Growth performance, nutrients digestibility, caecum microbiota, antioxidant status and immunity of broilers as influenced by kombucha fermented on white sugar or sugar beet molasses

Saleh Salehia,b, Amirali Sadeghi a and Ahmad Karimia

aDepartment of Animal Science, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran; bDepartment of Animal Science, Kurdistan Agricultural and Natural Resources Research and Education Centre (AREEO), Sanandaj, Iran

ABSTRACT
The purpose of this study was to evaluate the effects of fermented kombucha on green tea with white sugar or sugar beet molasses in broiler chickens. Birds (n = 448) were randomly allotted into seven treatments of four replicates each. Treatments were included: tap water with no kombucha (Control), water containing 3 ml/100 ml kombucha based on white sugar (SK3%), water containing 6 ml/100 ml kombucha based on white sugar (SK 6%), water containing 3 ml/100 ml kombucha based on sugar beet molasses (MK3%), water containing 6 ml/100 ml kombucha based on sugar beet molasses (MK6%). Except for SK3%, all birds received SK or MK in their drinking water had greater (p < 0.05) BWG than control. Feed intake increased (p < 0.05) in SK6% group in comparison to the control. All SK or MK treatments resulted in lower (p < 0.05) FCR in comparison with the control. Birds received the MK6% and SK6%+MK6% treatments showed greater (p < 0.05) apparent ileal digestibility of CP. ALT levels decreased (p < 0.05) in birds received all kombucha treatments. The SK and MK treatments decreased (p < 0.05) total coliform counts in caecum whereas the lactobacillus population increased (p < 0.05) in birds received 6% of SK, MK or their combination. The SK6%+MK6% groups had greater (p < 0.05) antibody titre against NDV. Plasma and liver activities of CAT and SOD were greater (p < 0.05) in kombucha treatments in comparison with the control group. In conclusion, kombucha fermented on white sugar or sugar beet molasses showed growth promoting effect in broilers and addition of 6 ml fermented kombucha on sugar beet molasses per 100 ml of drinking water result in the best performance.

HIGHLIGHTS
• Kombucha can improve performance in broilers
• Kombucha can improve intestinal morphology and caecum microbiota in broilers
• Kombucha can improve antioxidant status in broilers

Introduction
Following the elimination of antibiotics as growth stimulants from poultry diets in the European Union since 1 January 2006, (Castanon 2007), the poultry industry has faced an increase in disease outbreaks and mortality and reduced growth rates (Murugesan et al. 2015; Cowieson and Kluenter 2019). As a result, researchers have been trying to find suitable alternatives to antibiotics in order to prevent the economic losses caused by their elimination.

Kombucha is made by fermenting sweetened tea with a cellulose matrix of bacteria and yeast, and its metabolites include organic acids, minerals, vitamins, polyphenols, and other biologically active components (Neffe-Skocińska et al. 2017; Villarreal-Soto et al. 2018; Jakubczyk et al. 2020). Therefore, Kombucha could be considered as a suitable candidate for replacing growth-promoting antibiotics.

Most important metabolites of kombucha are organic acids that may exert beneficial effects. It has an organic acid content of about 16.75 g/L (Kaewkod et al. 2019; Jakubczyk et al. 2020) at 14–15 days of fermentation and the main organic acids in kombucha are acetic acid, gluconic acid, gluconic acid, lactic acid, malic acid, citric acid, pyruvic acid, succinic acid,
malonic acid, and usnic acid (Kumar and Joshi 2016; Neffe-Skocińska et al. 2017; Villarreal-Soto et al. 2018). Organic acids improve broiler performance by helping to create an acidic intestinal environment (Centeno et al. 2007; Abdel-Fattah et al. 2008; Nourmohammadi and Afzali 2013), a balance of intestinal microflora (Bourassa et al. 2018; Nguyen et al. 2018; Adhikari et al. 2020) and increased nutrient digestibility and nutrient utilisation resulting in enhanced protein and energy output (Khan and Iqbal 2016; Nguyen et al. 2018; Nguyen and Kim 2020).

Additionally, kombucha contain polyphenols and other biologically active components that might boost its beneficial effects in comparison to organic acids. In broilers, supplementation of kombucha fermented on white sugar improved the feed conversion ratio (Arani et al. 2014), body weight gain (Afsharmanesh and Sadaghi 2014) and boosted the immune system (Arani et al. 2014). Traditional carbon source for kombucha fermentation is sucrose and using of other sugars may influence the products of kombucha fermentation, especially its acid content. Sugar beet molasses could be an attractive source of sugar because of its low price and the presence of a number of components including minerals, organic compounds and vitamins, which are very useful for the fermentation process (Malbaša et al. 2008). It has been shown that total acidity, yield of biomass and lactic acid content were greater in kombucha fermented on sugar beet molasses, while in kombucha fermented on sucrose the acetic acid was greater (Malbaša et al. 2008). Thus, using kombucha prepared on different sugars may exert different effects in broilers. To our knowledge, no reports on using kombucha fermented based on sugar beet molasses in broilers has appeared in the literature. Therefore, the present study was conducted to evaluate the effects of kombucha fermented on sugar beet molasses (MK) alone or in combination with kombucha fermented on white sugar (SK) on growth performance, ileal digestibility of some nutrients, caecal microbiota, immune response and antioxidant status of broiler chickens.

Materials and methods

All procedures used in the present study have been approved by the Animal Care and Use Committee of the University of Kurdistan (Sanandaj, Iran).

Birds and housing

Four hundred and forty-eight day-old Ross 308 male broiler chickens were housed in a completely randomised design. Each treatment included 4 replicates and 16 chicks per replicate. Each pen was $140 \times 150$ cm, covered with wood shavings as litter and equipped with hanging water bottle and feeder. The broilers had ad libitum access to feed and water throughout the entire experiment period. A continuous lighting schedule was used during the first day after arrival then the broilers were exposed to a photoperiod of 23 h of light and 1 h of dark.

Preparation of kombucha

To prepare one litre of kombucha, 1.5 grams of green tea and 70 grams of white sugar or sugar beet molasses were used. First, water was boiled, and green tea was brewed in the boiled water for 10 minutes. After removing the tea pulp, sugar and molasses was added to the boiled water. After the temperature of the sweet tea cooled to $25 \degree C$, kombucha starter (liquid kombucha culture is usually taken from a previously fermented kombucha and needed to start kombucha fermentation) was added in the amount of 10% (v/v) as the starter. Next, 24 gr of symbiotic culture of bacteria and yeast (SCOBY) were added, and the kombucha cultures were stored under aseptic conditions. Fermentation was carried out by incubating the kombucha culture at $25 \degree C$ for 10 days.

Diets and treatments

In this study, the kombucha product was added to the birds’ drinking water, so all of the birds received the same feed. Basal diets were formulated to meet all nutrient recommendations based on rearing guidelines for Ross 308 broilers (Table 1).

The chickens received kombucha in drinking water in seven treatments in the following order: tap water with no kombucha (Control), water containing 3 ml/100 ml kombucha based on white sugar (SK3%), water containing 6 ml/100 mL kombucha based on white sugar (SK 6%), water containing 3 ml/100 mL kombucha based on sugar beet molasses (MK3%), water containing 6 ml/100 mL kombucha based on s sugar beet molasses (MK6%), SK 3%+MK 3% and SK 6%+MK 6%.

Experimental procedure

All birds were weighed at 1, 10, 24, and 42 d of ages on a group basis, and the feed intake of each pen was recorded during each growth period in order to calculate the feed conversion ratio. The amount of water intake per pen was measured during different growth
periods. Bird deaths and weights per pen were recorded daily, and the data was used for adjusting body weight and water intake. The average body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), and water intake (WI) were then measured from 1 to 10, 11 to 24, 25 to 42, and 1 to 42 d of age.

At 24 and 42 days of the experiment, two birds per replicate (16 chicks per treatment) were randomly selected, bled by wing vein puncture, weighed, and slaughtered by severing the jugular veins and carotid arteries. The following samples were taken for further experiments and studies.

Carcass traits and tissue sampling
At days 24 and 42, carcase weights, breast, thighs, liver, and abdominal fat were measured and expressed as g/100 g of bodyweight. The empty crop, proventriculus, gizzard, duodenum, jejunum, ileum, and caeca were also removed, weighed, and calculated as a percent of bodyweight. Similarly, the lengths of the duodenum, jejunum and ileum were measured (cm).

Water consumption and water-feed ratio
The amount of water consumed by chickens in different growth periods’ including 1–10, 11–24 and 25–42 days of age was measured, the amount of feed consumed per pen during each period was determined, and this data was used to estimate the ratio of water intake to feed intake.

Intestinal morphology
The segments sampled from the midpoint of the jejunum and ileum were used for measuring the villus height, crypt depth, villus width and for determining the villus surface area and villus height to crypt depth ratio (VH:CD). The separated pieces were flushed twice with a physiological saline solution (1% NaCl) in order to remove intestinal contents and placed in 10% formalin in a 0.1 mol/L phosphate buffer saline. The samples were then removed from the formalin and placed in the automatic processing machine, dehydrated through a series of graded ethanol baths in order to displace the water, cleared in xylene and infiltrated with paraffin. Sample tissue blocks 5 μm thick were taken from the jejunum and ileum by microtome and affixed to slides. A routine staining procedure was then carried out using haematoxylin and eosin, and a morphological examination of the samples was performed with an optical microscope (Olympus CX31, Tokyo, Japan).

Digesta pH
At 24 days of age, 1 gr of the contents of different parts of the gastrointestinal tract were taken from slaughtered birds in order to measure their pH. The contents of each section were mixed with 9 ml of distilled water and the pH was determined using a digital pH metre (Thompson and Hinton 1997).

Apparent ileal digestibility of the feed nutrients
At 24 days of age, birds received the experimental feeds with an added 0.3% of the indicator chromium oxide (Cr₂O₃) for 72 hours in order to estimate the apparent ileal digestibility. The ileal contents of slaughtered birds were collected by gentle squeezing, and the digesta samples were stored at −20°C until further analysis. The concentrations of Ca, P, and CP (N × 6.25, Micro-Kjeldahl) in diets and ileal samples were analysed according to the relevant laboratory procedures (AOAC 1990). The chromium oxide contents of the experimental diets and ileal digesta samples were determined according to Fenton and Fenton

Table 1. Ingredients and chemical composition of the basal diet fed to broilers, % as fed.

| Ingredients               | 1–10 days | 11–24 days | 25–42 days |
|---------------------------|-----------|------------|------------|
| Corn                      | 52.15     | 57.01      | 63.85      |
| Soybean meal (42.9% CP)   | 40.69     | 36.51      | 30.55      |
| Soybean oil               | 3.04      | 2.90       | 2.42       |
| Dicalcium phosphate       | 1.54      | 1.42       | 1.18       |
| Calcium carbonate         | 1.08      | 0.84       | 0.76       |
| Common salt               | 0.37      | 0.37       | 0.37       |
| Vitamin premix*           | 0.25      | 0.25       | 0.25       |
| Mineral premix*           | 0.25      | 0.25       | 0.25       |
| DL-methionine             | 0.31      | 0.24       | 0.21       |
| Lysine HCl                | 0.21      | 0.14       | 0.14       |
| L-Threonine               | 0.11      | 0.06       | 0.03       |

*Supplied per kg diet: 9000 U vitamin A, 3000 U vitamin D₃, 18 U vitamin E; 2 mg vitamin K₃; 1.8 mg thiamine; 6.6 mg riboflavin; 3 mg vitamin B₆; 0.015 mg vitamin B₇; 30 mg niacin; 1000 mg choline chloride; 300 mg vitamin C; 10 mg calcium D-pantothenate; 1 mg folic acid.

bSupplied per kg diet: 100 mg Mn; 50 mg Fe; 84.7 mg Zn; 10 mg Cu; 1 mg I; 15 mg Se.
The ileal digestibility of nutrients was then calculated using the formula shown below:

\[
\text{Digestibility (\%) } = \frac{1 - (Nf \times Cd)/(Nd \times Cf))}{C2} \times 100
\]

where:
- **Nf** = Nutrient concentration in excreta (%DM)
- **Nd** = Nutrient concentration in diet (%DM)
- **Cf** = Chromium concentration in excreta (%DM)
- **Cd** = Chromium concentration in diet (%DM)

**Caecal microbial populations**

On day 24 of the study, 0.5 g of caecal contents was removed from slaughtered birds and mixed with 1.5 g of glycerol solution (Russell and Hespell 1981). The samples were placed in a 2 mL microtubule and then frozen in a nitrogen tank and placed in a freezer at \(-24^\circ C\). Caecal contents samples were cultured in the laboratory according to Hung et al. (2012). Coliform bacteria were cultured in coliform agar (Merck 1.10426), and plates were placed in an incubator at 37\(^\circ C\) for 48 h. Lactobacillus strains were cultured in MRS-agar (Merck, 1.1066) and the plates were placed in an anaerobic jar (Merck 1.16387) with an anaerobic gas pack system (Anaerocult, Merck 1.13829) at 37\(^\circ C\) for 48 h. The microflora enumerations were expressed as log\(_{10}\) CFU per gram.

**Plasma biochemical parameters and antibody titre**

Concentrations of total cholesterol, triglyceride, high density lipoprotein (HDL), low density lipoprotein (LDL), glucose, uric acid and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity were analysed with a spectrophotometer (Hitachi U-2001 spectrophotometer, Tokyo, Japan). SOD activity was measured by the xanthine oxidase method by inhibition of nitro blue tetrazolium reduction and the change of absorbance at 560 nm (Sun et al. 1988). The activity of CAT was measured following the decrease in absorbance at 240 nm due to hydrogen peroxide decomposition (Aebi 1984). GPx activity was measured at 412 nm by quantifying the rate of oxidation of reduced glutathione to oxidised glutathione (Hafeman et al. 1974). The MDA level was determined with a wavelength of 532 nm in order to assess absorbance (Placer et al. 1966).

**Statistical analysis**

The collected data was submitted to analysis of variance using the General Linear Model (GLM) procedure of the SAS statistical package (SAS Institute Inc 2001). Differences among means were tested using Duncan’s multiple range test \((p<.05)\). The following model was used to study the effect of test materials on the investigated parameters:

\[y_{ij} = \mu + T_i + e_{ij}\]

where:
- \(y_{ij}\) = Observation for each dependent variable
- \(\mu\) = Overall mean
- \(T_i\) = Treatment effects
- \(e_{ij}\) = Random residual effects

**Results**

**Performance and carcass characteristics of broilers**

The effects of experimental treatments on BWG, FI and FCR are shown in Table 2. There was no significant difference among treatments with BWG during 1 to 10 d of age \((p>.05)\). During 11 to 24 d of age, other than the SK3%+MK3% treatment, the inclusion of SK and MK resulted in greater \((p<.05)\) BWG than that of the control group, and MK 6% caused the
Table 2. Effect of treatments on body weight gain (g), feed intake (g) and feed conversion ratio (g/g) in broiler chicks.

| Treatments          | Body weight gain (g) | Feed intake (g) | Feed conversion ratio (g/g) |
|---------------------|----------------------|-----------------|-----------------------------|
|                     | 1–10                 | 11–24           | 25–42                       | 1–42 | 1–10 | 11–24 | 25–42 | 1–42 |
| Control             | 176.5                | 609.9           | 1451.6                      | 2327.7 | 241.9 | 1005.6 | 3032.4 | 4279.9/c |
| SK 3%               | 175.0                | 670.7           | 1461.4                      | 2306.9/bc | 242.1 | 1064.2/ab | 3041.3 | 4309.4/c |
| SK 6%               | 175.1                | 718.0           | 1496.2                      | 2389.3/a | 242.8 | 1078.7 | 3089.0 | 4410.3/c |
| MK 3%               | 172.7                | 683.4           | 1490.0                      | 2364.5/bc | 247.9 | 1035.9/hed | 2974.6 | 4250.2/c |
| MK 6%               | 183.1                | 701.6           | 1537.8                      | 2422.3/a | 249.9 | 1060.5/hc | 3000.0 | 4310.3/c |
| SK3%+MK3%           | 173.3                | 642.1           | 1587.4                      | 2402.8/b | 240.7 | 1010.2/d | 3121.5 | 4372.2/c |
| SK6%+MK6%           | 181.2                | 676.2           | 1558.2                      | 2416.5/b | 239.7 | 1022.0/hd | 3069.4 | 4339.3/c |
| SEM                 | 2.300                | .0030           | .0040                       | .0005  | .9040 | .0030 | .0040 | .0005  |
| p Value             | .0020                | .0120           | .0140                       | .0090  | .0020 | .0120 | .0070 | .0001  |

*Kombucha prepared based on white sugar. **Kombucha prepared based on sugar beet molasses. *SEM: Standard error of means. #Means within columns with different superscripts are significantly different (p < .05).

Table 3. Effects of treatments on carcase yields and proportions of carcase parts to body weight at 42 d of age in broiler chicks.

| Treatments          | Item                           | Control | SK 3% | SK 6% | MK 3% | MK 6% | SK3%+MK3% | SK6%+MK6% | SEM |
|---------------------|--------------------------------|---------|-------|-------|-------|-------|-----------|-----------|------|
|                     | Body weight (g)                | 2397.50 | 2259.50 | 2453.50 | 2361.30 | 2532.00 | 2447.50 | 2510.00 | 47.310 | .95 |
|                     | Proportion of carcase parts to body weight (%) | 23.30 | 24.00 | 24.40 | 23.90 | 24.10 | 24.20 | 24.90 | .530 | .99 |
|                     | Breast                         | 17.80 | 18.10 | 18.50 | 17.90 | 18.40 | 19.60 | 18.50 | .230 | .47 |
|                     | Thigh                          | 0.52 | 0.54 | 0.50 | 0.49 | 0.49 | 0.62 | 0.55 | .015 | .35 |
|                     | Heart                          | 2.34 | 2.00 | 1.76 | 2.06 | 2.17 | 2.33 | 2.31 | .065 | .14 |
|                     | Spleen                         | 0.12 | 0.10 | 0.08 | 0.09 | 0.11 | 0.12 | 0.14 | .006 | .41 |
|                     | Proventricular                 | 0.53 | 0.41 | 0.42 | 0.53 | 0.42 | 0.38 | 0.43 | .018 | .11 |
|                     | Gizzard                        | 2.77 | 2.55 | 2.37 | 2.54 | 2.18 | 3.02 | 2.41 | .086 | .21 |
|                     | Pancreas                       | 0.26 | 0.22 | 0.23 | 0.23 | 0.25 | 0.23 | 0.23 | .009 | .72 |
|                     | Duodenum                       | 0.74 | 0.56 | 0.60 | 0.75 | 0.73 | 0.74 | 0.72 | .023 | .34 |
|                     | Jejunum                        | 1.69 | 1.73 | 2.26 | 2.02 | 2.47 | 2.57 | 2.03 | .083 | .09 |
|                     | Ileum                          | 1.60 | 0.47 | 0.54 | 0.63 | 0.57 | 0.72 | 0.50 | .034 | .53 |
|                     | Small intestine segments and caeca length (cm) | 35.00 | 28.50 | 36.50 | 36.80 | 36.50 | 36.50 | 47.30 | 1.840 | .27 |
|                     | Duodenum                       | 83.20 | 83.30 | 76.80 | 78.50 | 82.00 | 79.00 | 86.30 | 1.850 | .87 |
|                     | Jejunum                        | 88.00 | 83.30 | 84.30 | 96.80 | 94.50 | 98.30 | 94.80 | 2.200 | .37 |
|                     | Ileum                          | 19.80 | 19.50 | 22.00 | 22.30 | 20.50 | 22.00 | 19.00 | .710 | .83 |

*Kombucha prepared based on white sugar. **Kombucha prepared based on molasses. *SEM: Standard error of means.

The greatest BWG. During the 25–42 d of age, birds receiving SK3%+MK3% had greater (p < .05) body weight gain than control and those receiving the 3% kombucha in drinking water. From day 1–42, except for SK3%, all birds received SK or MK in their drinking water had greater (p < .05) BWG than control and the greatest BWG was belonged to birds received MK 6% in drinking water.

Feed intake was not significantly influenced by the experimental treatments during 1–10 and 25–42 d of age. During 11–24 d of age, the birds received SK3%, SK6% and MK6% treatments consumed more feed than control group (p < .05). However, during 1–42 d of age, only SK6% significantly (p < .05) increased the FI in comparison to the control treatment. There was no significant difference in the FCR among treatments during 1–10 d of age. During 11–24, 25–42 and 1–42 d of age, with the exception of the SK3% treatment, the inclusion of SK or MK in drinking water resulted in lower (p < .05) FCR in comparison with the control group and the lowest FCR in the entire experimental period was belonged to the birds that received MK6%.

The mortality rate of chickens was not affected by experimental treatments and was within the expected range (data not shown).

As shown in Table 3, the inclusion of kombucha in the drinking water had no significant effect on the carcase yields, and the relative weight of carcase parts and internal organs to body weight at 42 days of age (p > .05). Moreover, the weight and length of the small intestine segments and caecum were not affected by the kombucha treatments (p > .05).

Morphology of jejunum and ileum

The results in Table 4 show the intestinal morphology in broiler chicks at 24 days of age. Jejunal villus height was significantly increased (p < .05) in all treatments except for the SK3% treatment when compared to the control treatment and the greatest villus height were belonged to MK6% and SK6%+MK6%. Inclusion of MK
Table 4. Effect of treatments on intestinal morphology in broiler chicks at 24 days of age.

| Treatments         | Jejunum | Ileum |
|--------------------|---------|-------|
|                    | VHh     | WVi   | CDj | AVSAk | VH/CDl | VH | VW | CD | AVSA | VH/CD |
| Control            | 650c    | 144d  | 167 | 293e  | 3.90   | 571f  | 167g  | 163 | 300h  | 3.53   |
| SK 3%              | 665hi   | 161i  | 165 | 336j  | 4.05   | 600kl | 176m  | 162 | 332n  | 3.75   |
| SK 6%              | 750ih   | 159i  | 172 | 373j  | 4.43   | 664o   | 185p  | 179 | 385q  | 3.73   |
| MK 3%              | 682ah   | 159ai | 173 | 340b  | 3.97   | 617h   | 182ab | 157 | 352c  | 3.93   |
| MK 6%              | 772a    | 154b  | 192 | 374j  | 4.04   | 670a   | 180bc | 182 | 378c  | 3.73   |
| SK3%+MK3%          | 679ab   | 156bc | 164 | 332b  | 4.15   | 605c   | 184d  | 155 | 350e  | 3.95   |
| SK6%+MK6%          | 771f    | 154g  | 181 | 373j  | 4.27   | 692g   | 184h  | 177 | 400i  | 3.96   |
| SEMg               | 9.76    | 1.15  | 3.11 | 5.61  | 0.10   | 8.93   | 1.21  | 3.59 | 6.62  | 0.08   |
| p Value            | .001    | .001  | .169 | .001   | .001   | .001   | .244  | .001 | .801  |        |

*Kombucha prepared based on white sugar. **Kombucha prepared based on sugar beet molasses. †SEM: Standard error of means. aVillus height. bVillus width. cCrypt depth. dApparent villus surface area (×10^-3 µm²). eVillus height to crypt depth ratio. fMeans within columns with different superscripts are significantly different (p<.05).

Table 5. Effect of treatments on ileum nutrient contents and digestibility in broiler chicks at 24 days of age.

| Treatments         | Ileum nutrient content (%) | Ileum nutrient digestibility (%) |
|--------------------|---------------------------|---------------------------------|
|                    | Ash | Calcium | Phosphorus | CP | Ash | Calcium | Phosphorus | CP |
| Control            | 11.0  | 1.19   | 0.63   | 14.8e | 34.0  | 50.0   | 48.3   | 77.2e |
| SK 3%              | 10.2  | 1.17   | 0.64   | 13.8f | 38.4  | 50.4   | 50.3   | 79.6f |
| SK 6%              | 10.8  | 1.22   | 0.63   | 13.9g | 37.4  | 51.1   | 50.3   | 79.9g |
| MK 3%              | 10.4  | 1.23   | 0.66   | 13.9h | 39.9  | 50.2   | 48.4   | 79.4h |
| MK 6%              | 10.8  | 1.25   | 0.66   | 13.2i | 45.4  | 53.7   | 51.4   | 82.1i |
| SK3%+MK3%          | 11.1  | 1.21   | 0.64   | 14.3j | 35.7  | 50.5   | 48.7   | 78.2j |
| SK6%+MK6%          | 10.4  | 1.22   | 0.63   | 12.6k | 40.2  | 51.4   | 50.4   | 81.5k |
| SEMh               | 0.010  | 0.0150 | 0.007  | 0.220 | 1.110  | 0.640  | 0.690  | 0.430 |
| p Value            | .080  | .890   | .170   | .430  | .800   | .860   | .016   |        |

*Kombucha prepared based on white sugar. **Kombucha prepared based on molasses. †SEM: Standard error of means. aCrude protein. fMeans within columns with different superscripts are significantly different (p<.05).

Table 6. Effect of treatments on serum metabolites in broiler chicks at 24 days of age.

| Treatments         | Cholesterol mg/dL | Triglyceride mg/dL | HDL mg/dL | LDL mg/dL | Glucose mg/dL | Uric acid mg/dL | AST U/L | ALT U/L |
|--------------------|-------------------|-------------------|-----------|-----------|---------------|-----------------|---------|---------|
| Control            | 212.8a            | 137.0             | 90.3a     | 59.0a     | 273.0         | 6.6a            | 224.5a  | 14.4a   |
| SK 3%              | 196.5ab           | 124.0             | 90.8ab    | 53.3ab    | 252.3         | 5.6ab           | 202.5ab | 9.9ab   |
| SK 6%              | 175.8b            | 103.7             | 94.8b     | 46.8b     | 240.3         | 4.4b            | 194.5b  | 9.0b    |
| MK 3%              | 197.8abc          | 128.8             | 106.3abc  | 39.3abc   | 226.3         | 4.2bc           | 119.3d  | 9.2d    |
| MK 6%              | 198.0abc          | 117.3             | 109.8abc  | 35.5abc   | 214.5         | 4.9bc           | 117.8d  | 8.8b    |
| SK3%+MK3%          | 190.8abc          | 121.5             | 93.8abc   | 47.0abc   | 247.8         | 5.2bc           | 187.8abc| 11.5b   |
| SK6%+MK6%          | 185.5abc          | 112.3             | 101.3abc  | 43.3abc   | 221.5         | 4.2b            | 163.0c  | 9.1c    |
| SEMi               | 0.030             | 0.010             | 0.001     | 0.010     | 0.085         | 0.010           | 0.001   | 0.002   |
| p Value            | .0330             | .1010             | .0001     | 0.0010    | .0850         | .010           | .0001   | .0020   |

*Kombucha prepared based on white sugar. **Kombucha prepared based on molasses. †SEM: Standard error of means. fMeans within columns with different superscripts are significantly different (p<.05).

or SK lonely or in combination in drinking water increased (p<.05) the jejunal villus width and jejunal apparent villus surface area. In addition, kombucha treatments increased (p<.05) the ileal villus height (except for SK3% and SK3%+MK3%) and ileal apparent villus surface area when compared to the control group.

Ileum nutrient content and digestibility
Ileum nutrient content and digestibility are shown in Table 5. There were no significant differences in the ileum content of ash, Ca, and P. However the CP content of ileum was lower (p<.05) in birds received SK6%+MK6% in their drinking water. The digestibility of ash, Ca and P were not affected by kombucha treatments. MK6% and SK6%+MK6% treatments increased (p<.05) the ileal digestibility of CP. In addition, there was a significant (p<.05) numerical increase in the digestibility of ash, Ca, and P in MK6% treatments.

Serum biochemical parameters
The effects of experimental treatments on cholesterol, triglycerides, HDL, LDL, glucose, uric acid, AST, and ALT content of plasma are presented in Table 6. The addition of kombucha to the chickens’ drinking water significantly increased the plasma HDL and reduced the plasma LDL, uric acid and AST (except for SK 3%) compared to the control treatment (p<.05). Moreover, ALT levels decreased (p<.05) significantly in all
kombucha treatments compared to birds that received the control treatment. Triglycerides and glucose concentrations were not influenced by kombucha treatments \((p > 0.05)\). The plasma cholesterol level was significantly \((p \leq 0.05)\) reduced in SK6% and SK6%+MK6% treatments, but differences between other treatments and the control group were not observed.

**Caecal microbial populations and immune responses**

Table 7 shows the effects of treatments on caecal microflora (total coliform counts and lactobacillus population) and antibody titre against Newcastle disease virus (NDV). Inclusion of SK and MK in drinking water at all levels decreased \((p \leq 0.05)\) total coliform counts whereas the lactobacillus population increased \((p \leq 0.05)\) in birds received 6% of SK, MK or their combination in comparison with the control group. In addition, no significant \((p > 0.05)\) difference between treatments was observed with regard to antibody titre against NDV at 24 days of age, but at 42 days of age, the SK6%+MK6% and MK6% groups had greater \((p \leq 0.05)\) antibody titre against NDV compared to the control group.

**Antioxidant enzyme activities and lipid peroxidation**

Plasma and liver concentrations of SOD, CAT, GPx, MDA and TAC are reported in Table 8. Plasma and liver activities of CAT and SOD (except for SK3% treatment) were significantly \((p \leq 0.05)\) greater in all kombucha treatments compared with the control group. Moreover, the TAC of plasma and liver was significantly improved in birds receiving kombucha treatments compared to the control group and the greatest amount was belonged to MK6% group. Plasma MDA levels decreased \((p \leq 0.05)\) in the kombucha groups compared to the control and SK3% groups, and liver MDA levels in the experimental groups were lower \((p \leq 0.05)\) compared with the control group. Plasma GPx concentration was greater compared to that of the SK3%, SK3%+MK3% and control treatments, and the GPx activity in the liver was enhanced compared to that of the control treatment \((p \leq 0.05)\).

**Digesta pH**

As reported in Table 9, the addition of kombucha to drinking water significantly \((p \leq 0.05)\) reduced the pH of the jejunum compared to the control treatment. No significant difference was observed in the pH of the proventriculus, gizzard, duodenum, ileum, and caecum. However, the pH tended to decrease numerically in the kombucha groups as compared to the control group.

**Water intake and water-feed ratio**

As shown in Table 10, water intake and the water to feed ratio were not influenced by the addition of
kombucha to drinking water when compared to the control group. However, during 25–42 days of age, the water intake in SK3 + MK3 group was significantly (p<.05) greater than SK6% and SK6% + MK6%, and the water-feed ratio was significantly (p<.05) greater in SK3% and SK6% groups than SK6% + MK6% group.

Discussion

Kombucha contains a considerable amount of organic acids (Jakubczyk et al. 2020) and could exert antimicrobial and antioxidant properties (Villarreal-Soto et al. 2018; Kapp and Sumner 2019). Therefore, it could be considered as an acceptable alternative to growth-promoting antibiotics for improving the performance of broilers. The results of the present study revealed that BWG was greater in birds received 6 g/100 g of kombucha fermentation based on white sugar or sugar beet molasses alone or in combination. All kombucha treatments, except SK3%, improved FCR. Our findings are in agreement with those of Afsharmanesh and Sadaghi (2014) and Arani et al. (2014) who reported that using kombucha improved the growth rate and FCR in broiler chickens.

Kombucha consists of organic acids, vitamins, polyphenols, amino acids, antibiotics, and microelements (Jayabalan et al. 2014; Kaewkod et al. 2019; Jakubczyk et al. 2020) that may exert beneficial effects on broilers productive performance. Organic acids have beneficial effect on the gut flora. As seen in the present study (Table 7) inclusion of kombucha in chickens’ drinking water increased the lactobacillus population and decreased coliforms (Table 7), and improved CP digestibility (Table 5). The inhibition of pathogenic intestinal bacteria by kombucha may leading to the reduced metabolic needs and decreased the level of toxic bacterial metabolites, thereby increasing the availability of nutrients to the birds. Also, the greater count of favourable bacteria such as Lactobacillus can increase the enzyme activity in GIT of poultry (Chawla et al. 2013) and improve the digestibility and absorption of nutrients. In the present study, as it has been discussed later, kombucha increased the intestinal villus height (Table 4) and this is in parallel with digestive and absorptive capacity of intestine due to greater available surface area for nutrient absorption. Therefore, enhanced broiler performance following kombucha intake could be associated with its beneficial nutrients (especially organic acids and B vitamins) and above mentioned mechanisms.

Kombucha had no effect on the carcase yield and organ weights, which are in agreement with those of Afsharmanesh and Sadaghi (2014).

Intestine morphometric parameters such as villus height, villus width and villus surface area in the jejunum and ileum of broiler chicken were improved in birds received kombucha. This finding is in contrast with those of Afsharmanesh and Sadaghi (2014) who found no significant difference in histomorphological parameters in birds received kombucha. This could be attributed to the method of application and the amount of kombucha used in these two experiments, as they used lower concentration of kombucha in wheat based diet, however we used greater concentration in drinking water. Kombucha is a rich source of organic acids and some studies have reported the beneficial effects of organic acids on intestinal morphology (Emami et al. 2017; Sabour et al. 2019; Saleem et al. 2020). It has been hypothesised that organic acids reduce the growth of many pathogenic intestinal bacteria which decrease the infectious processes and the inflammatory reactions at the intestinal mucosa, which increases the villus height (Adil et al. 2010).

Calcium, phosphorus and total ash digestibility did not affected by kombucha treatments. However, the
inclusion of SK6%+MK6% and MK6% in drinking water improved ileal digestibility of CP when compared to the control group, and may have been responsible for greater BWG and lower FCR in these groups. This finding is in agreement with those of Afsharmanesh and Sadaghi (2014) who showed that apparent ileal digestibility of CP was greater for birds in the Kombucha treatment as compared to those in the control treatment. The greater protein digestibility in these groups may be due to the better histomorphological parameters of intestine in these groups. These groups also had greater counts of Lactobacillus which may increase the enzyme activity of GIT and decreased the level of toxic bacterial metabolites, causing an improvement in the ileal protein digestibility.

The addition of kombucha to the birds’ drinking water significantly increased the plasma HDL and reduced the plasma levels of cholesterol, LDL, uric acid, AST, and ALT in broiler chickens. Previous studies have reported the effect of kombucha on lowering blood levels of cholesterol, LDL, triglycerides, AST, and ALT, as well as its beneficial effect on lowering blood glucose and cholesterol and improving HDL in laboratory animals (Bellassoued et al. 2015; Hyun et al. 2016; Lee et al. 2019). In agreement with our results, Adriani et al. (2011) reported a decrease in LDL and total cholesterol and an increase in HDL in duck blood. The beneficial role of organic acids in reducing the blood lipid profile may be interpreted through their influence in decreasing the microbial intracellular pH. This inhibits the action of important microbial enzymes and forces the bacterial cell to use energy for the release of acid protons, leading to an intracellular accumulation of acid anions (Young and Foegeding 1993). Lower levels of AST and ALT may be due to glucuronic acid which is one of the most valuable, healthy Kombucha components. This acid has a detoxifying effect and can bind xenobiotics in liver and accelerate these substances excretion by kidneys (Neffe-Skocińska et al. 2017).

In the present experiment, birds received 6% kombucha had lower colliforms and greater lactobacillus counts as compared to the control treatment. Many studies have shown inhibitory effects of kombucha broth against several pathogenic microorganisms of Gram-positive and Gram-negative origin (Villarreal-Soto et al. 2018; Kaewkod et al. 2019; Kapp and Sumner 2019). This modulation in microbial population may be due to the presence of organic acids and polyphenols in kombucha. Furthermore, high organic acids content of kombucha has pH reducing property in gastrointestinal tract of the broiler chicken. In the present study, the digesta pH in the jejunum was reduced in kombucha treatments as compared to the control treatment. The reduced pH is suitable for the growth of favourable bacteria simultaneously hindering the growth of pathogenic bacteria which grow at greater pH. Although the lactobacillus counts increased in the caecum, but the pH value of caecum did not change. We did not measure the volatile fatty acids (VFAs) content of caecum. Maybe the changes in the VFAs was not enough to change the pH of caecum, though the pH value of caecum decreased numerically.

Our results showed that antibody titre against NDV improved in birds received SK6%+MK6% and MK6% treatments at 42 d of age. This finding is in agreement with those of Afsharmanesh and Sadaghi (2013) who reported greater antibody titre against SRBC in broilers fed with kombucha. In the present study addition of kombucha to drinking water increased the lactobacillus population and it has been shown that lactic acid bacteria can exert immune-stimulatory effects in the host innate immune response (Marteau and Rambaud 1993; Gaggia et al. 2010).

In the present study, inclusion of the Kombucha in drinking water improved the antioxidant status of broilers. Kombucha is a rich source of polyphenols and the antioxidant activity of kombucha polyphenolic compounds has been noted in several studies (Villarreal-Soto et al. 2018; Ahmed et al. 2020; Villarreal-Soto et al. 2020; Tanticharakunsiri et al. 2021). In the present study we used green tea for preparation of kombucha and green tea is a good source of flavonoids. Some studies have reported that kombucha prepared using green tea exhibits more antioxidant properties (Kaewkod et al. 2019; Jakubczyk et al. 2020). In our study, the kombucha made by sugar beet molasses showed greater antioxidant properties than those of made by white sugar. It has been reported that sugar beet molasses has antioxidant properties (Chen et al. 2015), and use of sugar beet molasses in the preparation of kombucha increased the phenolic components (Chen et al. 2017).

In the present study, the water intake and water to feed ratio were not affected by experimental treatments compared to the control group. However, during 25–42 days of age, the water intake in SK3+MK3 group was greater than SK6% and SK6%+MK6. Chickens respond to bitter, umami, sour, salty, and high concentration of sweet taste stimuli (Yoshida et al. 2021). This shows that adding kombucha to drinking water had no effect on its taste and palatability for broilers at early ages but in late ages, different
kinds and levels of kombucha may make different tastes for broilers.

**Conclusion**

Processed kombucha based on green tea with white sugar or sugar beet molasses was shown to improve the growth performance, intestinal morphology, blood parameters, caecum microbiota, ileal digestibility of some nutrients, immune responses and antioxidant status of broiler chickens, and it could be considered as a suitable alternative to growth-promoting antibiotics in broiler chickens. We recommend 6 ml fermented kombucha on sugar beet molasses per 100 ml of drinking water to achieve the best performance in broilers.

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**ORCID**

Amirali Sadeghi [http://orcid.org/0000-0003-4837-5664](http://orcid.org/0000-0003-4837-5664)

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