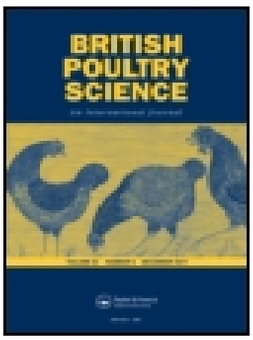

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Preliminary study on alleviation of heat-induced intestinal inflammation through compensatory effects of glucose oxidase

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ABSTRACT

1. The influence of glucose oxidase (GOD) supplementation on growth, gut inflammation and its compensatory effects in broilers was investigated before and after heat stress.
2. Before heat stress, one-day-old broilers were divided into two groups: the control (CON) and GOD (100 g/t complete feed) groups. On d 21, the CON group was equally divided into CON1 and CON2 groups, and heat stress (35°C) was applied to the CON2 and GOD groups for 8 h/day to the end of the study, d 27 of age. The chickens were either killed before heat stress and 2 d after heat stress for the determination of cytokines in the liver and ileum, serum antioxidant enzymes and ileal microbiota. Growth performance was determined before and 7 d after heat stress.
3. The GOD decreased Clostridiales and Enterobacteriaceae families of bacteria and increased ileal nuclear factor-κB, interleukin-1β, and interferon-γ ($P < 0.05$) before heat stress. The broilers exhibited compensatory effects, including increases in ileal sirtuin-1, heat shock protein 70 expression, liver nuclear factor erythroid 2-related factor 2 content, serum total antioxidant capacity and glutathione peroxidase level ($P < 0.05$). At 2 d after heat stress, inflammatory factors were increased in both the CON2 and GOD groups, but the levels were lower in the GOD than CON2 ($P < 0.05$). On d 7 after heat stress, GOS alleviated heat stress induced growth retardation ($P < 0.05$).
4. These data suggested that GOD supplementation in broiler diets before heat stress stimulated intestinal oxidative stress and produced a compensatory response, which prevented a rapid increase in intestinal inflammatory factors and helped to maintain growth performance under heat stress.

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KEYWORDS
Glucose oxidase; broiler; heat stress; cytokine; compensatory effect

Introduction

Glucose oxidase (GOD) is an aerobic dehydrogenase fermented by *Aspergillus niger, Penicillium notatum* and other fungi (Hatzinikolaou and Macris 1995; Hatzinikolaou et al. 1996). GOD can catalyse the oxidation of β-D-glucose to gluconic acid and hydrogen peroxide (H_2O_2) and simultaneously consumes a large amount of oxygen (Bankar et al. 2009). GOD is listed in the Chinese additive list (Announcement No. 318 of the Ministry of Agriculture), and its use to support animal growth has been well proven in practice (Pang et al. 2013; Wang et al. 2018; Wu et al. 2019). However, Chen et al. (2020) found that GOD increased the serum levels of inflammatory factors, such as immunoglobulin G, tumour necrosis factor-α, and interleukin-6 (IL-6) in Chuanzang black piglets. In the context of growth, increased intestinal proinflammatory cytokine levels leads to an acute phase response, resulting in loss of appetite (Gruys et al. 2006) and decreased growth (Humphrey and Klasing 2003). Therefore, increased intestinal inflammatory factor levels caused by the addition of GOD (Chen et al. 2020) might negatively affect growth performance, which is contradictory to the enhanced growth performance induced by GOD in practice.

The mechanism by which GOD improves animal growth performance in practice remains unclear, but may be associated with the hormesis mechanism. The hormesis mechanism states that proper stimulation (low-level) can cause organisms to develop self-defence mechanisms, thus facilitating the body to prepare for heavier stress loads (Southam and Ehrlich 1943; Schulz 1888). According to the hormesis mechanism, the addition of GOD might induce proper intestinal oxidative stress and stimulate a compensatory effect in the body, facilitating the body to prepare for heavier stress in practice, such as high density, toxic gases, cold or heat stress and infectious diseases.

Heat stress can lead to oxidative stress and nitrative stress in broilers (Lambert 2009; Hall et al. 2001) and has adverse effects on animal welfare (Nienaber and Hahn 2007) and growth performance (Nardone et al. 2010; Renaudeau et al. 2012).

The effects of supplementation of broilers with GOD before heat stress was studied to determine if it can protect the broilers from the adverse effects of heat stress through compensatory effects. The aim of this preliminary study was to provide new insight into the role of GOD in maintaining intestinal epithelial immune homeostasis in animals and the development of anti-stress strategies in practice.

Materials and methods

The animal care and use protocol in this study was approved by the institutional animal care and use committee of Qingdao Agricultural University.

Materials and animals

GOD (2000 U/g) was provided by the Qingdao GBW Group (Qingdao, China). The broilers (Ross 308) were purchased from a local farm (Qingdao, China). This study was performed in two environmentally controlled chicken rooms

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at the Qingdao GBW Group's research and development centre in December 2019. The study was performed in two stages. In total, 750, one-day-old broilers (375 male, 375 female) were divided into three groups, but initially there were only two treatments, the control (CON) group (n = 20 pen replicates/group; 25 chickens/replicate) and GOD group (n = 10 replicates/group; 25 chickens/replicate). The birds were kept in 1.5- × 1.5-m floor pens with fresh wood shavings and rice hull (1:1) and free access to pelleted diets and water throughout the entire experimental period. The CON group was divided into two groups (CON1 and CON2 with 10 replicates each; 25 chickens/replicate) on d 21, and heat stress was then applied to both groups. After heat stress, the study involved three groups. The temperature and relative humidity (RH) on d 1 were maintained at 35°C ± 1.1°C and 65% ± 5%, respectively. The temperature was decreased by 2.8°C per week until 26.7°C was reached on d 21, which was designated as the thermoneutral zone, along with an RH of 65% ± 5%. From d 21, broilers in the CON1 group were maintained at 26.7°C, whereas those in the CON2 and GOD groups were subjected to cyclic heat stress by exposing them to 35°C and an RH of 75% for 8 h/d from d 21 to the end of the study on d 27. The broilers in the CON, CON1, and CON2 groups received a corn-soybean meal diet with no antibiotic supplementation, and the broilers in the GOD group were fed the same diet supplemented with GOD (100 g/t feed) from d 1 to 20 before heat stress. After heat stress, a corn-soybean meal diet with no supplementation was given. GOD was added prior to pelleting, and diets were pelleted through a cold pellet press at a temperature of 60°C. The GOD content in the diet were analysed (0.21 and 0.23 U/g in starter and grower diets, respectively) using the Micro GOD Assay Kit from Shanghai Enzyme-linked Biotechnology Co. Ltd. (Shanghai, China). Formulated feed without antibiotics or GOD that met or exceeded the National Research Council (1994) requirements was used in this study (Table 1).

Table 1. Ingredients and calculated compositions of basal starter and grower diets.

Item	Starter (1 ~ 14 d)	Grower (15 ~ 22 d)
Ingredient g/kg		
Corn	570.3	652.6
Soybean meal	348.2	276.3
Palm oil	36.2	40.2
Vitamin-mineral premix[a]	3.4	3.2
Dicalcium phosphate	13.7	9.6
Salt	4.1	3.5
Limestone	11.0	10.5
Sodium bicarbonate	1.0	1.0
Methionine	3.3	3.3
Lysine	11.0	10.5
Choline chloride		
Calculated nutrients		
Metabolisable energy[b] MJ/kg	12.63	12.83
Crude protein g/kg	214.8	188.0
Crude fibre g/kg	24.9	23.8
Crude fat g/kg	53.2	49.3
Methionine g/kg	6.3	6.1
Methionine + Cystine g/kg	9.7	9.1
Lysine g/kg	13.3	12.0
Calcium g/kg	10.0	9.0
Total phosphorus g/kg	7.2	6.1
Available phosphorus g/kg	4.5	3.5

[a]Vitamin-mineral premix per kg of diet: cholecalciferol, 125 μg; retinol, 3,000 μg; α-tocopherol, 30 mg; cyanocobalamin, 30 mg; menadione, 1.5 mg; folic acid, 1.5 mg; choline, 500 mg; thiamine, 2.5 mg; pantothenic acid, 15 mg; pyridoxine, 5 mg; riboflavin, 6.5 mg; iron, 30 mg; zinc, 80 mg; copper, 8 mg; manganese, 100 mg; selenium, 0.15 mg; iodine, 1 mg.
[b]Metabolisable energy was calculated from data provided by the Feed Database of China (2016).

Growth performance

The birds were weighed before heat stress (d 20) and 7 d after (d 27) to determine body weight (BW), feed intake (FI) and feed conversion ratio (FCR). Mortality was recorded as it occurred, and the weight of dead chicks was determined where possible. Moreover, the European broiler index (EBI) was calculated according to the following formula:

$$\mathrm{EBI} = \text{survival rate}\,(\%) \times \text{live weight}\,(\mathrm{kg}) \times 100/\text{age}\,(\text{days}) \times \mathrm{FCR}.$$

Sample collection

Twelve chicks from each group were randomly selected and killed by exsanguination before heat stress (d 20) and two days after heat stress (d 22), respectively. As the parameters 2 d after heat stress was different from that at 7 d after heat stress, the latter data were not analysed in the present study, with the exception of growth performance. After decapitation, blood samples were taken directly from the neck vein into 5.0 ml centrifuge tubes and centrifuged at 2,000 × *g* at 4°C for 20 min. The serum was harvested and stored at −20°C for analysis of the serum antioxidant status. Ileal tissue samples obtained from near the caecum, and liver samples were removed and gently flushed with ice-cold saline in order to analyse the cytokine content. The ileal samples were snap-frozen in liquid nitrogen and stored at −80°C until they were assayed for sirtuin-1 (SIRT1) and heat shock protein 70 (HSP70). The digestive contents of the ileum near the caecum were collected and stored at −20°C to quantify total bacteria counts and those from the Enterobacteriaceae, Clostridiales and Bacteroides families.

Analysis of serum antioxidant status

Serum superoxide dismutase (SOD), total antioxidant capacity (TAOC), and glutathione peroxidase (GSHPx) were analysed by Shanghai Jining Industrial Co. Ltd. (Shanghai, China), and the assay kits for each were obtained from Shanghai Enzyme-linked Biotechnology Co. Ltd. (Shanghai, China). The SOD activity was measured using a xanthine oxidase method that monitors the inhibition of nitro blue tetrazolium reduction in the sample (Winterbourn et al. 1975). The TAOC was measured by a ferric reducing/antioxidant power assay (Benzie and Strain 1996) and detected at 520 nm using a spectrophotometer. The activity of GSHPx was detected with 5,5′-dithiobis-p-nitrobenzoic acid, and the change in absorbance at 412 nm was monitored (Hafeman et al. 1974). All assays were performed in accordance with the manufacturers' instructions.

Determination of cytokine production

The liver and intestinal samples were ground into a fine powder in liquid nitrogen, and the powder (0.3 g) was then added to 2.7 ml of sterile saline, homogenised, and centrifuged at 3,000 × *g* for 10 min at 4°C. The supernatant was assayed for the nuclear factor erythroid 2-related factor 2 (Nrf2) content in the liver samples and the nuclear factor-κB (NF-κB), interleukin-1β (IL-1β), and interferon-γ (IFN-γ) contents in the intestinal samples using commercial chicken

ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturers' protocols. The inducible nitric oxide synthase (iNOS) content in the supernatant of intestinal samples was analysed using an iNOS assay kit (Nanjing Jiancheng Bioengineering Institute). The content of iNOS is expressed as units/mg protein, and the contents of other cytokines are expressed as ng/mg protein. The concentration of protein was determined using a bicinchoninic acid protein assay kit (Beijing CWBio Inc., Beijing, China).

Quantitative real-time PCR analysis

Genomic DNA from the ileal samples was extracted using a DNA stool kit (TakaRa Bio, Shiga, Japan) following the manufacturers' instructions. The different primers and sequences used to quantify total bacteria and those from the Enterobacteriaceae, Clostridiales Bacteroides families are listed in Table 2. Quantitative real-time PCR (qRT-PCR) analysis was carried out with a 7500-fluorescence detection system (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions using a SYBR® Premix Ex Taq II kit (TakaRa Bio). The reaction mixture was 20 µl (2 µl of 1,200 ng/µl *cDNA*, 0.8 µl of 10 µmol/l forward and reverse primer, 10 µl of 2× SYBR Premix Ex Taq II, and 6.4 µl of RNase-free water). The cycling conditions were 95°C for 10 min, followed by 35 cycles at 95°C for 15 s, and then annealing and extension for 1 min at the temperature specific for each primer set. The efficiency of RT-PCR was calculated as previously described (Rasmussen 2001). Standard curves were constructed using the PCR product of the 16S rRNA gene of *Escherichia coli* (ATCC®25922™), *Bacteroides fragilis* (strain ATCC 25285), and *Clostridium butyricum* MIYAIRI 588® (CBM 588®). The PCR product was purified using a commercial DNA purification kit (TakaRa Bio), and the concentration was measured at 260 nm by a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The obtained products were sequenced (ABI 3100 Genetic Analyzer; PE Applied Biosystems, Warrington, UK) for confirmation. Standard curves were generated from 10-fold serial dilutions in water containing known concentrations of genomic bacteria DNA and used to quantify the copy number of the respective PCR reaction. Amplicons from *E. coli* (ATCC®25922™) were used for the quantification of Enterobacteriaceae and total bacteria, amplicons from *B. fragilis* (strain ATCC 25285) were used for the quantification of *Bacteroides* spp., and amplicons from *C. butyricum* MIYAIRI 588® (CBM 588®) were used for quantification of those in the Clostridiales family. The results were presented as the number of 16S rDNA copies per gram of fresh samples. A BLAST search in the NCBI database of the primers used for the quantification of taxa showed that many of the genera in each taxon could be amplified by the primers used (data not shown).

Western bolt analysis

Sample proteins (40 µg) were separated by SDS-PAGE in 12% polyacrylamide gels and then transferred onto nitrocellulose membranes in Tris-glycine buffer with 20% (vol/vol) methanol. The membranes were saturated with 5% (wt/vol) skim milk powder (Mengniu Dairy Co. Ltd., China) in Tris-buffered saline for 2 h at room temperature and incubated with diluted primary antibody overnight at 4°C. The primary antibodies used were rabbit anti-SIRT1 (1:1,000; XFS2035) and rabbit anti-HSP70 (1:1,000; XFS1980) (Xinfan Biotechnology, Shanghai, China). The specificity of antibodies was confirmed in a preliminary experiment. After several washes with Tris-buffered saline containing 0.1% Tween 20, the membranes were incubated in a 1:20,000 dilution of an anti-rabbit horseradish peroxidase-conjugated secondary antibody (Xinfan Biotechnology). They were then incubated with a 1:10,000 dilution of a horseradish peroxidase-conjugated mouse monoclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Kangchen Biotechnology, Shanghai, China) to normalise the results. The protein signals were detected by chemiluminescence with a WesternBright ECL substrate kit (Advansta, San Jose, CA) in accordance with the manufacturer's protocol. The density of the blotting bands was analysed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data were statistically analysed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). An unpaired Student's t-test was used to test differences between the CON and GOD groups before heat stress, whereas one-way analysis of variance followed by Duncan's test was used for comparison of more than two groups after heat stress. Data were presented as mean ± standard deviation. Statistical significance was set at $P < 0.05$.

Results

Growth performance

There were no significant differences in growth performance indices such as BW, FI, FCR, and EBI between the CON and GOD groups before heat stress ($P > 0.05$; Table 3). On d 7 after heat stress, the BW and FI significantly increased in the CON1 group, followed by the GOD group, and these parameters were lowest in the CON2 group ($P < 0.05$). The EBI was lower and the FCR was higher in the CON2 group than in the other groups ($P < 0.05$). There were no significant differences in the EBI or FCR between the GOD and CON1 groups ($P > 0.05$; Table 3).

Table 2. Primer sequences used for quantitative reverse-transcription PCR.

Target Gene	Primer Sequence	Fragment Length (bp)	Temperature	Reference
Clostridiales	F:5 - GCGTTATCCGGATTTAC-3′ R: 5 - ACACCTAGTATTCATCG-3′	286	60°C	Videnska *et al.* 2013
Bacteroides	F: 5 - GAGAGGAAGGTCCCCCAC-3′ R: 5 - CGCTACTTGGCTGGTTCAG-3′	108	60°C	Layton *et al.* 2006
Enterobacteriaceae	F: 5 - CGGTGTACCCGCAGAAGAAGCAC[FAM]G-3′ R: 5 - GCCTCAAGGGCACAACCTCCAAG-3′	368	55°C	Martinon *et al.* 2011
Total Bacteria	F: 5 - GCAGGCCTAACACATGCAAGTC-3′ R: 5 - CTGCTGCCTCCCGTAGGAGT-3′	314	60°C	Castillo *et al.* 2006

Table 3. Effect of GOD on growth performance of broilers under heat stress.

Treatment	BW, g/bird	FI, g/bird	FCR	EBI
Performance 0–20 days (BH)				
CON	963	1108	1.15	239
GOD	880	1088	1.24	201
SEM	4.0	53.6	0.034	9.7
P	ns	ns	ns	ns
Performance 0–27 days (7AH)				
CON1	1500[c]	2095[c]	1.40[a]	295[b]
CON2	1110[a]	1670[a]	1.51[b]	207[a]
GOD	1401[b]	1987[b]	1.42[a]	278[b]
SEM	60.1	65.7	0.020	14.2
P	*	*	*	*

BW, body weight; FI, feed intake; FCR, feed conversion ratio; EBI, European broiler index; CON, control; CON1, thermoneutral zone control; CON2, heat stress control; GOD, glucose oxidase group; BH, before heat stress; 7AH, 7 days after heat stress.
[a,b,c]Data in the column with different superscripts differ significantly. *P < 0.05; ns, not significant

Intestinal microbiota

The results showed that before heat stress, the number of total bacteria was not significantly different between the CON and GOD groups (P = 0.382). In contrast, the number of bacteria belonging to the Clostridiales, Enterobacteriaceae, and Clostridiales/Enterobacteriaceae families was significantly lower in the GOD group than in the CON group (P < 0.05; Figure 1(a–f)). At 2 d after heat stress, total bacteria were not significantly different among the groups (P = 0.114). Bacteria from the families Clostridiales, Bacteroides, Clostridiales/Enterobacteriaceae, and *Bacteroides*/Enterobacteriaceae were markedly lower in the CON2 and GOD groups than in the CON1 group (P < 0.05). Furthermore, those from the families Clostridiales/Enterobacteriaceae and *Bacteroides*/Enterobacteriaceae were significantly lower in the GOD group than in the CON2 group (P < 0.05) (Figure 1(a–f)).

Intestinal cytokines

Before heat stress, the levels of NF-κB, IL-1β, and IFN-γ in the GOD group were significantly higher than those in the CON group (P < 0.05; Figure 2(a–d)). At 2 d after heat stress, the levels of NF-κB, IL-1β, IFN-γ and iNOS were markedly higher in the CON2 and GOD groups than in the CON1 group (P < 0.05; Figure 2(a–d)). However, the levels of NF- κB, IL-1β, and IFN-γ were significantly lower in the GOD group than in the CON2 group (P < 0.05; Figure 2(a–d)).

Intestinal SIRT1 and HSP70 protein expression

The results showed that, before heat stress, GOD significantly increased the intestinal protein expression of SIRT1 and HSP70 in broilers (P < 0.05; Figure 3(a–c)). At 2 d after heat stress, the protein expression of SIRT1 and HSP70 was significantly higher in the CON2 and GOD groups than in the CON1 group (P < 0.05), and the protein expression of SIRT1 and HSP70 was significantly higher in the CON2 group than in the GOD group (P < 0.05; Figure 3(a–c)).

Liver Nrf2 content and plasma antioxidative enzymes

The results showed that, before heat stress, the Nrf2 level in the liver and the serum TAOC and GSHPx contents were significantly higher in the GOD group than in the CON group (P < 0.05; Figure 4(a–d)). At 2 d after heat stress, the Nrf2 content in the liver and the serum antioxidant enzyme contents were significantly increased in the GOD group, followed by the CON2 group (except SOD); the content in the CON1 group was the lowest (P < 0.05; Figure 4(a–d)).

Discussion

The mechanism of action of GOD might be related to hormesis, in which proper stimulation (low-level) allows organisms to develop a self-defence mechanism, which prepares the body for heavier stress (Schulz 1888; Southam and Ehrlich 1943). Yamaoka et al. (1991) reported that low-dose radiation activated the activity of GSHPx and SOD in the liver and argued that these enzymes expedited the elimination of free radicals, which protects the body from harm. Therefore, the following hypothesis was tested in the present study, whereby supplementation of broilers with GOD before heat stress can protect the broilers from the adverse effects of heat stress through compensatory effects. This preliminary investigation will provide a theoretical basis for the application of GOD in animal production.

Growth performance

Several studies have suggested that heat stress negatively affects the health and growth performance of broilers (Nienaber and Hahn 2007; Renaudeau et al. 2012). In the present study, heat stress significantly decreased growth performance indices including BW, FI, FCR, and EBI, whereas the addition of GOD in the diet improved the growth performance of broilers under high temperatures. Some studies suggested that gluconic acid, which is a key metabolite of GOD, can improve the growth performance of broilers (Biagi et al. 2006; Biggs and Parsons 2008). Wu et al. (2019) found that the addition of GOD can improve the apparent digestibility of nutrients and the activity of digestive enzymes in broilers.

Intestinal microbiota

The results showed that heat stress and GOD did not affect the numbers of total bacteria; however, heat stress and GOD reduced the numbers of bacteria in the families Clostridiales, Bacteroides, and Enterobacteriaceae. After heat stress, the ratios of Clostridiales/Enterobacteriaceae and Bacteroides/Enterobacteriaceae were markedly lower in the CON2 and GOD groups than in the CON1 group. A previous study

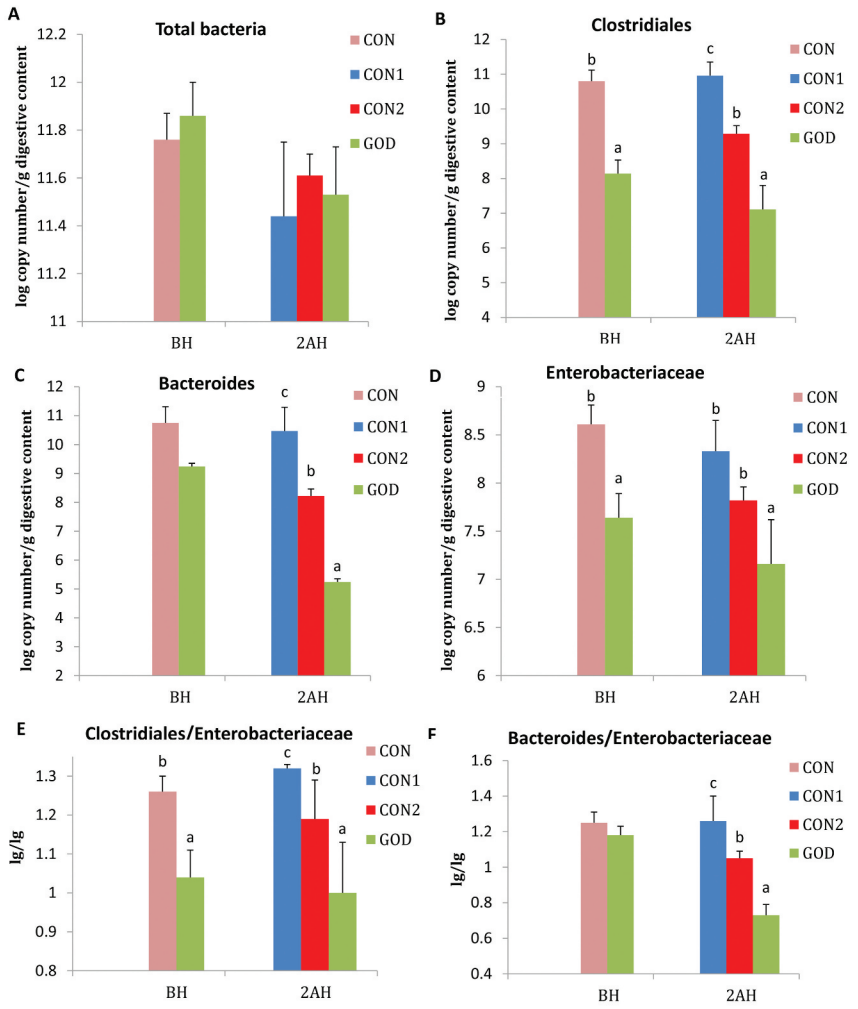

Figure 1. Real-time PCR analysis of the effect of GOD on intestinal (a) total bacteria, (b) Clostridiales, (c) Bacteroides, (d) Enterobacteriaceae, (e) Clostridiales/Enterobacteriaceae, and (f) Bacteroides/Enterobacteriaceae in broilers under heat stress (n = 12). Data are presented as the mean ± SD. Bars with different lowercase letters differ significantly ($P < 0.05$). CON, control; CON1, thermoneutral zone control; CON2, heat stress control; GOD, glucose oxidase group; BH, before heat stress; 2AH, 2 days after heat stress.

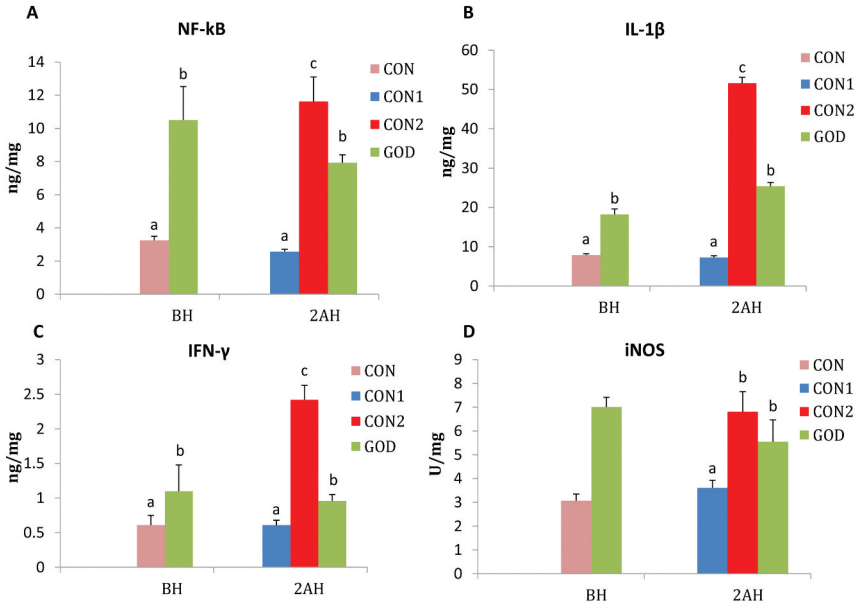

Figure 2. ELISA analysis of the effect of GOD on intestinal (a) nuclear factor- κB (NF- κB), (b) IL-1β, (c) interferon γ (IFN-γ), and (d) inducible nitric oxide synthase (iNOS) in broilers under heat stress (n = 12). Data are presented as the mean ± SD. Bars with different lowercase letters differ significantly ($P < 0.05$). CON, control; CON1, thermoneutral zone control; CON2, heat stress control; GOD, glucose oxidase group; BH, before heat stress; 2AH, 2 days after heat stress.

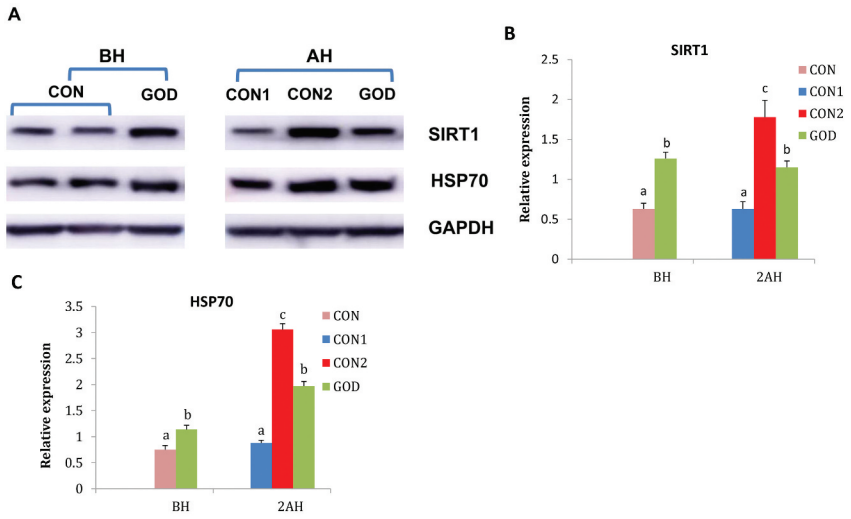

Figure 3. Western blot analysis of the effect of GOD on intestinal SIRT1 and HSP70 in broilers under heat stress (n = 12). (a) Representative western blot images of SIRT1, HSP70, and GAPDH protein expression in the ileal tissue. The intensities of (b) SIRT1 and (c) HSP70 were normalised to the corresponding GAPDH levels. All analyses were performed in triplicate. Data are presented as the mean ± SD. Bars with different lowercase letters differ significantly ($P < 0.05$). SIRT1, sirtuin-1; HSP70, heat shock protein 70; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CON, control; CON1, thermoneutral zone control; CON2, heat stress control; GOD, glucose oxidase group; BH, before heat stress; 2AH, 2 days after heat stress.

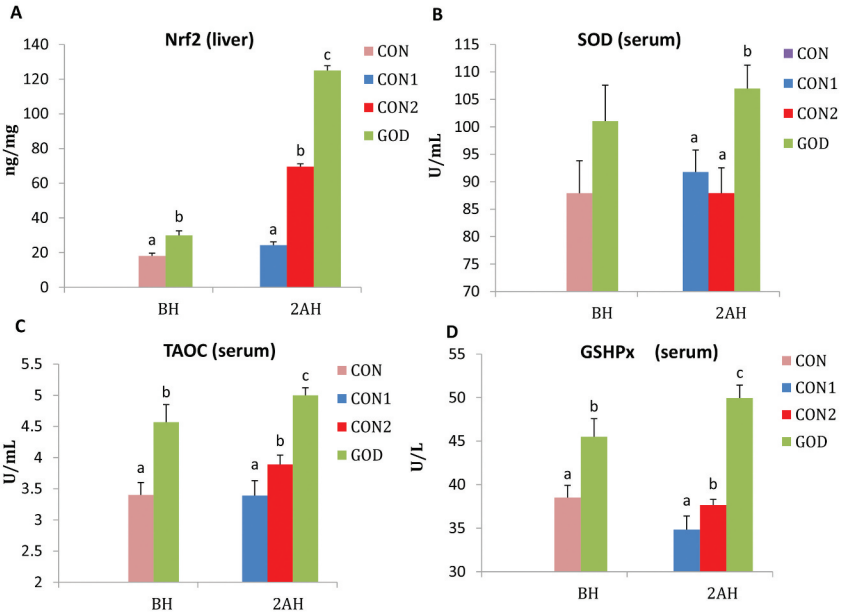

Figure 4. ELISA analysis of the effect of GOD on the (a) liver Nrf2 content and serum (b) SOD, (c) TAOC, and (d) GSHPx contents in broilers under heat stress (n = 12). Data are presented as the mean±SD. Bars with different lowercase letters differ significantly ($P < 0.05$). Nrf2, nuclear factor erythroid 2-related factor 2; SOD, superoxide dismutase; TAOC, total antioxidant capacity; GSHPx, glutathione peroxidase; CON, control; CON1, thermoneutral zone control; CON2, heat stress control; GOD, glucose oxidase group; BH, before heat stress; 2AH, 2 d after heat stress.

showed that obligate anaerobic bacteria such as *Clostridium* spp. populations are particularly sensitive to inflammatory reactive oxygen species (Frank et al. 2007). Unlike obligate anaerobic bacteria, Enterobacteriaceae such as *Salmonella typhimurium* can utilise tetrathionate formed by the reaction of reactive oxygen species with endogenous thiosulfate (Winter et al. 2010; Lopez et al. 2012, 2015). Therefore, in the present study, bacteria belonging to Clostridiales and Bacteroides families were more sensitive to heat stress and GOD, which could induce more oxidative stress (Lambert 2009; Hall et al. 2001) than bacteria belonging to the Enterobacteriaceae family. Some authors have indicated that GOD consumes a large amount of oxygen, providing an anaerobic environment for the survival of obligate anaerobic bacteria such as *Faecalibacterium prausnitzii* (Wu et al. 2019). However, these authors did not mention the effect of oxidative stress induced by GOD-catalysed H_2O_2 on gut microflora.

In the present study, obligate anaerobic bacteria were decreased in the GOD group. Other authors have considered that H_2O_2 produced by glucose metabolism catalysed by GOD is only harmful to pathogenic bacteria, and may be advantageous to the survival of beneficial bacteria (Wu et al. 2019; Kapat et al. 1998). These conclusions were different from those in the present study, where there was a decrease in the number of Enterobacteriaceae bacteria, including the

pathogens *Salmonella* spp. and *E. coli*, but the number from the Clostridiales family, which produce butyric acid, which is considered to benefit the host, simultaneously decreased. This suggested that H_2O_2 kills bacteria indiscriminately.

Intestinal inflammation and SIRT1 and HSP70 protein expression

Before heat stress, the addition of GOD increased the levels of intestinal inflammatory factors including NF-κB, IL-1β, and IFN-γ in the present study, and subsequent heat stress treatment greatly increased the levels of intestinal inflammatory factors in the CON2 and GOD groups ($P < 0.05$). However, after heat stress, the rate of increase in the intestinal inflammatory factors in the GOD group was significantly decreased compared with the CON2 group ($P < 0.05$). This suggested that GOD prevented rapid increases in the levels of inflammatory factors caused by heat stress. At the same time, the compensatory effect produced by increased SIRT1 and HSP70 protein expression was investigated. Other researchers have suggested that the compensatory effect may represent a general phenomenon that occurs when cells or organisms are exposed to risk factors, and activation of repair mechanisms will then be stimulated to protect against disease (Gori and Münzel 2012). The SIRT1 is a protein deacetylase that stimulates various cellular protective mechanisms, including autophagy and DNA repair (Martel et al. 2019). Additionally, the production of HSP70 under oxidative stress can protect the host from the deleterious cellular effects of reactive oxygen species (Lara and Rostagno 2013) by inhibiting the NF-κB signalling pathway (Calabrese et al. 2001) and increasing the activity of antioxidant enzymes, such as SOD (Liu et al. 2016). The results from the present study showed that the response of SIRT1 and HSP70 protein expression to heat stress and GOD was similar to inflammatory factors. These data suggested that, before heat stress, the addition of GOD increased stress levels in the animals and then strengthened the corresponding compensation by increasing the SIRT1 and HSP70 protein expression levels, which stimulated cellular protective mechanisms. After heat stress, GOD prevented rapid increases in the levels of inflammatory factors and SIRT1 and HSP70 protein expression caused by heat stress. However, the specific mechanism of these phenomena requires further investigation.

Veličković et al. (2019) found a significant increase in SIRT1 expression in the setting of fructose-induced inflammation, which suggested a compensatory rise in the level of SIRT1 to reduce the inflammation-related metabolic reactions after feeding rats high dietary fructose. Furthermore, Elibol and Kilic (2018) proposed that a significant increase in oxidative stress parameters might induce SIRT1 expression. This compensatory mechanism enhanced the antioxidant response to oxidative stress in patients with cardiovascular disease (Elibol and Kilic 2018).

Liver Nrf2 content and plasma antioxidative enzymes

This study investigated the liver Nrf2 content and plasma antioxidative enzymes. The Nrf2 is a critical leucine zipper transcription factor that plays an important role in cellular oxidative stress through antioxidant response element-mediated induction of several phase 2 detoxifying and antioxidant enzymes (Ueda et al. 2008; Negi et al. 2011; Chen et al. 2013). The results showed that Nrf2 content in the livers from the GOD group increased before and after heat stress, and this was accompanied by increased serum TAOC and GSHPx levels. This suggested that the Nrf2 protein expression level was increased by activation of the Nrf2–antioxidant response element signalling pathway and that the serum antioxidant enzyme contents subsequently increased. These findings were consistent with previous studies, whereby Zhang et al. (2020) found that GOD promoted the antioxidant capability in weanling piglets by upregulating the Nrf2/Keap1 pathway in the liver and jejunum. Interestingly, the heat stress treatment in the present study increased inflammatory factor levels in the CON2 group, and at the same time, Nrf2, TAOC, and GSHPx contents were significantly increased in this group during heat stress. These results indicated that heat stress stimulated a compensatory effect in the animals in the control group through the hormesis mechanism.

The effects of GOD supplementation relative to stressor events in broilers were investigated for the first time in this study, which provided new insights into its mechanism of action. However, the study had several limitations, firstly, only the short-term effects of heat stress were examined. A longer study might yield different results. Secondly, only heat stress was examined; thus, the findings of this study did not necessarily translate to other stressors. Third, only one GOD dosage (100 g/t complete feed) was evaluated because this is the normal level added in practice. Different inclusion rates in feed might produce varying levels of oxidative stress in birds. Therefore, further research is needed to more comprehensively determine the effect of GOD supplementation on the intestinal health of broilers.

In conclusion, before heat stress, the addition of GOD to broiler diets increased the intestinal oxidative stress and production of inflammatory factors, which activated the immune system and stimulated compensatory effects. Thus, GOD protects the animals from serious harm by preventing a rapid increase in intestinal inflammatory factors after heat stress.

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