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Research Article

Pathogen Control, and Digestion and Immunity Development in Broilers by Supplementing Drinkable Water with Waste Date Vinegar

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Abstract | To investigate the effects of supplementing drinkable water with waste date's vinegar (WDV) on the growth performance and digestive tract of broilers, two hundred 1-day-old broilers (Ross 308) were used. Chicks were randomly allocated to 5 experimental groups including supplementing 0 (control), 0.5, 1, and 1.5 % of WDV and 1 % industrial vinegar (IV) into drinkable water. Broilers body weight and food consumptions were measured at the trial's beginning and days 10, 21, 35 and 42 of experimental period. Moreover, one chick from every replicate was killed at days 21 and 42 to measure development of digestive tissues and morphology and microbiology of small intestine and evaluate the humoral immune responses (anti-SRBC antibody, IgG and IgM). Results showed that although the periodic body weight gain increased in all vinegar treatments compared to control (P<0.05), feed intake and feed conversion ratio were not affected by adding vinegar into water. Villus height was higher by use of 1% WDV compared to control and IV (P<0.05), but crypt depth was not different across groups. Also, ileum microbiota and humoral immune responses were not affected by treatments. Results indicated that supplementing drinking water with WDV and IV had positive effects on growth performance and use of 1% WDV developed histomorphology of jejunum in broilers.

Keywords | Acetic acid, Ileum microflora, Jejunum histomorphology, Performance, Probiotic.

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INTRODUCTION

Because of increasing demand for high quality broiler meat and decreasing tolerance for environmental contamination, lots of researches all over the world are encouraging efficient use of natural substances. Also, due to the ban of antibiotics in poultry nutrition, new projects are working on organic alternatives. Some of these organic alternatives which are potentially useful to inhibit pathogen growth and increase poultry performance include Probiotics (Patterson and Burkholder, 2003), non-traditional chemicals (Moore et al., 2006), bacteriophages (Higgins et al., 2005), acidifiers (Tasharofi et al., 2017), enzymes and etc. Some of these natural substances are not cited a lot in the scientific literatures, but are used locally. A good example for these locally used additives, is waste date vinegar (WDV) inclusion to water and/or diet of broilers in south of Iran. Annual production of waste date in Iran, which is not used by human, is 160000 tones that could be used in poultry diets after removing the kernels. Also, after some processing, WDV could be produced with the acetic acid as its main component. Acetic acid is one of the main short chain fatty acids produced by intestinal microbes, which can affect intestinal functions and metabolism (Bergman, 1990; Kishi et al., 1999; Lutz and Scharrer, 1991). Overall, organic acids make the gut circumstances unsuitable for pathogen bacteria due to pH lessen (a result of their free hydrogen proton) and gram-negative bacteria growth inhibition (a result of penetration into cytoplasm of these bacteria and oxidative phosphorylation prevention) (Luck-

stadt and Kuhlmann, 2013). In addition to these effects of WDV, it includes some beneficial bacteria such as *lactobacillus* spp. to improve performance, immunity and digestive tract in broiler chicks. A strikingly crucial event in the development of probiotics was the finding that newly hatched chicks could be protected against colonization by *Salmonella enteritidis*. This could happen by dosing a suspension of gut contents derived from healthy adult chickens which is called competitive exclusion. Also, use of probiotics containing *lactobacilli* inhibits the growth of *Salmonella enteritidis* and *Ecoli* in broilers (Murry et al., 2006). *Lactobacillus* of WDV as a probiotic could be settled in small intestine and as a result, decreases the infectious bacteria such as *salmonella* and *Ecoli*. The intestine seems to be the most fundamental organ for improving animal products. Activation of intestinal function of broilers might increase the meat products in response to an increasing demand for poultry protein (Ruttanavut et al., 2009). So, we decided to investigate how broiler performance and intestinal histology would be affected after feeding the WDV. In our previous project, we have supplemented WDV into diet to investigate intestinal histology and pathogen control of broilers and its findings are published (Tasharofi et al., 2017). In the present study, due to simplicity of use of water additives in poultry farms, impressions of adding WDV into drinkable water on body weight gain, feed consume and feed conversion ratio were examined in broiler chicks. Also, their jejunum villus height and crypt depth were measured and amounts of their ileum *lactobacillus* and *Ecoli*. were counted.

MATERIALS AND METHODS

Preparation of WDV

Almost one ton of fresh waste date was soaked in water, reduced to pulp, removed the kernels and then combined to water in ratio of approximately 1 waste date to 3 water to produce WVD before being used in current study.

Chemical and Microbial Analysis

Representative samples of WDV and IV were analyzed for percentages of acetic acid (titration method with a colored pH indicator) which were 2.6 and 10.4 respectively. Also, samples of WDV were tested for microorganism existence (microbial culture method) which showed lactobacillus, bacillus and mold existence with total account 4.5×10^5 cfu (Colony Forming Unit). Table 1 shows the chemical and microbial composition of IV and WDV. Also, it is noticeable that to ignore the effects of different percentages of acetic acid in two vinegars (Scharf and Malerich, 2010), during all experiment, IV was 4 times diluted with distilled water before use.

Animals, Diets and Experimental Design

Two hundred mixed sex one-day-old broilers (Ross 308) weighing 40±1.5 g were allocated to five experimental treatments in a balanced completely randomized design (n=4) with 20 pens (1.5×0.7 m^2 each) and 10 chicks in every pen. Treatments were as follows: Supplemented drinkable water with incremental levels of WDV [0 (control), 0.5, 1, and 1.5%] and 1% of IV. Table 1 presents the chemical composition of diet and all chicks had free access to feed and water (*ad-libitum*). Chicks were raised under similar environmental conditions based on Ross 308 management recommendations for 42 days (Aviagen, 2009). Before the beginning of experiments, all animals were vaccinated for bronchitis and routine vaccinations (i.e., newcastele {at days 8, 17 and 28} and gambro {at days 13 and 24}) were done during the growing period. Chicks were visited daily in a regular program for general health and some individual behaviors including illness, breath, anorexia and etc.

Performance

Experimental period lasted 42 days. The feed amounts offered and refused were measured periodically at days 10, 21, 35 and 42 for each pens to calculate feed intake (FI). Moreover, the chicks were weighed at these days after 2 hours of fasting to reduce the disputes arising from feed consumption and these weights were used to calculate the body weight (BW) changes and average periodic body weight gain (BWG) of chicks over the experimental time. By having these two measurements, feed conversion ratio (FCR) was calculated as feed consumed per unit of gain.

Digestive Tract Sampling and Analysis

At days 21 and 42 of experimental period, 4 chick from every treatment (from 2 pens: male and from 2 pens: female, with average weight ± 20 g) were sacrificed by cervical dislocation to measure relative weight of different parts of small intestine (weight {g organ/g live body weight} of duodenum, jejunum and ileum), morphology and microbiology of jejunum and ileum respectively. The experimental protocols were reviewed and approved by the Animal Care Committee of Research Institute of Animal Science, Iran.

Jejunum Morphology and Analysis

For histopathologic and morphometric analysis, 0.5 cm tissue samples from the jejunum of chicks mentioned above were obtained and fixed in 10% buffered formalin (100 mL of 40% form aldehyde, 4 g phosphate, 6.5 g dibasic sodium phosphate and 900 mL of distilled water) for 24 h and then the 10% buffered formalin were renewed. Tissues were dehydrated by transferring through a series of alcohols with increasing concentrations, placed into xylene and embedded in paraffin. A microtome was used to make 5 cuts that were 5 µm. The paraffin sections were stained with hematoxylin-eosin (Thompson and Applegate, 2006).

Table 1: Ingredients and chemical composition of diets and chemical and microbial composition of vinegars

Ingredients of diet (g/kg)	Diets per days			
	1-10	11-21	22-35	36-42
Corn	538	529	652	680
Soybean meal	400	355	288	260
Soybean oil	17.50	13.00	22.00	22.00
DL-Methionine	2.90	2.80	2.30	2.05
L-Lysine hydrochloride	1.00	1.15	1.05	1.10
Threonine	0.55	0.65	0.50	0.45
Di calcium phosphate	18.40	16.00	15.50	15.80
Calcium carbonate	11.60	10.00	9.60	9.60
Salt	3.20	3.00	2.80	2.80
Sodium bicarbonate	1.50	1.40	1.00	1.00
Vitamin Premix	2.50	2.50	2.50	2.50
Mineral Premix	2.50	2.50	2.50	2.50
Chemical composition of diet				
ME (Kcal/kg)	2910	2950	3070	3100
CP (%)	22.00	20.70	18.20	17.10
Ca (%)	1.03	0.90	0.85	0.85
Available P (%)	0.50	0.45	0.42	0.42
Lysine (%)	1.31	1.21	1.03	0.97
Methionine (%)	0.61	0.58	0.50	0.46
Methionine-Cysteine (%)	0.97	0.92	0.81	0.75
Threonine (%)	0.90	0.85	0.73	0.69
Na (%)	0.19	0.18	0.16	0.16
K (%)	0.95	0.88	0.76	0.72
Chemical and microbial composition of vinegars	Kind of vinegars			
Acid acetic (g/100ml)	IV		10.4	
Acid acetic (g/100ml)	WDV		2.6	
Total Count (cfu /g)	WDV		4.5×10^5	

The values were measured with a LEICA light microscope [using the LEICA Queen 550 software (Germany)]. Measurements of villus height, width and crypt depth were determined at a magnification of 10X. A minimum of 5 measurements per slide were made for each parameter and averaged into one value.

Ileum Microflora and Analysis

Digesta were obtained from ileum of chicks mentioned above, and collected in sterile bags to count *lactobacillus* and *E.coli*. Digesta samples were homogenized with 1 mL serum physiologic. Five µL aliquot was mixed with blood agar and eosin methylene blue (EMB) and incubated at 37° C for 24 h. After incubation, bacteria colonies were counted in selective agar media for enumeration of target bacterial groups. The microbial counts were determined as colony forming units (cfu) per gram of wet samples (Boyd and Mulvey, 2013).

Humoral Immune Response

The humoral immune responses were evaluated by hemagglutination inhibition (HI) method. At first, sheep red blood cells (SRBC) were collected and washed 3 times in PBS. The packed cells were brought to a 5% v/v solution in sterile PBS. Breast muscles of chicks were injected by 0.5 ml/chick SRBC at 14th day, followed by a booster injection at 35th day. chick's blood samples were taken 7 days after the first and second injections (21st and 42nd days). Then, plasma was stored at -20°C until tested. During the test, plasma was heat inactivated at 56°C for 30 min and then analyzed for total, mercaptoethanol-sensitive (IgM) and mercaptoethanol-resistant (IgG) anti-SRBC antibodies. Briefly, 25 µl of plasma was added to an equal amount of PBS in the first column of a wells U-shaped bottom microplate and serial dilution was then made and 25 µl of 1% SRBC suspension was added to each well. Total antibody titers were then read after 120 min of incubation at 37°C.

Table 2: Effect of IV and different levels of WDV on performance of broilers.

Parameters					Treatments	*P*-Value			SEM
	Control	1%IV	0.5%WDV	1%WDV	1.5%WDV				
						Treat × Period	Period	Treat	
BWG, g p^{-1}	495.36[b]	531.88[a]	534.23[a]	558.97[a]	538.71[a]	0.040	<0.0001	0.024	11.748
FI, g p^{-1}	1065.22	1017.55	1057.71	1057.72	1046.28	0.862	<0.0001	0.769	27.958
FCR	2.05	1.86	1.90	1.85	1.88	0.147	<0.0001	0.379	0.075

BWG, body weight gain per period; FI, feed intake per period; FCR, feed conversion ratio.

[a,b] Means within a row with different subscripts differ (P<0.05).

Table 3: Effect of IV and different levels of WDV on relative weight × 100 of different parts of small intestine of broilers.

Parameters	Treatments					*P*-Value	SEM
	Control	1%IV	0.5%WDV	1%WDV	1.5%WD		
21st day							
Relative weight of duodenum (g/g)	1.34	1.39	1.28	1.34	1.30	0.93	0.0000
Relative weight of jejunum (g/g)	2.50	2.75	2.45	2.40	2.57	0.58	0.0016
Relative weight of ileum (g/g)	2.01	2.22	1.68	2.09	2.18	0.26	0.0010
42nd day							
Relative weight of duodenum (g/g)	0.78	0.85	0.74	0.89	0.79	0.63	0.0007
Relative weight of jejunum (g/g)	1.58	1.77	1.51	1.76	1.61	0.31	0.0010
Relative weight of ileum (g/g)	1.41	1.61	1.35	1.44	1.49	0.59	0.0011

Relative weight, g weight of tissue per g live body weight of chick.

Table 4: Effect of IV and different levels of WDV on jejunum morphology and ileum microflora of broilers.

Parameters	Treatments					*P*-Value	SEM
	Control	1%IV	0.5%WDV	1%WDV	1.5%WD		
21st day							
Villus height (µm)	1059.84	1131.63	1157.04	1172.80	1154.87	0.49	47.362
Villus width (µm)	182.31	190.40	168.69	214.81	174.78	0.29	15.439
Crypt depth (µm)	195.18	189.62	202.59	204.60	225.11	0.55	15.355
Villus height/crypt depth	5.51	5.98	5.72	5.90	5.31	0.87	0.503
42nd day							
Villus height (µm)	1194.07[b]	1204.42[b]	1263.78[ab]	1308.43[a]	1248.00[ab]	0.02	24.104
Villus width (µm)	199.48	215.06	196.19	204.16	212.72	0.83	13.839
Crypt depth (µm)	245.80	245.58	255.13	215.73	257.40	0.50	17.838
Villus height/crypt depth	4.98	5.00	5.04	6.18	4.87	0.21	0.425
21st day							
Lactobacillus (Log cfu/g)	4.41	4.43	4.31	4.62	4.18	0.82	0.265
E.coli (Log cfu/g)	4.71	4.63	4.63	4.54	4.34	0.76	0.212
42nd day							
Lactobacillus (Log cfu/g)	4.59	4.57	4.39	4.68	4.30	0.77	0.235
E.coli (Log cfu/g)	4.67	4.48	4.48	4.38	4.25	0.83	0.252

[a,b,c] Means within a row with different subscripts differ (P<0.05).

The well immediately preceding a well with a distinct SRBC button was considered as the endpoint titer for agglutination. For IgG response, 25 µl of 0.02 M mercaptoethanol in PBS was used instead of PBS alone, followed by the pervious mentioned procedure. The difference between the total and IgG response was considered to be equal to the IgM antibody level (Khaleghi Miran et al., 2010).

Table 5: Effect of IV and different levels of WDV on humoral immune responses of broilers.

Parameters	Treatments					*P*-Value	SEM
	Control	1%IV	0.5%WDV	1%WDV	1.5%WD		
21st day							
Anti-SRBC titer	3.75	4.00	3.50	4.00	3.50	0.29	0.214
IgG	2.25	2.25	2.75	2.75	2.00	0.11	0.223
IgM	1.25	1.75	1.00	1.00	1.50	0.12	0.241
42nd day							
Anti-SRBC titer	6.75	6.00	6.00	6.00	6.50	0.64	0.442
IgG	4.75	4.25	4.25	4.00	4.50	0.82	0.469
IgM	2.00	1.50	2.00	2.25	1.75	0.68	0.376

Relative weight, g weight of tissue per g live body weight of chick.

Statistical Analysis

The data obtained from performance (BWG per period, FI per period and FCR) were analyzed using repeated measurements model in which the time series (1 to 10, 11 to 21, 22 to 35 and 36 to 42) covariance structure was modeled by using 4 different covariance structures for each variable tested and the means were compared using Tukey's multiple comparisons procedure. Other variables based on a completely randomized design were statistically analyzed using the GLM procedure of SAS (SAS, 2003), and the means were compared using Duncen's multiple comparisons procedure. Statistical models were as follows.

Repeated measurements model:

$Y_{ijk} = \mu+T_i+P_j+T_i*P_j+e_{ijk}$

μ: Overall mean T_i: Treatment

P_j: Time

T_i*P_j: Interaction between treatment and time

e_{ijk}: error

Completely randomized design:

$Y_{ij} = \mu+T_i+e_{ij}$

μ: Overall mean T_i: Treatment

e_{ij}: error

RESULTS

Animal Performance

Table 2 shows data of performance variables. Obviously, average periodic weight gain of control group was lower than other treatments (P<0.05), but FI and FCR were not affected by use of vinegar in the water.

Small Intestinal Growth

Data of growth (relative weight) of different parts of small intestine is presented in Table 3. All data (relative weights of duodenum, jejunum and ileum) showed no significant difference between the treatments.

Jejunum Histomorphology

Table 4 shows the intestinal morphology characteristics of broilers including villus height, villus width, crypt depth and ratio of villus height to crypt depth. As shown in this table, villus height increased linearly in control, IV and WDV treatments at 42nd day of growing period (P<0.05). This data indicated the beneficial impression of WDV use on villus height of broilers. None of other parameters like villus width, crypt depth and ratio of villus height to crypt depth were affected by adding vinegar to drinkable water.

Ileum Microbiota

According to Table 4, none of ileum microflora variables (including *lactobacillus* and *E.coli*. spp. count) were affected by supplementing water with WDV and IV. But numerically, their supplementation maintained the populations of unprofitable bacteria or potential pathogens (*E.coli*) at relatively low levels in the ileum's digesta in comparison with control group.

Humoral Immune Response

Based on obtained results by HI method in Table 5, none of humoral immune responses (Anti-SRBC titer, IgM and IgG) were impressed by use of IV and WDV.

DISSCUSION

The performance data are in line with results which reported significantly increase in BW by feeding vinegar (including 5% acetic acid) and probiotics in broilers (Kral et al., 2011) and supplementing organic acids into diet or water (Luckstadt and Kuhlmann, 2013 and Shanoon et al., 2018), and administrating probiotic into drinkable water (Karimi Torshizi et al., 2010). But some have reported that supplementing diet by bamboo vinegar solution does not affect the final BW, FI and FCR of ducks (Ruttanavut et al., 2009). Also, supplementing drinking water of broilers with a commercial organic acid (Chaveerach et al., 2004 and Hayajneh 2019) or commercial lactobacillus based probioticdose (Eckert et al., 2010) does not result to any significant affection on BW.

Acetic acid of vinegar reportedly increases gastric proteolysis and improves the digestibility of proteins and amino acids (Samanta et al., 2010) which leads to better performance of animals. In addition, this acid inhibits the growth of harmful intestinal bacteria which competes with the host animal for available nutrients (Dhawale, 2005). The combination of acetic acid and probiotic in WDV added to drinkable water is the main reason which controls the balance of intestinal microflora and positively affects intestinal functions and metabolism compared with IV.

Results of small intestine growth are in line with the findings that observed no changes in relative weight of different parts of small intestine of broilers derived from probiotic supplementation in diet (Seifi, 2013). On the other hand, use of butyric acid (Mahdavi and Torki, 2009), and acetic acid (Furuse et al., 1991), in broiler diet caused an increase of relative weights of jejunum and ileum.

Jejunum histomorphology results are in agreement with an increase of jejunum villus height and area on male broilers fed bamboo vinegar liquid (Ruttanavut et al., 2009).

It is well demonstrated that organic acid in vinegar increases the solubility of nutrients and improves the gastric proteolysis which develops digestibility of proteins and amino acids (Samanta et al., 2010). Also, probiotic produces antimicrobial substances that protect the villi and absorption surface against toxins (Pelicano et al., 2005), and mainly promotes secretion of digestive enzymes (Ledezma-Torres et al., 2015). All of these reasons lead to increase in the absorption of available nutrients, a mechanism that directly affects the recovery of the intestinal mucosa and increasing villus height and better intestinal functionality (Biernasiak and Slizewska, 2009).

Our findings about the ileum microbiota were not supported by the results that have reported decrease of adverse bacteria in gut microflora of broilers by use of probiotics (Decroos et al., 2004; Biernasiak and Slizewska, 2009; Mountzouris et al., 2010), and decrease of Gram negative bacteria in use of probiotic and organic acid by broiler chicks (Gunal et al., 2006). On the other hand, in line with our result, it has been reported that drinking water acidification causes no effect on count of anaerobic bacteria in broilers' gut (Chaveerach et al., 2004). It is generally documented that there are two basic mechanisms by which probiotics act to maintain a beneficial microbial population, including "competitive exclusion" and "immune modulation". Competitive exclusion involves competition for substrates, production of antimicrobial metabolites that inhibit the pathogens, and competition for attachment sites (Yang et al., 2009). Also, by directly interacting with gut mucosal immune system, probiotics can modulate either innate or acquired immunity, or both to protect the increase in amount of pathogens in gut (Dugas et al., 1999).

Some researches have shown that antibody levels in broilers (anti-SRBC, IgM and IgG) increase in response to probiotics (Haghighi et al., 2005; Mountzouris et al., 2010) and acidifiers (Elnaggar et al., 2017), but some have not seen any significant differences between humoral immune responses in broilers by probiotic usage (Yakhkeshi et al., 2012; Pourakbari et al., 2016). Natural antibodies in chicken may be reactive to self or foreign antigens, and there is an association between high specific antibody responsiveness and high levels of natural antibodies in serum (Haghighi et al., 2005). It is expected that broilers divert some of nutrients from growth to immune development (Mountzouris et al., 2010), and probiotics and acidifiers have been shown to have immunomodulatory activity, but in the present study, there were not any obvious different between humoral immune responses in broilers.

CONCLUSIONS

Results from current experiment indicates that adding WDV into drinking water increases the body weight gain of broiler chicks. In addition, intestinal morphology increases by implementation of WDV to drinking water of broilers. In conclusion, WDV can be supplemented to water of broilers to improve growth performance.

CONFLICT OF INTEREST

There is no conflict of interest.

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AUTHORS CONTRIBUTION

All authors contributed equally.

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