Betaine could help ameliorate transport associated water deprivation stress in broilers by reducing the expression of stress-related transcripts and modulating water channel activity

Abdulaziz A. Al-Abdullatif, Ahmad A. Al-Sagan, Elsayed O. S. Hussein, Islam M. Saadeldin, Gamaleldin M. Suliman, Mahmoud M. Azzam, Saud I. Al-Mufarrej and Rashed A. Alhotan

Department of Animal Production, King Saud University, Riyadh, Saudi Arabia; King Abdulaziz City for Science & Technology, Riyadh, Saudi Arabia

ABSTRACT
This study evaluated the effects of betaine and pre-slaughter transport of broilers on meat quality, blood parameters, selected water channels (AQP) and stress-related transcripts. Ross 308 broilers (n = 336) were housed in 56-floor pens with seven replicates per treatment. In a 4 × 2 design, broilers were either supplemented with betaine in the feed (0.2%), in drinking water (1 or 2 mL/L), or un-supplemented, and then were transported for 5-min or 4-h pre-slaughter. The results revealed that broilers transported for long-distance had elevated blood glucose pre-slaughter, and their breast meat was characterised with lower initial pH, higher ultimate pH, higher 0-h post-mortem temperature, and lower water holding capacity than those transported for short-distance. Moreover, there was a two-way interaction effect between betaine and transport on the expression of brain genes (Proopiomelanocortin, AQP1, AQP4), muscle glucocorticoid receptor (GR), hepatic AQP3 and AQP9 and intestinal AQP3. Long-distance transport upregulated the relative expression of the muscle AQP9 and downregulated the hepatic GR. Furthermore, betaine in the water at 2 mL upregulated the muscle AQP9 and hepatic GR. In conclusion, betaine could ameliorate the water-deprivation stress associated with transportation by reducing the expression of stress-related transcripts and modulating water channels without improving broiler meat quality.

HIGHLIGHTS
- Long-distance transport of broilers is stressful, as evidenced by rising blood glucose, high temperature of fresh meat, and expression of stress-related transcripts.
- Betaine could ameliorate the water-deprivation stress associated with transport by reducing the expression of stress-related transcripts and modulating water channels.

Introduction
The pre-processing handling of broiler chickens represents a critical step in the broiler production cycle. The importance of this step can be attributed to the fact that chickens are subjected to potential stressors, including feed and water withdrawal, catching, crating, transportation, and lairage. These stressors can cause a series of physiological and metabolic changes, leading to significant economic losses due to increased mortality (Xing et al. 2015; Vecerek et al. 2016), body weight loss (Pan et al. 2018) and inferior meat quality (Zhang C et al. 2019). However, the magnitude of the economic losses is dependent on several factors like environmental temperature and time duration during pre-processing handling. Although the effects of the pre-processing stressors are additives, transportation alone can be detrimental to chickens, especially when transporting for long distances under hot conditions. Pre-slaughter transport stress has been reported to trigger numerous physiological and metabolic changes such as elevated plasma corticosterone concentrations (Zhang et al. 2009), increased heterophil to lymphocyte ratio (Yalçın and Guler 2012), and altered meat fatty acid profile (Zanetti et al. 2011). Several dietary supplements have been suggested to ameliorate the adverse effects of transport stress such as guanidino...
acetic acid, *Forsythia suspensa* extract, resveratrol and L-threonine (Zhang et al. 2017; Pan et al. 2018; Zhang C et al. 2019; Zhang L et al. 2019).

Betaine is a natural effective osmolyte found in high concentrations in plants exposed to drought and high osmotic stress. In situations where cells are under high osmotic pressure, water molecules move out of the cells towards the high solute (e.g. Na\(^+\)) concentrations outside the cells (Yancey 2005). Consequently, the cells will eventually dehydrate and shrink in size, leading to cell death. To overcome extensive water leakage, osmolytes such as betaine accumulate inside the cells causing water retention and avoiding cell shrinkage (Zúñiga and Corcuera 1987). Furthermore, betaine is known for its methyl donor and antioxidant properties (Metzler-Zebeli et al. 2009; Ganesan et al. 2010). Betaine has shown promising results in improving poultry performance under heat stress (Park and Kim 2017; Shakeri et al. 2018). However, betaine supplementation to broiler chickens exposed to pre-slaughter transport stress is poorly investigated. Therefore, the objectives of this study were to investigate the effects of supplemental betaine on selected stress parameters, expression of water channel proteins, and meat quality of broiler chickens exposed to pre-slaughter transport stress.

**Materials and methods**

**Birds and housing**

All the procedures followed were approved by the Research Ethics Committee at King Saud University (Riyadh, Saudi Arabia; Ethics Reference No: KSU-SE-20-54). A total of 336 male broiler chicks (Ross 308) were obtained from a commercial hatchery and reared until 21 days of age. Chicks were raised in floor pens in an environmentally controlled house with a bell drinker and a hopper feeder in each pen. Chicks were provided with 24 h of light per day during the experiment. House initial temperature was set at 32 °C and then the temperature was gradually reduced to reach 24 °C at 21 days of age and was maintained around this point until the end of the experiment. A starter (0–21 days) and finisher (21–32 days) diets were formulated to meet the nutrition recommendations of Ross (Ross Broiler, 2019; Aviagen) and were fed in mash form (Table 1).

**Experimental design and treatment design**

The experimental design was completely randomised. At 21 days of age, chicks were weighed, randomly based on BW (907 ± 5 g), and housed in 56-floor pens with six chicks each. There were four dietary treatments, and each treatment was applied to 14 replicate pens: (1) supplementation of 0.2% of betaine in finisher feed only (21–32 days); (2) betaine supplementation in drinking water only (1 mL/L) 72 h pre-slaughter; (3) betaine supplementation in drinking water only (2 mL/L) 72 h pre-slaughter; (4) betaine neither supplemented in feed nor in drinking water; Betaine was added to feed as Betafin\(^\circledR\) (DuPont Industrial Biosciences, Marlborough, UK), whereas a liquid betaine (AtcoBeet, ATCO PHARMA; Menoufi, Egypt) was added to drinking water. The liquid betaine product provides 380 mL of betaine (as Actibeet L) per 1000 mL of the product (the rest is water and citric acid). This will make the actual betaine added for treatment two 380 mL per one L of drinking water (or ~0.04%) and treatment three 760 mL per one L of drinking water (or ~0.08%). The liquid betaine supplier recommends that the product be added at a concentration of 1 mL/L. A double dose was used to test if the requirement of betaine increase with stress.

| Ingredient (%) (as fed basis) | Starter (0–21 days) | Finisher (21–32 days) |
|------------------------------|---------------------|-----------------------|
| Yellow corn                  | 56.32               | 60.36                 |
| Soybean meal (48%)           | 37.60               | 32.32                 |
| Vegetable oil               | 2.28                | 3.96                  |
| Dicalcium phosphate          | 1.62                | 1.38                  |
| Limestone                    | 1.02                | 0.92                  |
| DI-Methionine                | 0.31                | 0.28                  |
| L-Lysine HCL                 | 0.15                | 0.12                  |
| L-Threonine                  | 0.09                | 0.06                  |
| Common salt                  | 0.40                | 0.40                  |
| Vitamin premixa              | 0.10                | 0.10                  |
| Mineral premixb              | 0.10                | 0.10                  |
| Calculated Composition       | 100.00              | 100.00                |
| Metabolizable energy, kcal/kg| 3050                | 3200                  |
| Crude protein, %             | 22.25               | 20.00                 |
| Calcium, %                   | 0.92                | 0.81                  |
| Available phosphorus, %      | 0.46                | 0.41                  |
| Digestible lysine, %         | 1.22                | 1.06                  |
| Digestible methionine, %     | 0.62                | 0.56                  |
| Digestible total sulphur AA, %| 0.91               | 0.83                  |
| Digestible threonine, %      | 0.81                | 0.71                  |

\(^a\) Vitamin premix provided the following (per kg of diet): Vitamin B1, 2 mg; Niacin, 50 mg; Vitamin B2, 6 mg; Pantothenic Acid, 15 mg; Vitamin B12, 16.0 μg; Vitamin B6, 3 mg; Biotin, 150 μg; Folic Acid, 1.75 mg; Vitamin K3 (MNB), 3 mg; Vitamin D3 (cholecalciferol), 5000 U; Vitamin A (retinol acetate), 10,000 U; Vitamin E (Dl-alpha-tocopheryl acetate), 50 U; Total antioxidants, 50 mg.

\(^b\) Trace mineral premix provides the following in milligrams per kilogram of diet: Manganese (Oxide), 120; Zinc (Oxide), 100; Iron (Sulphate), 40; Copper (Sulphate), 16; Iodine (Potassium Iodide), 1.25; Selenium (Sodium Selenite) 0.30.
At 32 days of age, a second factor, which is transportation, was included in the design. Half of the replicates were transported by a truck for a long-distance (4h) while the other half were transported for short-distance (5 min.). The short-distance group was transported gently in crates by hand from the pens to an adjacent processing building. This makes the final treatment design a 4 × 2 with seven replicates per treatment combination (n = 56 total). Feed was removed from all groups eight hours before catching, but water access was available ad libitum to the birds. After catching, 28 plastic crates were placed randomly in a truck for transportation. The transportation started at 8:00 AM and ended at 12:00 PM with an average speed of 80 km/h. The weather was sunny, with an average relative humidity of 27%, with min and max temperatures of 17°C and 23°C.

**Measurements and sample collection**

Chicks and feed were weighed at 21 and 32 days of age to calculate body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR). At 32 days of age, two birds were randomly selected from each transport crate for processing (14 per treatment, 112 total). Birds were slaughtered by cutting the jugular vein, carotid artery, and windpipe using a sharp knife. Blood was collected from each bird at slaughter and placed in sterilised tubes and was left to coagulate at room temperature. The blood samples were then centrifuged at 2,328 × g for 5 min to separate serum. The serum samples were then kept in sterilised Eppendorf tubes and stored at −20°C for later analysis. Fresh samples (≈1 g) from the brain (hypothalamus), intestinal ileum (≈2 cm distal to Meckel’s diverticulum), right pectoralis major, and liver were collected from the birds immediately at slaughter. The tissue samples were then washed with normal saline to remove any contaminants, placed in sterilised tubes filled with RNAlater solution (RNAlater, Ambion, Austin, TX), and then stored at a temperature of −80°C for later RNA extraction. Hot carcases were individually packed in plastic bags and kept in a cooler (≈4°C) overnight before deboning. Weights of hot eviscerated carcase, chilled carcase, wings, breasts (pectoralis major and minor), and saddles (legs and backs) were taken. The left pectoralis major and minor muscles were stored in a cooler for meat quality measurements.

**Meat quality measurements**

**pH and temperature values**

Initial (i) and ultimate (u) pH and temperature values were recorded 20 min and 24 h (at 4°C) post-mortem, respectively using a portable waterproof pH metre dedicated in meat measurements (Model: HI-99,163; Hanna Instruments, Woonsocket, Rhode Island, USA). The pH metre is fitted with a specialised FC2323 probe with a stainless steel blade that is inserted directly into the breast muscle. The mean value of two readings from each parameter was recorded.

**Colour components**

The initial (i) and ultimate (u) colour components of the CIELAB Colour System: L* (lightness), a* (redness), and b* (yellowness) were determined directly on breast muscles 20 min and 24 h (at 4°C) post-mortem, respectively, using a Chroma Metre (CR-400 Konica Minolta, Tokyo, Japan). The breast muscle was held at room temperature (22°C) for approximately 30 min to allow for blooming before recording the ultimate colour components. The mean value of the two readings was recorded.

**Water-holding capacity**

Water-holding capacity (WHC) was measured according to the technique outlined by Wilhelm et al. (2010). Two replicate pieces of approximately 2 g were taken from the pectoralis muscle and then cut into small cubes. The cubes were then placed between filter papers and two Plexiglas plates and left under a weight of 10 kg for 5 min. Finally, the cubes were weighed to calculate the WHC values as the difference between the initial and final weights.

**Cooking loss**

A commercial indoor countertop grill was used for cooking muscle samples at 200°C to reach an internal temperature of 70°C. A thermocouple thermometer probe (Ecoscan Temp JKT; Thermo Scientific, Eutech Instruments Pte Ltd 7 Gul Circle, level 2 M, Keppel Logistic Building, Singapore 629563) was inserted into the centre of the muscle to monitor temperature changes. The cooking loss was then calculated by taking the difference between the weights of the sample before and after cooking and then dividing it by the initial weight of the sample (Al-Owaimer et al. 2014).

**Shear force and texture profile analysis**

The cooked samples used in determining cooking loss were reused to assess the shear force, according to Wheeler et al. (2005). The samples were first left to cool down at room temperature (22°C), then five-round cores, measuring 1.27 cm in diameter, were extracted from each sample (parallel to the longitudinal muscle fibers) using a manual coring device.
Shear force was determined using a texture analyser (TA.HD-Stable Micro Systems, Surrey, UK) fitted with a Warner-Bratzler attachment with a 200 mm/min crosshead speed. On the other hand, texture profile analysis (TPA) variables: hardness, cohesiveness, springiness, and chewiness were determined using a digital homogeniser (Ultra Turrax; IKA-Werke, Staufen, Germany) equipped with a compression-plate attachment.

**Myofibril fragmentation index**

Myofibril fragmentation index (MFI) was determined, as described by Al-Owaimer et al. (2014). Approximately 4 g of breast muscle samples were minced with scissors and then homogenised using a digital homogeniser (Ultra Turrax; IKA-Werke, Staufen, Germany) with 40 mL of cold isolating MFI buffer. The absorbance of a 0.5 mg/mL solution was read at 540 nm using a spectrophotometer (HACH DR/3000, Germany) with 40 mL of cold isolating MFI buffer. The absorbance was determined by multiplying the absorbance value by the dilution factor (200).

**Blood biochemical analysis**

Concentrations of serum albumin, uric acid, glucose, triglycerides, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured. These biochemical indices were analysed by a spectrophotometric analyser (Randox, London, UK) using reagent kits (Randox, London, UK) according to the manufacturer’s instructions.

**RNA isolation, cDNA synthesis and real-time polymerase chain reaction**

Total RNA extraction from brain, kidney, intestine, and liver samples was performed according to Hassan et al. (2018) using PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. RNA concentration and purity were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher, Waltham, MA, USA), and the values were estimated at 230 and 260 nm (acceptable ratios were above 1.80). Afterward, the total RNA was reverse-transcribed into complementary DNA (cDNA) utilising a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s protocol. The analysis of the Real-time PCR was carried out using Power SYBR™ Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), in which β-actin was used as the internal control. Gene-specific primer sequences (Table 2) were utilised to amplify the transcripts of chicken β-actin (ACTB), proopiomelanocortin (POMC), glucocorticoid receptor (GR), aquaporins (AQ1, AQ3, AQ4 and AQ9). The qRT-PCR was done using 20 μL reactions that contained 10 μL of 2X Power SYBR™ Green PCR Master Mix, 1 μL of each primer (10 pmol), 6 μL of H2O, and a 2 μL cDNA template. The analysis was performed with the following cycling conditions: 15 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. The Melt-curve analysis was accomplished (from 65 to 95°C, using 0.5°C temperature increments with a 5 s hold at each step) using Applied Biosystems 7500 real-time PCR machine (Applied Biosystems, Carlsbad, CA, USA). The comparative $2^{-ΔΔCt}$ method (Livak and Schmittgen 2001) was followed to calculate the relative fold-change in the expression of target genes. The value of ΔΔCt is the difference between the mean ΔCt of the control and the treatment. The ΔCt denotes the difference between the mean Ct of the gene of interest and the ACTB for each sample.

**Statistical analysis**

Fl, BWG and FCR data were analysed in a one-way ANOVA using the GLM procedure of SAS software 9.2 (SAS Institute, 2010). Processing performance, meat quality, biochemical blood indices, and the relative gene expression data were analysed in a two-way ANOVA using the GLIMMIX procedure of SAS. The effects of betaine supplementation, transport distance, and any possible interactions were evaluated. The CORR procedure of SAS was used to calculate Pearson correlation coefficients (r) between the means of various mRNA transcript expressions. Differences were considered significant when $p \leq .05$. When
pronounced significant, means were separated using Tukey’s Studentized Range (HSD) Test. Each pen was considered as an experimental unit.

Results

Growth performance

FI, BWG, and FCR from 21–32 days of age, measured in the birds supplemented with betaine, are presented in Table 3. The results of the present study showed that there were significant effects (p = .004) of treatment on FI, the birds supplemented with 0.2% of betaine in feed consumed less feed compared to the other treatments. Betaine supplementation in feed or water did not significantly affect BWG (Table 3). However, FCR was reduced (p = .001) around 8–10 points when broilers were supplemented with betaine in feed compared to betaine supplementation in drinking water and no supplementation, with no differences among the latter groups. Mortality rates were not significantly different among treatment groups.

Processing performance

The effects of betaine supplementation and pre-slaughter transport on broiler processing performance at 32 days of age are shown in Table 4. There was no significant interaction between betaine supplementation and pre-slaughter transport on the live BW, eviscerated carcass, or any of the chicken carcase portions measured. Similarly, the main effects of betaine

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**Table 3. Effect of betaine supplementation on broiler performance at 32 days of age.**

| Variable          | Betaine supplementation1,2,3 | p-Value | SEM  |
|-------------------|------------------------------|---------|------|
|                   | 0.2% in feed | None | 1 mL in water | 2 mL in water |
| Feed intake (g/bird) | 1555.35a | 1616.46b | 1637.17b | 1623.38b |
| Weight gain (g/bird) | 1119.1 | 1103.3 | 1114.4 | 1093.0 |
| FCR (g/g)          | 1.39a | 1.47b | 1.47b | 1.49b |

1Treatment groups were supplemented with betaine at 0.2% of finisher feed, 1 mL/L of drinking water 72 h pre-slaughter, 2 mL/L of drinking water 72 h pre-slaughter or non-supplemented.
2Values are means of 14 replicate pens per treatment.
3Means within a row with no common superscript differ significantly (p ≤ .05).

**Table 4. Effect of betaine supplementation and pre-slaughter transport on broiler processing performance at 32 days of age.**

| Betaine1 | n2 | Live BW, g | Eviscerated carcass3, % | Wings4, % | Pectoralis major4, % | Pectoralis minor5, % | Saddles4.5, % |
|----------|----|------------|------------------------|-----------|---------------------|---------------------|-----------------|
| Short    |    |            |                        |           |                     |                     |                 |
| 0.2% in feed | 7 | 1998 | 72.67 | 9.59 | 29.66 | 5.56 | 39.43 |
| None     | 7 | 1945 | 72.53 | 9.77 | 28.74 | 5.55 | 40.22 |
| 1 mL in water | 7 | 2014 | 72.68 | 9.99 | 29.80 | 5.65 | 39.22 |
| 2 mL in water | 7 | 1936 | 72.26 | 9.99 | 28.62 | 5.70 | 40.01 |
| Long     |    |            |                        |           |                     |                     |                 |
| 0.2% in feed | 7 | 1962 | 72.13 | 9.85 | 28.93 | 5.67 | 39.52 |
| None     | 7 | 1859 | 72.03 | 9.78 | 29.83 | 5.72 | 39.04 |
| 1 mL in water | 7 | 1849 | 72.98 | 9.97 | 29.38 | 5.68 | 39.69 |
| 2 mL in water | 7 | 1998 | 72.82 | 9.82 | 29.43 | 5.62 | 39.38 |
| SEM 48.00 | 0.39 | 0.16 | 0.50 | 0.13 | 0.47 |

1Treatment groups were supplemented with betaine at 0.2% of finisher feed, 1 mL/L of drinking water 72 h pre-slaughter, 2 mL/L of drinking water 72 h pre-slaughter or non-supplemented. Half of the pens were then cooped and transported for 4 h pre-slaughter whereas the other half were transported for a very short distance to the slaughter house for processing.
2Number of replicate pens with 2 broiler chickens selected from each pen for processing.
3Calculated as a percentage of live BW.
4Calculated as a percentage of chilled eviscerated carcass.
5Legs and backs combined.

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supplementation and pre-slaughter transport on the measurements were not significant.

**Meat quality**

There was no two-way interaction between betaine supplementation and pre-slaughter transport on pH, temperature, or colour components of broiler breast meat at 32 days of age (Table 5). Furthermore, none of the main effects of betaine were significant for any of these parameters. The main effect of transport on fresh meat pH was significant as the birds transported for a long distance had lower meat pH than the birds transported for a short distance (6.29 vs. 6.34; \( p = .047 \)). Surprisingly, the meat pH after 24 h of slaughter was lower for the short transported birds than the long transported ones (6.09 vs. 6.15; \( p = .019 \)). In addition, meat temperature behaved similarly as the long transported birds had higher meat temperature immediately post-slaughter (29.15 vs. 26.96; \( p < .001 \)) and lower temperature 24 h post-slaughter compared to the short transported birds (13.03 vs. 15.08; \( p < .001 \)). There was no effect of supplemental betaine, pre-slaughter transport or their two-way interaction on water holding capacity, cooking loss, myofibril fragmentation index, shear force, and components of texture profile analysis of broiler meat, except the main effect of transport on water holding capacity with long transported birds having lower value (21.98% vs. 24.30%; \( p = .008 \)) than the short transported birds (Table 6).

**Blood biochemical parameters**

The statistical analysis revealed no interaction between betaine supplementation and pre-slaughter transport on the selected blood parameters (Table 7). Out of the main effects tested, only the effect of transport on blood glucose was significant (\( p < .001 \)), with the long transported birds having higher glucose levels than the short transported birds (209.24 vs. 182.93 mg/dL).

**Expression of the water channel and stress-related transcripts**

Betaine in either feed (0.2%) or water (2 mL/L) increased the expression of brain AQP1 particularly in the short-distance group, while in long-distance groups, AQP1 showed no differences but increased when compared with the short-distance control and 1 mL supplemented group (Table 8). On the other hand, muscle AQP1 was not changed with any treatment (Table 8). Conversely, betaine significantly decreased liver AQP3, and this effect was aggravated.

### Table 5. Effect of betaine supplementation and pre-slaughter transport on meat quality of broiler chickens at 32 days of age.

| Betaine1 | Post-mortem pH | Post-mortem temp. | Initial colour components3 | Ultimate colour components3 |
|----------|----------------|-------------------|---------------------------|---------------------------|
|          | 0 h | 24 h | 0 h | 24 h | Lia | aia | bi | Lu | au | bu |
| Transport distance | | | | | | | | | | |
| Short | 0.2% in feed | 7 | 6.39 | 6.09 | 26.96 | 14.87 | 47.50 | 3.77 | 3.57 | 50.19 | 5.34 | 6.46 |
| None | 7 | 6.30 | 6.09 | 26.87 | 15.04 | 46.64 | 4.07 | 3.69 | 48.87 | 6.64 | 7.43 |
| 1 mL in water | 7 | 6.31 | 6.10 | 27.06 | 15.70 | 47.37 | 4.66 | 3.59 | 49.79 | 6.50 | 6.90 |
| 2 mL in water | 7 | 6.36 | 6.07 | 26.96 | 14.71 | 46.50 | 4.43 | 3.67 | 48.99 | 6.09 | 7.44 |
| Long | 0.2% in feed | 7 | 6.31 | 6.16 | 28.99 | 13.17 | 46.36 | 4.26 | 3.07 | 51.00 | 6.04 | 6.36 |
| None | 7 | 6.31 | 6.16 | 29.43 | 13.49 | 47.01 | 4.03 | 3.99 | 50.16 | 5.69 | 6.30 |
| 1 mL in water | 7 | 6.24 | 6.11 | 28.87 | 13.03 | 46.03 | 4.23 | 3.34 | 49.34 | 6.23 | 6.56 |
| 2 mL in water | 7 | 6.27 | 6.17 | 29.33 | 12.41 | 45.76 | 3.89 | 3.01 | 50.20 | 5.44 | 5.90 |
| SEM | 0.04 | 0.04 | 0.58 | 0.72 | 0.74 | 0.32 | 0.38 | 1.00 | 0.47 | 0.57 |
| Main effect means | | | | | | | | | | |
| Short | 28 | 6.34a | 6.09b | 26.96b | 15.08a | 47.00 | 4.23 | 3.63 | 49.46 | 6.14 | 7.06 |
| Long | 28 | 6.29b | 6.15a | 29.15b | 13.03b | 46.33 | 4.10 | 3.35 | 50.18 | 5.85 | 6.28 |
| SEM | 0.02 | 0.02 | 0.29 | 0.36 | 0.37 | 0.16 | 0.19 | 0.50 | 0.24 | 0.28 |

1Treatment groups were supplemented with betaine at 0.2% of finisher feed, 1 mL/L of drinking water 72 h pre-slaughter, 2 mL/L of drinking water 72 h pre-slaughter or non-supplemented. Half of the pens were then cooped and transported for 4 h pre-slaughter whereas the other half were transported for a very short distance to the slaughter house for processing.

2Number of replicate pens with 2 broiler chickens selected from each pen for processing.

3Li: lightness; a: redness; b: yellowness.

Means within a column with no common superscript differ significantly (\( p < .05 \)).
by supplementation with water in a dose and trans-
portation-dependent manner (Table 8). Hepatic AQP9
showed a significant increase in betaine-supplemented
and short-distance transportation birds, while it was
not affected by the water supplementation with 2 mL
dosage in long-distance transportation (Table 8).

Table 6. Effect of betaine supplementation and pre-slaughter transport on meat quality of broiler chickens at 32 days of age.

| Transport distance | Betaine | n² | WHC³, % | Cooking loss, % | MFI⁴ | Shear force, N | Hardness, N | Springiness⁵ | Cohesiveness⁶ | Chewiness, N |
|--------------------|---------|----|---------|----------------|------|---------------|-------------|--------------|---------------|--------------|
| Short              | 0.2% in feed | 7  | 25.33   | 27.41          | 90.46| 6.36          | 7.00        | 0.71         | 0.40          | 2.23         |
|                    | None    | 7  | 24.57   | 26.37          | 91.90| 6.21          | 7.69        | 0.76         | 0.43          | 2.50         |
|                    | 1 mL in water | 7  | 24.20   | 31.73          | 92.00| 5.97          | 6.83        | 0.74         | 0.40          | 2.21         |
|                    | 2 mL in water | 7  | 23.10   | 24.17          | 102.83| 6.11         | 7.19        | 0.70         | 0.41          | 2.26         |
| Long               | 0.2% in feed | 7  | 22.64   | 27.70          | 95.39| 6.76          | 6.77        | 0.70         | 0.41          | 2.06         |
|                    | None    | 7  | 22.16   | 28.67          | 100.33| 6.41         | 6.57        | 0.71         | 0.40          | 1.99         |
|                    | 1 mL in water | 7  | 21.57   | 28.94          | 100.23| 5.87         | 6.70        | 0.73         | 0.43          | 2.19         |
|                    | 2 mL in water | 7  | 21.53   | 28.06          | 94.41| 6.86          | 9.17        | 0.70         | 0.41          | 2.90         |
| SEM                |         | 1.20 | 2.86    | 5.63            | 0.33 | 0.67          | 0.02        | 0.02         | 0.27          |

Main effect means

| Transport distance | Betaine | n² | WHC³, % | Cooking loss, % | MFI⁴ | Shear force, N | Hardness, N | Springiness⁵ | Cohesiveness⁶ | Chewiness, N |
|--------------------|---------|----|---------|----------------|------|---------------|-------------|--------------|---------------|--------------|
| Short              | 0.2% in feed | 28 | 24.30  | 27.42          | 94.30| 6.16          | 7.18        | 0.73         | 0.41          | 2.30         |
|                    | None    | 28 | 21.98  | 28.34          | 97.59| 6.48          | 7.30        | 0.71         | 0.41          | 2.28         |
|                    | 1 mL in water | 28 | 21.54  | 28.94          | 100.23| 5.87         | 6.92        | 0.73         | 0.41          | 2.19         |
|                    | 2 mL in water | 28 | 21.53  | 28.06          | 94.41| 6.86          | 9.17        | 0.70         | 0.41          | 2.90         |
| SEM                |         | 0.60 | 2.86    | 5.63            | 0.33 | 0.67          | 0.02        | 0.02         | 0.27          |

Main effect means

| Transport distance | Betaine | n² | WHC³, % | Cooking loss, % | MFI⁴ | Shear force, N | Hardness, N | Springiness⁵ | Cohesiveness⁶ | Chewiness, N |
|--------------------|---------|----|---------|----------------|------|---------------|-------------|--------------|---------------|--------------|
| Short              | 0.2% in feed | 14 | 23.99  | 27.56          | 92.92| 6.56          | 6.89        | 0.71         | 0.41          | 2.14         |
|                    | None    | 14 | 23.36  | 27.52          | 96.11| 6.31          | 7.13        | 0.74         | 0.41          | 2.24         |
|                    | 1 mL in water | 14 | 22.89  | 28.94          | 96.41| 5.92          | 6.76        | 0.74         | 0.41          | 2.20         |
|                    | 2 mL in water | 14 | 22.31  | 26.11          | 98.62| 6.49          | 8.18        | 0.70         | 0.41          | 2.58         |
| SEM                |         | 0.85 | 2.03    | 3.98            | 0.24 | 0.48          | 0.01        | 0.01         | 0.19          |

Main effect means

| Source of variation | p-Values |
|--------------------|----------|
| Transport          | .008     |
| Betaine            | .554     |
| Transport x Betaine| .964     |

Table 7. Effect of betaine supplementation and pre-slaughter transport on blood biochemical indices of broilers at 32 days of age.

| Transport distance | Betaine | n² | Albumin, g/dL | Uric acid, mg/dL | Glucose, mg/dL | Triglycerides, mg/dL | ALT³, U/L | AST³, U/L |
|--------------------|---------|----|---------------|-----------------|--------------|---------------------|-----------|-----------|
| Short              | 0.2% in feed | 7  | 1.49          | 1.9             | 178.18       | 118                 | 9         | 271       |
|                    | None    | 7  | 1.56          | 1.8             | 173.83       | 119                 | 8         | 251       |
|                    | 1 mL in water | 7  | 1.54          | 1.9             | 193.80       | 115                 | 8         | 264       |
|                    | 2 mL in water | 7  | 1.58          | 1.9             | 185.89       | 120                 | 8         | 238       |
| Long               | 0.2% in feed | 7  | 1.54          | 1.9             | 212.24       | 115                 | 8         | 264       |
|                    | None    | 7  | 1.48          | 1.9             | 202.41       | 121                 | 8         | 268       |
|                    | 1 mL in water | 7  | 1.48          | 1.9             | 208.34       | 116                 | 8         | 256       |
|                    | 2 mL in water | 7  | 1.51          | 2.3             | 213.95       | 117                 | 7         | 256       |
| SEM                |         | 0.04 | 0.1    | 6.03           | 0.73          | 4.26               | 0.5       | 8         |

Main effect means

| Source of variation | p-Values |
|--------------------|----------|
| Transport          | .118     |
| Betaine            | .829     |
| Transport x Betaine| .301     |

Table 8. Effect of water supplementation on hepatic AQP9 expression in broiler chickens at 32 days of age.

| Treatment | AQP9 expression |
|-----------|-----------------|
| Control   | 0.23            |
| 1 mL      | 0.28            |
| 2 mL      | 0.32            |

1Treatment groups were supplemented with betaine at 0.2% of finisher feed, 1 mL/L of drinking water 72 h pre-slaughter, 2 mL/L of drinking water 72 h pre-slaughter or non-supplemented. Half of the pens were then cooped and transported for 4 h pre-slaughter whereas the other half were transported for a very short distance to the slaughter house for processing.

2Number of replicate pens with 2 chickens selected from each pen for processing.

3Water holding capacity.

4Myofibril fragmentation index.

5Unitless.

Means within a column with no common superscript differ significantly (P ≤ 0.05).

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Table 8. Effect of betaine supplementation and pre-slaughter transport on gene expression of broiler chickens at 32 days of age.

| Betaine1 | n2 | Brain3 | Muscle3 | Liver3 | Intestine3 |
|---------|----|--------|---------|--------|------------|
| Distance | | POMC | AQP1 | AQP4 | GR | AQP1 | AQP9 | GR | AQP3 | AQP9 | AQP3 |
| 0.2% in feed | 5 | 1.00a | 1.00bc | 1.00ab | 1.00bc | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00b |
| None | 5 | 0.73c | 0.66c | 0.89b | 0.43b | 0.92 | 1.04 | 0.72 | 0.89b | 0.53b | 0.51bc |
| 1 mL in water | 5 | 2.25a | 0.61a | 0.82b | 1.11cd | 1.11 | 1.33 | 0.86 | 1.34bc | 0.43a | 0.64a |
| 2 mL in water | 5 | 2.68b | 0.79ab | 1.11ab | 1.62a | 1.07 | 1.30 | 0.96 | 1.21ab | 0.41a | 0.87a |
| Long | | | | | | | | | | | |
| 0.2% in feed | 5 | 0.98a | 1.46a | 0.82ab | 0.68a | 1.07 | 1.21 | 0.44 | 0.97cd | 0.86a | 1.98bc |
| None | 5 | 4.54b | 1.26bc | 1.23bc | 0.63a | 0.97 | 1.16 | 0.11 | 0.33cd | 1.02b | 0.39bc |
| 1 mL in water | 5 | 2.71a | 1.22bc | 1.19bc | 0.51cd | 1.10 | 1.19 | 0.21 | 0.52ab | 0.98a | 2.28 |
| 2 mL in water | 5 | 1.60a | 1.02bc | 1.12ab | 0.70cd | 1.12 | 1.74 | 0.33 | 0.62a | 0.53ab | 2.26 |
| SEM | 0.09 | 0.09 | 0.09 | 0.08 | 0.07 | 0.10 | 0.07 | 0.07 | 0.05 | 0.11 |

Main effect means

| Source of variation | p-Values | Distance | Betaine | Distance × Betaine |
|---------------------|----------|----------|---------|------------------|
| Distance | <.001 | <.001 | .034 | <.001 | .399 | <.001 | <.001 | <.001 | <.001 |
| Betaine | <.001 | .011 | .122 | <.001 | .093 | <.001 | <.001 | <.001 | <.001 |
| Distance × Betaine | <.001 | .018 | .007 | <.001 | .963 | .054 | .926 | <.001 | <.001 |

1Treatment groups were supplemented with betaine at 0.2% of finisher feed, 1 mL/L of drinking water 72 h pre-slaughter, 2 mL/L of drinking water 72 h pre-slaughter or non-supplemented. Half of the pens were then cooped and transported for 4 h pre-slaughter whereas the other half were transported for a very short distance to the slaughter house for processing.

2Number of replicate pens with 2 broiler chickens selected randomly from each pen for blood sampling.

3Proopiomelanocortin (POMC), glucocorticoid receptor (GR), aquaporin (AQP).

Meanwhile, muscle AQP9 showed no differences between treatments except its increase in the 2 mL betaine-supplemented group (Table 8). Brain expression of AQP4 showed no differences among the groups (Table 8). Interestingly, POMC was significantly increased in the control long-distance transportation group; however, betaine completely restored its expression in the feed-supplemented group and partially restored its expression in the water-supplemented group, except for the long-distance 1 mL betaine-supplemented group (Table 8). Moreover, hepatic GR was decreased in the long-distance transportation group, and betaine supplementation increased its expression in the increased dosage groups (0.2% in feed and 2 mL in water) (Table 8). Conversely, muscle GR was significantly decreased in the betaine-supplemented groups either in feed or in water (Table 8). On the other hand, betaine supplementation in water increased the expression of intestinal AQP3, except for the long-distance 1 mL supplemented group (Table 8).

The correlation analysis in the Supplementary Table S1 revealed that the expression of the brain AQP4 was positively correlated with the expression of brain POMC ($r = 0.74$, $p = 0.037$). Similarly, hepatic AQP3 expression was positively correlated with the expression of hepatic GR ($r = 0.88$, $p = 0.004$). In addition, muscle AQP1 and AQP9 were found to be positively correlated ($r = 0.79$, $p = 0.045$). No other significant correlations between the genes of interest were observed.

**Discussion**

Betaine supplementation in feed during the 12-day finisher period in the present study resulted in a significant reduction in feed intake without affecting the weight gain of chickens, leading to improved feed efficiency compared to water supplementation of betaine and no supplementation. In general, the influence of supplemental betaine on the live performance of broiler chickens is inconsistent in the literature. Several researchers reported no influence of betaine supplementation on broilers (Esteve-Garcia and Mack 2000; Park and Kim 2019), while others reported some positive effects on performance (Shakeri et al. 2018; Ghasemi and Nari 2020). Our results are partially in agreement with those of Shakeri et al. (2018) and Chen et al. (2018), who found supplemental betaine improved the FCR of broilers. Broiler chickens may or may not respond to betaine as stated above and the lack of response to betaine could be attributed to a variety of factors such as betaine dose, betaine feeding duration, feeding form (liquid vs. in-feed), heat challenge, and diet adequacy in methyl donors. In the
present study, feeding liquid betaine has no advantage in improving live performance compared to in-feed supplementation during the finisher period, which is most likely due to the short-term feeding of liquid betaine (72 h) compared to 12 days of in-feed supplementation.

Pre-processing handling of broiler chickens is a crucial step in broiler production that can considerably influence carcase quality and induce many physiological changes, mostly when broilers are transported for long distances to the processing plant. Transport over four hours in the present study has not significantly influenced the processing performance of broilers. One hypothesis tested here was the potential ameliorating effect of supplemental betaine when broilers are transported for long distances. The logic behind this hypothesis is that during long-distance transportation in hot weather, chickens could be dehydrated, and betaine accumulation in the body could help, as an osmolyte, minimise water loss. However, there was no betaine and transport distance interaction affecting any of the processing performance measurements. Although not significant, chickens transported for the long-distance have lost 56 g compared to those transported for the short distance. Pan et al. (2018) reported a significant reduction in the live BW of broilers transported for three hours’ pre-slaughter. Similarly, Zhang C et al. (2019) observed inferior BW for broilers after being transported pre-slaughter. The reduction in the BW after transportation can be attributed to dehydration and tissue mobilisation for energy (Warriss et al. 1990).

In commercial settings, broiler chickens are fasted for 8–12 h prior to the expected time of processing to minimise the risk of contamination with faecal materials during processing. Transportation and lairage at the processing plant could add more time to the fast- ing period. During fasting, chickens utilise the glycogen content in the liver and muscle as a source of energy, which results in a significant reduction in glycogen content with time (Yue et al. 2010; Sturkie 2012). Moreover, other metabolic processes like lipolysis of stored triglyceride and oxidation of tissue amino acids provide energy, leading to increased blood uric acid due to amino acid oxidation (Hu et al. 2016). In the present study, uric acid was not significantly changed due to transport. Interestingly, blood glucose was significantly elevated in the long transported group compared to the short transported group, suggesting a stress response activated by the hypothalamic–pituitary–adrenal axis (Puvadolpirod and Thaxton 2000; Hazard et al. 2005; Shini et al. 2009).

Our results revealed that chickens transported for four hours had lower initial breast muscle pH immediately post-slaughter than the chickens transported for 5 min period (6.29 vs. 6.34). Interestingly, the ultimate pH of the breast meat at 24 h post-slaughter behaved differently as the value related to the short-distance group dropped lower than the long-distance group (6.09 vs. 6.15). Our initial pH results are in agreement with those of Dos Santos et al. (2017) and Zheng et al. (2020), who reported similar findings. When broilers are subjected to transport stress, anaerobic glycolysis of muscle glycogen is accelerated, leading to lactic acid accumulation, which then contributes to the decreased meat pH post-mortem (Savenije et al. 2002). The observation that the ultimate pH of breast muscle for the chickens transported for a short distance is lower than that of the long-distance group was reported previously (Dos Santos et al. 2017). This is reportedly associated with the stressed birds having a rapid pH decline and are slaughtered before having sufficient time to restore the muscle glycogen reserves (Aberle et al. 2001). In general, lower meat pH values are associated with poor water-holding capacity, and this was observed in the long transported group, where there was a 2.32% reduction in the water holding capacity (24.3 vs. 21.98%). The long transported group had about an 8% increase in 0 h post-mortem breast temperature, indicating heat stress. Environmental conditions contribute significantly to the pre-slaughter transport stress in poultry and could lead to a considerable loss in performance, especially when it coincides with long-distance transportation.

The current gene expression results showed variable responses of different aquaporins to the distance of transportation with or without betaine supplementation. Several reports showed different behaviours of AQP expression in response to osmolytes concentrations; renal AQP2 and AQP3 increased with water restriction, while no effects were observed on AQP4 (Terris et al. 1995). Paradoxically, cortisol regulates the expression of AQP1 in different manners; mRNA and protein levels were increased in the intestine but decreased in the kidney in response to the hormone (Martinez et al. 2005; Maclver et al. 2009). Our results showed that even within the same organ, the response of AQP to transport distance stress and betaine supplementation is different, and this finding coincides with previous patterns observed by (Martinez et al. 2005; Maclver et al. 2009).

POMC is the precursor of pituitary melanocyte-stimulating hormone (α-MSH), adrenocorticotropic hormone (ACTH), and β-endorphin and is considered as a
marker of entire body stress condition (Gerets et al. 2000; Liu et al. 2020). The results indicated that POMC expression was increased with long-distance transportation; however, betaine supplementation in feed restored its expression and relieved this stress. We may speculate the effects of betaine through regulating the intracellular osmotic pressure and AQP4 expression in the brain (Umenishi and Schrier 2006); however, further investigation is required in this regard. The correlation analysis showed a direct correlation ($r = 0.74$) between POMC and AQP4 expressions.

The elevation of plasma glucose in long-distance transportation coincides with the increase of POMC expression because stress response and glucocorticoids exert a hyperglycaemic effect through inhibiting glucose uptake by the muscles and through glycolysis in the liver (McIntyre et al. 2012). Moreover, muscle GR was increased in the short-distance transport, indicating the acute response of muscle metabolism to the transportation stress, while adaptive response through the liver was noticed in the long-transportation distance (McIntyre et al. 2012; Finsterwald and Alberini 2014). Interestingly, betaine ameliorated the alterations in GR that might be due to the regulations of AQP expression. Correlation analysis indicated that muscle AQP1 is strongly correlated with AQP9 ($r = 0.79$), while hepatic AQP3 is strongly correlated with GR ($r = 0.88$) with a negative correlation with AQP9 ($r = -0.6$). Therefore, the interplay between GR and selective expression of AQP and POMC might be the possible way for betaine actions. However, further investigations are required in this regard.

Conclusions

In conclusion, supplemental betaine did not influence any of the processing performance or meat quality characteristics of transported broilers pre-slaughter. However, betaine could ameliorate the stressful effects of transport on POMC and GR expression and maintain cellular osmosis through osmolyte interactions with aquaporins expression, particularly the intestinal, hepatic, brain aquaporins (AQP1, AQP3 and AQP9). Our findings also showed different behaviours of the various aquaporins to transportation stress and betaine supplementation, which requires further investigation about the molecular mechanisms underlying this regulation.

Ethical approval

The protocol followed in this study was reviewed and approved by the Research Ethics Committee at King Saud University (Riyadh, Saudi Arabia; Ethics Reference No: KSU-SE-20-54).

Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

Islam M. Saadeldin http://orcid.org/0000-0002-7633-730X
Gamaleldin M. Suliman https://orcid.org/0000-0001-9865-1589

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