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Evaluation of Essential Oil Mixture Overuse on Gut Health and Some Immune Parameters in Laying Japanese Quail (*Coturnix Coturnix Japonica*)

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Abstract. This experiment was conducted to investigate the effect of relatively overuse of patent essential oil mixture (EOM) continuously added to drinking water for a period of 6 weeks in Japanese quails on microbial colonization in iliocecal content, some morphometric characteristic and some immune parameters. Experimental birds were divided into 3 groups, control (G1) and two treatment groups (G2 and G3) which received essential oil mixture at a rate of 1 and 2ml per liter, respectively. Results of total bacterial count (TBC), E.coli, Streptococcus and Lactobacillus counts revealed no significant difference ($p \leq 0.05$) between different groups, however there was a trend toward increase counts in treated birds. Also the same pattern was observed in pH values of different intestinal segments. On the other hand, villus height of jejunum and ilium revealed significant decrease ($p \leq 0.05$) in both treatments accompanied with decrease in the digestibility percentage of crude protein and dry matter of fecal content. Carcass yield showed non-significant decrease in G3. Treated groups showed non-significant decrease in both post first and second dose responses to Newcastle disease virus vaccine in comparison with control. Cell-mediated immune response to phytohaemagglutinin (PHA) revealed no significant difference between treated groups and control. On conclusion levels of essential oil mixture used for a period of 6 weeks showed no beneficial effect on carcass yield or reduction of bacterial count in the intestine with no improvement of the immunity; on contrast it has a negative effect on villus height of jejunum and ilium and use of natural products should be evaluated from both beneficial levels and economic importance of levels used.

Keywords: Essential Oil Mixture (EOM), Bacterial colonization, Intestinal morphology, Immunity, Protein digestibility, Laying Japanese quails.

1. Introduction

Essential oils (EOs) are complex mixtures of secondary metabolites consisting of low-boiling-point phenylpropenes and terpenes [1].They have received attention in recent years as potential 'natural' alternatives replacing antibiotic as growth promoters (AGPs) in animal diets due to their positive effect on growth performance, intestinal colonization of bacteria and welfare [2]. The Essential oil mixtures (EOM) have shown some beneficial activities such as antioxidant [3], enzymatic effects [4], digestion stimulating [5], anti-heat stress and immune system activity [6]. The beneficial role of EOs in the modulation of intestinal

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microbiota is associated with health and immunity, and as a consequence affects growth performance and welfare [2]. Development of the morphometric characteristics of gastrointestinal tract is greatly enhanced by the diet of animal [7]. Heightened villi are often associated with intestinal health and improved nutrient absorption [8].

Generally essential oils are very potent molecules and must be used in small quantities. Adversely they can affect the function of intestinal microflora, can cause allergies, suppress feed intake and can be stored in tissues. With the proper usage most of essential oils are recognized as GRAS (generally recognized as safe**).** [9**]**

Most of the studies carried out in this field have shown positive effects of phytogenic products on broiler performance, this may lead to abuse using of these natural products by producers claiming its safety, therefore further research is needed to explore in detail and establish the optimal application of such additives including their optimal dosage level in the feed in order to obtain maximum effects [10] and their action, metabolic pathway. [11]. the present study was carried out to spotlight and investigate whether long term use of essential oil mixture have beneficial or harmful effect on colonization of intestinal microflora some intestinal morphology, carcass characteristics, intestinal content digestibility and some immune response parameters.

2. Material and Methods

2.1. Experimental Birds

Forty weeks-old laying Japanese quail (*Coturnix coturnix japonica*) were divided into 3 groups (n= 30/group). First group (G1) served as a control group at which birds received tap water without any additives. Birds in the other two groups received essential oil mixture in the drinking water for 6 weeks at a rate of 1 and 2 ml per liter of drinking water (G2 and G3, respectively). Birds were given ad libitum access to feed and water and housed in individual layer cages. Titanium dioxide 5 g/kg was incorporated in bird's feed as a dietary marker for digestibility. Birds were vaccinated against New Castle disease by using avian Lassota strain (IZO S.P.A, Italy) at 4 weeks of the experiment then duplication of the dose was performed at 5 weeks of the experiment. Virus vaccines were diluted in distilled water and birds were vaccinated intraocularly. Birds were held until they swallowed the vaccine to ensure vaccination.

2.2. Essential Oils Mixture Composition

EOM was generously donated from A.M. Trading Co., Egypt and is registered commercially under the name of Golden rose. The product contains mixture of EOM and mainly consists of the following; sweet almond oil (20%), olive oil (10%), soya bean oil (5%), lavender oil (15%), eucalyptus oil (5%), coconut oil (15%), pepper mint oil (10%), sesamum seed oil (7%) and citrus oil (13%). The main active components of this oil mixture are monoterpene hydrocarbons including anethole 16.96%, limonene 14.78%, eucalyptol (cineole) 10.55%, trans-traumatic acid 2.11%, menthol 1.67%, eugenol 1.38%, alpha pinene 1.24% and beta pinene 1.24%.

2.3. Enumeration of Intestinal Microflora

Iliocecal contents of 5 slaughtered birds from each group were collected aseptically at the end of the experiment. One gram of ilio-caecal contents was transferred into a sterile test tube, containing 9 ml sterile nutrient broth and then diluted by tenfold serial dilution then 10 µl of mixed diluted sample using vortex (WiseMix, VM-10, Korea) was plated using drop plate technique described by [12] onto the following media: Standard plate count agar (Difco, USA) for total bacterial count (TBC) ;EMB agar (Lab M, LAB061, UK) for E. coli,; Selective streptococci agar (Biolife, Italia) for streptococci, De Man-Rogosa-Sharpe agar (MRS, Lab M, LAB093, UK) for lactobacillus count. Total aerobic bacteria and streptococcus were cultivated at 37℃ for 24–48 h and E. coli was cultivated at 44℃ for 48 hr. Lactobacilli were incubated in 3% CO2 atmosphere at 37℃ for 48–72 h. The results were expressed as arithmetical means ± SE (in log10 cfu/g).

2.4. Carcass Yield and Intestinal Characteristic Parameters (Micrometry and pH)

At the end of the study 5 birds from each treatment were randomly chosen, slaughtered and body weight was measure after feather removal and evisceration was measured using digital balance. The pH value of the

intestinal (duodenum, jejunum and ilium) contents was determined with a pH meter (Jenway, 370 pH meter, U.K).) whereas ileal contents from each replicate were pooled and homogenized for immediate pH measurement.

Intestinal specimens (duodenum, jejunum and ilium) of the control and treated groups were immediately removed and fixed in 10% neutral formalin saline, dehydrated in serially ascending alcohol, embedded in paraffin wax. Tissue specimens were sectioned and stained with hematoxilin and eosin stains [13]. Sections were examined microscopically and elucidated for measuring villous height (VH) , crypt depth (CD) and villus height to crypt depth ratio(VH: CD) of duodenum, jejunum, ileum.

2.5. Apparent Ileal Digestibility

The method of [14] modified by [15] was used for analysis of digestibility. Briefly, contents from 1 cm below Meckel,s diverticulum to 4 cm above the ileo-cecal junction were gently removed. Ileal contents were freeze dried then were used for determination of ileal protein digestibility. 0.1 g freeze-dried digesta sample was ashed and dissolved in 7.4 M sulphuric acid. Hydrogen peroxide (30%vol.) was subsequently added, resulting in the typical orange color the intensity of which was dependent upon the titanium concentration. Aliquots of the solutions obtained and of similarly prepared standard solutions were analyzed using a spectrophotometer and measuring the absorbance at 410 nm [14].

Table 1: Composition and calculated chemical analysis of Japanese quail layers basal diets.

Ingredients	%	Ingredients	%	Calculated composition	
Ground yellow corn	62.62	Limestone (38% Ca)	5.75	Crude protein (%)	20.0
Soya bean meal (45%)*	16.3	DL – Methionine (purity 98%)	0.1	ME (kcal per kg)	2900.0
Corn gluten (60%)	11.1	Lysine (purity 98%)	0.2	Calorie/protein ratio(C/P)	145
Wheat bran	2	Iodized sodium chloride	0.3	Calcium (%)	2.5
Dical.Phosphate (22%Ca&19%P)	1.33	Mineral& Vitamins premix**	0.3	Phosphorus (%)	0.35

* Determined values

** Each 3 kg contain the following vitamins and minerals:
Vit. A 15 mIU, vit. D3 2 mIU, vit. E 1000mg, vit. k3 1000mg, vit. B1 1000mg, vit. B2 5000mg, vit. B6 1500mg, vit. B12 10mg, biotin 50mg, pantothinic acid 10g, nicotinic acid 30g, folic acid 1000mg, manganese 60g, zinc 50g, iron 30g, copper 4g, iodine 300mg, selenium 100mg, cobalt 100mg, carrier(CaCO3) to 3kg. (Golden premix- Selim Pharm Elasher, Egypt. patch No. 8181, production 3- 2010

2.6. Humoral Immune Response to New Castle Disease Virus (ND) Vaccine

At 7 days post each vaccination blood samples from 5 birds per each group were collected from the jugular vein. Serum was collected, and frozen (-20^{0}C) until analyzed for the antibody titers of New castle disease virus using ELISA New castle disease virus antibody test kit (AffinitiTech, LTD , Bentoville,USA).

2.7. Lymphoproliferative Response to Phytohemagglutinin (PHA-P).

Cell mediated immune response (CMI) was assessed by the cutaneous basophilic hypersensitivity (CBH) test in vivo using phytohaemagglutinin (PHA-P code number L8754 – lectin from Phaseolus vulgaris from Sigma-Aldrich) according to the method described by [16] as follow:

5mg of the lyophilized powder were diluted in 5mL of phosphate buffered saline solution (PBS) to obtain a dose of 100µg/0.1mL per bird. PHA-P was injected (100 µg/100 µL/bird) intradermally into the web of the left wing of 5 birds in each group at 26th day of the experiment and at 40th day of the experiment. 0.1mL PBS was injected in the other wing as a control

The thickness of the wing webs were measured before PHA-P injection and then at 24 hrs after injection using a micrometer [17]. The swelling response was calculated as the percentage increase in wing web thickness from the pre injection thickness.

The results were used to calculate response as indicated by [18] as following:

Response = post-PHA-P injection thickness of the left wing – pre-PHA-P injection thickness of the left wing (mm) (1)

PBS control response = post-PBS injection thickness of the left wing – pre-PBS injection thickness of the right wing (mm) (2)

Therefore, cell reaction at each evaluation time was calculated as:

$$CBH = (1) – (2) \quad (3)$$

2.8. Statistical Analysis

Results are expressed as means ± standard error (SE) for each group. Groups were tested for differences by performing the ANOVA and fisher's least protected significance test, also Correlation Coefficient and Factor Analysis were performed using IBM SPSS software computer program version 16, NY, USA (Inc., 1989-2010). Differences were considered statistically significant at $p<0.05$.

3. Results

Table 2 illustrates the results of average of bacterial count of iliocecal contents after 6 weeks of the experiment. Results revealed no significant difference between control and treatment groups. Pathogenic bacteria counts (E. coli and Streptococci) revealed different patterns of increase or decrease indicating no effect of treatments. There was a trend toward increase in the beneficial bacterial count (Lactobacillus) in treated birds as compared to control group in a dose dependent manner.

Morphometric Analysis and pH of the small intestine of the birds at 6 weeks post treatment are shown in Table 3. Duodenum showed no significant difference in pH, villus height (VH) between groups, but there was a significant ($p\leqslant0.05$) decrease in G3 (2ml/L EOM) followed by G2 (1ml/L EOM) in crypt depth, at the same time non-significant improvement was observed in VH:CD in both groups (G2 and G3). Jejunal pH showed different pattern other than duodenum as there was significant increase ($p\leqslant0.05$) in G2 and non-significant increase in G3 in comparison to control, significant decrease ($p\leqslant0.05$) was observed in both treatments G2 and G3 in jejunal VH and CD. Same pattern in VH and CD as jejunum was shown in ilium with non-significant change in both pH and VH: CD.

Tabulated data in Table 4 revealed the factor loading for a rotated factor solution for bacterial count, intestinal characteristics and pH to analyze the association between these parameters. Factor I in G1 had strong positive association between bacterial counts (E.coli, Streptococcus and lactobacillus), duodenal crypt depth, jejunal and ilial villus height crypt depth ratio and ilial villus height. The controlled factors in Factor II in G1 was duodenal villus height (0.992) and jejunal pH (0.98) which show strong positive association with TBC, VH of duodenum and jejunum. Factor I in G2 had a very clear strong positive factor loadings of duodenal (morphology and pH) and pH of ilium which are considered as determinable factors strongly correlated positively with Streptococcus and lactobacillus counts (0.81 and 0.991, respectively) and negatively correlated with E,coli count. Meanwhile, factor II in G2 had strong positive association between TBC and E.coli count with pH of jejunum and ilium. In G3 factor I characterized by very strong factor loading (1) of both TBC and VH:CD of jejunum with pH of duodenum (0.997) which are considered controlled factors for factor I, there were strong positive factor loadings with VH of both jejunum and ilium and CD of duodenum. On one hand, moderate association in factor (1) in G3 was observed in Lactobacillus count, on the other hand strong negative association was observed in E.coli and Streptococcus counts. Factor I in G3 characterized by strong positive loading of pH and CD of both jejunum and ilium.

Data illustrated Table 5 revealed that there was a significant decrease ($p\leqslant0.05$) in both digestibility of Crude protein and dry matter digestibility of treated birds in comparison with control group. At the same time carcass yield (g) showed almost same result in both control and 1 ml EOM treated birds, in the same time 2 ml EOM treated birds had a trend toward decrease carcass yield though differences were not statistically significant.

Data tabulated in Table 6 Indicted that there were no significant differences in antibody responses between 3 groups in both responses (week after first and second duplicated vaccination dose), however second dose response decreased significantly as compared to primary response in all groups $P<0.05$. Results

of skin thickness as a response to PHA injection in wing web revealed no significant difference between the EOM treated group and control group at 4 weeks and the end of the 6weeks experimental period.

Table 2: Bacterial count in iliocecal contents of the birds at 6 weeks post treatment

Treatments	Bacterial count LOG10(CFU/gm)			
	TBC	E.coli	Streptococcus	Lactobacillus
Control	8.48±0.18	7.52±0.3	7.57±0.42	7.63±0.45
1 ml /L EOM	8.39±0.11	6.6±0.49	7.96±0.12	8.1±0.11
2 ml /L EOM	8.64±0.11	7.21±0.29	8.23±0.05	8.41±0.02

Mean within column having different superscript are statistically significant (P≤ 0.05)

Table 3: Small intestine morphology and pH of laying quails at 6 weeks post treatment

Intestinal sections	Parameters	Control	1 ml/L EOM	2ml/L EOM
Duodenum	pH	6.27±0.05	6.24±0.07	6.23±0.06
	VH	528.47^{a}±29.64	526.56^{a}±21.86	496.13^{a}±42
	CD	533.66^{a}±20.24	412.97^{b}±6.12	347.62^{c}±22.77
	VH:CD	0.99±0.05	1.27±0.04	1.5±0.21
Jejunum	pH	6.11^{b}±0.13	6.39^{a}±0.06	6.2^{ab}±0.02
	VH	739.65^{a}±32.79	580.22^{b}±34.33	598.88^{b}±15.1
	CD	485.84^{a}±35.28	413.59^{b}±10.01	385.92^{b}±12.13
	VH:CD	1.57±0.17	1.44±0.07	1.57±0.03
Ilium	pH	6.05±0.2	6.26±0.29	6.63±0.2
	VH	700.62^{a}±13.22	539.00^{b}±10.9	567.17^{b}±9.45
	CD	471.47^{a}±8.82	402.32^{b}±16.41	390.66^{b}±26.41
	VH:CD	1.49±0.05	1.36±0.06	1.48±0.12

Mean within row having different superscript are statistically significant (P≤ 0.05)

Table 4: Varimax rotated factor loading matrix for bacterial count, intestinal characteristics and pH

Groups		Control		1ml/L EOM		2ml/L EOM	
Factors		I	II	I	II	I	II
Bacterial count	TBC	0.417	0.909	0.554	0.833	1	0.019
	E.coli	0.91	-.415-	-.477-	0.879	-.987-	0.162
	Streptococcus	0.903	0.43	0.81	-.587-	-.976-	0.217
	Lactobacillus	0.912	0.411	0.991	-.135-	0.546	-.838-
Duodenum	pH	-.022-	-1.000-	0.951	0.309	0.997	-.079-
	VH	0.124	0.992	0.998	-.069-	-.994-	-.111-
	CD	0.963	0.271	0.951	0.31	0.94	0.342
	VH:CD	-.591-	0.807	0.96	-.278-	-.963-	-.268-
Jejunum	pH	-.198-	0.98	-.106-	0.994	0.524	0.851
	VH	0.49	0.872	-.380-	-.925-	0.807	0.591
	CD	-.997-	-.074-	0.131	-.991-	-.626-	0.78
	VH:CD	0.783	0.621	-.571-	-.821-	1	-.001-
Ilium	pH	0.58	0.814	-.488-	0.873	0.274	0.962
	VH	0.935	-.354-	0.88	0.476	0.971	-.239-
	CD	-.974-	-.228-	0.918	-.398-	-.058-	0.998
	VH:CD	0.988	-.156-	-.871-	0.491	0.369	-.929-
% Explained variance		52.76	47.24	53.3	47.7	63.36	36.64
Cumulative % of variance		52.76	100	53.3	100	63.36	100

Strong loading values ≥ 0.75, moderate loading values (0.5-0.75) and weak loading values 0.5–0.3)[19]

Table 5: Digestibility of intestinal content with carcass yield at the end of experiment between different groups

Parameters	Control	1ml/L EOM	2ml/L EOM
Crude protein digestibility% (Digestion coefficient of CP)	88.288^{a}+0.382	84.01^{b}+ 0.552	79.209^{c}+ 0.58
Dry matter digestibility% (Digestion coefficient of DM)	94.05^{a} +0.15	92.83^{b}+ 0.154	89.95^{c}+ 0.23
Carcass yield (g)	231.4±12.28	230.8±12.96	206.6±7.65

Mean within row having different superscript are statistically significant (P≤ 0.05)

Table 6: Humeral and cellular immune response

Immune response	Groups	Control	1ml/L EOM	2ml/L EOM
Humeral immune response against NDV	1wk post 1st dose	63.46^{a}±12.8	42.51^{a}±2.06	46.1^{a}±4.26
	1wk post 2nd dose	30.47^{b}±3.75	31.36^{b}±2.34	32.54^{b}±2.77
Cellular immune response against PHA	4 wks.	0.43±0.05	0.53±0.04	0.48±0.1
	6 wks.	0.48±0.15	0.47±0.1	0.44±07

Mean within row having different superscript are statistically significant (P≤ 0.05)

4. Discussion

Of the main benefits of any product improving gastrointestinal tract health are reduction of pathogenic microorganisms counts as well as increasing counts of beneficial ones. Interaction of the active ingredients of herbs with other nutrient and the balanced microbes in gastrointestinal tract makes the evaluation of the possible antimicrobial action of herbs and spices in *in vivo* situations hard [9]. In our study it is clear that in spite of non-significant change in bacterial count in iliocecal content between the EOM treated groups and control, there was a trend toward decrease in E.coli count in G2 followed by G3, and these distribution of bacterial count could be attributed to the constituents of the EOM which contain some ingredients which have antimicrobial effect such as limonene or cinnamaldehyde and this result is in consistent with [20] who reported that the membranes of *E. coli* grown in the presence of limonene or cinnamaldehyde showed changes in long chain fatty acid profile. Also presence of lavender oil could have a moderate antimicrobial activity against different bacterial species of lavender [21]. On the other hand, lactobacillus count revealed a trend toward increase in G3 followed by G2 as compared to control group which might be attributed to the presence of EOM which could enhance the growth of lactobacilli, and so increases the ratio of lactobacilli to enterobacteria and this result is strengthened with results of [22] who reported that herbs and spices have both antimicrobial activity, and modulate the composition of microbial population by prebiotic activity.

The pH of different segments of small intestine could have an explanation in bacterial modulation in the iliocecal content at which in G2, streptococcus and lactobacillus counts strongly associated with pH of duodenum while E.coli count was negatively associated, at the same time TBC and E.coli count positively correlated to pH of jejunum and ilium. In G3, TBC is a controlled factor which is strong positive associated with the pH of duodenum, also TBC negatively associated with E.coli count and moderately correlated with lactobacillus count. The pH of the matrix in which the EOs are found affects their hydrophobicity which will enhance their interaction with the bacterial cell membrane, and therefore, affect the antibacterial action of EOs across the different intestinal segments. So pH considered as ideal candidates as intestinal bacterial modulators [2].

Small intestine morphology did not reveal significant improvement in both EOM treatments in comparison with control group, results revealed shorter villus height in jejunum and ilium in both EOM treatments which could be explained due to presence of some irritant ingredients in the EOM in higher doses such as menthol which found in peppermint in much smaller proportions and could causes hypersensitivity reaction [23], abdominal pain [24], loss of weight and hepatocellular changes [25] which does not enhance villus height or improve nutrient absorption [26]. This result is in agreement with [27] who stated that changes in intestinal morphology such as shorter villi and deeper crypts have been associated with the presence of toxins or higher tissue turnover. There are some herbs that may cause toxicity when they are administrated in large quantities [16].

Significant decrease of digestibility% of both crude protein and dry matter in G3 followed by G2 and non-significant decrease of carcass yield in G3 could be attributed to significant decrease in villus height of

different intestinal segment in both treatments especially in higher dose (G3) as the height of intestinal villi is associated with the capacity of the bird to absorb nutrients from feed [28]. Regarding carcass yield our results is in partial agreement with [29] who found no significant improvement in carcass yield of quails when use essential oil mixture (EOM) at 48 mg/kg diet.

Results of humeral immune response against ND vaccine is in agreement with [30] who demonstrated that an EOM blend was not effective in improving the humoral immune response of layer hens as measured serum IBDV, NDV, and IBV titers. Significant decrease in antibodies after duplication of the vaccinal dose at 5 weeks of the experiment could be due to the high resistance of the quail to avian form NDV vaccine, which produce low response in the 3 groups when compared to the first vaccination response. In addition the type of vaccine might had determinable effect as in this study we used inactivated live vaccine and results could be strengthen by results obtained by [31] who found that commercially available ND LaSota strain inactivated vaccine for chickens induced a high antibody response in Japanese quails. Also their results showed that Japanese quails produced a moderated antibody response when vaccinated with commercially available live vaccines (Ulster 2C, B1 and LaSota) for chickens against Newcastle disease without any clinical signs of post vaccinal reactions.

It is clear that none of the two levels used in this study supported the immune system and boost antibody titers or cell mediated immune response to PHA. As improvement of the immunity is a result of improvement and enhancement of intestinal morphology and any deterioration effect on intestinal morphometric characteristics will intern be reflected on performance and immunity. Also antibody responses of laying hens is influenced by age-related decline rather than feed additive-based interference [32].

5. Conclusion

Our study indicated that EOM treatments had affected the studied parameters into two ways. The first way was in the form of no significant changes on the microbial colonization in iliocecal content, pH of intestinal segments and immune parameters compared to the control group. The second way was harmful effect which was clear in the form of shortening of villus height of jejunum and ilium with decrease of digestibility% of crude protein and dry matter in intestinal content in both treatments. Considering dose and period of using changing from those recommended by the manufacture (0.25ml per liter for 3-5 days) into higher dose of 1 and 2ml/L using of natural products should not be looked at as a safe additive regardless of levels used and periods of application. Also different natural products mixtures should be evaluated for their safety margin levels in different animal species.

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