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Effect of rearing temperature on physiological measures and antioxidant status of broiler chickens fed stevia (*Stevia rebaudiana* B.) leaf meal and exogenous xylanase

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ABSTRACT

Background: The global climate is warming. Heat stress, as a result of high ambient temperatures, may negatively impact physiology and reduce growth performance of poultry. Stevia is a perennial shrub indigenous to South America where its phytochemical extracts have been used as a natural sweetener for hundreds of years. Its physiological effects, including antioxidant properties, on poultry are well known, however, the translation of these to improved growth performance is variable. Combining stevia with a commercial xylanase to enhance feed digestibility could therefore form a feeding strategy to partially mitigate the negative impact of rearing birds under high ambient temperatures.

Purpose: The study aimed to compare the growth performance, dietary energy and nutrient availability, oxidative status, gastrointestinal tract development, and caecal short chain fatty acid concentration; at two ambient rearing temperatures, when feeding diets containing stevia and exogenous xylanase, alone or in combination, to broiler chickens.

Study design/Methods: Day-old chicks (n = 105) were reared in a single floor pen following breeder recommendations for the first 7 days, whereupon birds (n = 96) were randomly allocated to one of four experimental diets (negative control, stevia at 20 g/kg diet, xylanase at 100 FXU/kg diet, stevia at 20 g/kg diet + xylanase at 100 FXU/kg diet), in one of two environmental conditions (high ambient temperature at 32 ± 2 °C or regular rearing at breeder recommendations), in a 2 × 2 × 2 factorial design.

Results: Rearing birds at high ambient temperature reduced daily feed intake ($p = 0.02$). Birds fed stevia and reared at regular temperature had similar weight gain to birds reared in high ambient temperatures, although birds on the control diet housed at regular temperatures had the greatest weight gain ($P < 0.05$). Exogenous xylanase improved overall dietary metabolisable energy and improved nitrogen retention in the high ambient temperature group only ($P < 0.05$). Dietary stevia reduced caecal digesta butyric acid: acetic acid at regular temperature, but xylanase increased the butyric acid concentration at high ambient temperature ($P < 0.05$).

Abbreviations: XYL, xylanase; STE, stevia; FXU, xylanase units; HT, high ambient temperature; RT, regular ambient temperature; RH, relative humidity; FI, feed intake; WG, weigh gain; FCR, feed conversion ratio; GIT, gastrointestinal tract; PG, proventriculus and gizzard; DM, dry matter; GE, gross energy; AMEn, apparent metabolizable energy corrected for nitrogen retention; NR, nitrogen retention; GSH-Px, glutathione peroxidase; SCFAs, short chain fatty acids; AA, acetic acid; BA, butanoic acid; PA, pentanoic acid; PRA, propanoic acid; ANOVA, analysis of variance; T°C, temperature.

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[1] All authors contributed equally to writing and reviewing this paper. VP and SPR were involved in conceptualization, data curation, formal analysis, funding acquisition. SCM, VP and AGA were further involved in editing. IMW, SE, KK, AEJ, FD and SOD conducted laboratory and other analysis.

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Dietary stevia increased ($P < 0.001$) the hepatic carotenoid concentrations and xylanase improved ($P < 0.05$) hepatic vitamin E concentrations.

Conclusions: Rearing temperature is an important environmental factor in broiler production. Exogenous xylanase supplementation can increase feed efficiency and dietary metabolisable energy. Feeding xylanase or stevia improves hepatic antioxidant status in broilers by increasing hepatic vitamin E and carotenoids, respectively, suggesting that either may be effective in counteracting oxidative stress.

1. Introduction

The global climate is changing. Global temperatures have risen approximately 1.0 °C since pre-industrialised times and are predicted to reach 1.5 °C by 2052 or earlier (IPCC 2018). Heat stress, as a result of higher ambient temperatures may negatively impact physiology and reduce growth performance of poultry (Pirgozliev et al., 2020; Woods et al., 2020, 2021). The associated oxidative stress is implicated in reduced bird welfare and carcass quality (Quinteiro-Filho et al., 2010). Heat stress is therefore one of the most challenging environmental conditions affecting commercial poultry, broilers in particular, and it causes a significant loss of revenue each year (Woods et al., 2020). For commercial production, cooling and ventilation systems are often used to overcome the issues of high ambient temperatures, however, there are economic considerations for these technologies. Whilst free-range rearing systems have been used for laying hens for many years, the idea of using them in broiler production is increasing in popularity. In free-range broiler settings, it is difficult to control environmental temperatures and humidity to the degree currently available in most modern indoor broiler facilities. Alternative techniques to commercial cooling and ventilation systems to aid management of birds in high ambient temperature free-range systems needs further research.

The use of supplementary antioxidants in poultry feed is an important topic, particularly with the rising global temperatures associated with climate change (Pirgozliev et al., 2015a, 2019a). Stevia (*Stevia rebaudiana*, Bertoni; STE) is a perennial shrub indigenous to South America where it has been used as a natural sweetener for hundreds of years. The sweetening property of STE and its extracts (stevioside and ribaudioside A) are well recognised and used in human diets worldwide (Geuns et al., 2003; Geuns, 2008). Stevia (and extracts) have been used in poultry diets but with inconsistent effects on growth performance variables (Wood et al., 1996; Geuns et al., 2003; Atteh et al., 2008). There are also various physiological effects of stevia and its extracts including: insulinotropic activity, hypotensive and diuretic effects and it also possesses antimicrobial properties (summarised by Atteh et al., 2008). Research by Stoyanova et al. (2011) suggested that stevioside might also be involved in antioxidant defence mechanisms to help survive stresses.

After phytase, exogenous xylanase (XYL) is the most used enzyme in poultry production (Bedford, 2018), as it improves not only productive performance but also hepatic antioxidative status of birds (Pirgozliev et al., 2015b). However, information on combining STE as a phytochemical antioxidant and to improve gut health and XYL as a digestibility enhancer in diets is not available. Diets formulated specifically for use in high ambient temperature environments may enhance the antioxidant status of animals but could also slow the depletion levels of tissue antioxidants. Supplementing diets with STE and / or XYL could be a viable option in this regard for the poultry industry, however, there are currently no reported studies comparing the growth performance response, nutrient and energy availability or antioxidant status to STE in combination with exogenous XYL of broilers reared under standard and high ambient temperatures. The main objectives of this study were to compare broiler antioxidative status and performance when the birds were fed diets, with or without STE and / or exogenous XYL supplementation, when raised at two different temperature regimes (T °C) of the breeder recommended temperature curve (27 °C reducing to 21 °C) and a temperature of 32 ± 2 °C.

2. Materials and methods

2.1. Experimental diets

A wheat-soya based basal diet that met breeder's recommendations for growing broilers (Aviagen Ltd., Edinburgh, UK) was mixed. Two experimental diets were prepared from the basal diet that included either 20 g/kg of milled dry STE leaf or 20 g/kg of milled dry grass pellets, in order to provide the same dietary dilution (Table 1). The STE plant is from cultivar Stela and was produced at the Agricultural Institute in Shumen, Bulgaria, during the 2019 growing year. The grass pellets were obtained from Target Feeds Ltd (Whitchurch, UK). The diets were supplied with 20 g/kg of acid insoluble ash, a feed grade diatomaceous earth (Multi-Mite®, Wiltshire, UK). Both diets were then split into two batches and one part of each diet was supplemented with *Aspergillus oryzae* commercial preparation of *endo*-1,4-beta-xylanase at 100 g/kg (100 FXU/kg, Ronozyme WX, DSM, Switzerland), resulting in four diets in total. All diets were fed as mash.

2.2. Husbandry and sample collection

The experiment was conducted at the National Institute of Poultry Husbandry and approved by the Research Ethics Committee of Harper Adams University (UK). A total of 105 female Ross 308 birds were purchased from a commercial hatchery (Cyril Bason Ltd, Craven Arms, UK), allocated to a single floor pen and offered a proprietary wheat-based broiler starter feed formulated to meet Ross 308 nutrient requirements (Aviagen Ltd., Edinburgh, UK). Birds were reared during the first week according to breeder recommendations: 30 °C on arrival, decreasing gradually to 27 °C. At 7d age, 96 of the birds, excluding ill and malformed, were allocated at random to the four experimental diets. Each diet was fed to eight pens (0.36 m^2 floor area; 3 birds per pen), which were allocated to four rooms, two pens in each room, following randomisation. Each of the pens had a solid floor and were equipped with an individual feeder and drinker. Feed and water were offered *ad libitum* to birds throughout the experiment. The T°C in two of the rooms was maintained at 32 ± 2 °C (HT), and the T°C in the other two rooms was gradually reduced from 27 °C to 21 °C by 21 d age (following Ross 308 guidance; regular temperature, RT). The relative humidity (RH) in the HT rooms was maintained at 50% (±3%) by heating water in 50 L Buffalo Manual Fill Water Boilers (Nisbets Plc., Bristol, UK). In the RT rooms there were no humidity control, and RH was 52% on average, varying between 47 and 62%. A standard industry lighting programme for broilers (Aviagen Ltd, Edinburgh, UK) was used. Birds and feed were weighed at the beginning (day 7) and end (day 21) of the experiment to determine average daily feed intake (FI), average daily weight gain (WG) and feed conversion ratio (FCR) on a pen basis. From 17 to 21 d age, the solid floor of each pen was replaced with a wire mesh. Excreta were collected each day until the end of the experiment, stored in a fridge, later dried at 60 °C and milled through a 0.75 mm screen. At the end of the study, one bird per pen selected at random, was weighed then electrically

Table 1
Ingredient composition (g/kg 'as fed') of the experimental diets.

Dietary ingredients	Stevia	Basal
Stevia	20.0	–
Grass meal	–	20.0
Wheat	630.0	630.0
Soybean meal (48% CP)	219.7	219.7
Soybean meal (full fat)	50.0	50.0
Vegetable oil	20.0	20.0
Dicalcium phosphate	14.5	14.5
Limestone	12.5	12.5
NaCl	2.7	2.7
Lysine	2.7	2.7
Methionine	3.9	3.9
Acid Insoluble Ash	20.0	20.0
Vitamin mineral premix[1]	4.0	4.0
	1000	**1000**
Calculated analysis (as fed)		
Crude Protein g/kg	204	199
ME MJ/kg	12.42	12.42
Crude Fat g/kg	43.9	43.1
Ca g/kg	9.5	9.5
Available P g/kg	4.5	4.4
Lysine g/kg	12.2	12.1
Methionine + Cysteine g/kg	9.7	9.7

[1] Provided per kg feed: 2160 μg retinol, 75 μg cholecalciferol; 25 mg α-tocopherol, 1.5 mg menadione, 5 mg riboflavin, 8 mg pantotenic acid, 10 μg cyanocobalamin, 1.5 mg pyridoxine, 1.5 mg thiamine, 0.5 mg folic acid, 30 mg niacin, 60 μg biotin, 0.8 mg I, 10 mg Cu, 80 mg Fe, 0.3 mg Se, 80 mg Mn, 80 mg Zn (Target Feeds Ltd., Whitchurch, UK).

stunned and killed by exsanguination. Blood was obtained in heparin coated tubes from the jugular vein during exsanguination. The organs from the gastrointestinal tract (GIT), including proventriculus and gizzard (PG), duodenum, pancreas, jejunum, ileum, caeca, liver and the spleen were weighed and processed as previously described (Pirgozliev et al., 2020).

2.3. Validation of methods (Quality Assurance)

Stevioside and rebaudioside in the dry STE leaf were determined as previously described (Geuns et al., 2003). Non-starch polysaccharides in STE, grass meal and diet were determined as described by Englyst et al. (1994). The antioxidants in STE, grass meal and liver, including vitamin E, coenzyme Q_{10} and total carotenoids, were determined as previously described (Karadas et al., 2014).

2.4. Analysis of feed, excreta, caecal digesta and blood

Dry matter (DM) in feed and excreta samples was determined by drying of samples in a forced draft oven at 105 °C to a constant weight (AOAC, 2000; method 934.01). Crude protein (6.25 × N) in samples was determined by the combustion method (AOAC, 2000; method 990.03) using a LECO FP-528 N (Leco Corp., St. Joseph, MI). Oil (as ether extract) in diets was extracted with diethyl ether by the ether extraction method (AOAC, 2000; method 945.16) using a Soxtec system (Foss Ltd., Warrington, UK). The gross energy (GE) value of feed and excreta samples was determined in a bomb calorimeter (model 6200; Parr Instrument Co., Moline, IL) with benzoic acid used as the standard. Acid insoluble ash in feed and excreta was determined as explained by Van Keulen and Young (1977). Dietary apparent metabolisable energy corrected for nitrogen retention (AMEn) and retention coefficients were determined as described elsewhere (Abdulla et al., 2016; Pirgozliev et al., 2020; Woods et al., 2020).

2.5. Biochemical, histology and short chain fatty acid analysis

The glutathione peroxidase assay in blood was performed using a Ransel GSH-Px kit (Randox Laboratories Ltd., UK) that employs the method based on that of Paglia and Valentine (1967). The short chain fatty acid (SCFA) concentrations, including acetic acid (AA), butanoic acid (BA), pentanoic acid (PA) and propanoic acid (PRA), in poultry caecal digesta were determined by using an Agilent 5973 N GC/MS equipped with an Agilent 6890 N GC and an Agilent 7683 autosampler. Jejunum samples for histology collected as described by Pirgozliev et al. (2020) were dehydrated in increasing grades of ethyl alcohol (70%, 80%, 90% and 99.8%). Samples were embedded in paraffin wax, sectioned to 5 μm and four gut segments were fixed on each slide. Morphometric measurements were determined on 20 intact well-oriented villus–crypt units for each bird as previously described (Bancroft and Gamble, 2008).

2.6. Statistical analysis

Data were analysed using Genstat (19th edition) statistical software (IACR Rothamstead, Hertfordshire, UK). Comparisons for the main effects (and their interactions) of STE, XYL and T°C were performed by the general ANOVA procedure using a split-plot 2 × 2 × 2 factorial design. The main plots were the four rooms that were each randomly allocated to one of the two temperatures. The pens within each room were the sub-plots and these were randomly allocated to one of the four dietary treatments. The statistical analysis used the following matrix model:

$$Y_{ijkl} = \mu + A_i + N_{l(i)} + B_j + C_k + (BC)_{jk} + (AB)_{ij} + (AC)_{ik} + (ABC)_{ijk} + \varepsilon_{l(ijk)}$$

where

μ_i = Grand mean
A_i = Fixed effect of temperature
$N_{l(i)}$ = Whole plot (room) error
B_j = Fixed effect of STE
C_k = Fixed effect of XYL
$(BC)_{jk}$ = Fixed interaction of STE and XYL
$(AB)_{ij}$ = Fixed interaction of temperature and STE
$(AC)_{jk}$ = Fixed interaction of temperature and XYL
$(ABC)_{ijk}$ = Fixed three-way interaction of temperature, STE and XYL
$\varepsilon_{l(ijk)}$ = Split-plot error

Data were checked for normal distribution. A protected LSD test was used to separate differences in interaction means ($P < 0.05$). Means for interactions are only included in tables when P-values were significant.

3. Results

The determined composition of the dry STE leaves, milled grass pellets and the basal diet is presented in Table 2. Compared to grass meal, the STE sample contained three times less soluble NSP, 3.5 times more vitamin E and 25 times more total carotenoids. Adding 20 g STE in the diet provided 9 g of carotenoids. The CP and CF content in the diet agreed with breeder's recommendations.

3.1. Growth performance, AMEn and nutrient retention coefficients

There was no mortality and all birds were healthy throughout the study period. Rearing birds at HT reduced daily FI ($P = 0.015$), tended ($P = 0.091$) to reduce WG and did not influence FCR ($P > 0.05$) (Table 3). Feeding xylanase reduced FCR (improved feed efficiency) ($P = 0.007$), but did not significantly ($P > 0.05$) change daily FI and WG. There was a T°C by STE interaction for daily WG, as rearing birds at RT without STE improved daily WG ($P = 0.047$). Feeding XYL improved AMEn ($P = 0.045$) by 0.34 MJ and tended ($P = 0.057$) to

Table 2
Nutritional analysis of stevia, grass meal and the basal diet (analysis were performed in duplicates).

Determined values	Stevia	Grass meal	Basal diet
Dry matter (g/kg)	881	919	878
Gross energy (MJ/kg)	17.94	15.33	17.94
Crude protein (g/kg)	119.2	152.4	212.9
Crude fat (g/kg)	39.8	9.6	40.2
Stevioside (mg/kg)	76.1	nd	nd
Rebaudioside (mg/kg)	48.1	nd	nd
Starch (g/kg)	nd	nd	381
Non-starch polysaccharide/ total (g/kg)	152	407	93
Non-starch polysaccharide/ soluble (g/kg)	107	358	61
Non-starch polysaccharide/ insoluble (g/kg)	45	49	32
Coenzyme Q_{10} (μg/g)	3.1	2.2	0.6
Total carotenoids (μg/g)	469.4	18.8	1.0
Vitamin E (μg/g)	44.8	12.6	34.0

nd = not determined.

improve dietary DMR (Table 3). There was a T°C x XYL interaction (P = 0.006) on NR as XYL improved NR at HT only.

3.2. Gastrointestinal tract/ organ growth and jejunal villus morphometry

Rearing temperature (T°C) did not directly influence the growth of the GIT organs (P > 0.05) (Table 4). However, there was an interaction between T°C and XYL on the relative weight of the liver, as feeding XYL at RT increased its weight compared to rearing at HT (P = 0.028). Dietary XYL reduced the weight of PG (P = 0.027) and ileum (P = 0.050), and tended (P = 0.071) to reduce overall GIT weight. Feeding STE increased the weight of the pancreas (P = 0.016) and the ileum (P = 0.017). The results on jejunal villus morphometry are presented in Table 5. There was a T°C by XYL interaction (P = 0.049), where rearing birds at HT with XYL reduced the thickness of the jejunal wall. There was also STE × XYL interaction (P = 0.037), where feeding diets with STE and XYL increased the thickness of the jejunal wall. There was also a tendency (P = 0.051) for an interaction on crypt depth between T°C and STE.

3.3. Caecal volatile fatty acids production

The results of VFA concentration in caecal digesta are presented in Table 6. Overall, T°C did not influenced the VFA concentration. However, there was a T°C by XYL interaction (P = 0.030) as the highest BA production was in the caeca of birds fed XYL and reared at HT. Dietary XYL tended (P = 0.055) to increase the BA: AA. Feeding STE tended (P = 0.097) to reduce BA production. There was T°C by STE interaction as birds reared at RT and fed STE had reduced (P = 0.006) BA: AA.

3.4. Hepatic and blood antioxidant concentrations

Table 7 contains the results of the hepatic and blood antioxidant analysis. The liver of birds reared at HT had a lower concentration (P = 0.031) of vitamin E compared to birds reared at RT. Feeding

Table 3
Effect of bird rearing temperature (T°C) and dietary stevia (STE) and xylanase (XYL) inclusion on feed intake (FI), weight gain (WG), feed conversion ratio (FCR), N-corrected apparent metabolisable energy (AMEn), dry matter (DMR), nitrogen (NR) and fat (FR) retention coefficients, when fed to broiler chickens from 7 to 21 d age.

Treatment	FI (g)	WG (g)	FCR (g:g)	AMEn (MJ/kg DM)	DMR	NR	FR
T°C							
HT	704	440	1.611	12.76	0.681	0.612	0.747
RT	822	489	1.689	13.40	0.725	0.666	0.720
SEM	10.2	11.2	0.0485	0.0609	0.0399	0.0479	0.0362
STE							
No	769	476	1.625	13.14	0.707	0.643	0.730
Yes	756	452	1.674	13.02	0.698	0.635	0.737
SEM	12.6	9.7	0.0207	0.114	0.0063	0.0077	0.0132
XYL							
No	764	453.6	1.693	12.91	0.694	0.630	0.729
Yes	760	473.2	1.606	13.25	0.712	0.648	0.738
SEM	12.6	9.7	0.0207	0.114	0.0063	0.0077	0.0132
T°C × STE							
HT -	701	437[a]	1.623	12.73	0.682	0.614	0.736
HT +	706	442[a]	1.599	12.79	0.680	0.609	0.758
RT -	836	514[b]	1.627	13.54	0.733	0.671	0.724
RT +	806	462[a]	1.750	13.25	0.716	0.661	0.716
SEM	16.2	14.8	0.0527	0.619	0.0404	0.0485	0.0385
T°C × XYL							
HT -	722	441	1.651	12.53	0.665	0.586[a]	0.741
HT +	685	437	1.571	12.99	0.697	0.637[b]	0.753
RT -	806	466	1.736	13.29	0.723	0.673[c]	0.718
RT +	836	510	1.642	13.51	0.727	0.659[bc]	0.722
SEM	16.2	14.8	0.0527	0.619	0.0404	0.0109	0.0385
STE × XYL							
No -	773	461	1.689	13.03	0.703	0.639	0.728
No +	767	491	1.562	13.25	0.712	0.646	0.732
Yes -	757	448	1.698	12.79	0.684	0.620	0.730
Yes +	755	456	1.651	13.25	0.711	0.650	0.743
SEM	17.9	13.7	0.0292	0.161	0.0090	0.0109	0.0187
Probabilities							
T°C	0.015	0.091	0.375	0.535	0.517	0.506	0.650
STE	0.470	0.106	0.107	0.468	0.291	0.491	0.731
XYL	0.809	0.165	0.007	0.045	0.057	0.113	0.655
T°C × STE	0.355	0.047	0.190	0.291	0.396	0.842	0.442
T°C × XYL	0.074	0.098	0.811	0.455	0.126	0.006	0.826
STE × XYL	0.972	0.417	0.181	0.461	0.333	0.310	0.824
T°C × STE × XYL	0.252	0.191	0.262	0.661	0.836	0.381	0.533

SEM = pooled standard errors of mean; RT = regular rearing temperature following breeder's recommendations; HT = high rearing temperature of 32 ± 2 °C; Dietary AMEn, DMR, NR and FR were determined between 17 and 21 d age.

Table 4
Effect of bird rearing temperature (T°C) and dietary stevia (STE) and xylanase (XYL) inclusion on the relative organ weight expressed as the percent of body weight of proventriculus and gizzard (PG), duodenum, pancreas, jejunum, ileum, caeca, liver, total gastrointestinal tract (GIT) and spleen of 21 d old broiler chickens.

Treatment	PG	Duodenum	Pancreas	Jejunum	Ileum	Caeca	Liver	GIT	Spleen
T°C									
HT	2.81	1.15	0.42	1.95	1.57	0.66	2.61	8.56	0.08
RT	2.93	1.18	0.47	2.09	1.75	0.68	2.87	9.10	0.09
SEM	0.130	0.034	0.019	0.051	0.068	0.010	0.123	0.266	0.010
STE									
No	2.80	1.19	0.41	2.05	1.57	0.71	2.73	8.71	0.09
Yes	2.94	1.14	0.48	1.99	1.76	0.64	2.75	8.95	0.08
SEM	0.079	0.043	0.020	0.058	0.052	0.034	0.067	0.190	0.005
XYL									
No	3.00	1.17	0.46	2.08	1.74	0.64	2.65	9.09	0.09
Yes	2.74	1.15	0.43	1.97	1.59	0.70	2.83	8.58	0.09
SEM	0.079	0.043	0.020	0.058	0.052	0.034	0.067	0.190	0.005
T°C × STE									
HT -	2.72	1.16	0.36	1.93	1.43	0.69	2.65	8.30	0.09
HT +	2.91	1.13	0.48	1.97	1.72	0.63	2.58	8.82	0.08
RT -	2.88	1.21	0.46	2.16	1.71	0.72	2.81	9.13	0.09
RT +	2.98	1.15	0.49	2.02	1.80	0.65	2.92	9.08	0.09
SEM	0.152	0.055	0.028	0.077	0.085	0.035	0.140	0.327	0.011
T°C × XYL									
HT -	2.92	1.14	0.42	2.06	1.63	0.63	2.63[a]	8.81	0.09
HT +	2.71	1.15	0.42	1.83	1.52	0.69	2.60[a]	8.30	0.08
RT -	3.09	1.20	0.50	2.08	1.85	0.65	2.66[ab]	9.36	0.09
RT +	2.77	1.16	0.45	2.10	1.66	0.72	3.07[b]	8.85	0.09
SEM	0.152	0.055	0.028	0.077	0.085	0.035	0.140	0.327	0.011
STE × XYL									
No -	2.82	1.19	0.41	2.08	1.67	0.68	2.65	8.85	0.09
No +	2.77	1.19	0.41	2.01	1.47	0.74	2.82	8.58	0.09
Yes -	3.18	1.16	0.51	2.06	1.81	0.61	2.65	9.32	0.09
Yes +	2.70	1.12	0.46	1.93	1.70	0.67	2.85	8.57	0.08
SEM	0.112	0.061	0.029	0.082	0.073	0.048	0.094	0.268	0.007
Probabilities									
T°C	0.592	0.554	0.188	0.188	0.201	0.254	0.288	0.283	0.610
STE	0.203	0.396	0.016	0.521	0.017	0.154	0.872	0.393	0.345
XYL	0.027	0.745	0.297	0.215	0.050	0.208	0.059	0.071	0.523
T°C × STE	0.700	0.837	0.178	0.295	0.204	0.929	0.353	0.301	0.682
T°C × XYL	0.629	0.707	0.432	0.138	0.618	0.882	0.028	0.987	0.390
STE × XYL	0.065	0.728	0.474	0.776	0.554	0.992	0.900	0.385	0.273
T°C × STE × XYL	0.420	0.952	0.527	0.862	0.081	0.599	0.205	0.295	0.103

SEM = pooled standard errors of mean; RT = regular rearing temperature following breeder's recommendations; HT = high rearing temperature of 32 ± 2 °C.

XYL increased the vitamin E concentration in the liver of birds (P = 0.007), and dietary STE increased (P < 0.001) hepatic carotenoids. Feeding STE and XYL simultaneously reduced (P = 0.044) blood haemoglobin and tended (P = 0.061) to reduce GSH-Px in blood.

4. Discussion

This study evaluated the effects of dietary STE and XYL, alone and in combination, when fed to broiler chickens reared at high and standard T°C. Studying the impact of temperature is important as large variations in the temperature of poultry houses during summer months globally are increasing due to climate change, which may have negative welfare and health implications for poultry. The performance of the birds in the current study was below that of breed standards but similar to that previously obtained at our facility in other studies (Pirgozliev et al., 2020; Yang et al., 2020).

4.1. Growth performance, AMEn and nutrient retention coefficients

High temperature is usually associated with low growth performance in modern broiler strains (Woods et al., 2020; Pirgozliev et al., 2020). In this study, birds reared at a temperature of 32 ± 2 °C responded with a 16.7% reduction in FI and 15% reduction in growth rate compared to those reared at RT, which agrees with published reports (Woods et al., 2020, 2021; Pirgozliev et al., 2020). Similar to Wood et al. (1996) and Geuns et al. (2003), feeding STE did not change FI. Atteh et al. (2008) also did not find evidence that STE, or stevioside affected feed intake of broilers during the starter feeding phase (0–14 d age), but STE fed birds had lower WG and feed efficiency, similar to the birds fed STE in RT in the reported study.

Rearing T°C did not change the coefficients of DMR, FR and AMEn values, which agrees with Pirgozliev et al. (2020) but feeding XYL at HT improved NR. This suggests that the reduced FI of broilers kept at 32 ± 2 °C most likely reflect a reduced heat production of digestion (Pirgozliev et al., 2015a) and is not related to utilisation of nutrients. However, the research on the ability of broilers to utilise dietary energy and nutrients when exposed to high T°C is inconsistent (Bonnet et al., 1997; Habashy et al., 2017), thus further studies are warranted.

Similar to previous reports (Pirgozliev et al., 2010, 2019b), feeding XYL increased dietary AMEn and improved feed efficiency, suggesting a reduction in gut viscosity and improved gut health (Yang et al., 2020). In accordance with Atteh et al. (2008), feeding STE did not impact dietary AMEn and nutrient retention coefficients and there were no XYL by STE interactions. Dietary AME is a measurement of the available energy of carbohydrates, fats and proteins, thus there is not a surprise that dietary STE would not greatly impact dietary ME status.

4.2. Gastrointestinal tract/ organ growth and jejunal villus morphometry

The results of the organ growth agreed with published reports (Abdulla et al., 2016, 2017; Yang et al., 2020). In the opposite of

Table 5
Effect of bird rearing temperature (T °C) and dietary stevia (STE) and xylanase (XYL) inclusion on the jejunal villus morphometry of 21 d old broiler chickens.

Treatment	Villus height (μm)	Villus width (μm)	Crypt depth (μm)	Height/depth	Intestinal wall (μm)
T°C					
HT	1415	126	147	9.9	125
RT	1504	113	137	11.3	139
SEM	34.0	3.5	13.4	0.96	13.5
STE					
No	1487	122	146	10.4	135
Yes	1432	116	138	10.8	129
SEM	23.5	2.5	5.9	0.50	3.2
XYL					
No	1467	121	136	11.2	137
Yes	1452	117	148	10.0	127
SEM	23.5	2.5	5.9	0.50	3.2
T°C × STE					
HT -	1415	130	142	10.3	129
HT +	1416	121	152	9.4	121
RT -	1559	113	150	10.5	141
RT +	1448	111	124	12.2	136
SEM	34.0	3.5	13.4	0.961	13.9
T°C × XYL					
HT -	1408	130	149	9.8	135[a]
HT +	1423	121	146	9.9	115[b]
RT -	1527	111	124	12.5	138[a]
RT +	1481	114	150	10.2	138[a]
SEM	23.5	2.5	5.9	0.504	13.9
STE × XYL					
No -	1476	122	146	10.4	145[a]
No +	1498	121	147	10.4	124[b]
Yes -	1458	119	127	12.0	128[b]
Yes +	1406	113	149	9.7	129[b]
SEM	23.5	2.5	5.9	0.504	4.5
Probabilities					
T°C	0.208	0.116	0.640	0.390	0.553
STE	0.124	0.129	0.330	0.552	0.224
XYL	0.646	0.344	0.192	0.142	0.051
T°C × STE	0.118	0.314	0.051	0.094	0.746
T°C × XYL	0.377	0.101	0.109	0.117	0.049
ST × XYL	0.288	0.458	0.244	0.122	0.037
T°C × STE × XYL	0.743	0.247	0.496	0.667	0.310

SEM = pooled standard errors of mean; RT = regular rearing temperature following breeder's recommendations; HT = high rearing temperature of 32 ± 2 °C.

recent research (Pirgozliev et al., 2020; Woods et al., 2020), rearing temperature did not affect the relative weight of the GIT, as the relative wet weight of the liver only increased in XYL fed birds reared at RT. Feeding STE extract to rats increased the pancreas weight (Misra et al., 2011), which was also associated with revitalising the insulin secreting cells. In poultry, an increased pancreatic weight may also be associated with reduced pancreatic enzymes secretion due to presence of trypsin inhibitors and/or tannins in diets (Abdulla et al., 2016). Although feeding STE at RT in the reported study reduced weigh gain and feed efficiency, there were no reductions in nutrient retention coefficients, thus indicating no reduction in pancreatic enzyme secretion. Atteh et al. (2008) did not find an effect on pancreas weight of chickens fed STE.

Abdulla et al. (2017) also reported a reduced weight of the PG when feeding a mixture of enzymes containing XYL to broilers. In the reported study, the reduction in ileum weight of birds fed XYL diets paralleled the increased AMEn and feed efficiency. Reduction in relative size or weight of small intestine usually coincides with increased digestive efficiency associated with age (Yang et al., 2020) and/or enzyme use (Abdulla et al., 2017). This may also explain the reduced ileal wall thickness in birds fed XYL at HT. The increased ileum weight of birds fed STE diets mirrored the increased pancreas weight and tendency of reduced feed efficiency. In general, if the efficiency of digestion is consistently suboptimal, whether due to ingredient quality, microbial interaction or anti-nutritive factors, the GIT responds by increasing in both size (surface area) and digestive enzyme output (Abdulla et al., 2017; Bedford, 2018). Villus morphometry can be used to assess the integrity of the small intestine of birds reared at HT (Santos et al., 2015). The exposure of birds, at a similar age to those in this study, to heat stress (39 °C) led to shorter villus, decreased villus:crypt ratio, villus denudation and crypt damage (Santos et al., 2015). The lack of significant changes in villus morphometry in our study suggest that the temperature of 32 ± 2 °C did not provoke heat and dehydration stress and subsequent gut damage.

4.3. Hepatic and blood antioxidant concentrations

High ambient temperature beyond the range of the thermoneutral zone is recognised as a very potent stressor that can trigger various biological responses including poor performance (Pirgozliev et al. 2020; Woods et al. 2020, 2021). The reduced hepatic vitamin E content in the birds reared at HT may be associated with the reduced synthesis and bioavailability of vitamin E. However, the lack of changes in blood GSH-Px suggests that this may not be sensitive enough to detect changes in the antioxidant status in poultry or the temperature in this study did not invoke heat stress.

Stevia contains a very high quantity of carotenoids; thus, the increased total hepatic carotenoid concentration might be expected, further supporting the view that STE may counteract oxidative stress (Stoyanova et al., 2011). It has previously been discussed that although the diet is the main determinant of the carotenoid composition in liver tissue, feed supplements other than carotenoids (e.g. phytase, XYL and plant extracts) can affect the efficiency of carotenoid assimilation from the diet and subsequently, their accumulation in the liver (Pirgozliev et al., 2010, 2015b; Karadas et al., 2014).

Table 6
Effect of bird rearing temperature (T°C) and dietary stevia (STE) and xylanase (XYL) inclusion on the caecal digesta concentration of short chain fatty acids (ppm) in 21 d old broiler chickens.

Treatment	Acetic acid	Butanoic acid	Pentanoic acid	Propanoic acid	BA: AA
T°C					
HT	618	121.2	5.1	33.0	0.203
RT	387	76.9	4.6	26.4	0.207
SEM	92.6	23.30	0.95	2.42	0.0179
STE					
No	504	102.1	5.2	30.4	0.213
Yes	501	96.0	4.6	29.0	0.196
SEM	59.2	12.84	0.90	3.19	0.0231
XYL					
No	511	89.3	4.7	30.2	0.181
Yes	493	108.9	5.1	29.2	0.228
SEM	59.2	12.84	0.90	3.19	0.0231
T°C × STE					
HT -	624	113.1	5.2	31.6	0.176^{ad}
HT +	612	129.3	5.1	34.5	0.230^{ac}
RT -	383	91.1	5.2	29.2	0.250^{bc}
RT +	390	62.7	4.1	23.6	0.163^{d}
SEM	110.0	26.60	1.31	4.01	0.0292
T°C × XYL					
HT -	595	96.5^{a}	4.2	32.2	0.167
HT +	641	145.9^{b}	6.1	33.9	0.238
RT -	428	82.0^{a}	5.1	28.2	0.195
RT +	345	71.8^{a}	4.1	24.6	0.218
SEM	110.0	26.60	1.31	4.01	0.0292
STE × XYL					
No -	498	84.2	5.0	29.4	0.182
No +	509	120.1	5.4	31.5	0.244
Yes -	524	94.4	4.4	31.1	0.180
Yes +	478	97.7	4.8	27.0	0.212
SEM	83.8	18.15	1.27	4.52	0.0326
Probabilities					
T°C	0.130	0.198	0.643	0.111	0.847
STE	0.965	0.639	0.497	0.669	0.479
XYL	0.765	0.142	0.646	0.760	0.055
T°C × STE	0.878	0.097	0. 608	0.195	0.006
T°C × XYL	0.289	0.030	0.119	0.410	0.300
STE × XYL	0.631	0.218	0.979	0.342	0.535
T°C × STE × XYL	0.228	0.060	0.884	0.172	0.539

SEM = pooled standard errors of mean; ST = standard rearing temperature following breeder's recommendations; HT = high rearing temperature of 32 ± 2 °C; L = linear response; Q = quadratic response; BA: AA = Butanoic to Acetic acid ratio.

Increased viscosity of intestinal digesta, sometime attributed to high pentosane wheat, may result in more inefficient mixing of digesta and movement of solutes, with a resultant depression in nutrient digestibility (Bedford, 2018) and reduced hepatic antioxidant concentration (Pirgozliev et al., 2016). High digesta viscosity may also provoke more nutrient oxidation in GIT. In addition, supplementary XYL may not only reduce digesta viscosity, but also possess some prebiotic activity (released xylooligosachharides)/ microbiome modifications that may result in improved antioxidant status of the birds.

4.4. Caecal volatile fatty acids production

The T°C by XYL interaction regarding caecal BA concentration in the reported study is challenging to interpret. Birds subjected to high ambient temperature usually decrease the intestinal counts of *Lactobacillus* and *Bifidobacterium*. These probiotic microbes have well recognised "health promoting" properties (Song et al., 2014). However, the lack of changes in overall caecal SCFA concentrations agrees with those reported by Pirgozliev et al. (2020b), who reared birds at the same age and the same temperatures, but fed maize based diets.

The beneficial effects of exogenous XYL as a supplement in the broiler feed has been explained through several mechanisms. The most studied presently is the impact of dietary NSP and the generation of xylooligosaccharides with potential prebiotic effect (Bedford, 2018). One mechanism by which prebiotics may exert protective effects is through the modulation of the gut microbiota, e.g. selectively stimulating growth and proliferation of "health promoting" gut microbes, like *Bifidobacterium*, and subsequent production of short chain fatty acids following fermentation (Nettleton et al., 2019). Indeed, Lee et al. (2017) reported that feeding XYL to broilers encouraged caecal colonisation of *Bifidobacterium spp* with subsequent greater acetic and butyric acid production. Xylanase supplementation also lowered the proportion of branched VFA in the caeca, suggesting suppressed protein fermentation (Lee et al., 2017). Increased supply of fermentable carbohydrates to the caeca, as well as an enhanced efficiency of protein utilisation by xylanase-fed birds at HT in the reported study may account in part for the increased BA fermentation and improvement in feed efficiency. However, this conclusion remains highly speculative because there was not the same beneficial effect of XYL in birds reared at RT.

Information on the impact of STE on caecal SCFA is limited and contradictive. In rats, feeding STE increased caecal acetate and valerate, but did not change butyrate concentration (Nettleton et al., 2019). In broilers, feeding STE however caused a decrease in the total concentration and a change in the profile of SCFA (Atteh et al., 2008). In the current study, feeding STE at RT tended to reduce BA, in agreement with Atteh et al. (2008), although feeding STE at HT did not affect the caecal SCFA concentrations. Short chain fatty acids are the primary products of carbohydrate fermentation, and have multiple effects on host energy metabolism and the GIT microbiota (den Besten et al., 2013). Dietary STE caused a decrease in the BA and AA to BA ratio, suggesting not only a change in number but also a

Table 7
Effect of bird rearing temperature (T°C) and dietary dietary stevia (STE) and xylanase (XYL) inclusion on the hepatic coenzyme Q_{10}, vitamin E (Vit E), carotenoids, haemoglobin (HB) and blood plasma glutathione peroxidase (GSH-Px) and in 21 d old broiler chickens.

Treatment	Liver wet (g)	Liver dry (g)	DM liver	Q_{10} (μg/g)	Vit E (μg/g)	Carotenoids (μg/g)	HB (g/dl)	GSH-Px (g HB/L)
T°C								
HT	17.0	4.5	0.262	241	57	4.7	116	628
RT	18.7	4.8	0.257	253	87	4.6	114	664
SEM	0.57	0.20	0.0028	20.5	3.8	0.45	3.3	13.9
STE								
No	17.4	4.8	0.260	242	74	2.9	117	662
Yes	18.4	4.5	0.259	252	70	6.4	114	631
SEM	0.62	0.16	0.0024	12.5	5.9	0.33	1.7	22.1
XYL								
No	17.4	4.6	0.261	242	60	4.6	116	653
Yes	18.37	4.7	0.258	252	84	4.7	115	639
SEM	0.62	0.16	0.0024	12.5	5.9	0.33	1.7	22.1
T°C × STE								
HT -	17.5	4.6	0.262	239	56	2.7	116	645
HT +	16.6	4.3	0.261	242	59	6.7	117	612
RT -	19.2	5.0	0.259	245	93	3.1	118	679
RT +	18.3	4.7	0.256	262	80	6.2	111	649
SEM	0.84	0.25	0.0036	24.0	7.0	0.56	3.7	26.1
T°C × XYL								
HT -	17.2	4.5	0.261	232	51	4.6	119	645
HT +	16.9	4.4	0.262	250	64	4.7	114	612
RT -	17.6	4.6	0.262	253	69	4.6	112	661
RT +	19.9	5.0	0.253	253	105	4.7	116	667
SEM	0.84	0.25	0.0036	24.0	7.0	0.56	3.7	26.1
STE × XYL								
No -	18.1	4.7	0.260	229	61	2.9	115^{ab}	638
No +	18.6	4.8	0.261	256	88	2.8	119^{a}	686
Yes -	16.7	4.4	0.263	256	58	6.3	117^{ab}	668
Yes +	18.2	4.6	0.255	247	81	6.6	111^{b}	593
SEM	0.88	0.23	0.0033	17.7	8.4	0.47	2.4	31.3
Probabilities								
T°C	0.166	0.321	0.374	0.709	0.031	0.973	0.727	0.212
STE	0.326	0.252	0.615	0.587	0.576	<0.001	0.225	0.332
XYL	0.278	0.431	0.280	0.611	0.007	0.814	0.797	0.666
T°C × STE	0.963	0.933	0.765	0.698	0.344	0.408	0.121	0.954
T°C × XYL	0.157	0.277	0.175	0.622	0.173	0.941	0.074	0.532
STE × XYL	0.566	0.772	0.234	0.326	0.815	0.643	0.044	0.061
T°C × STE × XYL	0.940	0.749	0.440	0.551	0.500	0.842	0.539	0.485

SEM = pooled standard errors of mean; RT = regular rearing temperature following breeder's recommendations; HT = high rearing temperature of 32 ± 2 °C.

change in the types of microbes that may be present in the ceca. The aforementioned reductions of SCFA may indicate appropriate changes in the diversity of microbes responsible for their production but require confirmation by determining the microbial population in the caeca of treated broilers. An important question is whether SCFA levels are an accurate predictor of fermentation in the caeca of birds?

The concentration of SCFA does not only depend on the availability of fermentable substrates and microbial fermentation, but also on other factors including SCFA absorption (Bautil et al., 2019). Expressed in relation to ME intake, the SCFA contributed up to 8% of energy needs of the chicken (Jørgensen et al., 1996). It is however difficult to relate these changes to the overall growth performance of broilers. The observations in this study suggests that dietary XYL increases overall feed efficiency and dietary metabolisable energy, although changes in SCFA production were not consistent. Similarly, changes in performance and SCFA production due to dietary STE did not follow a consistent pattern.

The reduction in the concentrations of digesta BA and AA: BA of STE fed birds at RT, and the observed differences in XYL fed birds, may be due to their differential absorption rate in the ceca. Thus, it may be challenging to relate these changes to broiler growth performance and dietary energy availability. In the present study, feeding STE at RT reduced BA production by 31%, WG and feed efficiency by 10% and 7.5%, respectively, and reduced AMEn by 2% only. Research by Bautil et al. (2019) showed that the capacity of intestinal microbiota to degrade carbohydrates in the hindgut increases as the broiler ages, thus results obtained with young birds (e.g. 21 d old females) might be more challenging to interpret. Taken together, it is important, however, to understand the implications of using point-in-time measurements for evaluating differences between treatments, particularly since sugar and SCFA levels are not static (Lee et al., 2017).

5. Conclusions

This experiment has confirmed the expected biological effects of high ambient temperature. However, dietary xylanase increased hepatic vitamin E and stevia increased the hepatic carotenoid content in birds. Therefore, a strategy of supplementing the diets of birds subjected to high ambient temperatures with xylanase and stevia may be applied. It should be noted that if ambient temperatures decrease, then stevia may cause a reduction in growth rate. Xylanase supplementation was without this issue in this study.

Ethics approval

The Research Ethics Committee of Harper Adams University (UK) approved the experiment. This manuscript complies with the ARRIVE guidelines (Kilkenny et al., 2010).

Data and model availability statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request, subject to restrictions and conditions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Given his role as Current Research in Biotechnology Editor-in-Chief, Atanas G. Atanasov had no involvement in the peer review process of this article and have had no access to information regarding its peer review process.

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