The effects of herbal plants on *Mucin 2* gene expression and performance in ascetic broilers

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Abstract:

BACKGROUND: The mucus layer plays an important role as an intermediate for the protection of the gut against acidic chyme, digestive enzymes, and pathogens; in addition, it acts as a lubricant and facilitator of nutrient transportation. Phytogenic compounds seem to promote intestinal mucus production. **OBJECTIVES:** The current study was conducted to investigate the effects of low and high levels of energy and amino acids in combination with turmeric, thyme, and cinnamon on chicken performance and expression of *mucin* 2 gene. **METHODS:** The eight experimental groups consisted of diluted and condensed diet with and without the addition of 5g/kg of each turmeric, thyme, and cinnamon to the diet. Chicken performance was recorded. Expression analysis of the *mucin* 2 gene was carried out by quantitative RT-PCR. **RESULTS:** Body weight gain, feed intake, FCR, and mortality rate were not affected by diets ($p>0.05$). A significant ($p<0.05$) reduction of the *mucin* 2 gene expression was observed in chickens fed by condensed diet; however, the expression increased by supplementation of turmeric, thyme, and cinnamon. **CONCLUSIONS:** These results, in addition to the function of herbs in increasing the activity of some enzymes which is possibly related to the mucin biological pathways, showed that the application of turmeric, thyme, and cinnamon could be useful in poultry diets. It appears that supplementation of turmeric, thyme, and cinnamon could increase *mucin* 2 gene expression in the small intestine, and this can improve intestinal digestive function and defense.

Introduction

Over the last decade, the importance of gastro-intestinal tract health in broiler chicken was increasingly studied due to its contribution to their overall health and performance (Mountzouris et al., 2007; Rehman et al., 2007). The use of antibiotics at sub-therapeutic levels have been a basis of the poultry industry for the control of subclinical diseases, maintenance of gut health, and growth promotion for a number of decades (Mathew et al., 2007). The emergences of antibiotic resistant strains of pathogens have raised some restrictions on antibiotic use in food animals. In Europe, sub-therapeutic use of antibiotics in poultry rearing has been phased out since 2006. In accordance with these restrictions, the use of phytogenic feed additives, which comprise a wide variety of herbs, has recently gained increasing interest, especially for use in poultry.

Numerous studies have demonstrated anti-oxidative and anti-microbial efficacy of phytogenic compounds in vitro; however, in vivo experimental evidences are still quite limited (Denli et al., 2004). In addition, it was hypothesized that phytogenic

compounds may specifically enhance the activities of digestive enzymes and promote intestinal mucus production (Moghaddam et al., 2011).

The epithelium of the intestinal tract is covered mainly by a layer of mucus composed of mucin glycoproteins that are synthesized and secreted by goblet cells. The mucus layer plays an important role in protecting the gut against acidic chyme, digestive enzymes, and pathogens; mucin also acts as lubricant and facilitated nutrition transport between the luminal contents and the epithelial cells (Montagne et al., 2004). In humans, mucins are now categorized into three distinct families according to the structure of the protein product which are gel-forming (*Mucin* 2, *Mucin* 5AC, *Mucin* 5B and *Mucin* 6), soluble (Mucin 7), and membrane-bound (*Mucin* 1, *Mucin* 3, *Mucin* 4 and *Mucin* 12) (Moniax et al., 2001). *Mucin* 2 is the major intestinal *mucin* gene that was initially isolated from a human jejunum cDNA library (Sadasivan et al., 2011).

In the current study, we investigate the effects of two different kinds of diets (lower and higher levels of energy and protein according to the Arian strain recommendation) and three kinds of herbal plants (turmeric, thyme, and cinnamon) on performance traits and *mucin* 2 gene expression.

Materials and Methods

Broiler management: 960 one day old Arian chickens were randomly divided into eight groups of 120 chicks; with four replicates of 30 chicks assigned to each replicate. The chicks were reared for 42 days on wood shavings under standard conditions and provided adlibitum access to feed and water. Formulation of diluted and condensed diets is shown in table 1. Treatment groups were as follows:1) diluted diet 2) condensed diet; 3) diluted diet + 5 g/kg turmeric; 4) condensed diet + 5 g/kg turmeric; 5) diluted diet + 5 g/kg thyme; 6) condensed diet + 5 g/kg thyme; 7) diluted diet + 5 g/kg cinnamon; and 8) condensed diet + 5 g/kg cinnamon (Table 2). Herbal plants used in this experiment were obtained from a commercial source as dry powder.

Traits measured and tissue sampling: Body weights were measured weekly. Total feed intake was measured per pen weekly and mortality rate was recorded daily. FCR was measured and adjusted for mortality. On day 42, six birds from each treatment were slaughtered and their intestine (jejunum) segments were removed and immediately were frozen at -80°C.

Real-time RT-PCR: Total RNA was isolated from the jejunum samples using the RNXTM (Plus) (RN7713C, Cinnagen Inc., Tehran, Iran) according to the manufacturer's instructions. The RNA samples were aliquoted into four replicates and stored at -80°C until analysis.

RNA was reverse transcribed to cDNA using a RevertAidTM first strand cDNA synthesis kit (K1622, Fermentas). All RNA samples were reverse transcribed simultaneously for minimizing the variations. The cDNA samples were stored at -80°C until analysis. Real-time PCR was performed using universal SYBER Green PCR master mix (RR350Q, Takara) in Corbett Science Rotor-Gene 3000 sequence detection system (Qiagen). The PCR was performed in a reaction volume of 25 µL containing the reagents at the following final concentrations: 1X Universal SYBER Green PCR master mix (2X), forward primer 10 µM, reverse primer 10 µM and 2µL of cDNA sample. The cycling profiles used for three genes were: 1 cycle at 95°C for 5 min, 40 cycles PCR (denaturation at 95°C for 30s; annealing at 63°C for 30s and extension at 72°C for 30s) followed by a final extension at 72°C for 5 min. The specific primers for intestinal *mucin* 2 are shown in table 3. This primer was designed by using the reference sequence of the gene in Gallus gallus.

In each run, a negative control, a calibrator sample, cDNA samples, and endogenous control (GAPDH) were included. GAPDH samples were analyzed in duplicate and the target genes were analyzed in triplicate. The relative gene expression was quantified by the $\Delta\Delta C_t$ method. ΔC_t was calculated by subtracting the Ct amount of *mucin* 2 gene from Ct of GAPDH for each sample, then $\Delta\Delta C_t$ was calculated from subtract ΔC_t of each treatment of ΔC_t control. We selected the diluted diet as control and then $2^{-\Delta\Delta C_t}$ was calculated.

Statistical analysis: Data were analyzed using GLM procedures of SAS software (SAS, 2006).

Results

Performance measurements: Performance traits

Table 1. Composition of basal diet fed to broilers. [(1)]Premix provided the following per kilogram of diet: vitamin A(vitamin A acetate) 9000 U; vitamin B1, 1.8 mg; vitamin B2, 6.6 mg; niacin, 30 mg; vitamin B6, 3 mg; Calcium pantothenate, 10 mg; Folic Acid, 1 mg; B12, 0.015 mg; 0.1 mg D-biotin, vitamin D3 2000 U; vitamin E 18 U; vitamin K2, 2 mg; Choline chloride, 500 mg; manganese (Manganese oxide), 100 mg; zinc, 100 mg; iron (Iron sulfate, 7H2O), 50 mg; copper (copper sulfate, 5H2O), 10 mg; iodine (Calcium iodine), 1 mg; selenium (Sodium selenite), 0.2 mg.

Ingredients	1 to 21 days age		21 to 42 days age	
	Condensed diet	Diluted diet	Condensed diet	Diluted diet
Corn	55.09	49.9	60.31	54.53
Soybean meal	38	36.5	34	34.7
Fish meal	1	5	-	1.9
Soybean oil	1.5	4.83	1.45	5
Dicalcium phosphate	1.9	1.63	1.78	1.63
Oyster shell	1.19	0.94	1.11	1.01
Baking soda	0.04	0.05	0.09	0.05
Salt	0.33	0.33	0.33	0.33
L - Met	0.28	0.22	0.26	0.22
L - Lys	0.07	-	0.07	0.03
Vitamins and minerals[(1)]	0.6	0.6	0.6	0.6

Table 2. Nutrient analysis of diets.

Chemical composition of diets	1 to 21 days age		21 to 42 days age	
	Diluted diet	Condensed diet	Diluted diet	Condensed diet
Crude protein, %	22	23.5	20	21.07
MEn, (kcal/kg)	2850	3050	2910	3100
Calcium, %	1	1	0.9	0.9
Threonine, %	0.85	0.92	0.76	0.81
Methionine + cysteine, %	0.99	1	0.9	0.91
Lysine, %	1.27	1.37	1.13	1.20
Available phosphorus, %	0.5	0.5	0.45	0.45
Sodium %	0.17	0.18	0.17	0.17
Anion Cation Balance	241	243	230	230

of broilers are presented in table 4. Chickens fed condensed diet showed more body weight gain than those fed by diluted diet. Body weight gain had no differences in chickens fed by various herbal plants. Supplementation of diluted diet with turmeric, thyme, and cinnamon had no effect on body weight gain as well as condensed diet with turmeric. Feed intake, FCR, and the rate of mortality were not significantly affected by diet concentration and adding the herbs to each diet ($p>0.05$).

***Mucin* 2 mRNA expression:** The expression of *mucin* 2 gene in the chicken jejunum, was significantly reduced in chickens fed condensed diet compared with those fed diluted diet. Addition of turmeric, thyme, and cinnamon to both basal diets increased the expression of *mucin* 2 mRNA in jejunum of chickens (Table 4).

Discussion

The prohibition of antibiotic use in poultry feed has forced investigators to research growth promoting alternatives (Marcincák et al., 2011). These alternatives are greatly favored in the poultry industry. Turmeric (*Curcuma Longa*) thyme (*Thymus vulgaris*) and cinnamon (Cinnamomum verum) are among alternatives for growth promoting antibiotics. The major components of turmeric, thyme, and cinnamon are curcumin, thymol, and carvacrol and trans-cinnamyl acetate and β-caryophyllene, respectively. It has been demonstrated that all of these components have antioxidative properties (Toghyani et al., 2010).

In this study we observed that the application of condensed diet increased body weight gain in comparison to the diluted diet. Supplementation of turmeric, thyme, and cinnamon to each of the diluted and turmeric to the condensed diet (interaction effect) had no effect on body weight gain ($p>0.05$). It was expected that supplementation of diets with herbs would stimulate the growth performance of broilers (Al-Kassie, 2009; Toghyani et al., 2011). The bio-active substances of these herbs may improve feed digestibility, the gut microbial balance, and excitation of digestive enzymes and thus improve growth performance in broilers without affecting FCR (Sadeghi et al., 2012). These herbs also may improve safety in host animals and increase availability of nutrients in the intestine for absorption; thereby resulting in animals to grow better and decreasing incidences of disease and mortality. Some unknown parameters may have interacted with the herbs such as basal diet, age, strain, and environmental conditions that affect the herbal plant.

The results of the present study are in agreement with the previous observations that indicated no effect of these herbs ($p<0.05$) on body weight gain, feed intake, or feed conversion ratio in broilers (Mehala and Moorthy, 2008; Toghyani et al., 2010).

Table 3. Primers used for RT-PCR analysis of chicken mRNAs.

Target (Accession No.)	Primer	Sequence (5'➔ 3')	Size of PCR product (bp)	T_m °C
GAPDH	Forward	TGAAGGGTGGTGCTAAGCGTG	288	66
(NM_204305.1)	Reverse	GGATGATGTTCTGGGCAGCAC		
MUC2	Forward	CTGTTGTGGATGGGCGGATTG	157	66
(XM_421035.2)	Reverse	CCAAACTTGCTGTCCAGCTCC		

Table 4. Measured parameters for performance of broiler chickens from 1 to 42 days of age. (a, b and c) Means with no common superscripts differ significantly ($p<0.05$). MSE is mean squared error.

Dietary treatment	Body weight gain, (g/bird)	Feed intake, (g/bird)	Feed conversion ratio, (g/g)	Mortality%
Diluted diet	57.20^b	106.60	1.90	3.80
Condense diet	60.00^a	107.10	1.80	3.90
None herbs	58.50	109.40	1.90	3.90
Turmeric	57.90	105.80	1.80	3.50
Thyme	59.10	106.00	1.80	4.30
Cinnamon	58.90	106.10	1.80	3.80
Diluted diet without any herbs	56. 57	107.61	1.90	3.25
Condense diet without any herbs	60.40	111.20	1.84	4.50
Diluted diet + 5 g/kg diet turmeric	56.70	107.57	1.90	4.00
Condensed diet + 5 g/ kg diet turmeric	59. 11	104.00	1.76	3.11
Diluted diet + 5 g/ kg diet thyme	57.85	104.44	1.80	4.25
Condensed diet + 5 g/ kg diet thyme	60.31	107.50	1.78	4.25
Diluted diet + 5 g/ kg diet cinnamon	57.50	106.77	1.86	3.75
Condensed diet+ 5 g/ kg diet cinnamon	60.31	105.52	1.74	3.75
MSE	4.77	36.78	0.01	0.53

Table 5. Relative quantification using the comparative CT method. (a, b and c) Means with no common superscripts differ significantly ($p<0.05$). MSE is mean squared error.

Dietary treatment	*Mucin* 2 (Average C_T)	GAPDH (Average C_T)	ΔC_T (MUC2- GAPDH)	$\Delta\Delta C_T$ (ΔC_T- $\Delta C_T{}^0$)	Fold change in *mucin* 2 gene
Diluted diet	15.50	12.48	3.02	0	1.00^a
Condense diet	21.02	12.95	8.08	5.52	0.03^b
Diluted diet + 5 g/kg diet turmeric	14.97	12.54	2.43	-0.17	1.50^c
Condensed diet + 5 g/ kg diet turmeric	17.35	14.43	2.92	-0.03	1.07^a
Diluted diet + 5 g/ kg diet thyme	16.15	13.74	2.41	-0.18	1.52^c
Condensed diet + 5 g/ kg diet thyme	16.76	14.58	2.18	-0.25	1.78^d
Diluted diet + 5 g/ kg diet cinnamon	16.87	14.73	2.14	-0.26	1.84^d
Condensed diet+ 5 g/ kg diet cinnamon	15.98	13.85	2.13	-0.26	1.85^d
MSE					0.028

However, extracted oil from thyme and cinnamon improved body weight gain, feed intake, and feed conversion ratio in broiler diets (Al-Kassie, 2009). Supplementing of thyme and cinnamon to each of the diets increased body weight of broilers ($p<0.05$), which was in agreement with some studies (Ocak et al., 2008; Toghyani et al., 2010) but is not concordant with the others (Tekeli et al., 2009; Rahimi et al., 2011).

Carbohydrates, proteins, and specific amino acids such as threonine have been demonstrated to alter mucin secretion and may interact directly with goblet cells or with the enteric nervous system to elicit changes in mucin secretion (Smirnov et al., 2005; Smirnov et al., 2006; Horn et al., 2009; Moghaddam et al., 2011). *Mucin* 2 gene expression enhanced after starvation in chickens (Smirnov et al., 2004). There was no effect of threonine on intestinal goblet cell

density or *mucin* 2 mRNA abundance for broilers (Horn et al., 2009; Moghaddam et al., 2011). The expression 5 pattern of the *mucin* 2 gene in chickens fed antibiotic growth promoter (AGP) or a probiotic product were greater than the observation in controls (Smirnov et al., 2005).

Thus, changes of the mucus layer would be expected to influence nutrient digestion processes. Supplementation of turmeric, thyme and cinnamon to both basal diets increased the expression of *mucin* 2 mRNA in jejunum of chickens. Any component, dietary or environmental, that induces changes in *mucin* gene expression has the potential to affect the integrity of the mucus layer and nutrient absorption. Reduction of the *mucin* 2 gene expression in jejunum of broiler chickens fed condensed diet may be related to the decrease of the mRNA stability. Several lines of evidence indicated that the regulation of mRNA stability, in response to external stimuli, changes the gene expression (Cheadle et al., 2005; Barnett et al., 2007). Stability of *mucin* 2 mRNA can be influenced by the initiation factor 5A, which affects the turnover of mRNA. Cytokines, growth factors, and bacterial products or any conditions that affect differentiation of goblet cells can also affect *mucin* 2 gene expression. It is possible that bioactive substances of these herbs may influence the HapA concentration that is an extracellular proteinase and increases secretion and accumulation of *mucin* 2 gene in the gastrointestinal tract. The bioactive substances also may alter the activity of transcription factors such as GATA4 and Fox1 that regulates *mucin* 2 gene expression in broiler chickens (Van der Sluis et al., 2004; Van der Sluis et al., 2008). Supplementation herbs to broiler chickens' diets could change the mucin expression and nutrient utilization.

Our results showed that supplementation of turmeric, thyme, and cinnamon enhanced the *mucin* 2 gene expression in jejunum of broiler chickens, and thus it may influence its protective properties and nutrient absorption. Application of these herbs that could promote *mucin* 2 gene expression can be useful for poultry.

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تأثیر گیاهان دارویی بر بیان ژن *mucin 2* و عملکرد جوجه‌های گوشتی آسیتی

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چکیده

زمینهٔ مطالعه: موسین نقش مهمی در حفاظت لایهٔ مخاطی مجاری گوارشی در برابر اسید معده، آنزیم‌های گوارشی و پاتوژن‌ها با ایجاد سطح لغزنده و تسهیل جذب مواد مغذی ایفا می‌کند. به نظر می‌رسد ترکیبات گیاهی در افزایش ترشح موسین نقش داشته باشند. **هدف:** این تحقیق به منظور بررسی تأثیر دو سطح بالا و پایین انرژی و پروتئین جیره به همراه مکمل گیاهان دارویی زردچوبه، آویشن و دارچین بر عملکرد جوجه‌های گوشتی و بیان ژن *mucin 2* انجام گرفت. **روش کار:** هشت تیمار آزمایشی شامل جیرهٔ غلیظ و رقیق بدون مکمل و به همراه ۵g/kg مکمل گیاهان دارویی شامل زردچوبه، آویشن و دارچین بود. عملکرد جوجه‌ها در طول دورهٔ پرورش ثبت شد و بیان ژن *mucin 2* به روش PCR Real Time بررسی شد. **نتایج:** براساس نتایج تفاوت معنی‌داری در میزان خوراکی مصرفی، افزایش وزن روزانه و ضریب تبدیل خوراک در بین تیمارهای مختلف مشاهده نشد ($p>۰/۰۵$). کاهش معنی‌داری در بیان ژن *mucin 2* در جوجه‌های تغذیه شده با جیرهٔ غلیظ مشاهده شد ($p>۰/۰۵$)، اگرچه افزودن مکمل گیاهان دارویی به هر دو جیرهٔ پایه (رقیق و غلیظ) بیان این ژن را در بافت رودهٔ جوجه‌ها افزایش داد ($p>۰/۰۵$). **نتیجه‌گیری نهایی:** مواد مؤثر بر موجود در این گیاهان دارویی ممکن است از طریق افزایش فعالیت آنزیم‌های دخیل در مسیر تولید موسین، بیان ژن *mucin 2* را در روده افزایش می‌دهد. افزودن مکمل زردچوبه، آویشن و دارچین به جیرهٔ جوجه‌های گوشتی می‌تواند با افزایش موسین، فرایند هضم خوراک و سیستم ایمنی پرنده را بهبود می‌بخشد.

واژه‌های کلیدی: جوجه‌های گوشتی، دارچین، بررسی بیان ژن *Mucin 2*، آویشن، زردچوبه

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