp and 10^6 cfu of C perfringens/ml/bird. Birds of Treatment 3 were deprived of EBP treatment and administered the same challenge at 28 d of age. Results showed that birds of Treatment 2, and at the end of the first life cycle of Eimeria (34 days of age) had higher average weight gain, lower means of oocyst count and intestinal lesion score, a reduction in transcribed IL-8 chemokine produced by the Intraepithelial lymphocytes (IEL) of the duodenum ($p<0.05$), ileum ($p<0.05$), and cecum ($p>0.05$), associated with higher serum nitrite at challenge age (28 d.), compared to birds of Treatment 3.

Conclusions

The EBP-treatment was able to improve the weight gain during the incubation period of the challenge, reducing the multiplication of oocysts and associated lesion score, and increasing the plasma nitrite, while reducing the IL-8 generated by intestinal IEL.

Significance and Impact of Study

This study provides a data that will pave

the way for future transformation in poultry practices towards the use of immunopotentiators in control of economic poultry coccidiosis, in attempts to comply with the customers demand for safe poultry products that are devoid of synthetic coccidiostat residues.

INTRODUCTION

Coccidiosis is the most important protozoan disease affecting the poultry industry worldwide, resulting in an annual loss of more than 4 billion US dollars.[1,2] Eighty percent of the losses are due to mortality, reduced weight, and inefficient feed conversion in broilers.[3] The continuous use of coccidiostats in feed resulted in the emergence of drug-resistant strains of Eimeria spp.[4] Furthermore, there is an increased concern of consumers about synthetic coccidiostat residues in the poultry products due to their negative effect on human health.[5] This triggered the need for alternative control of avian coccidiosis by different approaches that are compiled in a review article by Huyghebaert and coworkers in 2011, including the approach of immunopotentiation by natural products.[6-8]

In addition, poultry coccidiosis is proved to enhance a secondary infection by Clostridium perfringens, the cause of necrotic enteritis (NE), requiring the supplementation of feed by antimicrobials.[9,10]

NE is controlled by antimicrobial Growth Promoters (AGPs) such as bacitracin, avoparcin, avilamycin, and some ionophores, such as narasin and monensin.[11-14] However, the role of AGPs in the emergence of antimicrobial resistance in human zoonotic organisms has been documented,[15-17] and consequently, the European Union decided to ban them, effective January 1, 2006.[18] Unfortunately, the banning of AGPs in chickens increased the frequency of coccidiosis and clostridial infections,[19] and urged for the need of investing in research related to alternative products against prevalent economic diseases in poultry. [6,7,20-22]

Certain active ingredients of the Echinacea plant are known to activate the innate immune response through macrophage activation in mice and rats;[23,24] the modulation of the macrophage-derived cytokine, and the activation of the polymorphonuclear leukocytes and natural killer cells by Echinacea ingredients were also documented in mice and humans,[25,26] and further confirmation of their immunopotentiating effects were reported.[27,28]

The first documented attempt[29] was able to establish the dose of Echinacea pupurea roots in broilers' feed that resulted in the induction of immunopotentiation, manifested by reduction of immunosuppression due to Infectious Bursal Disease, and in lowering the infectivity by Salmonella Enteritidis. There are a few reports on the use of Echinacea in horses,[30] swine,[31] cattle,[32] and another two documentations in poultry.[33,34]

Allen in 2003 studied the effect of Echinacea purpurea (EP) ground root-inclusion in poultry feed on immunity and protection against coccidiosis. The inclusion rate of the root in feed was similar to that established previously by Barbour et al in the year 2000.[29] The birds' weight gain before challenge with Eimeria spp, the causative protozoa of coccidiosis, were improved by the EP treatment compared to that in birds treated with the live Immunocox® vaccine alone. In addition, the EP was able to protect the challenged broilers at an age of 28 days against weight gain suppression and development of gross lesions.

The purpose of this research is to study the impact of intermittent administration of Echinacea Based Preparation (EBP) in drinking water of broilers, at ages of 1-3, 8-10, 15-17, and 22-24 days, on performance and immunomodulation against a multivalent challenge at 28 d with viable oocysts of four Eimeria spp. and C perfringens.

MATERIALS AND METHODS

Experimental Design

A total of 60 day-old Cobb birds were divided into three treatments, with 20 birds per each treatment. Birds of Treatment 1 were administered the EBP in drinking water,

and deprived of challenge. The standardized EBP administration was based on its major ingredient, namely the providing of 0.44 mg alkamides/liter of drinking water. The intermittent treatment with EBP in drinking water was at the ages of 1-3, 8-10, 15-17, and 22-24 days.

Birds of Treatment 2 were administered the EBP at the same intermittent intervals, followed by an intra-esophageal challenge with four Eimeria spp at 28 d, namely, Eimeria acervulina (7.5×10^4), Eimeria tenella (7.5×10^4), Eimeria maxima (7.5×10^4), and Eimeria necatrix (1.5×10^4), and with a viable count of C perfringens equivalent to 10^6/ml/bird.

Birds of Treatment 3 were deprived of EBP treatment and administered the same challenge at 28 d of age. It is worth noting that the management and handling of birds was performed according to the Institutional Animal Care and Use Committee regulations at the American University of Beirut.

Performance Parameter

The performance parameter included the weight gain. The birds in the different treatments were weighed individually at 28 d (age at challenge) and at 34 d (6 days post challenge, the age at which the first Eimeria life cycle ended).

Protection Parameters

The protection parameters included the recording of the lesion scores and the oocyst counts in the four intestinal organs of each bird. Birds of all treatments were sacrificed at the age of 34 days (6 days post challenge) and the lesion scores were recorded in the duodenum, jejunum, ileum and cecum. The score ranged between 0 (no gross lesions), 1 (mild inflammation), 2 (moderate inflammation) and 3 (severe inflammation). The average oocyst counts of Eimeria spp in each of the four intestinal organs was determined by a previously documented protocol.[35,36] In parallel, part of each of the four intestinal organs was collected for recovery of the intestinal Intraepithelial Lymphocytes (IEL), aiming at quantification of transcribed genes of specific cytokines and chemokines, as described under the below 'immunomodulation' section.

Immunomodulation Parameters

The immunomodulation parameters included the Plasma Nitrite and cytokines produced by the Intraepithelial lymphocytes (IEL) of the 4 intestinal organs.

Plasma Nitrite

Blood samples were taken from birds of each of the three treatments at the ages of 28 and 34 days, and their plasma were collected for quantitation of the NO2-level , according to the documented method of Allen & Teasdale, 1994.[37] This parameter helps in evaluation of the immunomodulation of phagocytosis, which relates to their immune injuries and consequently to the development of intestinal lesions. The analysis was performed according to the manufacturer instructions of the "Griess Reagent Kit" (Invitrogen Molecular Probes Co., Eugene, Oregon, USA).

Cytokines of the intestinal IEL

Other modulation parameters were assessed by q-PCR for transcribed cytokines genes produced by the intestinal Intraepithelial lymphocytes (IEL), namely, the IL-6, IL-12, IL-15, and IL-8. The Glyceraldehyde 3-phosphate dehydrogenase (GADPH) gene was included as a reference Housekeeping gene. Briefly, the IELs of each of the four intestinal organs were purified on a discontinuous Percoll Density Gradient,[38] adjusted to a count of 1.5×10^6 cells/ml, and their RNA were extracted by TRIzol (Sigma, St. Louis, MO). Reverse transcription was performed by an Avian Reverse Transcriptase (Sigma, St. Louis, MO). The real time q-PCR was optimized for each Interleukin and for the HouseKeeping gene (GAPDH), using forward and reverse oligonucleotide primers sequences, and optimized cycling conditions were presented in Tables 1 and 2, respectively. The real time q-PCR included the SYBR® Green JumpStartTMTaqReadyMixTM (Sigma, St Louis, MO), and the amplification was accomplished by the C1000 Torch Thermocycler (BioRad, 2000 Alfred Nobel Drive, Hercules, CA). The $2^{-\Delta\Delta Ct}$

Table 1. *Sequence of the oligonucleotide primers used in Real-time q-PCR*

RNA target	Primer sequences		Size of PCR product (bp)
	Forward	Reverse	
GAPDH[1]	5'GGTGGTGCTAAGCGTGTTAT3'	5'ACCTCTGTCATCTCTCCACA3'	264
IL-6	5'CAAGGTGACGGAGGAGGAC3'	5'TGGCGAGGAGGAGGGATTTCT3'	254
IL-8	5'GGCTTGCTAGGGGAAATGA3'	5'AGCTGACTCTGACTAGGAAACTGT3'	200
IL-12	5'AGACTCCAATGGGCAAATGA3'	5'CTCTTCGGCAAATGGACAGT3'	274
IL-15	5'TCTGTTCTTCTGTTCTGAGTGATG3'	5'AGTGATTTGCTTCTGTCTTTGGTA3'	243

[1]*GADPH is the housekeeping gene Glyceraldehyde 3- phosphate dehydrogenase*.

method was adopted in analysis of the relative changes in gene expression.[39,40]

Statistics

The means of the above defined production and immunomodulation parameters were compared among the treatments by Analysis Of Variance (ANOVA), followed by Tukey's test, and the reporting of significant differences among the means was at a P value of < 0.05.

RESULTS

Performance

The performance in weight gain during the 6 days of Eimeria spp and C perfringens incubation in the differently treated broilers is shown in Table 3. The control birds in Treatment 1, administered intermittently the EBP and deprived of challenge, had the highest weight gain of 527g. Birds of Treatment 2, EBP-treated and challenged, had more average weight gain of 460.6 g compared to the average weight gain obtained by birds of Treatment 3 (415 g), that were similarly challenged but deprived of EBP.

Protection

The data related to protection by EBP, against the multiplication of the challenging Eimeria-oocysts in the four intestinal organs of differently treated broilers, is shown in Table 4. The controls in Treatment 1 had no oocyst counts in their intestinal organs, reflecting the proper isolation standards followed in this experiment. The mean oocyst counts in the four organs of the intestine were consistently lower in the EBP-treated and challenged birds of Treatment 2 compared to that of the birds that were similarly challenged, but deprived of EBP (Treatment 3). This reduction in intestinal oocysts of broilers in Treatment 2 could be the reason behind having better weight gain, compared to that of birds in Treatment 3 that were deprived of EBP, but similarly challenged. The lower multiplication of oocysts in birds of Treatment 2 was associated generally with lower means of lesion scores compared to birds of Treatment 3 (Table 5).

Table 2. *Optimized cycling conditions of the Real-time q-PCR for the amplification of GAPDH and target genes of four interleukins*

RNA target	Cycling conditions
GAPDH	95°C for 3 min, and 40 cycles of 95°C for 10s, 55°C for 30s
IL-6	95°C for 3 min, and 40 cycles of 95°C for 10s, 59.8°C for 30s
IL-8	95°C for 3 min, and 40 cycles of 95°C for 10s, 57.9°C for 30s
IL-12	95°C for 3 min, and 40 cycles of 95°C for 10s, 59.3°C for 30s
IL-15	95°C for 3 min, and 40 cycles of 95°C for 10s, 57.6°C for 30s

[1]*GADPH is the housekeeping gene Glyceraldehyde 3- phosphate dehydrogenase*

Immuno-modulation Parameters

The assessed immunomodulation parameters included the plasma nitrite and IEL-cytokines.

Table 3. *Weight gain during the 6 days of Eimeria spp. and C. perfringens incubation in the differently treated broilers*

Treatments	EBP[1]	Challenge[2]	Weight gain between 28-34d. of age (g)
1	+	-	527.5
2	+	+	460.6
3	-	+	415.0

[1] *Treatment with Echinacea-based preparation (EBP) at the following intermittent intervals of 1-3, 8-10, 15-17, and 22-24 days of age*
[2] *Multivalent challenge at 28d. of age with four Eimeria spp. and C. perfringens*

Plasma Nitrite

The plasma nitrite level in micro-molar at 28 d (age at challenge) and 34 days (end of the first life cycle of Eimeria) is shown in Table 6. Birds of Treatments 1 and 2 received a similar intermittent treatment by EBP in water before testing the nitrite in their blood at 28 d of age . However, birds of Treatment 3 were deprived of this treatment. This resulted in similar respective mean nitrite level at 28 d of age in birds of Treatments 1 and 2, namely, 6.3 and 6.6 μmolar, that were almost double the mean plasma level in birds of Treatment 3 that were deprived of EBP. The cessation of EBP-treatment at the age of 24 days led to decay of nitrite in the plasma of birds at day 34, with an apparent significant decline shown in birds of Treatment 2 ($p<0.05$) .

Cytokines

The pro-inflammatory mean level of transcribed IL-8 in the Intraepithelial Lymphocytes (IEL) of each of the four intestinal organs, at the end of the 6 d- period of incubation of the multivalent challenge in broilers is shown in Table 7. There was a consistent down regulation by EBP treatment of the IL-8 transcription by the IEL of three out of four intestinal organs in broilers of Treatment 2 compared to the EBP-deprived broilers of Treatment 3, namely, in the duodenum, ileum, and cecum.

On the contrary, there was a high Jejunal IL-8 transcription in birds of Treatment 2 compared to those of Treatment 3 , which was associated with low means of oocyst count and lesion score in the Jejunum of Treatment 2-birds compared to that in birds of Treatment 3 (Tables 4 and 5). The transcription of IL-6, IL-12, and IL-15, in relation to normalization by the control birds of treatment 1, was not detectable by the q-PCR system that was applied on the IEL of the chicken intestinal organs.

DISCUSSION

Production

The improvement in production of the birds by the EBP in the face of the multivalent challenge by four Eimeria spp and the high viable count of C perfringens is most likely due to the documented enhancement of

Table 4. *Mean Oocyst counts in four organs of each bird at the end of 6 days of incubation period following challenge with Eimeria spp. and C. perfringens*

Treatments	EBP[1]	Challenge[2]	Mean oocyst count/gram of intestinal organ			
			Duodenum	Jejunum	Ileum	Cecum
1	+	-	0.00	0.00	0.00	0.00
2	+	+	3.40×10^4	6.1×10^5	0.50×10^4	5.10×10^4
3	-	+	6.60×10^4	6.7×10^5	1.13×10^5	8.60×10^4

[1] *Intermittent treatment with Echinacea-Based Preparation (EBP) at the following ages of 1-3, 8-10, 15-17, and 22-24 d.*
[2] *Multivalent challenge at 28d. of age with four Eimeria spp. and C. perfringens*

Table 5. *A trend in reduction of intestinal lesion score by intermittent administration of EBP to broilers challenged with Eimeria spp. and C. perfringens*

Treatments	EBP[1]	Challenge[2]	Mean oocyst count/gram of intestinal organ			
			Duodenum	Jejunum	Ileum	Cecum
1	+	-	1.0[a]	0.6[a]	0.1[a]	0.0[a]
2	+	+	1.0[a]	1.4[b]	0.8[b]	0.6[b]
3	-	+	1.0[a]	1.9[b]	1.1[b]	0.9[b]

[1]Evaluation of lesions based on score of 0 to 3
[2]Intermittent treatment with Echinacea-Based Preparation (EBP) at the following ages of 1-3, 8-10, 15-17, and 22-24 d.
3Multivalent challenge at 28d. of age with four Eimeria spp. and C. perfringens
[a,b]Means in a column followed by different alphabet superscripts are significantly different (p<0.05)

Table 6. *Plasma Nitrite level (μM) at challenge time and at the end of 6 days-incubation period*

Treatments	EBP[1]	Challenge[2]	Mean Nitrite level (μM) at age (d.)		
			28	34	Difference between Nitrite level at 34 and 28d of age
1	+	-	6.3[a,3]	0.6[a,3]	5.7
2	+	+	6.6[a,3]	1.6[a,4]	5.0
3	-	+	3.7[a,3]	2.0[a,3]	1.7

[1]Intermittent treatment with Echinacea-Based Preparation (EBP) at the following ages of 1-3, 8-10, 15-17, and 22-24 d.
[2]Multivalent challenge at 28d.of age with four Eimeria spp. and C. perfringens
[a,b]Means in a column followed by same Alphabetic superscript are insignificantly different (p>0.05)
[3,4]Means in a row, followed by different Arabic numerical superscripts, are significantly different (p<0.05)

Table 7. *Pro-inflammatory IL-8 levels produced by intraepithelial lymphocytes (IEL) of each of the four intestinal organs at the end of 6 days of incubation period following challenge with Eimeria spp. and C. perfringens*

Treatments	EBP[2]	Challenge[3]	Normalized[1] means of IL-8 relative to means of controls in Treatment 1			
			Duodenum	Jejunum	Ileum	Cecum
2	+	+	0.2[a]	$(8.3x10^{11})^{a}$	3.1[a]	0.17[a]
3	-	+	$(1.3x10^{3})^{b}$	$(2.6x10^{2})^{a}$	$(4.5x10)^{b}$	$(1.1x10^{3})^{a}$

[1]Normalized amount of IL-8 = 2–ΔΔCt , where ΔΔCt = (Ct of target (IL-8) – Ct of GAPDH)Treatment – (Average Ct of target (IL-8) – Average Ct of GAPDH) Control Treatment 1.
[2]Intermittent treatment with Echinacea-Based Preparation (EBP) at the following ages of 1-3, 8-10, 15-17, and 22-24 d.
[3]Multivalent challenge at 28d. of age with four Eimeria spp. and C. perfringens
[a,b]Means in a column followed by different alphabet superscript are significantly different (p<0.05)

the immune system response by the EBP components to the virulence factors carried by the etiologic agents present in the challenge.[23-28] This improvement in performance of these broilers is also in agreement with data of previous reports, documenting the benefits of Echinacea purpurea in performance of broilers against different challenges.[29,33]

Protection

The lower multiplication of Eimeria spp oocysts in birds of Treatment 2 was associated generally with lower means of lesion scores compared to birds of Treatment 3 that were similarly challenged but deprived of EBP treatment (Table 5). This is in agreement with previous workers who correlated the level of intestinal oocysts multiplication in chicken to the degree of intestinal lesions, absorption efficiency of nutrients, and weight gain.[41,42]

Immunomodulation

Plasma Nitrite

The experimental design of this work allowed birds of Treatments 1 and 2 to receive a similar intermittent treatment by EBP in drinking water, before testing their plasma nitrite at 28 d of age. However, birds of Treatment 3 were deprived of this treatment. The EBP-administered birds of treatments 1 and 2 resulted in similar respective mean nitrite level at 28 d of age, almost double the level obtained in birds of Treatment 3, that were deprived of EBP. This Nitrite data indicate the effect of EBP on activation of phagocytes that produce the nitrite,[43] which could have helped in the decrease in oocysts multiplication of birds in Treatment 2 compared to that of Treatment 3.

The cessation of EBP-treatment at the age of 24 days led to decay of nitrite in the plasma at day 34 in all treatments. This decay in Nitrite level could be due to utilization of induced nitrite by the multivalent challenge. Previous literature points at the engagement of the nitrite molecule in infiltrating infectious agents within the host.[44,45]

Cytokines

Data showed a consistent down regulation of the IL-8 transcription by EBP treatment in most intestinal organs of broilers in Treatment 2 compared to the EBP-deprived broilers of Treatment 3. This down regulation in Il-8 is most likely due to the lower injury created by the diminished replication of the oocysts in the these intestinal organs (Table 4), thus reducing the inflammatory reactions in these sites, as revealed by the reduced lower intestinal lesion scores shown in birds of Treatment 2 (Table 5).

On the contrary, the high Jejunal IL-8 transcription in birds of Treatment 2 compared to those of Treatment 3 was associated with low means of oocyst count and lesion score in the Jejunum of Treatment 2-birds compared to that in birds of Treatment 3 (Tables 4 and 5). The literature documented that chickens inoculated orally with C perfringens following a primary challenge with E necatrix had significantly increased numbers of C perfringens especially in the jejunum and ileum where the endogenous stages of E necatrix take place.[46] This documented research could explain the cause of observing a high Il-8 level in the Jejunum of birds belonging to Treatment 2. It is worth noting that gross lesions are mainly due to the injury created by the host immune response to infection and not merely by the protozoal reproduction, as documented by the literature of Williams in the year 2003.[47]

The transcription of IL-6, IL-12, and IL-15, in relation to normalization by the control birds of Treatment 1, was not detectable by the q-PCR system that was applied on the IEL of the chicken intestinal organs. This reflects the cessation of transcription of the genes of these cytokines under the multivalent challenge, a phenomenon that could be related to molecules produced by the combination of etiologic agents that affect the regulation of these genes.[48,49] The dynamics of cytokines transcription under multivalent challenges will be the subject of future investigation in the area of epigenetic

of coccidiosis/necrotic enteritis interaction.

In conclusion, the EBP-treatment was able to improve the weight gain of broilers during the incubation period of the multivalent challenge, to have a trend in reducing the multiplication of oocysts and lesion score, and a trend in increasing the plasma nitrite, and in reducing IL-8 produced by the IEL of most intestinal organs.

Future investigations will extend the treatment by EBP through the incubation period of the challenge, attempting to sustain the plasma nitrite level, and to increase the inhibition of the oocysts multiplication and its related lesion scores in order to reach in the future into a replacement strategy of synthetic coccidiostats by natural alternatives, to meet the respective challenges of banning of drugs, while satisfying the consumers' demand for safe poultry products.

ACKNOWLEDGEMENT

This work was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant No. 130-1021-D1435. The authors, therefore, acknowledge with thanks DSR technical and financial support. In addition, the authors thank EWABO co. in Germany for providing their continuous support.

Conflict of Interests: There is no conflict of interest among authors and any other party.

REFERENCES

1. Williams, R.B: A compartmentalised model for the estimation of the cost of coccidiosis to the world's chicken production industry. *Int. J. Parasitol*. 29 (8), 1209-1229. 1999
2. Shirley, M.W., Ivens, A., Gruber, A., Madeira, A.M., Wan, K.L., Dear P.H. and Tomley, F.M.: The Eimeria genome projects: a sequence of events. *Trends Parasitol.* 20(5), 199-201. 2004
3. Dalloul, R.A. and Lillehoj, H.S.: Recent advances in immunomodulation and vaccination strategies against coccidiosis. *Avian Dis*. 49 (1), 1-8. 2005
4. Ruff, M.D. and Danforth, H.D.: Resistance of coccidian to medications. Proc. XX World's Poultry Congress, II, 427-430. 1996
5. McDougald, L.R. and Seibert, B.P.: Residual activity of anticoccidial drugs in chickens after withdrawal of medicated feeds. *Vet. Parasitol.*74, 91-99. 1998
6. Dalloul, R.A., Lillehoj, H.S., lee, J.S., Lee, S.H., and Chung, K.S.: Immunopotentiating effect of Fomitella fraxinea-derived lectin on chicken immunity and resistance to coccidiosis. *Poultry Sci*. 85, 446-451. 2006
7. Lillehoj, H.: Immunopotentiating effect of a Fomitella fraxinea-derived lectin on chicken immunity and resistance to coccidiosis. US Patent, Publication number 20070036817 A1. 2007
8. Barbour, E.K., Bragg, R.R., Karrouf, G., Iyer, A., Azhar, E., Harakeh, S., and Kumosani, T.: Control of eight predominant Eimeria spp. involved in economic coccidiosis of broiler chicken by a chemically characterized essential oil. *J. Appl. Microbiol*., 118, 583-591. 2014
9. Al-Sheikhly, F. and Al-Saieg, A.: Role of Coccidia in the occurrence of necrotic enteritis of chickens. *Avian Dis.* 24, 324-333. 1980
10. Hafez, H.M.: Enteric diseases of poultry with special attention to Clostridium perfringens. *Pak. Vet*. J., 31, 175-184. 2011
11. Elwinger, K., Schneitz, C., Berndtson, E., Fossum, O., Teglöf, B. and Engström, B.: Factors affecting the incidence of necrotic enteritis, ceacal carriage of Clostridium perfringes and bird performance in broiler chickens. *Acta Vet. Scand.* 33, 369-378. 1992
12. Vissiennon, T., Kröger, H., Khöler, T. and Kliche, R.: Effect of avilamycin, tylosin, and ionophore anticoccidials on Clostridium perfringens entertoxaemia in chickens. *Berliner und Münchener Tierärztliche Wochenschrift* 113, 9-13. 2000
13. Martel, A., Devriese, L.A., Cauwerts, K., De Gussem, K., Decostere, A., Haesebrouck, F. Susceptibility of Clostridium perfringens strains from broilers chickens to antibiotics and anticoccidials. *Avian Pathol*. 33, 3-7. 2004
14. Wieczorek, K., Kania, I. and Osek, J. Prevalence and antimicrobial resistance of Campylobacter spp. isolated from poultry carcasses in Poland. *J. Food Prot.* 76 (8), 1451-1455. 2013
15. Wegener, H.C., Aarestrup, F.M., Jensen, L.B., Hammerum, A.M., and Flemming, B.: Use of antimicrobial growth promoters in food animals and Enterococcus faecium resistance to therapeutic antimicrobial drugs in Europe. *Emerg. Infect. Dis.,* 5, 329-335. 1999
16. ASM (American Society of Microbiology): First International Meeting on 'Antimicrobial resistance in zoonotic bacteria and foodborne pathogens'. Conference of ASM, Finland/Norway Halls, Radisson SAS Scandinavian Hotel, Copenhagen, Denmark, June 15-18. 2008
17. EFSA (European Food Safety Authority): The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals, and food in 2012. *EFSA Journal*, 12, 3590-3926. 2014
18. European Commission: Regulation 1831/2003/EC on additives for use in animal nutrition, replacing Directive 70/524/EEC on additives in feed stuffs. 2005
19. Casewell, M., Friis, C., Marco, E., McMullin, P., and Phillips, I.: The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *J. Antimicrob.*

20. Van Immerseel, F., Rood, J.I., Moore, R.J. and Titball, R.W.: Rethinking our understanding of the pathogenesis of necrotic enteritis in broilers. *Trends Microbiol.*, 17, 32-36. 2009

21. Huyghebaert, G., Ducatelle, R., and Van Immerseel, F.P.: An update on alternatives to antimicrobial growth promoters for broilers. *The Vet. J.,* 187, 182-188. 2011

22. Barbour, E.K., Shaib, H., Azhar, E., Kumosani, T., Iyer, A., Harakeh, S., and Bragg, R.R.: Modulation by essential oil of vaccine response and production improvement in chicken challenged with velogenic Newcastle Disease Virus. *J. Appl. Microbiol*., 115, 1278-1286. 2013

23. Stimple, M., Proksch, A., Wagner, H., and Lohmann-Matthes, M.L. Macrophage activation and induction of macrophage cytoxicity by purified polysaccharide fractions from the plant Echinacea purpurea. *Infect. Immun.*, 46, 845-849. 1984.

24. Goel, V., Chang, C., Slama, J.V., Barton, R., Bauer, R., Gahler, R., Basu, T.K.: Alkylimides of Echinacea purpurea stimulate alveolar macrophage function in normal rats. *Int. Immunopharmacol*., 2: 381-387. 2002

25. See, D.M., Broumand, N., Sahl, L., Tilles, J.G.: In vitro effects of Echinacea and ginseng on natural killer and antibody-dependent cell cytotoxicity in healthy subjects and chronic fatigue syndrome or acquired immunodeficiency syndrome patients. *Immunopharmacology*, 35: 229-235. 1997

26. Currier, N.L., Miller, S.C.: Echinacea purpurea and melatonin augment natural killer cells in leukemic mice and prolong lifespan. *J Altern Complement Med*, 7: 241- 25. 2001

27. Percival S.S.: Use of Echinacea in medicine. *Biochem.Pharmacol*., 60, 155-158. 2000

28. Barrett, B.: Medicinal properties of Echinacea: a critical review. *Phytomedicine* 10, 66-86. 2003

29. Barbour, E.K., Husseini, S.S., Eid, A., Saker, W. and Hamadeh, S.K.: A holistic approach for growth promotion, protection against Salmonella Enteritidis, and immunopotentiation against infectious bursal disease virus in broiler chickens. *J. Am. Holistic Vet. Med. Assoc.*19, 19-24. 2000

30. O'Neill, W., McKee, S. and Clark, A.F.: Immunological and haematinic consequences of feeding a standardized Echinacea (Echinacea augustifolia) extract to healthy horses. *Equine Vet. J.*, 34, 222-227. 2002

31. Stahl, M., Reifenberg, K., Okpanyi, S., Losch, U.: Porcine granulocyte functions: evaluation and modulation. *J. Vet. Med*., 37: 261-267. 1990

32. Schuberth, H.J., Riedel-Caspari, G. and Leibold, W.: Flow cytometric testing of immunological effects of a phytomedicinal combination (equimun) and its compounds on bovine leucocytes. *J. Vet. Med. A. Physiol. Pathol. Clin. Med.*, 49, 291-298. 2002

33. Allen, P.C.: Dietary supplementation with Echinacea and development of immunity to challenge infection with coccidia. *Parasitol. Res*., 91, 74-78.

34. Böhmer, B.M., Salisch, H., Paulicks, B.R. and Roth, F.X.: Echinacea purpurea as a potential immunostimulatory feed additive in laying hens and fattening pigs by intermittent application. *Livestock Sci*. 122, 81-85. 2009

35. Holdsworth, P.A., Conway, D.P., McKenzie, M.E., Dayton, A.D., Chapman, H.D., Mathis, G.F., Skinner, J.T., Mundt, H.C., Williams, R.B.: World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for evaluating the efficacy of anticoccidial drugs in chickens and turkeys. *Vet. Parasitol.*, 121: 189-212. 2004

36. Conway, D.P., Mckenzie, M.E.: Poultry coccidiosis: diagnostic and testing procedures, 3rd ed. Blackwell Publishing Professional, Ames, Iowa, USA. (pp: 1-164). 2007

37. Allen, P.C., Teasdale, D.: Plasma nitrite increases during Eimeria acervulina infections in chickens. *FASEB J*., 8: A490. 1994

38. Chai, J.Y. and Lillehoj, H.S.: Isolation and functional characterization of chicken intestinal intraepithelial lymphocytes showing natural killer cell activity against tumor target cells. *Immunology* 63, 111-117. 1988

39. Livak, K.J. and Schmittgen, T.D.: Analysis of relative gene expression data using real-time quantitative PCR and the 22-DDCT Method. *Methods* 25, 402-408. 2001

40. Schmittgen, T.D. and Livak, K.J.: Analyzing real-time PCR data by the comparative CT method. *Nat Protoc*, 3 (No.6), 1101-1107. 2008

41. Conway, D.P., Sasai, K., Gaafar, S.M., Smothers, C.D.: Effects of different levels of oocyst inocula of Eimeria acervulina, E. tenella, and E. maxima on plasma constituents, packed cell volume, lesion scores, and performance in chickens. *Avian Dis*., 37: 118-123. 1993

42. Assis, R.C., Luns, F.D., Beletti, M.E., Assis, R.L., Nasser, N.M., Faria, E.S., Cury, M.C.: Histomorphometry and macroscopic intestinal lesions in broilers infected with Eimeria acervulina. *Vet. Parasitol*., 168: 185-189. 2010

43. Allen, P.C., Anderson, M., and Danforth, H.D.: Use of Echinacea as a feed additive to enhance protection against coccidiosis. US Patent, Publication number US 6767546 B1. 2004

44. Brouet, I., Ohshima, H.: Curcumin, an anti-tumour promoter and anti-inflammatory agent, inhibits induction of nitric oxide synthase in activated macrophages. *Biochem. Biophys. Res. Commun*., 206: 533-540. 1995

45. Dimier-Poisson I.H., Soundous, Z., Naciri, M., Bout, D.T., Quere P.: Mechanisms of the Eimeria tenella growth inhibitory activity induced by concanavalin A and reticuloendotheliosis virus supernatants with interferon gamma activity in chicken macrophages and fibroblasts. *Avian Dis*., 43: 65-74. 1999

46. Baba, E., Ikemoto, T., Fukata, T., Sasai, K., Arakawa, A. and McDougald, L.R.: Clostridial population and the intestinal lesions in chickens infected

with Clostridium perfringens and Eimeria necatrix. *Vet. Microbiol* 54, 301-308. 1997

47. Williams, R.B.: Anticoccidial vaccination: the absence or reduction of numbers of endogenous parasites from gross lesions in immune chickens after virulent coccidial challenge. *Avian Pathol.* 32, 535-543. 2003

48. Kim W.H., Jeong, J., Park, A.R., Yim, D., Kim, S., Chang, H.H., Yang, S-H., Kim, D-H., Lillehoj, H.S. and Min, W.: Down-regulation of chicken-17 receptor A during Eimeria infection. *Infect. Immun.* Doi:10.1128/IAI.02141-14. 2014

49. Zhou, H, Gong, J., Brisbin, J., Yu, H., Sarson, A.J., Si, W., Sharif, S. and Han, Y.: Transcriptional profiling analysis of host response to Clostridium perfringens infection in broilers. *Poultry Sci*., 88, 1023-1032. 2009