

## Effects of a feed additive blend on broilers challenged with heat stress

Regiane R. Santos, Ajay Awati, Petra J. Roubos-van den Hil, Theo A. T. G. van Kempen, Monique H. G. Tersteeg-Zijderfeld, Peter A. Koolmees, Coen Smits & Johanna Fink-Gremmels

To cite this article: Regiane R. Santos, Ajay Awati, Petra J. Roubos-van den Hil, Theo A. T. G. van Kempen, Monique H. G. Tersteeg-Zijderfeld, Peter A. Koolmees, Coen Smits & Johanna Fink-Gremmels (2019) Effects of a feed additive blend on broilers challenged with heat stress, Avian Pathology, 48:6, 582-601, DOI: [10.1080/03079457.2019.1648750](https://doi.org/10.1080/03079457.2019.1648750)

To link to this article: <https://doi.org/10.1080/03079457.2019.1648750>



© 2019 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group



Published online: 07 Aug 2019.



Submit your article to this journal [↗](#)



Article views: 2441




View related articles [↗](#)



View Crossmark data [↗](#)

## Effects of a feed additive blend on broilers challenged with heat stress

Regiane R. Santos <sup>a,b</sup>, Ajay Awati<sup>c</sup>, Petra J. Roubos-van den Hil<sup>c</sup>, Theo A. T. G. van Kempen<sup>c,d</sup>,  
Monique H. G. Tersteeg-Zijderfeld<sup>a</sup>, Peter A. Koolmees<sup>a</sup>, Coen Smits<sup>c</sup> and Johanna Fink-Gremmels<sup>a</sup>

<sup>a</sup>Institute for Risk Assessment Sciences, Division of Veterinary Pharmacy, Pharmacotherapy and Toxicology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands; <sup>b</sup>Animal Sciences Post-graduation Program, Federal University of Pará, Belém, Pará, Brazil; <sup>c</sup>Trouw Nutrition R&D, Boxmeer, Netherlands; <sup>d</sup>Department of Animal Science, North Carolina State University, Raleigh, NC, USA

### ABSTRACT

We evaluated a blend of medium-chain fatty acids (MCFA), organic acids, and a polyphenol antioxidant on gut integrity. Eighty Ross Broilers were exposed to 20–22°C (control – normothermic) or to 35–39.5°C (heat stress) for eight hours a day for a period of 1 or 5 days. Birds were fed a standard diet, or a diet supplemented with the test blend. Thereafter, birds were euthanized, and intestinal sections were excised for morphological, morphometric and gene expression analyses. Blood samples were collected for glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase (GSH-Px) activity and trolox equivalent antioxidant capacity (TEAC) determination. Heart and liver tissues were used to quantify the expression of heat shock proteins 60 and 70 (HSP60 and HSP70, respectively) and inhibitor of kappa light chain gene enhancer in B cells alpha (IKBA). The jejunum was the most sensitive intestinal section, where heat stress modulated the expression of HSP70, of the inflammatory markers IKBA, interleukin 8 (IL-8), interferon gamma (IFN $\gamma$ ), and toll-like receptor 4 (TLR4). Moreover, expression of tight junctions (CLDN1, ZO1 and ZO2) and nutrient transporters (PEPT1 and EAAT3) was modulated especially in the jejunum. In conclusion, the feed additive blend protected intestines during heat stress from the decrease in villus height and crypt depth, and from the increase in villus width. Especially in the jejunum, heat stress played an important role by modulating oxidative stress and inflammation, impairing gut integrity and nutrient transport, and such deleterious effects were alleviated by the feed additive blend.

### RESEARCH HIGHLIGHTS

- Jejunum is the most sensitive intestinal segment during heat stress.
- Heat stress affects the expression of tight junctions and nutrient transporters.
- Feed management helps to alleviate the disturbances caused by heat stress.
- A blend of MCFA, organic acids and a polyphenol protects broilers under heat stress.

### ARTICLE HISTORY

Received 10 November 2018  
Accepted 23 July 2019

### KEYWORDS

Intestines; MCFA; organic acids; phenolic compounds; poultry; thermic stress

## Introduction

Heat stress is a worldwide problem, resulting in compromised bird welfare and economic losses due to decreased meat production and even bird mortality (Lara and Rostagno, 2013). This problem is aggravated by global warming resulting in more extreme climates (Tanizawa *et al.*, 2014). At the same time, genetic progress in broilers resulting in more rapid growth, and consequently higher heat production by the bird itself (Havenstein *et al.*, 2003), increases the sensitivity of birds to high environmental temperatures.

Birds dissipate heat via evaporative cooling from the respiratory tract, i.e. panting (Marder and Arad, 1989), which induces dehydration associated with oxidative stress and subsequent damage of the intestinal epithelium (Gu *et al.*, 2012). Impairment of the intestinal barrier occurs because of a disturbance in the tight junction network. This may result in diminished absorption of

nutrients, increased risk of bacterial translocation, and activation of the inflammatory cascade (Dokladny *et al.*, 2006). Feed supplementation with organic acids is one of the strategies of managing intestinal health by inhibiting bacterial growth via modulation of pH and microbiota in the intestinal tract. Medium-chain fatty acids (MCFA) decrease the host response to inflammation (Carlson *et al.*, 2015) and are candidates for use as supplements in the feed to counteract the effects of heat stress. Oxidative stress in the intestines can be lowered by providing locally effective antioxidants, such as plant polyphenols. It is known that under thermal stress, plants increase the biosynthesis of phenolic compounds as an acclimation mechanism (Rivero *et al.*, 2001). These phenolic compounds play an important role due to their antioxidant, anti-inflammatory, and antimicrobial activities, and increase glutathione peroxidase activity in heat-challenged broilers (Akbarian *et al.*, 2016).

**CONTACT** Regiane R. Santos  [RSantos@schothorst.nl](mailto:RSantos@schothorst.nl), [regianers@hotmail.com](mailto:regianers@hotmail.com)  Schothorst Feed Research PO Box 533, 8200 AM, Lelystad, Netherlands  
 Supplemental data for this article can be accessed at <https://doi.org/10.1080/03079457.2019.1648750>

© 2019 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group  
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Based on the above-mentioned information, we hypothesized that a blend of MCFA, organic acids and a phenolic plant extract (Presan FY<sup>®</sup>) may support the intestinal health of broilers challenged with heat stress. Furthermore, we aimed to gain knowledge on the effects of heat stress, period of heat exposure (1 or 5 days; 8 consecutive hours per day), and diet on broiler intestinal function. To assess this, intestinal integrity and functioning were evaluated by morphological and morphometric analysis of the duodenum, jejunum and ileum. Furthermore, we measured the mRNA expression of markers for tight junction proteins (claudin 1 and 5 – CLDN1 and CLDN5; zona occludens 1 and 2 – ZO1 and ZO2), inflammation (inhibitor of kappa light chain gene enhancer in B cells alpha – IKBA; interleukin 8 – IL-8; interferon gamma – IFN $\gamma$ ; and toll-like receptors 2 and 4 – TLR2 and TLR4), transforming growth factor beta (TGF $\beta$ ), nutrient transporters (excitatory amino-acid transporter 3 – EEAT3; peptide transporter 1 – PEPT1; sodium-dependent glucose transporter – SGLT) and oxidative stress (Haem oxygenase – HMOX; Heat Shock Proteins 60 and 70 – HSP60 and HSP70) in the small intestine. The mRNA expression of stress indicators (HMOX, HSP60 and HSP70) was also measured in the liver and myocardium.

## Materials and methods

### Birds and treatments

Ross Broilers ( $n = 80$ ) were obtained at the age of 14 days from a commercial broiler farm. Birds were housed in special bird units with a light programme of 16 h light (04:00–20:00) per 24 h. On day 17, the birds weighed an average of  $956 \pm 13$  g (ranging of 944–970 g) and were randomly allocated to treatments and adapted to the individual feed mixtures for a period of 7 days, when they reached a mean body weight of  $1600 \pm 40$  g. Thereafter, each group was exposed to normal (20–22°C) or elevated (35–39.5°C for 8 h a day) temperature programmes for a period of 1 or 5 days and euthanized immediately after exposure, i.e. at days 1 or 5 (under the same temperature schedule) for tissue and blood sampling. Feed and water were given *ad libitum*. The experimental protocol was established in line with the prerequisites of the use of birds in research (DIRECTIVE 2010/63/EU) and had been approved prior to the onset of the experimental trials by the Animal Welfare Committee of the University of Veterinary Medicine Hannover (competent ethics committee of the university) on the uses of animals in research according to BGI. I S. 1105/2.

### Feed

The feed for the experimental period was delivered by Trouw Nutrition (Putten, the Netherlands) and consisted

of a standard broiler feed that meets commercial practice recommendations. It was based on wheat, soybean meal, soybean oil, corn, minerals, amino acids and vitamins (see detailed composition at Supplementary Table 1). The standard feed was either not supplemented, or supplemented with a commercially available gut health additive (Presan FY<sup>®</sup>, Trouw Nutrition), which is based on MCFA (C8, C10 and C12), organic acids and a phenolic plant extract.

### Sampling

As described above, the experimental protocol contained two distinct sampling points: 50% of the birds per group were euthanized immediately after 1-day heat challenge, and the remaining birds were euthanized after 5 days of heat stress. At both sampling points the body weight was recorded, and samples of liver, intestines and heart tissue were preserved for qRT-PCR analysis (described below). Blood samples were taken from each bird, after which plasma was collected and stored at  $-20^{\circ}\text{C}$  for further analyses. Immediately after euthanasia, the small intestine was removed, and approximately 2-cm segments of the duodenum, jejunum and ileum were taken from the middle of each part. Two pieces per segment were collected, of which one piece was fixed in formalin 10% and embedded in paraffin for histology, and the other piece was snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA extraction for qRT-PCR.

### Blood plasma analysis

Glucose-6-phosphate dehydrogenase (G6PD) concentration (U/g Hb) in total blood was measured with an assay kit from Trinity biotech (Wicklow, Ireland; no. 345-B). Plasma glutathione peroxidase (GSH-Px) activity (nmol NADP/min/ml) was measured by using a kit from Merck (Darmstadt, Germany; no. 353919). Plasma trolox equivalent antioxidant capacity (TEAC, mM) was measured by using an assay kit from Sigma (Saint Louis, MO, USA; no. CS0790).

### Histological analyses and scores: morphology and morphometry

Four micrometer transverse sections were cut and every 10th section was kept and stained with haematoxylin–eosin. At least five samplings were performed for each tissue. Morphological and morphometrical analyses were performed as previously described by Santos *et al.* (2015). In brief, the degree of mucosal damage was determined by applying the Chiu/Park scale (Quaedackers *et al.*, 2000):

- Degree 0: intact without visible damage;

- Degree 1: damage in sub-epithelial space at villus tips;
- Degree 2: extension of sub-epithelial space with moderate lifting;
- Degree 3: massive lifting down the sides of villi with some denuded villi;
- Degree 4: denuded villi with dilated capillaries;
- Degree 5: disintegration of lamina propria;
- Degree 6: crypt injury;
- Degree 7: trans-mucosal infarction;
- Degree 8: transmural infarction

To statistically compare the degrees among the treatments, a composite score per treatment was determined by averaging the score from each bird. For this, the percentage of villi with a specific degree was multiplied by its respective degree. This calculation was performed for each degree per treatment and the sum obtained was considered the composite score.

Morphometrical parameters were recorded using an image-analyzing system (Olympus, Tokyo, Japan), coupled to a microscope (Olympus). The villus height, top, middle and basal width of the villi, crypt depth, as well as epithelial cell, villi and serosa areas from morphologically normal villi were measured. A minimum of 15 villi per sample were measured. For this, villi having lamina propria were measured from the tip to base of the villi. Crypt depth was defined as the invagination between two villi. The number of villi per unit of serosa area and the mucosal (villus) surface area per unit serosa area were estimated.

### Quantitative RT-PCR

RNA from samples of liver, heart and intestines was isolated using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions, and total RNA was quantified by spectrophotometer (Nanodrop ND-1000, Thermo Scientific, Wilmington, DE, USA). Subsequently, 1 µg of extracted total RNA was reverse transcribed with the iScript™ cDNA Synthesis kit (BIO-RAD, Hercules, CA, USA). The cDNA was diluted to a final concentration of 30 ng/µl. Primers, as presented in Table 1 (Mott *et al.*, 2008; Lorda-Diez *et al.*, 2010; Osselaere *et al.*, 2013), were commercially produced (Eurogentec, Maastricht, the Netherlands). The primers used were selected based on specificity and efficiency by qPCR analysis of dilution series of pooled cDNA at a temperature gradient (55–65°C) for primer-annealing and subsequent melting curve analysis. The reaction mixture for the qPCR containing 10 µl of the diluted cDNA was mixed with 15 µl iQSYBR Green Supermix (Bio Rad Laboratories Inc., USA), forward and reverse primers (final concentration of 0.4 pmol/µl for each primer) and sterile water according to the manufacturer's instructions. qPCR was

performed using the MyIQ single-color real-time PCR detection system (Bio-rad) and MyiQ System Software Version 1.0.410 (Bio Rad Laboratories Inc., USA). Amplification efficiency was determined for each plate using linregPCR (Ruijter *et al.*, 2009). Data were analysed using the efficiency-corrected Delta-Delta-Ct method (Pfaffl, 2001). The fold-change values of the genes of interest were normalized using the geometric mean of the fold-change values of two housekeeping genes. Housekeeping genes were tested for all the test conditions after which the most stable housekeeping genes were selected using the geNorm software. The most stable housekeeping genes had an M-value between 0.2 and 0.5. To determine if the inclusion of an additional housekeeping gene was required, the cut-off value for variation was set at 0.2. The fold-change values of the target genes were normalized using two housekeeping genes: hypoxanthine-guanine phosphoribosyl transferase (HPRT) and hexose-6-phosphate dehydrogenase (H6PD). The mRNA expression level of proteins involved in stress i.e. HMOX, HSP60 and HSP70 were evaluated in the liver, myocardium and intestines. Furthermore, mRNA expression of different markers in the duodenum, jejunum and ileum were measured: tight junction proteins (CLDN1, CLDN5, ZO1 and ZO2), inflammatory markers such as IKBA, IL-8, IFNγ, TLR2, TLR4, the marker of the transforming growth factor, TGFβ, and the nutrient transporters EEAT3, PEPT1, and SGLT.

### Statistical analysis

The data analysis for this paper was performed using SAS software, Version 9.1 of the SAS System (SAS Institute Inc., Cary, NC, USA). Data were compared using a 2 × 2 × 2 factorial design (Heat Stress × Diet × Day), where two-way ANOVA and LSmeans was applied. Differences were considered significant when  $P < 0.01$ , except for differences in gene expression, for which  $P < 0.001$  was used.

## Results

### Bird inspection

Birds were inspected every day for clinical signs of disease or discomfort. During heat exposure, the birds were mostly panting, with wing spreading and squatting close to the ground. Activity was regained immediately when room temperature fell below 30°C. After 1-day heat stress challenge, birds fed standard diet (body weight 1537 g) were numerically lighter than those receiving standard diet at normothermic conditions (body weight 1618 g), but no significant differences were observed among the groups. After 5 days of consecutive heat stress challenge, a significant decrease

**Table 1.** Primers used for the quantification of genes of interest (GOI) and housekeeping gene (HKG) expression.

Genes	Accession no.	Primer	Sequence
HKG			
H6PD	XM_425746.2	Forward	GGAGAACCAGCACTTCTTAGAC
		Reverse	GGGTTCCAGCAATCCACTG
HPRT	NM_204848.1	Forward	CGTTGCTGTCTCTACTTAAGCAG
		Reverse	GATATCCCACACTTCGAGGAG
GOI			
CLDN1	NM_001013611	Forward	CTGATTGCTTCCAACCCAG
		Reverse	CAGGTCAAACAGAGGTACAAG
CLDN5	NM_204201	Forward	CATCACTTCTCCTTCGTCAGC
		Reverse	GCACAAAGATCTCCAGGTC
EAAT3	XM_424930	Forward	TGCTGCTTGGATTCCAGTGT
		Reverse	AGCAATGACTGTAGTGCAGAAGTAATATATG
HMOX1	NM_205344	Forward	CTTGCCACAAGGAGTGTAAAC
		Reverse	CATCCTGCTTGTCTCTCAC
HSP60	NM_001012916	Forward	GCCAATAGTCACCGCAAAC
		Reverse	GCAACAACCTGAAGACCAAC
HSP70	NM_001006685	Forward	CTCTCATCAAGCGTAACACCAC
		Reverse	CTCTCACCTTCATACACTGGAC
IKBA	NM_001001472	Forward	CTGTTGTGCAATACCTGCTGTC
		Reverse	CAGGTCTGAGTTCTGAAGGTCTAC
IL-8	NM_205018	Forward	CACGTTTCAGCGATTGAACCT
		Reverse	GACTCCACATTCTTGCACTG
IFN $\gamma$	NM_205149	Forward	CAAGTCCCAGTGAACGAC
		Reverse	GCAATTGCATCTCCTCTGAGAC
PEPT1	AY029615.1	Forward	CCCCTGAGGAGGATCACTGTT
		Reverse	CAAAAGAGCAGCAGCAACGA
SGLT	AJ236903.1	Forward	TGCTCTCTGGCAAGAACATGTC
		Reverse	GGGCAAGAGCTTCAGGTATCC
TGF $\beta$ 2	NM_205454.1	Forward	CTCAGTGGCAGGAATGTGC
		Reverse	CGAGGTTGGACTCTCTGTGC
TLR2	NM_204278	Forward	CCTGCAACGGTCATCTCAG
		Reverse	GTCTCAGGGCTGTCTTTCAG
TLR4	NM_001030693	Forward	CTGACCTACCCATCGGACAC
		Reverse	GCCTGAGAGAGGTCAGGTTG
ZO1	XM_413773	Forward	CTTCAGGTGTTCTCTTCTCCTC
		Reverse	CTGTGGTTTCATGGCTGGATC
ZO2	NM_204918	Forward	CGGCAGCTATCAGACCCTC
		Reverse	CACAGACCAGCAAGCCTACAG

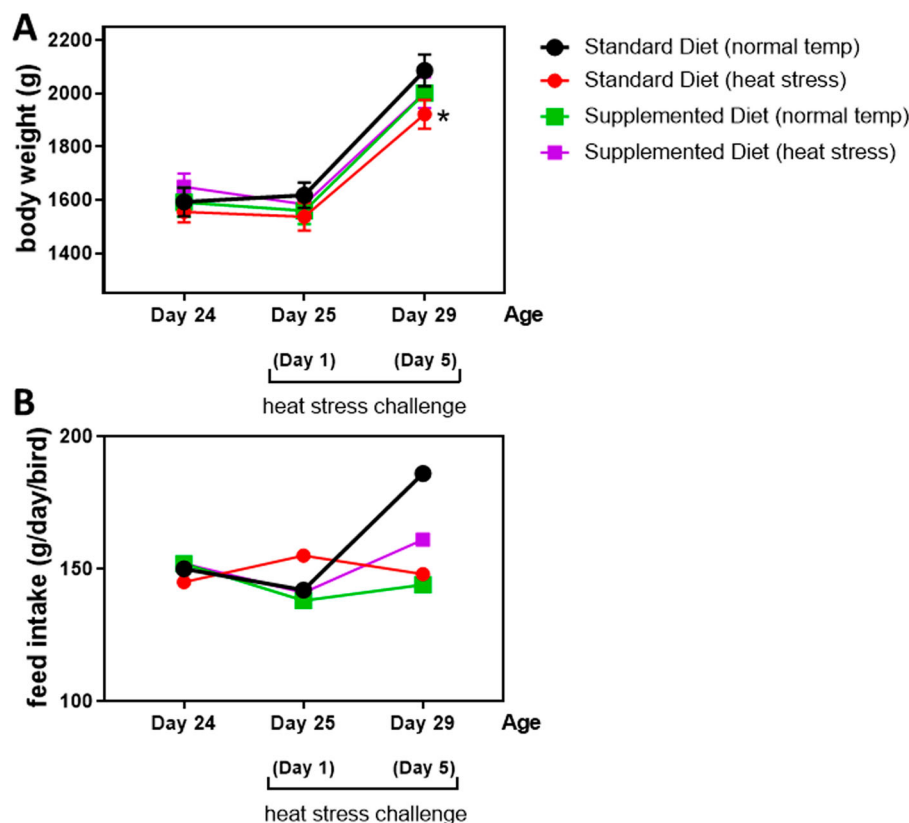
in body weight was observed in the birds fed standard diet (1922 g) when compared with those fed standard diet under normal temperature (2086 g; [Figure 1\(a\)](#)).

The mean body weight of the birds fed supplemented diet did not differ from any other treatment regardless of the presence (2003 g) or absence (2001 g) of heat

**Table 2.** Mean ( $\pm$  SEM) levels of glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase (GSH-Px) activity and trolox equivalent antioxidant capacity (TEAC) in blood/plasma from birds fed with standard or supplemented diets under either heat stress challenge or normothermic conditions.

Treatment	G6PD (U/g Hb)		GSH-Px (nmol/min/ml)		TEAC (mM)	
<b>Day 1</b>						
<i>Normal temperature</i>						
Standard diet	6.03 $\pm$ 1.56	e	7.13 $\pm$ 3.69	ab	0.23 $\pm$ 0.05	b
Supplemented diet	4.19 $\pm$ 1.32	cd	9.87 $\pm$ 4.64	b	0.17 $\pm$ 0.08	b
<i>Heat stress</i>						
Standard diet	1.59 $\pm$ 1.26	a	14.36 $\pm$ 4.84	c	0.06 $\pm$ 0.04	a
Supplemented diet	3.44 $\pm$ 1.98	bc	9.86 $\pm$ 5.34	b	0.17 $\pm$ 0.07	b
<b>Day 5</b>						
<i>Normal temperature</i>						
Standard diet	5.33 $\pm$ 1.48	de	5.83 $\pm$ 3.62	a	0.25 $\pm$ 0.15	b
Supplemented diet	3.80 $\pm$ 1.94	bcd	10.62 $\pm$ 3.82	b	0.24 $\pm$ 0.14	b
<i>Heat stress</i>						
Standard diet	2.36 $\pm$ 1.14	ab	4.69 $\pm$ 3.21	a	0.23 $\pm$ 0.13	b
Supplemented diet	3.15 $\pm$ 1.54	abc	8.06 $\pm$ 2.46	ab	0.19 $\pm$ 0.09	b
<i>Normal temperature</i>						
Standard diet	4.81 $\pm$ 1.58	b	8.36 $\pm$ 3.94		0.22 $\pm$ 0.11	
<i>Heat stress</i>						
Standard diet	2.63 $\pm$ 1.48	a	9.24 $\pm$ 3.96		0.16 $\pm$ 0.08	
Supplemented diet	3.77 $\pm$ 1.36		8.00 $\pm$ 3.84		0.20 $\pm$ 0.09	
Supplemented diet	3.64 $\pm$ 1.70		9.60 $\pm$ 4.07		0.19 $\pm$ 0.10	
Day 1	3.76 $\pm$ 1.53		10.31 $\pm$ 4.63	b	0.16 $\pm$ 0.06	a
Day 5	3.66 $\pm$ 1.53		7.30 $\pm$ 3.28	a	0.23 $\pm$ 0.13	b
<i>Effects of:</i>						
Heat stress $\times$ Diet $\times$ Day	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD
Heat stress	<0.0001	1.68	<0.0001	3.59	0.007	0.10
Diet	<0.0001	0.88	0.414	2.14	0.025	0.05
Day	0.807	1.01	0.137	2.12	0.947	0.05
	0.842	1.01	0.005	2.04	0.008	0.05

Note: a–e Different lower-case letters within a column indicate significant differences among treatments ( $P < 0.01$ ).



**Figure 1.** Mean body weight and daily feed intake of the birds fed standard or supplemented diets at the start of the experiment (day 24) and after 1-day (25-days of age) or 5 days (29-days of age) heat stress challenges. \*Differs significantly ( $P < 0.05$ ) from birds fed standard feed under normal temperature.

stress challenge (Figure 1(a)). Feed intake was measured for each group and the individual daily intake was estimated. Although no statistical analysis was possible, unchallenged birds fed the standard diet had the numerically highest feed intake (Figure 1(b)).

### Markers of cellular oxidative stress

Oxidative stress markers were assessed in plasma/blood samples (Table 2). There was a significant interaction of heat stress, diet and day on the G6PD levels, which were not affected by the diet or day of assessment alone. When birds were fed the standard diet, G6PD levels were significantly decreased in blood after one heat stress challenge (day 1) (1.59 U/g Hb) when compared to birds fed standard diet under normal temperature (6.03 U/g Hb), and such difference was maintained after 5 days heat stress. No changes were observed when birds were fed supplemented diets regardless of heat stress challenge, where G6PD levels were 4.19 and 3.44 U/g Hb at day 1, and 3.80 and 3.15 U/g Hb at day 5.

There was a significant interaction of heat stress, diet and day on the GSH-Px activity. A significant increase of GSH-Px activity (14.36 nmol/min/ml) was observed directly after 1-day of heat stress challenge in broilers fed standard diet compared to normothermic conditions (7.13 nmol/min/ml). However, after 5 days of heat stress, GSH-Px activity in plasma from

birds fed standard diet decreased significantly (4.69 nmol/min/ml) when compared with 1-day heat stress. Birds fed supplemented diet presented GSH-Px activity similar to normothermic conditions, regardless of heat stress challenge (Table 2).

The TEAC levels were affected by the interaction of heat stress, diet and day. After 1-day of heat stress challenge, broilers fed standard diet presented a significant decrease in TEAC when compared to all the other treatments. After 5 days of heat stress, this difference was no longer observed. Again, birds fed supplemented diet presented TEAC levels similar to normothermic conditions, regardless of heat stress challenge (Table 2).

### Morphological and morphometrical analyses of intestinal sections

#### Chiu/Park scale

All intestinal segments from birds at thermoneutral conditions were predominantly normal (50–80%) and, when presenting damage, this was limited to damage degree 1, i.e. impairment in the sub-epithelial space at villus tips, independent of the analysis period. However, 1- or 5-days heat stress challenge led to a significant increase in the severity (degree 2–3) of villus damage in duodenum and jejunum from birds fed standard diet. Birds fed supplemented diet and

**Table 3.** Mean ( $\pm$  SEM) degree of villus damage in the duodenum, jejunum and ileum from non-stressed and heat stressed birds.

	Duodenum		Jejunum		Ileum	
<b>Day 1</b>						
<i>Normal temperature</i>						
Standard diet	0.4 $\pm$ 0.2	a	0.7 $\pm$ 0.2	a	0.2 $\pm$ 0.1	
Supplemented diet	0.7 $\pm$ 0.2	ab	0.5 $\pm$ 0.3	a	0.3 $\pm$ 0.1	
<i>Heat stress</i>						
Standard diet	2.2 $\pm$ 0.9	c	1.7 $\pm$ 0.4	c	0.6 $\pm$ 0.3	
Supplemented diet	1.0 $\pm$ 0.3	b	1.2 $\pm$ 0.2	b	0.6 $\pm$ 0.1	
<b>Day 5</b>						
<i>Normal temperature</i>						
Standard diet	0.8 $\pm$ 0.3	ab	0.7 $\pm$ 0.3	ab	0.3 $\pm$ 0.3	
Supplemented diet	0.8 $\pm$ 0.2	ab	0.6 $\pm$ 0.3	ab	0.5 $\pm$ 0.5	
<i>Heat stress</i>						
Standard diet	2.8 $\pm$ 0.7	d	2.0 $\pm$ 0.5	c	0.5 $\pm$ 0.5	
Supplemented diet	1.0 $\pm$ 0.6	b	1.0 $\pm$ 0.4	b	0.2 $\pm$ 0.3	
Normal temperature	0.7 $\pm$ 0.2	a	0.6 $\pm$ 0.3	a	0.3 $\pm$ 0.3	
Heat stress	1.8 $\pm$ 0.6	b	1.5 $\pm$ 0.4	b	0.5 $\pm$ 0.3	
Standard diet	1.6 $\pm$ 0.5	b	1.3 $\pm$ 0.4	b	0.4 $\pm$ 0.3	
Supplemented diet	0.9 $\pm$ 0.3	a	0.8 $\pm$ 0.3	a	0.4 $\pm$ 0.3	
Day 1	1.1 $\pm$ 0.4		1.0 $\pm$ 0.3		0.4 $\pm$ 0.2	
Day 5	1.3 $\pm$ 0.5		1.1 $\pm$ 0.4		0.4 $\pm$ 0.4	
<i>Effects of:</i>	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD
Heat stress $\times$ Diet $\times$ Day	<0.001	0.46	<0.001	0.29	0.122	0.31
Heat stress	<0.001	0.32	<0.001	0.19	0.065	0.15
Diet	<0.001	0.37	<0.001	0.26	0.897	0.16
Day	0.388	0.40	0.846	0.28	0.631	0.16

Note: Results are expressed as composite score determined by the amplification of the percentage of each degree per treatment by the amplification of the fraction of each degree.

a-c: different lower-case letters within a column indicate significant differences among treatments within each intestinal section ( $P < 0.01$ ).

subjected to 1- or 5-days heat challenge showed the same duodenum villus damage levels as birds raised at normal temperature. However, the jejunum from birds fed supplemented diet and submitted to 1-day heat stress presented a significant increase in damage degree, followed by a decrease after 5 days of heat stress challenge (Table 3).

### Morphometrical analysis

**Duodenum.** A significant decrease in the absorption surface area of the duodenum was observed, demonstrated by decreased villus height and villus width, in birds fed standard diet and submitted to heat stress for 1-day. After 5 days of heat stress, the villus width was significantly increased together with villus height and serosa width in challenged birds fed standard diet. Feeding birds a supplemented diet did not avoid decrease in villus length after 1-day heat stress, but avoided the decrease in villus width. After 5 days of heat stress, villus height from birds fed supplemented diet did not differ from those challenged birds fed standard diet (Table 4).

**Jejunum.** One-day heat stress resulted in a significant decrease of the jejunum absorption area (villus height), decrease in enterocyte proliferation (crypt depth), and increase of the villus and serosa width in birds fed standard diet. These effects were counteracted by the supplemented diet, except for serosa width that remained increased. After 5 days of heat stress, birds fed standard diet presented a significant increase in villus and serosa width and a decrease in crypt depth. Feeding birds with

supplemented diet during these 5 days decreased crypt depth (Table 5).

**Ileum.** Birds fed standard diet and challenged with 1-day heat stress presented a significantly increased serosa width, and this effect was not counteracted by the supplemented diet. Importantly, birds fed supplemented diet after 1-day at normothermic conditions, presented a significant decrease in villus height. After 5 days of heat stress, birds fed standard diet presented a significant decrease of the villus height and villus area:epithelial cells area ratio, as well as increased serosa width. A recovery in both villus height and serosa width was observed in birds challenged for 5 days when fed supplemented diet (Table 6).

### Gene expression

#### Oxidative stress markers

All the three tested oxidative stress markers were regulated by the interaction heat stress  $\times$  day  $\times$  diet, regardless of the evaluated tissue. Table 7 depicts data from liver and heart, while Table 8 depicts data from the intestinal sections. After 1-day heat stress, birds fed standard diet presented HSP70 up-regulation in the liver, heart and jejunum. Similarly, HMOX was up-regulated in the heart and ileum and down-regulated in the jejunum. On the other hand, HSP60 was up-regulated in the liver and down-regulated in the jejunum from broilers fed standard diet and exposed for 1 day to heat stress. Such effects were counteracted by diet supplementation. After 5 days of heat stress HMOX was up-regulated only in the duodenal tissue,

**Table 4.** Morphometrical analysis (mean  $\pm$  SEM) of duodenum after 1-day or 5-days heat stress challenge.

	Villus height ( $\mu\text{m}$ )		Villus width ( $\mu\text{m}$ )		Villus area ( $\text{mm}^2$ )		Crypt depth ( $\mu\text{m}$ )		Epithelial cells area ( $\text{mm}^2$ )		Serosa width per villus ( $\mu\text{m}$ )		Crypt depth: villus height (ratio)		Villus area: epithelial area (ratio)	
<b>Day 1</b>																
<i>Normal temperature</i>																
Standard diet	1597 $\pm$ 166	c	269 $\pm$ 98	bcd	366 $\pm$ 163		459 $\pm$ 72		239 $\pm$ 113		219 $\pm$ 20	ab	3.56 $\pm$ 0.66		1.54 $\pm$ 0.12	
Supplemented diet	1182 $\pm$ 136	a	222 $\pm$ 43	ab	242 $\pm$ 63		499 $\pm$ 119		154 $\pm$ 37		223 $\pm$ 45	ab	2.48 $\pm$ 0.62		1.57 $\pm$ 0.11	
<i>Heat stress</i>																
Standard diet	1116 $\pm$ 140	a	198 $\pm$ 33	a	216 $\pm$ 38		394 $\pm$ 59		139 $\pm$ 29		184 $\pm$ 29	a	2.93 $\pm$ 0.78		1.57 $\pm$ 0.13	
Supplemented diet	1263 $\pm$ 228	ab	226 $\pm$ 36	ab	266 $\pm$ 78		464 $\pm$ 89		174 $\pm$ 55		229 $\pm$ 32	abc	2.85 $\pm$ 0.92		1.54 $\pm$ 0.13	
<b>Day 5</b>																
<i>Normal temperature</i>																
Standard diet	1242 $\pm$ 122	a	252 $\pm$ 48	abc	241 $\pm$ 58		454 $\pm$ 119		162 $\pm$ 36		207 $\pm$ 53	a	2.94 $\pm$ 0.91		1.49 $\pm$ 0.10	
Supplemented diet	1199 $\pm$ 193	a	277 $\pm$ 73	bcd	295 $\pm$ 116		504 $\pm$ 135		191 $\pm$ 76		270 $\pm$ 63	cd	2.54 $\pm$ 0.80		1.55 $\pm$ 0.09	
<i>Heat stress</i>																
Standard diet	1420 $\pm$ 225	b	304 $\pm$ 68	cd	322 $\pm$ 99		499 $\pm$ 63		186 $\pm$ 85		253 $\pm$ 68	bc	2.91 $\pm$ 0.69		1.90 $\pm$ 0.60	
Supplemented diet	1257 $\pm$ 233	ab	316 $\pm$ 71	d	327 $\pm$ 125		401 $\pm$ 109		208 $\pm$ 88		305 $\pm$ 67	d	3.32 $\pm$ 1.03		1.60 $\pm$ 0.16	
Normal temperature	1305 $\pm$ 154		255 $\pm$ 66		286 $\pm$ 100		479 $\pm$ 111		186 $\pm$ 66		230 $\pm$ 45		2.88 $\pm$ 0.75		1.54 $\pm$ 0.11	
Heat stress	1264 $\pm$ 207		261 $\pm$ 52		283 $\pm$ 56		440 $\pm$ 80		177 $\pm$ 64		243 $\pm$ 49		3.00 $\pm$ 0.86		1.65 $\pm$ 0.26	
Standard diet	1344 $\pm$ 163		256 $\pm$ 62		286 $\pm$ 90		452 $\pm$ 78		181 $\pm$ 65		216 $\pm$ 43	a	3.08 $\pm$ 0.76		1.63 $\pm$ 0.24	
Supplemented diet	1225 $\pm$ 198		260 $\pm$ 56		282 $\pm$ 96		467 $\pm$ 113		182 $\pm$ 64		256 $\pm$ 52	b	2.80 $\pm$ 0.84		1.57 $\pm$ 0.12	
Day 1	1289 $\pm$ 168		229 $\pm$ 53	a	272 $\pm$ 86		454 $\pm$ 85		176 $\pm$ 59		214 $\pm$ 32	a	2.96 $\pm$ 0.75		1.56 $\pm$ 0.12	
Day 5	1279 $\pm$ 193		287 $\pm$ 65	b	296 $\pm$ 100		464 $\pm$ 107		187 $\pm$ 71		259 $\pm$ 63	b	2.93 $\pm$ 0.86		1.64 $\pm$ 0.14	
<i>Effects of:</i>																
	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD
Heat stress $\times$ Diet $\times$ Day	<0.001	165.4	<0.001	55.5	0.017	89.61	0.089	88.39	0.059	62.82	<0.001	44.66	0.072	0.724	0.014	0.215
Heat stress	0.427	102.1	0.719	31.8	0.892	48.05	0.090	45.23	0.554	32.96	0.339	26.63	0.522	0.378	0.051	0.113
Diet	0.019	98.93	0.764	31.8	0.866	48.04	0.510	45.95	0.994	33.03	<0.001	25.18	0.129	0.373	0.302	0.115
Day	0.848	102.5	<0.001	29.0	0.321	47.75	0.654	46.02	0.538	32.95	<0.001	24.80	0.871	0.379	0.167	0.115

Note: a–e: different lower-case letters within a column indicate significant differences among treatments ( $P < 0.01$ ).



**Table 5.** Morphometrical analysis (mean ± SEM) of jejunum after 1-day and 5-days heat stress challenge.

	Villus height (µm)		Villus width (µm)		Crypt depth (µm)		Villus area (mm <sup>2</sup> )		Epithelial cells area (mm <sup>2</sup> )		Serosa width per villus (µm)		Crypt depth: villus height (ratio)		Villus area: epithelial area (ratio)	
<b>Day 1</b>																
<i>Normal temperature</i>																
Standard diet	1471 ± 218	e	160 ± 35	a	504 ± 129	d	203 ± 37		116 ± 25		154 ± 32	a	3.09 ± 0.93		1.85 ± 0.64	
Supplemented diet	1013 ± 194	a	202 ± 40	bc	458 ± 132	bcd	170 ± 39		107 ± 27		187 ± 22	ab	2.31 ± 0.49		1.60 ± 0.16	
<i>Heat stress</i>																
Standard diet	1162 ± 286	ab	230 ± 48	cd	383 ± 62	ab	229 ± 84		146 ± 52		240 ± 55	cd	3.14 ± 1.05		1.56 ± 0.08	
Supplemented diet	1418 ± 149	de	179 ± 28	ab	448 ± 81	bcd	224 ± 54		142 ± 39		207 ± 34	bc	3.26 ± 0.75		1.59 ± 0.18	
<b>Day 5</b>																
<i>Normal temperature</i>																
Standard diet	1373 ± 256	cde	185 ± 42	ab	530 ± 101	d	191 ± 37		133 ± 38		177 ± 28	ab	2.70 ± 0.88		1.54 ± 0.53	
Supplemented diet	1228 ± 190	bcd	216 ± 45	bcd	478 ± 132	cd	174 ± 28		119 ± 21		197 ± 37	b	2.74 ± 0.81		1.47 ± 0.10	
<i>Heat stress</i>																
Standard diet	1177 ± 222	abc	248 ± 59	d	397 ± 88	abc	248 ± 71		164 ± 45		245 ± 57	d	3.13 ± 1.01		1.51 ± 0.09	
Supplemented diet	1192 ± 271	abc	227 ± 36	cd	329 ± 101	a	206 ± 49		135 ± 36		205 ± 26	b	3.86 ± 1.17		1.53 ± 0.07	
Normal temperature	1271 ± 215		191 ± 41	a	492 ± 124	b	185 ± 35	a	119 ± 28	a	179 ± 32	a	2.71 ± 0.78	a	1.62 ± 0.38	
Heat stress	1237 ± 232		221 ± 43	b	389 ± 83	a	227 ± 65	b	147 ± 43	b	224 ± 43	b	3.35 ± 0.77	b	1.55 ± 0.11	
Standard diet	1296 ± 246		206 ± 46		454 ± 95		218 ± 57		140 ± 40		204 ± 43		3.02 ± 0.97		1.62 ± 0.34	
Supplemented diet	1213 ± 201		206 ± 37		428 ± 87		194 ± 43		126 ± 31		199 ± 30		3.04 ± 0.81		1.55 ± 0.13	
Day 1	1266 ± 212		193 ± 38		449 ± 101		207 ± 54		128 ± 36		197 ± 36		2.95 ± 0.81		1.65 ± 0.27	
Day 5	1243 ± 235		219 ± 46		434 ± 106		205 ± 46		138 ± 35		206 ± 37		3.11 ± 0.97		1.51 ± 0.20	
<i>Effects of:</i>	<i>P-value</i>	<i>LSD</i>	<i>P-value</i>	<i>LSD</i>	<i>P-value</i>	<i>LSD</i>	<i>P-value</i>	<i>LSD</i>	<i>P-value</i>	<i>LSD</i>	<i>P-value</i>	<i>LSD</i>	<i>P-value</i>	<i>LSD</i>	<i>P-value</i>	<i>LSD</i>
Heat stress × Diet × Day	<0.001	202.7	<0.001	37.9	<0.001	94.5	0.019	47.3	0.023	32.6	<0.001	34.1	0.021	0.81	0.218	0.28
Heat stress	0.562	116.6	0.006	21.0	<0.001	48.1	<0.001	23.6	<0.001	16.2	<0.001	18.1	0.003	0.41	0.336	0.14
Diet	0.157	115.3	0.983	22.1	0.342	53.1	0.058	24.8	0.122	17.2	0.654	20.8	0.904	0.43	0.335	0.14
Day	0.696	116.7	0.018	21.3	0.579	53.3	0.881	25.4	0.265	17.3	0.398	20.7	0.470	0.43	0.053	0.14

Note: a–e: different lower-case letters within a column indicate significant differences among treatments ( $P < 0.01$ ).

**Table 6.** Morphometrical analysis (mean  $\pm$  SEM) of ileum after 1-day or 5-days heat stress challenge.

	Villus height ( $\mu\text{m}$ )		Villus width ( $\mu\text{m}$ )		Crypt depth ( $\mu\text{m}$ )		Villus area ( $\text{mm}^2$ )		Epithelial cells area ( $\text{mm}^2$ )		Serosa width per villus ( $\mu\text{m}$ )		Crypt depth: villus height (ratio)		Villus area: epithelial area (ratio)	
<b>Day 1</b>																
<i>Normal temperature</i>																
Standard diet	788 $\pm$ 121	c	143 $\pm$ 35		285 $\pm$ 78		101 $\pm$ 35		65 $\pm$ 22		145 $\pm$ 37	a	2.90 $\pm$ 0.77		1.56 $\pm$ 0.10	a
Supplemented diet	669 $\pm$ 95	ab	158 $\pm$ 20		248 $\pm$ 66		111 $\pm$ 25		72 $\pm$ 19		159 $\pm$ 26	a	2.86 $\pm$ 0.78		1.57 $\pm$ 0.08	a
<i>Heat stress</i>																
Standard diet	759 $\pm$ 89	bc	189 $\pm$ 42		206 $\pm$ 30		121 $\pm$ 31		79 $\pm$ 18		197 $\pm$ 42	c	3.76 $\pm$ 0.65		1.53 $\pm$ 0.10	a
Supplemented diet	787 $\pm$ 81	c	188 $\pm$ 22		266 $\pm$ 101		131 $\pm$ 17		88 $\pm$ 13		193 $\pm$ 19	bc	3.24 $\pm$ 0.89		1.50 $\pm$ 0.15	a
<b>Day 5</b>																
<i>Normal temperature</i>																
Standard diet	897 $\pm$ 166	d	176 $\pm$ 52		284 $\pm$ 68		133 $\pm$ 20		77 $\pm$ 10		156 $\pm$ 34	a	3.24 $\pm$ 0.65		1.73 $\pm$ 0.21	b
Supplemented diet	723 $\pm$ 131	abc	174 $\pm$ 21		210 $\pm$ 35		107 $\pm$ 15		70 $\pm$ 8		157 $\pm$ 37	a	3.54 $\pm$ 0.92		1.53 $\pm$ 0.10	a
<i>Heat stress</i>																
Standard diet	632 $\pm$ 100	a	208 $\pm$ 70		235 $\pm$ 105		128 $\pm$ 83		82 $\pm$ 56		198 $\pm$ 55	c	3.60 $\pm$ 1.38		1.59 $\pm$ 0.14	a
Supplemented diet	773 $\pm$ 102	c	178 $\pm$ 40		218 $\pm$ 54		127 $\pm$ 29		85 $\pm$ 19		163 $\pm$ 37	ab	3.77 $\pm$ 1.01		1.48 $\pm$ 0.10	a
Normal temperature	770 $\pm$ 128		163 $\pm$ 32	a	257 $\pm$ 62		113 $\pm$ 24		71 $\pm$ 15		154 $\pm$ 34	a	3.13 $\pm$ 0.78		1.60 $\pm$ 0.12	
Heat stress	738 $\pm$ 93		190 $\pm$ 44	b	231 $\pm$ 73		127 $\pm$ 40		84 $\pm$ 27		188 $\pm$ 38	b	3.59 $\pm$ 0.98		1.52 $\pm$ 0.12	
Standard diet	769 $\pm$ 119		178 $\pm$ 50		253 $\pm$ 70		121 $\pm$ 42		76 $\pm$ 27		174 $\pm$ 42		3.38 $\pm$ 0.86		1.60 $\pm$ 0.14	
Supplemented diet	738 $\pm$ 102		174 $\pm$ 26		235 $\pm$ 64		119 $\pm$ 22		79 $\pm$ 15		168 $\pm$ 30		3.35 $\pm$ 0.90		1.52 $\pm$ 0.11	
Day 1	751 $\pm$ 97		169 $\pm$ 30		251 $\pm$ 69		116 $\pm$ 27		76 $\pm$ 18		174 $\pm$ 31		3.19 $\pm$ 0.77		1.54 $\pm$ 0.11	
Day 5	757 $\pm$ 125		184 $\pm$ 46		237 $\pm$ 66		124 $\pm$ 37		79 $\pm$ 23		169 $\pm$ 41		3.54 $\pm$ 0.99		1.58 $\pm$ 0.14	
<i>Effects of:</i>	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD
Heat stress $\times$ Diet $\times$ Day	<0.001	101.1	0.041	36.7	0.345	84.8	0.444	33.9	0.425	22.3	0.005	33.1	0.157	0.81	0.002	0.11
Heat stress	0.289	59.0	0.005	18.4	0.232	42.5	0.105	16.7	0.232	10.8	<0.001	16.7	0.026	0.40	0.024	0.06
Diet	0.301	59.0	0.683	19.4	0.422	42.7	0.854	17.0	0.567	11.2	0.479	18.2	0.905	0.42	0.010	0.06
Day	0.848	59.4	0.112	19.1	0.499	42.8	0.381	16.9	0.654	11.2	0.588	18.2	0.096	0.41	0.143	0.06

Note: a–d: different lower-case letters within a column indicate significant differences among treatments ( $P < 0.01$ ).

**Table 7.** Mean ( $\pm$  SEM) relative mRNA expression of genes encoding markers for oxidative stress (HMOX, HSP60, HSP70) in the liver and heart.

	Liver						Heart					
	HMOX		HSP60		HSP70		HMOX		HSP60		HSP70	
<b>Day 1</b>												
<i>Normal temperature</i>												
Standard diet	1.03 $\pm$ 0.55	b	1.05 $\pm$ 0.24	a	0.93 $\pm$ 0.60	ab	1.00 $\pm$ 0.35	a	1.00 $\pm$ 0.33	b	1.00 $\pm$ 0.31	a
Supplemented diet	1.01 $\pm$ 0.33	b	1.63 $\pm$ 1.01	a	1.39 $\pm$ 0.57	ab	1.82 $\pm$ 1.04	ab	0.96 $\pm$ 0.22	b	1.70 $\pm$ 0.74	a
<i>Heat stress</i>												
Standard diet	1.22 $\pm$ 0.47	b	7.32 $\pm$ 2.76	c	4.06 $\pm$ 1.58	d	4.62 $\pm$ 1.79	c	1.07 $\pm$ 0.29	b	14.61 $\pm$ 4.03	b
Supplemented diet	1.04 $\pm$ 0.32	b	4.39 $\pm$ 1.53	b	1.89 $\pm$ 0.67	bc	2.94 $\pm$ 1.28	b	0.89 $\pm$ 0.24	b	4.16 $\pm$ 2.10	a
<b>Day 5</b>												
<i>Normal temperature</i>												
Standard diet	1.00 $\pm$ 0.21	b	1.03 $\pm$ 0.50	a	1.09 $\pm$ 0.42	ab	1.00 $\pm$ 0.95	a	1.00 $\pm$ 0.69	b	1.00 $\pm$ 0.36	a
Supplemented diet	0.55 $\pm$ 0.16	ab	0.51 $\pm$ 0.22	a	0.56 $\pm$ 0.40	a	0.82 $\pm$ 0.74	a	0.33 $\pm$ 0.08	a	3.68 $\pm$ 1.46	a
<i>Heat stress</i>												
Standard diet	1.23 $\pm$ 0.77	b	1.34 $\pm$ 1.06	a	2.65 $\pm$ 1.23	c	1.52 $\pm$ 1.33	a	0.41 $\pm$ 0.16	a	11.31 $\pm$ 3.40	b
Supplemented diet	0.42 $\pm$ 0.07	a	0.42 $\pm$ 0.11	a	0.91 $\pm$ 0.31	ab	1.58 $\pm$ 1.17	a	0.45 $\pm$ 0.34	a	2.75 $\pm$ 1.32	a
Normal temperature	0.90 $\pm$ 0.31		1.06 $\pm$ 0.49	a	0.99 $\pm$ 0.50	a	1.16 $\pm$ 0.77	a	0.82 $\pm$ 0.33		1.85 $\pm$ 0.47	a
Heat stress	0.98 $\pm$ 0.41		3.37 $\pm$ 1.37	b	2.38 $\pm$ 1.70	b	2.70 $\pm$ 1.39	b	0.71 $\pm$ 0.26		8.21 $\pm$ 2.71	b
Standard diet	1.12 $\pm$ 0.50	b	2.69 $\pm$ 1.14		2.18 $\pm$ 0.96		2.05 $\pm$ 1.11		0.87 $\pm$ 0.37		6.98 $\pm$ 2.03	
Supplemented diet	0.75 $\pm$ 0.22	a	1.74 $\pm$ 0.72		1.19 $\pm$ 0.49		1.79 $\pm$ 1.06		0.66 $\pm$ 0.22		3.07 $\pm$ 1.41	
Day 1	1.07 $\pm$ 0.42		3.60 $\pm$ 1.39	b	1.07 $\pm$ 0.86		2.60 $\pm$ 1.12	b	0.98 $\pm$ 0.27	b	5.37 $\pm$ 1.80	
Day 5	0.80 $\pm$ 0.30		0.83 $\pm$ 0.47	a	0.80 $\pm$ 0.59		1.22 $\pm$ 1.05	a	0.55 $\pm$ 0.32	a	4.69 $\pm$ 1.64	
<i>Effects of:</i>												
	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD
Heat stress $\times$ Diet $\times$ Day	<0.001	0.374	<0.001	1.373	<0.001	0.996	<0.001	1.221	<0.001	0.304	<0.001	3.579
Heat stress	0.458	0.217	<0.001	1.099	<0.001	0.610	<0.001	0.721	0.242	0.195	<0.001	2.351
Diet	<0.001	0.202	0.148	1.206	0.003	0.647	0.522	0.797	0.030	0.190	0.004	2.610
Day	0.012	0.209	<0.001	1.075	0.025	0.663	<0.001	0.737	<0.001	0.170	0.622	2.750

Note: a–d: different lower-case letters within a column indicate significant differences among treatments ( $P < 0.001$ ).

**Table 8.** Mean ( $\pm$  SEM) relative mRNA expression of genes encoding markers for oxidative stress (HMOX, HSP60, HSP70) in the intestinal sections.

	Duodenum						Jejunum						Ileum						
	HMOX		HSP60		HSP70		HMOX		HSP60		HSP70		HMOX		HSP60		HSP70		
<b>Day 1</b>																			
<i>Normal temperature</i>																			
Standard diet	1.0 $\pm$ 0.6	a	1.0 $\pm$ 0.3	b	1.0 $\pm$ 0.4	abc	1.0 $\pm$ 0.7	c	1.0 $\pm$ 0.4	b	1.0 $\pm$ 0.6	a	1.0 $\pm$ 0.4	a	1.0 $\pm$ 0.4	ab	1.0 $\pm$ 0.5	b	
Supplemented diet	4.5 $\pm$ 1.0	d	2.1 $\pm$ 0.7	c	1.6 $\pm$ 0.4	cd	0.8 $\pm$ 0.2	bc	0.5 $\pm$ 0.2	a	0.6 $\pm$ 0.1	a	0.8 $\pm$ 0.2	a	0.5 $\pm$ 0.1	a	0.6 $\pm$ 0.2	ab	
<i>Heat stress</i>																			
Standard diet	1.0 $\pm$ 0.7	a	1.3 $\pm$ 0.5	b	0.5 $\pm$ 0.2	a	0.4 $\pm$ 0.2	b	0.4 $\pm$ 0.2	a	2.1 $\pm$ 0.4	b	2.5 $\pm$ 0.2	b	0.5 $\pm$ 0.3	a	0.5 $\pm$ 0.2	ab	
Supplemented diet	1.6 $\pm$ 0.9	ab	1.3 $\pm$ 0.7	b	1.8 $\pm$ 0.8	cd	0.9 $\pm$ 0.3	bc	0.7 $\pm$ 0.1	ab	0.5 $\pm$ 0.1	a	0.4 $\pm$ 0.3	a	0.4 $\pm$ 0.2	a	0.5 $\pm$ 0.2	ab	
<b>Day 5</b>																			
<i>Normal temperature</i>																			
Standard diet	1.0 $\pm$ 0.1	a	1.0 $\pm$ 0.3	b	1.0 $\pm$ 0.4	ab	1.0 $\pm$ 0.3	c	1.0 $\pm$ 0.4	b	1.0 $\pm$ 0.6	a	1.0 $\pm$ 0.4	a	1.0 $\pm$ 0.4	ab	1.0 $\pm$ 0.4	b	
Supplemented diet	2.5 $\pm$ 1.0	bc	0.2 $\pm$ 0.1	a	1.1 $\pm$ 0.5	bc	0.6 $\pm$ 0.3	b	2.7 $\pm$ 0.5	c	3.7 $\pm$ 0.8	c	0.4 $\pm$ 0.3	a	1.8 $\pm$ 0.4	c	0.3 $\pm$ 0.2	a	
<i>Heat stress</i>																			
Standard diet	3.0 $\pm$ 1.0	c	1.9 $\pm$ 0.3	bc	1.5 $\pm$ 0.8	bcd	0.7 $\pm$ 0.3	bc	2.5 $\pm$ 0.4	c	3.6 $\pm$ 0.7	c	0.6 $\pm$ 0.4	a	2.7 $\pm$ 0.9	d	2.3 $\pm$ 0.5	c	
Supplemented diet	1.0 $\pm$ 0.5	a	0.5 $\pm$ 0.3	a	1.2 $\pm$ 0.4	bc	0.1 $\pm$ 0.0	a	0.6 $\pm$ 0.3	ab	3.2 $\pm$ 0.7	c	2.4 $\pm$ 0.2	b	1.5 $\pm$ 0.4	bc	1.9 $\pm$ 0.8	bc	
<i>Normal temperature</i>	2.2 $\pm$ 0.7		1.1 $\pm$ 0.4		1.2 $\pm$ 0.4		0.8 $\pm$ 0.4		1.3 $\pm$ 0.4		1.6 $\pm$ 0.5	a	0.8 $\pm$ 0.3	a	1.1 $\pm$ 0.4		0.7 $\pm$ 0.3		
<i>Heat stress</i>	1.6 $\pm$ 0.8		1.2 $\pm$ 0.5		1.3 $\pm$ 0.6		0.5 $\pm$ 0.2		1.0 $\pm$ 0.3		2.3 $\pm$ 0.5	b	1.5 $\pm$ 0.3	b	1.3 $\pm$ 0.5		1.2 $\pm$ 0.4		
Standard diet	1.5 $\pm$ 0.6		1.3 $\pm$ 0.4		1.0 $\pm$ 0.5		0.8 $\pm$ 0.4		1.2 $\pm$ 0.4		1.9 $\pm$ 0.6		1.3 $\pm$ 0.4		1.3 $\pm$ 0.5		1.2 $\pm$ 0.4		
Supplemented diet	2.4 $\pm$ 0.9		1.0 $\pm$ 0.5		1.4 $\pm$ 0.4		0.6 $\pm$ 0.2		1.1 $\pm$ 0.3		2.0 $\pm$ 0.4		1.0 $\pm$ 0.3		1.0 $\pm$ 0.3		0.8 $\pm$ 0.4		
Day 1	1.5 $\pm$ 0.8		1.4 $\pm$ 0.6	b	1.0 $\pm$ 0.5		0.8 $\pm$ 0.4		0.6 $\pm$ 0.2	a	1.0 $\pm$ 0.3	a	1.2 $\pm$ 0.3		0.6 $\pm$ 0.3	a	0.6 $\pm$ 0.3	a	
Day 5	2.4 $\pm$ 0.7		0.9 $\pm$ 0.3	a	1.4 $\pm$ 0.5		0.6 $\pm$ 0.2		1.7 $\pm$ 0.4	b	2.9 $\pm$ 0.7	b	1.1 $\pm$ 0.3		1.7 $\pm$ 0.5	b	1.4 $\pm$ 0.5	b	
<i>Effects of:</i>	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	
Heat stress $\times$ Diet $\times$ Day	<0.001	0.978	<0.001	0.404	<0.001	0.502	<0.001	0.384	<0.001	0.396	<0.001	1.038	<0.001	0.696	<0.001	0.653	<0.001	0.503	
Heat stress	0.091	0.701	0.339	0.333	0.552	0.291	0.256	0.342	0.157	0.439	0.045	0.741	<0.001	0.825	0.437	0.451	0.010	0.375	
Diet	0.011	0.685	0.097	0.329	0.005	0.277	0.165	0.306	0.479	0.443	0.889	0.761	0.013	0.859	0.231	0.449	0.037	0.380	
Day	0.696	0.714	<0.001	0.312	0.974	0.292	0.221	0.307	<0.001	0.380	<0.001	0.639	0.002	0.790	<0.001	0.377	<0.001	0.351	

Note: a–e: different lower-case letters within a column indicate significant differences among treatments ( $P < 0.001$ ).

while HSP60 was up-regulated in the jejunum and ileum from broilers fed standard diet. Again, such effects were not observed when broilers were fed supplemented diet during heat stress challenge. Remarkably, broilers fed standard diets and exposed to 5 days of heat stress kept the up-regulated levels of HSP70 in the liver, heart, jejunum, and ileum. This time, diet supplementation was not sufficient to avoid HSP70 up-regulation in the jejunum after 5 days of heat stress challenge.

### Inflammatory markers

Table 9 depicts data from liver and heart, while Tables 10–12 depict data from the intestinal sections. An interaction diet  $\times$  day  $\times$  heat stress was observed when assessing the expression of markers for inflammation in the present study. IKBA was up-regulated in both liver and heart from birds fed a standard diet and submitted to 1-day heat stress (Table 9). Neither heat stress or test diet affected the expression of inflammatory markers in the duodenum, except for the up-regulation of IKBA when the birds were fed supplemented diet at day 1 (Table 10). The most significant changes in the expression of inflammatory markers were observed in the jejunum (Table 11). For instance, after 1-day heat stress, broilers fed standard diet presented down-regulation of IKBA, IL-8, and TLR4. When broilers were fed standard diet and submitted to 5 days heat stress, the jejunum presented mRNA expression of IL-8 and TLR4 similar to unchallenged broilers, with only a down-regulation in IFN $\gamma$  and up-regulation of IKBA. This down- and up-regulation of, respectively, IFN $\gamma$  and IKBA was also observed in

broilers fed supplemented diet (Table 11). Up-regulation of IL-8 and IFN $\gamma$  was observed in the ileum from broilers fed standard diet submitted to 1 and 5 days heat stress, respectively (Table 12).

### Tight junction proteins

Tables 13–15 depict data from duodenum, jejunum and ileum, respectively. The significant alterations in the expression of mRNA of tight junction proteins were mostly observed in the jejunum, with minor changes in the duodenum and ileum. The ZO1 up-regulation in the duodenum was observed after 5 days heat stress challenge in birds fed standard diet, or in birds fed supplemented diet under normothermic conditions (Table 13). After 5 days of heat stress being fed standard diet, CLDN1 was up-regulated in the jejunum. This up-regulation was also observed in the jejunum from broilers kept under normal temperature for 5 days and fed supplemented diet. Relative mRNA expression of both ZO1 and ZO2 was affected by heat stress only when this challenge was repeated for 5 days. Importantly, ZO1 was also up-regulated in the jejunum from broilers fed supplemented diet for 5 days under normal temperature (Table 14). In the ileum, ZO1 was up-regulated after 5 days heat stress regardless the diet, while ZO2 was down-regulated at day 1 and up-regulated at day 5, regardless of the diet and temperature of exposure (Table 15).

### Growth factor and glucose, amino acid and peptide transporters

Tables 16–18 depict data from duodenum, jejunum and ileum, respectively. The mRNA expression of TGF $\beta$  was not affected during the present study. In the duodenum, PEPT1 was up-regulated when broilers fed standard diet were submitted to 5 days heat stress. This effect was not observed at 1-day heat stress exposure, which was characterized by the up-regulation of SGLT regardless of the diet or housing temperature (Table 16). PEPT1 was down-regulated while EEA3 was up-regulated in the jejunum from broilers fed standard diet and submitted to 1-day heat stress. After 5 days of heat stress no effect was observed on PEPT1 expression in the jejunum. However, EAAT3 remained up-regulated including in the jejunum from heat-stressed broilers fed supplemented diet (Table 17). Expression of nutrient factors was not affected in the ileum (Table 18).

### Discussion

We have characterized the intestinal responses to heat stress at two different time-points, immediately after exposure of broiler chickens to a single challenge (day 1) and after 5 days heat stress challenge (day 5). Furthermore, two different feed conditions

**Table 9.** Mean ( $\pm$  SEM) relative mRNA expression of IKBA in the liver and heart from broilers.

	Liver		Heart	
<b>Day 1</b>				
<i>Normal temperature</i>				
Standard diet	1.0 $\pm$ 0.8	abc	1.0 $\pm$ 0.5	bc
Supplemented diet	1.4 $\pm$ 0.6	bc	0.6 $\pm$ 0.3	ab
<i>Heat stress</i>				
Standard diet	2.8 $\pm$ 0.6	d	4.0 $\pm$ 0.8	d
Supplemented diet	1.3 $\pm$ 0.5	abc	1.3 $\pm$ 0.6	c
<b>Day 5</b>				
<i>Normal temperature</i>				
Standard diet	1.3 $\pm$ 0.4	bc	1.0 $\pm$ 0.3	bc
Supplemented diet	0.5 $\pm$ 0.2	a	0.4 $\pm$ 0.1	a
<i>Heat stress</i>				
Standard diet	1.5 $\pm$ 0.8	b	1.1 $\pm$ 0.4	bc
Supplemented diet	0.5 $\pm$ 0.2	ab	0.6 $\pm$ 0.2	ab
Normal temperature	1.1 $\pm$ 0.5		0.7 $\pm$ 0.3	a
Heat stress	1.5 $\pm$ 0.5		1.8 $\pm$ 0.5	b
Standard diet	1.7 $\pm$ 0.7		1.8 $\pm$ 0.5	b
Supplemented diet	0.9 $\pm$ 0.4		0.7 $\pm$ 0.3	a
Day 1	1.6 $\pm$ 0.6		1.7 $\pm$ 0.6	b
Day 5	1.0 $\pm$ 0.4		0.8 $\pm$ 0.3	a
<i>Effects of:</i>				
	<i>P</i> -value	LSD	<i>P</i> -value	LSD
Heat stress $\times$ Diet $\times$ Day	<0.001	0.932	<0.001	0.561
Heat stress	0.090	0.537	<0.001	0.503
Diet	0.004	0.517	<0.001	0.500
Day	0.010	0.523	<0.001	0.510

Note: a–d: different lower-case letters within a column indicate significant differences among treatments ( $P < 0.001$ ).

**Table 10.** Mean ( $\pm$  SEM) relative mRNA expression of genes encoding markers for inflammation (IKBA, IFN $\gamma$ , IL8, TLR2, TLR4) in the duodenum.

	IKBA		IL8		IFN $\gamma$		TLR2		TLR4	
<b>Day 1</b>										
<i>Normal temperature</i>										
Standard diet	1.0 $\pm$ 0.4	a	1.0 $\pm$ 0.4		1.0 $\pm$ 0.6		1.0 $\pm$ 0.6		1.0 $\pm$ 0.4	
Supplemented diet	2.2 $\pm$ 0.9	b	1.1 $\pm$ 0.8		1.4 $\pm$ 0.7		1.3 $\pm$ 0.3		1.4 $\pm$ 0.2	
<i>Heat stress</i>										
Standard diet	1.0 $\pm$ 0.4	a	0.6 $\pm$ 0.5		1.3 $\pm$ 0.5		0.7 $\pm$ 0.3		1.3 $\pm$ 0.2	
Supplemented diet	2.6 $\pm$ 0.8	b	1.4 $\pm$ 0.9		1.9 $\pm$ 0.9		1.3 $\pm$ 0.2		0.8 $\pm$ 0.6	
<b>Day 5</b>										
<i>Normal temperature</i>										
Standard diet	1.0 $\pm$ 0.2	a	1.0 $\pm$ 0.6		1.0 $\pm$ 0.4		1.0 $\pm$ 0.3		1.0 $\pm$ 0.5	
Supplemented diet	1.7 $\pm$ 0.4	a	1.7 $\pm$ 0.8		1.2 $\pm$ 0.6		1.0 $\pm$ 0.4		1.6 $\pm$ 0.9	
<i>Heat stress</i>										
Standard diet	0.9 $\pm$ 0.3	a	1.4 $\pm$ 0.9		0.4 $\pm$ 0.2		0.8 $\pm$ 0.4		0.8 $\pm$ 0.3	
Supplemented diet	0.6 $\pm$ 0.2	a	0.7 $\pm$ 0.4		0.6 $\pm$ 0.5		0.5 $\pm$ 0.2		1.2 $\pm$ 0.8	
Normal temperature	1.5 $\pm$ 0.5		1.2 $\pm$ 0.7		1.1 $\pm$ 0.6		1.1 $\pm$ 0.4		1.2 $\pm$ 0.5	
Heat stress	1.3 $\pm$ 0.4		1.0 $\pm$ 0.7		1.1 $\pm$ 0.5		0.8 $\pm$ 0.3		1.0 $\pm$ 0.5	
Standard diet	1.0 $\pm$ 0.4	a	1.0 $\pm$ 0.6		0.9 $\pm$ 0.4		0.9 $\pm$ 0.4		1.0 $\pm$ 0.4	
Supplemented diet	1.8 $\pm$ 0.6	b	1.2 $\pm$ 0.7		1.3 $\pm$ 0.7		1.0 $\pm$ 0.3		1.2 $\pm$ 0.6	
Day 1	1.7 $\pm$ 0.7	b	1.0 $\pm$ 0.7		1.4 $\pm$ 0.7		1.1 $\pm$ 0.4		1.1 $\pm$ 0.4	
Day 5	1.0 $\pm$ 0.3	a	1.2 $\pm$ 0.7		0.8 $\pm$ 0.4		0.8 $\pm$ 0.3		1.1 $\pm$ 0.6	
<i>Effects of:</i>										
Heat stress $\times$ Diet $\times$ Day	<0.001	0.454	0.001	0.727	0.001	1.182	0.001	0.314	0.001	0.537
Heat stress	0.222	0.372	0.372	0.488	0.361	0.702	0.007	0.189	0.166	0.311
Diet	<0.001	0.335	0.244	0.486	0.103	0.694	0.001	0.182	0.168	0.311
Day	<0.001	0.342	0.004	0.464	0.001	0.636	0.010	0.190	0.001	0.290

Note: a–e: different lower-case letters within a column indicate significant differences among treatments ( $P < 0.001$ ).

were tested: standard feed or the same feed supplemented with a commercial gut health additive rich in MCFA, organic acids and a phenolic compound from a plant extract (Presan FY). The decreases in feed intake and body weight gain due to heat stress were expected and observed. However, due to the number of birds used, these are only indicative parameters. The focus of this study was to evaluate the effect of diet, day of challenge, and heat stress on selected functional blood parameters, gut morphology and mRNA expression of markers of oxidative stress, inflammation,

tight junction proteins and transporters of glucose, amino acids and peptides.

The regulatory enzyme G6PD, present in red blood cells, is essential for providing the major part of NADPH, which promotes glutathione regeneration, protecting the cells against oxidative damage (Zhang *et al.*, 2016). This enzyme is the only NADPH-producing enzyme activated due to oxidative stress (Filosa *et al.*, 2003). After 1-day heat stress challenge, increased activity of GSH-PX in response to oxidative stress was observed, demanding a higher production of NADPH

**Table 11.** Mean ( $\pm$  SEM) relative mRNA expression of genes encoding markers for inflammation (IKBA, IFN $\gamma$ , IL8, TLR2, TLR4) in the jejunum.

	IKBA		IL8		IFN $\gamma$		TLR2		TLR4	
<b>Day 1</b>										
<i>Normal temperature</i>										
Standard diet	1.0 $\pm$ 0.5	b	1.0 $\pm$ 0.4	c	1.0 $\pm$ 0.5	c	1.0 $\pm$ 0.5		1.0 $\pm$ 0.6	c
Supplemented diet	0.5 $\pm$ 0.1	ab	0.3 $\pm$ 0.2	ab	0.4 $\pm$ 0.1	ab	0.7 $\pm$ 0.2		0.1 $\pm$ 0.1	a
<i>Heat stress</i>										
Standard diet	0.2 $\pm$ 0.0	a	0.2 $\pm$ 0.1	a	0.6 $\pm$ 0.5	bc	1.4 $\pm$ 0.4		0.1 $\pm$ 0.0	a
Supplemented diet	1.0 $\pm$ 0.2	b	0.1 $\pm$ 0.1	a	0.2 $\pm$ 0.1	ab	0.6 $\pm$ 0.2		0.2 $\pm$ 0.1	ab
<b>Day 5</b>										
<i>Normal temperature</i>										
Standard diet	1.0 $\pm$ 0.3	b	1.0 $\pm$ 0.7	c	1.0 $\pm$ 0.4	c	1.0 $\pm$ 0.3		1.0 $\pm$ 0.4	c
Supplemented diet	2.1 $\pm$ 0.7	b	1.0 $\pm$ 0.7	c	0.3 $\pm$ 0.2	ab	1.7 $\pm$ 0.9		0.5 $\pm$ 0.3	bc
<i>Heat stress</i>										
Standard diet	2.9 $\pm$ 0.7	c	0.8 $\pm$ 0.3	c	0.3 $\pm$ 0.2	ab	0.8 $\pm$ 0.4		0.5 $\pm$ 0.3	c
Supplemented diet	6.4 $\pm$ 0.9	d	0.8 $\pm$ 0.4	bc	0.2 $\pm$ 0.1	a	1.1 $\pm$ 0.8		0.6 $\pm$ 0.3	c
Normal temperature	1.2 $\pm$ 0.4		0.8 $\pm$ 0.5		0.7 $\pm$ 0.3	b	1.1 $\pm$ 0.5		0.6 $\pm$ 0.4	
Heat stress	2.6 $\pm$ 0.5		0.5 $\pm$ 0.2		0.3 $\pm$ 0.3	a	1.0 $\pm$ 0.5		0.4 $\pm$ 0.2	
Standard diet	1.3 $\pm$ 0.4		0.8 $\pm$ 0.4		0.7 $\pm$ 0.4	b	1.0 $\pm$ 0.4		0.6 $\pm$ 0.3	
Supplemented diet	2.5 $\pm$ 0.5		0.5 $\pm$ 0.4		0.3 $\pm$ 0.1	a	1.0 $\pm$ 0.5		0.3 $\pm$ 0.2	
Day 1	0.7 $\pm$ 0.2	a	0.4 $\pm$ 0.2	a	0.6 $\pm$ 0.3		0.9 $\pm$ 0.3		0.3 $\pm$ 0.2	
Day 5	3.1 $\pm$ 0.7	b	0.9 $\pm$ 0.5	b	0.4 $\pm$ 0.2		1.1 $\pm$ 0.6		0.7 $\pm$ 0.3	
<i>Effects of:</i>										
Heat stress $\times$ Diet $\times$ Day	<0.001	1.011	<0.001	0.502	<0.001	0.352	0.002	0.779	<0.001	0.313
Heat stress	0.002	0.934	0.019	0.277	<0.001	0.205	0.353	0.275	0.009	0.211
Diet	0.019	0.962	0.131	0.283	<0.001	0.197	0.936	0.277	0.006	0.211
Day	<0.001	0.821	<0.001	0.267	0.253	0.219	0.141	0.273	0.002	0.207

Note: a–d: different lower-case letters within a column indicate significant differences among treatments ( $P < 0.001$ ).

**Table 12.** Mean ( $\pm$  SEM) relative mRNA expression of genes encoding markers for inflammation (IKBA, IFN $\gamma$ , IL8, TLR2, TLR4) in the ileum.

	IKBA		IL8		IFN $\gamma$		TLR2		TLR4	
<b>Day 1</b>										
<i>Normal temperature</i>										
Standard diet	1.0 $\pm$ 0.4		1.0 $\pm$ 0.5	b	1.0 $\pm$ 0.2	b	1.0 $\pm$ 0.4		1.0 $\pm$ 0.4	
Supplemented diet	0.5 $\pm$ 0.2		0.6 $\pm$ 0.3	ab	0.9 $\pm$ 0.4	b	0.8 $\pm$ 0.2		0.6 $\pm$ 0.2	
<i>Heat stress</i>										
Standard diet	0.5 $\pm$ 0.3		3.6 $\pm$ 0.5	c	0.9 $\pm$ 0.4	ab	0.9 $\pm$ 0.5		1.2 $\pm$ 0.5	
Supplemented diet	0.5 $\pm$ 0.2		0.8 $\pm$ 0.4	b	0.3 $\pm$ 0.2	a	1.0 $\pm$ 0.4		0.4 $\pm$ 0.2	
<b>Day 5</b>										
<i>Normal temperature</i>										
Standard diet	1.0 $\pm$ 0.2		1.0 $\pm$ 0.3	b	1.0 $\pm$ 0.6	b	1.0 $\pm$ 0.6		1.0 $\pm$ 0.8	
Supplemented diet	0.5 $\pm$ 0.2		0.3 $\pm$ 0.2	a	0.9 $\pm$ 0.3	ab	1.3 $\pm$ 0.5		0.4 $\pm$ 0.2	
<i>Heat stress</i>										
Standard diet	1.6 $\pm$ 0.5		1.1 $\pm$ 0.6	b	2.6 $\pm$ 0.8	c	1.7 $\pm$ 0.7		1.2 $\pm$ 0.8	
Supplemented diet	1.2 $\pm$ 0.5		0.7 $\pm$ 0.5	ab	1.3 $\pm$ 0.4	b	0.9 $\pm$ 0.3		0.8 $\pm$ 0.5	
<i>Normal temperature</i>										
Standard diet	0.8 $\pm$ 0.2		0.7 $\pm$ 0.3	a	1.0 $\pm$ 0.4		1.0 $\pm$ 0.4		0.8 $\pm$ 0.4	
<i>Heat stress</i>										
Standard diet	1.0 $\pm$ 0.4		1.5 $\pm$ 0.5	b	1.3 $\pm$ 0.5		1.1 $\pm$ 0.5		0.9 $\pm$ 0.5	
Supplemented diet	0.7 $\pm$ 0.3		0.6 $\pm$ 0.4	a	0.8 $\pm$ 0.3		1.0 $\pm$ 0.4		0.5 $\pm$ 0.3	
Day 1	0.7 $\pm$ 0.3		1.5 $\pm$ 0.4		0.8 $\pm$ 0.3	a	0.9 $\pm$ 0.4		0.8 $\pm$ 0.4	
Day 5	1.1 $\pm$ 0.4		0.8 $\pm$ 0.4		1.4 $\pm$ 0.5	b	1.2 $\pm$ 0.5		0.9 $\pm$ 0.6	
<i>Effects of:</i>										
	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD
Heat stress $\times$ Diet $\times$ Day	0.002	0.380	<0.001	0.415	<0.001	0.519	0.020	0.435	0.002	0.453
Heat stress	0.114	0.334	<0.001	0.443	0.094	0.566	0.285	0.443	0.229	0.458
Diet	0.025	0.318	<0.001	0.409	0.005	0.554	0.145	0.441	0.005	0.425
Day	0.004	0.314	0.002	0.448	<0.001	0.544	0.008	0.433	0.675	0.461

Note: a–e: different lower-case letters within a column indicate significant differences among treatments ( $P < 0.001$ ).

via G6PD. It seems that after 1-day of heat stress, the G6PD activity was already depleted as its activity was decreased. This decrease also remained during 5 days of heat stress, decreasing the source of NADPH for GSH-Px activity. This may explain why GSH-Px increased after 1-day heat stress with a subsequent decrease after 5 days of heat stress. Interestingly, the TEAC returned to normal levels, suggesting that the bird counteracted oxidative stress via non-enzymatic pathways. These blood and plasma alterations were not observed in broilers fed supplemented diets and submitted to heat stress. This effect may be attributed to the presence of a polyphenol in the tested additive. Indeed, it was shown before that feed supplementation with polyphenols improved the redox capacity in broilers (Dong *et al.*, 2015), and resulted in an increased total number of red blood in heat stress challenged broilers (Aengwanich & Suttajit, 2013).

Morphological and morphometrical analyses of the intestinal tract confirmed that jejunum was the most sensitive intestinal section to heat stress followed by duodenum and ileum, as reported before (Santos *et al.*, 2015). These findings may reflect a higher sensitivity of the jejunum to heat stress associated with a decreased cell proliferation of the intestinal cells. Self-renewing of the villi depends on the proliferation of the stem cells present in the crypt, which replace apoptotic cells in the villus (Tan & Barker, 2014). In a recent study, Xiaofang *et al.* (2018) showed that intestinal cell proliferation is decreased in the jejunum, but not in the duodenum or ileum from broilers exposed to chronic heat stress (7 days). To compensate villus shortening, morphological changes such as an increase in villus width were observed, as intestinal

villi can rapidly adapt their morphology and function with the aim to maintain the absorption of nutrients (Incharoen, 2013). These alterations were observed in the jejunum after 1 and 5 days of heat stress, and in the duodenum after 5 days of heat stress. It was remarkable that duodenal villus width decreased after 1-day of heat stress challenge, but increased after 5 days of heat stress. These variations show that intestinal segments react differently to heat stress, and parameters other than morphology should also be taken into account when evaluating heat stress and nutritional management for improving the entire intestinal function. Previous experiments had shown that a combination of organic acids with MCFA increases villus height and crypt depth without increasing villus width (Baltic *et al.*, 2017). It is therefore assumed that also in the current experiments the blend of organics acids and MCFA contributed to the maintenance of intestinal functions by supporting enterocyte proliferation under the conditions of heat stress. The mRNA expression of oxidative stress markers helped to evaluate the effects of heat stress on the liver, myocardium and intestines in more detail. Heat shock proteins (HSPs) are chaperones involved in the maintenance of protein structure in cells under stress. HSP70 enhances cell thermo-tolerance by stabilizing hydrophobic regions in proteins avoiding their denaturation, while HSP60 is mostly involved in protein folding and cyto-protection against apoptosis and necrosis (Takada *et al.*, 2010). It is also known that HSPs are involved in cellular defence mechanisms during adverse conditions such as exposure to heat (Parsell & Lindquist, 1993). Therefore, increased HSP level makes the bird more resistant to heat stress or

**Table 13.** Mean ( $\pm$  SEM) relative mRNA expression of genes encoding markers for tight junctions (CLDN1, CLDN5, ZO1 and ZO2) in the duodenum.

	CLDN1		CLDN5		ZO1		ZO2	
<b>Day 1</b>								
<i>Normal temperature</i>								
Standard diet	1.0 $\pm$ 0.3		1.0 $\pm$ 0.7		1.0 $\pm$ 0.3	b	1.0 $\pm$ 0.7	
Supplemented diet	0.6 $\pm$ 0.2		0.5 $\pm$ 0.3		1.0 $\pm$ 0.3	b	0.1 $\pm$ 0.1	
<i>Heat stress</i>								
Standard diet	0.9 $\pm$ 0.5		0.4 $\pm$ 0.2		0.9 $\pm$ 0.3	b	1.3 $\pm$ 1.2	
Supplemented diet	1.9 $\pm$ 1.5		1.2 $\pm$ 0.8		1.7 $\pm$ 0.7	c	0.5 $\pm$ 0.4	
<b>Day 5</b>								
<i>Normal temperature</i>								
Standard diet	1.0 $\pm$ 0.3		1.0 $\pm$ 0.3		1.0 $\pm$ 0.3	b	1.0 $\pm$ 0.7	
Supplemented diet	1.4 $\pm$ 0.6		2.1 $\pm$ 1.2		2.5 $\pm$ 0.4	d	1.2 $\pm$ 1.0	
<i>Heat stress</i>								
Standard diet	0.6 $\pm$ 0.3		2.6 $\pm$ 1.2		2.5 $\pm$ 0.4	d	0.9 $\pm$ 0.7	
Supplemented diet	1.3 $\pm$ 0.6		1.1 $\pm$ 0.5		0.5 $\pm$ 0.2	a	1.3 $\pm$ 0.9	
Normal temperature	1.0 $\pm$ 0.4		1.2 $\pm$ 0.6		1.4 $\pm$ 0.3		0.8 $\pm$ 0.6	
Heat stress	1.1 $\pm$ 0.6		1.3 $\pm$ 0.7		1.4 $\pm$ 0.4		1.0 $\pm$ 0.8	
Standard diet	0.9 $\pm$ 0.4		1.3 $\pm$ 0.6		1.3 $\pm$ 0.3		1.0 $\pm$ 0.8	
Supplemented diet	1.3 $\pm$ 0.7		1.2 $\pm$ 0.7		1.4 $\pm$ 0.4		0.8 $\pm$ 0.6	
Day 1	1.1 $\pm$ 0.6		0.8 $\pm$ 0.5		1.1 $\pm$ 0.4	a	0.7 $\pm$ 0.6	
Day 5	1.1 $\pm$ 0.5		1.7 $\pm$ 0.8		1.6 $\pm$ 0.3	b	1.1 $\pm$ 0.8	
<i>Effects of:</i>								
	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD
Heat stress $\times$ Diet $\times$ Day	0.002	0.607	0.001	0.699	<0.001	0.367	0.012	0.706
Heat stress	0.421	0.341	0.750	0.520	0.906	0.362	0.442	0.381
Diet	0.013	0.033	0.425	0.519	0.763	0.362	0.208	0.378
Day	0.860	0.342	0.001	0.452	0.007	0.346	0.042	0.372

Note: a–d: different lower-case letters within a column indicate significant differences among treatments ( $P < 0.001$ ).

different stressors. Not surprisingly, the most remarkable findings in the present study were related to HSP70 expression, which was up-regulated in the liver, heart and jejunum of broilers fed standard diet and exposed to heat stress for 1-day. After 5 days of challenge, HSP70 was also up-regulated in the ileum. The absence of HSP70 up-regulation in challenged birds fed supplemented diet indicates that these birds were not suffering sufficient stress conditions to activate the HSPs. HMOX was up-regulated in the ileum and down-regulated in the jejunum. It has been shown that HMOX

promotes cell resistance against oxidative stress (He *et al.*, 2001), explaining the greater sensitivity of the jejunum to heat stress than the duodenum and ileum.

Heat stress modulated inflammatory markers in the tissues. IKBA was up-regulated in the heart and liver of broilers fed standard diet and exposed to heat stress for 1-day, and in the jejunum from those birds challenged with 5 days heat stress. Such up-regulation was even higher in the jejunum from birds fed supplemented diet. Up-regulation of IKBA depicts the down-regulation of NF $\kappa$ B, a driver of inflammation, which is

**Table 14.** Mean ( $\pm$  SEM) relative mRNA expression of genes encoding markers for tight junctions (CLDN1, CLDN5, ZO1 and ZO2) in the jejunum.

	CLDN1		CLDN5		ZO1		ZO2	
<b>Day 1</b>								
<i>Normal temperature</i>								
Standard diet	1.0 $\pm$ 0.7	a	1.0 $\pm$ 0.4		1.0 $\pm$ 0.7	a	1.0 $\pm$ 0.7	ab
Supplemented diet	0.3 $\pm$ 0.3	a	0.5 $\pm$ 0.1		0.1 $\pm$ 0.1	a	0.2 $\pm$ 0.1	a
<i>Heat stress</i>								
Standard diet	0.2 $\pm$ 0.1	a	1.3 $\pm$ 0.3		0.1 $\pm$ 0.0	a	0.2 $\pm$ 0.1	a
Supplemented diet	0.8 $\pm$ 0.6	a	0.4 $\pm$ 0.2		0.2 $\pm$ 0.1	a	0.2 $\pm$ 0.0	a
<b>Day 5</b>								
<i>Normal temperature</i>								
Standard diet	1.0 $\pm$ 0.8	a	1.0 $\pm$ 0.7		1.0 $\pm$ 0.5	a	1.0 $\pm$ 0.5	ab
Supplemented diet	7.6 $\pm$ 1.6	c	1.4 $\pm$ 0.3		5.5 $\pm$ 1.7	c	5.1 $\pm$ 1.9	c
<i>Heat stress</i>								
Standard diet	5.4 $\pm$ 1.8	b	1.9 $\pm$ 0.7		2.6 $\pm$ 1.2	b	1.8 $\pm$ 0.6	b
Supplemented diet	0.9 $\pm$ 0.5	a	0.8 $\pm$ 0.6		7.3 $\pm$ 2.1	d	1.0 $\pm$ 0.4	ab
Normal temperature	2.5 $\pm$ 0.9		1.0 $\pm$ 0.4		1.9 $\pm$ 0.8		1.8 $\pm$ 0.8	
Heat stress	1.8 $\pm$ 0.8		1.1 $\pm$ 0.5		2.5 $\pm$ 0.9		0.8 $\pm$ 0.6	
Standard diet	1.9 $\pm$ 0.9		1.3 $\pm$ 0.5		1.2 $\pm$ 0.6		1.0 $\pm$ 0.5	
Supplemented diet	2.4 $\pm$ 0.8		0.8 $\pm$ 0.3		3.3 $\pm$ 1.0		1.6 $\pm$ 0.6	
Day 1	0.6 $\pm$ 0.4	a	0.8 $\pm$ 0.2		0.3 $\pm$ 0.2	a	0.4 $\pm$ 0.2	a
Day 5	3.7 $\pm$ 1.2	b	1.3 $\pm$ 0.6		4.1 $\pm$ 1.0	b	2.2 $\pm$ 0.9	b
<i>Effects of:</i>								
	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD
Heat stress $\times$ Diet $\times$ Day	<0.001	1.282	0.003	0.712	<0.001	1.407	<0.001	0.985
Heat stress	0.310	1.315	0.396	0.379	0.349	1.330	0.014	0.808
Diet	0.449	1.318	0.021	0.357	0.002	1.250	0.143	0.829
Day	<0.001	1.114	0.003	0.359	<0.001	1.036	<0.001	0.728

Note: a–d: different lower-case letters within a column indicate significant differences among treatments ( $P < 0.001$ ).



**Table 15.** Mean ( $\pm$  SEM) relative mRNA expression of genes encoding markers for tight junctions (CLDN1, CLDN5, ZO1 and ZO2) in the ileum.

	CLDN1	CLDN5	ZO1	ZO2
<b>Day 1</b>				
<i>Normal temperature</i>				
Standard diet	1.0 $\pm$ 0.6	1.1 $\pm$ 0.5	1.0 $\pm$ 0.4	1.0 $\pm$ 0.6
Supplemented diet	0.6 $\pm$ 0.2	0.5 $\pm$ 0.2	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1
<i>Heat stress</i>				
Standard diet	0.5 $\pm$ 0.2	1.3 $\pm$ 0.5	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1
Supplemented diet	1.6 $\pm$ 0.5	0.4 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1
<b>Day 5</b>				
<i>Normal temperature</i>				
Standard diet	1.0 $\pm$ 0.5	1.0 $\pm$ 0.2	1.0 $\pm$ 0.5	1.0 $\pm$ 0.6
Supplemented diet	1.6 $\pm$ 0.6	1.7 $\pm$ 0.7	5.5 $\pm$ 1.6	2.1 $\pm$ 0.6
<i>Heat stress</i>				
Standard diet	1.4 $\pm$ 0.6	1.9 $\pm$ 0.8	2.6 $\pm$ 0.9	1.8 $\pm$ 0.8
Supplemented diet	0.9 $\pm$ 0.5	0.8 $\pm$ 0.4	7.2 $\pm$ 1.3	1.0 $\pm$ 0.4
<i>Normal temperature</i>				
Standard diet	1.1 $\pm$ 0.5	1.1 $\pm$ 0.5	1.9 $\pm$ 0.4	1.8 $\pm$ 0.5
Supplemented diet	1.1 $\pm$ 0.5	1.1 $\pm$ 0.5	2.5 $\pm$ 0.8	0.8 $\pm$ 0.4
<i>Heat stress</i>				
Standard diet	1.0 $\pm$ 0.5	1.3 $\pm$ 0.5	1.2 $\pm$ 0.5	1.0 $\pm$ 0.5
Supplemented diet	0.9 $\pm$ 0.5	0.9 $\pm$ 0.4	3.3 $\pm$ 0.8	1.6 $\pm$ 0.3
Day 1	0.7 $\pm$ 0.4	0.8 $\pm$ 0.4	0.4 $\pm$ 0.2	0.4 $\pm$ 0.2
Day 5	1.2 $\pm$ 0.6	1.4 $\pm$ 0.5	4.1 $\pm$ 0.9	2.2 $\pm$ 0.6
<i>Effects of:</i>				
	<i>P</i> -value	LSD	<i>P</i> -value	LSD
Heat stress $\times$ Diet $\times$ Day	0.003	1.720	0.001	0.742
Heat stress	0.603	1.403	0.922	0.376
Diet	0.385	1.399	0.023	0.364
Day	0.001	1.248	0.004	0.357

Note: a–d: different lower-case letters within a column indicate significant differences among treatments ( $P < 0.001$ ).

commonly activated shortly after heat stress (Nivon *et al.*, 2012). It seems that the birds were able to cope with the stress in vital organs such heart and liver during stress, but not in the jejunum after 5 days challenge, where IKBA was up-regulated only when broilers were fed supplemented diet. The supplement tested in the present study is composed of MCFA, organic acids and plant extracts rich in polyphenols, all of them able to suppress NF $\kappa$ B signalling (Sahin *et al.*, 2010; Kim *et al.*, 2014; Carlson *et al.*, 2015; Burt *et al.*, 2016; Ebrahimzadeh *et al.*, 2018), which was confirmed herein by the up-regulation of IKBA, a NF $\kappa$ B suppressor. This is also confirmed by the up-regulation of IKBA in the duodenum of birds fed supplemented diet, regardless of the stress challenge. Heat stress did not lead to the modulation of inflammatory markers in the duodenum and ileum to the same extent as in the jejunum. These findings are in line with the results of the morphological analysis showing that duodenum and ileum were the less sensitive intestinal sections to heat stress. In the jejunum a down-regulation of TLR4 and IL-8 immediately after 1-day heat stress challenge was observed, followed by a decrease in IFN $\gamma$  mRNA expression after 5 days heat stress. This effect was observed in all challenged groups, regardless of the diet provided. TLR4 can be down-regulated after acute heat stress and is indicative of innate immune system disturbances under conditions of stress (Du *et al.*, 2012). Also, as a response to stress, IL-8 enhances cell proliferation mainly in the crypts to stimulate the repair of intestinal injury (Zachrisson *et al.*, 2001). This mechanism probably explains its up-regulation in the ileum and not in the jejunum, the most heat stress-sensitive intestinal section. Chronic heat stress

leads to a decrease in the natural killer and TH1 cells (Nagai & Iriki, 2001), which are the main producers of IFN $\gamma$ , explaining its down-regulation after 5-day heat stress. The inability of the supplemented diet to prevent IFN $\gamma$  down-regulation can be explained by the fact that this blend does not act non-specifically against all inflammatory pathways.

Tight junctions are the first line barrier against the passage of luminal toxic compounds and pathogens into the circulation by preventing intestinal paracellular transport (Ulluwishewa *et al.*, 2011). These junctions are mainly transmembrane protein complexes of claudins (CLDNs) and the cytosolic proteins zonula occludens, e.g. ZO1 and ZO2. One-day of heat stress was not enough to modulate the expression of these tight junctions. However, after 5 days of heat stress, birds fed standard diet presented an up-regulation of CDLN1 and ZO1 in the jejunum. The same up-regulation was observed in the jejunum of birds fed 5 days supplemented diet under normothermic conditions. Once more, these results confirm that the jejunum is the most sensitive intestinal section to heat stress (Santos *et al.*, 2015; Varasteh *et al.*, 2015). This up-regulation is most likely an adaptation/nutritional strategy to avoid intestinal leakage. It is important to note that tight junctions are dynamic structures interacting not only with antigens and pathogens but also with nutrients, ions and water (Ulluwishewa *et al.*, 2011). Therefore, fluctuation in expression and assembly of tight junctions due to stress, as well as feed supplementation, is expected. A decrease in mRNA expression is generally observed under optimal conditions of gut health with low regenerative needs, whereas an increase is indicative for epithelial cell injury and tissue repair.

**Table 16.** Mean ( $\pm$  SEM) relative mRNA expression of genes encoding markers for growth factor (TGF $\beta$ ) and nutrient transporters (PEPT1, SGLT and EAA3) in the duodenum.

	TGF $\beta$		PEPT1		SGLT		EAA3	
<b>Day 1</b>								
<i>Normal temperature</i>								
Standard diet	1.0 $\pm$ 0.6		1.0 $\pm$ 0.4	ab	1.0 $\pm$ 0.7	a	1.0 $\pm$ 0.6	
Supplemented diet	1.4 $\pm$ 0.5		1.0 $\pm$ 0.3	ab	5.3 $\pm$ 1.0	c	2.1 $\pm$ 0.9	
<i>Heat stress</i>								
Standard diet	0.9 $\pm$ 0.2		0.8 $\pm$ 0.4	a	4.5 $\pm$ 1.2	c	2.1 $\pm$ 0.7	
Supplemented diet	1.0 $\pm$ 0.4		2.0 $\pm$ 0.8	b	3.0 $\pm$ 0.9	b	1.5 $\pm$ 0.3	
<b>Day 5</b>								
<i>Normal temperature</i>								
Standard diet	1.3 $\pm$ 0.7		1.0 $\pm$ 0.5	ab	1.1 $\pm$ 0.4	a	1.0 $\pm$ 0.2	
Supplemented diet	0.7 $\pm$ 0.4		1.4 $\pm$ 0.6	ab	1.7 $\pm$ 0.6	a	1.5 $\pm$ 0.5	
<i>Heat stress</i>								
Standard diet	0.9 $\pm$ 0.4		6.1 $\pm$ 2.2	c	1.6 $\pm$ 0.4	a	2.9 $\pm$ 0.8	
Supplemented diet	2.0 $\pm$ 1.2		0.8 $\pm$ 0.5	a	1.0 $\pm$ 0.2	a	2.2 $\pm$ 0.4	
<i>Normal temperature</i>								
Standard diet	1.1 $\pm$ 0.6		1.1 $\pm$ 0.5		2.3 $\pm$ 0.7		1.4 $\pm$ 0.6	
Heat stress	1.2 $\pm$ 0.6		2.4 $\pm$ 1.0		2.5 $\pm$ 0.7		2.1 $\pm$ 0.6	
<i>Standard diet</i>								
Standard diet	1.0 $\pm$ 0.5		2.2 $\pm$ 0.9		2.1 $\pm$ 0.7		1.7 $\pm$ 0.6	
<i>Supplemented diet</i>								
Supplemented diet	1.3 $\pm$ 0.6		1.3 $\pm$ 0.5		2.8 $\pm$ 0.7		1.8 $\pm$ 0.5	
<i>Day 1</i>								
Day 1	1.1 $\pm$ 0.4		1.2 $\pm$ 0.5		3.5 $\pm$ 1.0	b	1.7 $\pm$ 0.6	
<i>Day 5</i>								
Day 5	1.2 $\pm$ 0.7		2.3 $\pm$ 1.0		1.3 $\pm$ 0.4	a	1.9 $\pm$ 0.5	
<i>Effects of:</i>								
	<i>P-value</i>	<i>LSD</i>	<i>P-value</i>	<i>LSD</i>	<i>P-value</i>	<i>LSD</i>	<i>P-value</i>	<i>LSD</i>
Heat stress $\times$ Diet $\times$ Day	0.009	0.686	<0.001	1.138	<0.001	0.865	0.010	0.810
Heat stress	0.479	0.373	0.004	0.885	0.545	0.830	0.087	0.416
Diet	0.197	0.370	0.046	0.910	0.088	0.816	0.840	0.450
Day	0.387	0.372	0.017	0.900	<0.001	0.679	0.215	0.445

Note: a–c: different lower-case letters within a column indicate significant differences among treatments ( $P < 0.001$ ).

Not only the intestinal barrier is affected by heat stress, but its effect on growth factors and transporters of glucose, amino acids and peptides should be considered. Transport of di- and tri-peptides from the intestinal lumen into the enterocytes is performed by the peptide transporter 1 (PEPT1), which is most highly expressed in the duodenum, but also by the jejunum and ileum (Gilbert *et al.*, 2007). Under feed restriction, PEPT1 is up regulated as means to uptake any available nutrient in the intestinal lumen (Madsen & Wong, 2011). PEPT1 down-regulation in the jejunum, on the

other hand, may indicate the inability of the jejunum to adapt to heat stress with the same efficiency as the duodenum. The up-regulation of the amino acid transporter in the jejunum, therefore, appears as an alternative to cover the PEPT1 down-regulation. Glucose is absorbed across the apical sodium-dependent glucose transporter 1 (SGLT) system, which was also assessed by mRNA expression. Garriga *et al.* (2006) previously reported the increase in apical glucose transport as an adaptation to heat stress. In the present study, not only one episode of heat stress increased SGLT

**Table 17.** Mean ( $\pm$  SEM) relative mRNA expression of genes encoding markers for growth factor (TGF $\beta$ ) and nutrient transporters (PEPT1, SGLT and EAA3) in the jejunum.

	TGF $\beta$		PEPT1		SGLT		EAA3	
<b>Day 1</b>								
<i>Normal temperature</i>								
Standard diet	1.0 $\pm$ 0.6		1.0 $\pm$ 0.1	bc	1.0 $\pm$ 0.5		1.1 $\pm$ 0.6	a
Supplemented diet	1.1 $\pm$ 0.8		0.5 $\pm$ 0.2	ab	1.8 $\pm$ 1.2		2.6 $\pm$ 0.9	bc
<i>Heat stress</i>								
Standard diet	0.5 $\pm$ 0.4		0.2 $\pm$ 0.1	a	0.6 $\pm$ 0.1		3.1 $\pm$ 0.5	c
Supplemented diet	0.4 $\pm$ 0.3		0.5 $\pm$ 0.2	ab	0.9 $\pm$ 0.5		1.5 $\pm$ 0.4	ab
<b>Day 5</b>								
<i>Normal temperature</i>								
Standard diet	1.0 $\pm$ 0.2		0.9 $\pm$ 0.3	bc	1.0 $\pm$ 0.3		1.0 $\pm$ 0.1	a
Supplemented diet	1.5 $\pm$ 0.6		1.1 $\pm$ 0.3	c	1.6 $\pm$ 0.8		1.5 $\pm$ 0.3	ab
<i>Heat stress</i>								
Standard diet	1.4 $\pm$ 0.8		2.0 $\pm$ 0.3	c	0.6 $\pm$ 0.4		2.9 $\pm$ 0.7	bc
Supplemented diet	1.1 $\pm$ 0.4		1.3 $\pm$ 0.2	c	0.7 $\pm$ 0.5		2.1 $\pm$ 0.9	b
<i>Normal temperature</i>								
Standard diet	1.2 $\pm$ 0.6		0.9 $\pm$ 0.2		1.4 $\pm$ 0.7		1.6 $\pm$ 0.5	
<i>Heat stress</i>								
Standard diet	0.9 $\pm$ 0.5		1.0 $\pm$ 0.2		0.7 $\pm$ 0.4		2.4 $\pm$ 0.6	
<i>Standard diet</i>								
Standard diet	1.0 $\pm$ 0.5		1.0 $\pm$ 0.2		0.8 $\pm$ 0.3		2.0 $\pm$ 0.6	
<i>Supplemented diet</i>								
Supplemented diet	1.0 $\pm$ 0.5		0.9 $\pm$ 0.2		1.3 $\pm$ 0.8		2.0 $\pm$ 0.6	
<i>Day 1</i>								
Day 1	0.8 $\pm$ 0.5		0.6 $\pm$ 0.2	a	1.1 $\pm$ 0.6		2.1 $\pm$ 0.6	
<i>Day 5</i>								
Day 5	1.2 $\pm$ 0.5		1.4 $\pm$ 0.3	b	1.0 $\pm$ 0.5		1.9 $\pm$ 0.5	
<i>Effects of:</i>								
	<i>P-value</i>	<i>LSD</i>	<i>P-value</i>	<i>LSD</i>	<i>P-value</i>	<i>LSD</i>	<i>P-value</i>	<i>LSD</i>
Heat stress $\times$ Diet $\times$ Day	0.002	0.397	<0.001	0.668	0.001	0.441	<0.001	0.901
Heat stress	0.002	0.171	0.468	0.366	0.001	0.166	0.028	0.515
Diet	0.704	0.182	0.385	0.365	0.014	0.195	0.186	0.526
Day	0.001	0.219	<0.001	0.319	0.455	0.221	0.088	0.522

Note: a–c: different lower-case letters differ significantly among treatments within the columns ( $P < 0.001$ ).

**Table 18.** Mean ( $\pm$  SEM) relative mRNA expression of genes encoding markers for growth factor (TGF $\beta$ ) and nutrient transporters (PEPT1, SGLT and EAA3) in the ileum.

	TGF $\beta$	PEPT1	SGLT	EAA3
<b>Day 1</b>				
<i>Normal temperature</i>				
Standard diet	1.0 $\pm$ 0.6	1.0 $\pm$ 0.5	1.0 $\pm$ 0.5	1.0 $\pm$ 0.5
Supplemented diet	1.1 $\pm$ 0.6	0.7 $\pm$ 0.3	0.9 $\pm$ 0.4	1.3 $\pm$ 0.9
<i>Heat stress</i>				
Standard diet	1.1 $\pm$ 0.8	1.2 $\pm$ 0.7	0.8 $\pm$ 0.3	1.0 $\pm$ 0.3
Supplemented diet	1.0 $\pm$ 0.5	1.0 $\pm$ 0.4	0.7 $\pm$ 0.3	0.7 $\pm$ 0.4
<b>Day 5</b>				
<i>Normal temperature</i>				
Standard diet	1.0 $\pm$ 0.6	1.0 $\pm$ 0.5	1.0 $\pm$ 0.4	1.0 $\pm$ 0.5
Supplemented diet	0.9 $\pm$ 0.7	0.4 $\pm$ 0.2	0.8 $\pm$ 0.4	1.5 $\pm$ 0.9
<i>Heat stress</i>				
Standard diet	2.1 $\pm$ 1.4	1.1 $\pm$ 0.8	1.2 $\pm$ 0.8	1.5 $\pm$ 0.8
Supplemented diet	1.2 $\pm$ 0.7	1.1 $\pm$ 0.8	0.7 $\pm$ 0.4	1.7 $\pm$ 0.7
<i>Normal temperature</i>				
Standard diet	1.0 $\pm$ 0.6	0.8 $\pm$ 0.4	0.9 $\pm$ 0.4	1.2 $\pm$ 0.7
Heat stress	1.3 $\pm$ 0.9	1.1 $\pm$ 0.7	0.8 $\pm$ 0.5	1.2 $\pm$ 0.6
Standard diet	1.3 $\pm$ 0.9	1.1 $\pm$ 0.7	1.0 $\pm$ 0.5	1.1 $\pm$ 0.5
Supplemented diet	1.0 $\pm$ 0.6	0.8 $\pm$ 0.4	0.8 $\pm$ 0.4	1.3 $\pm$ 0.7
Day 1	1.0 $\pm$ 0.6	1.0 $\pm$ 0.5	0.8 $\pm$ 0.4	1.0 $\pm$ 0.5
Day 5	1.3 $\pm$ 0.9	0.9 $\pm$ 0.5	0.9 $\pm$ 0.5	1.4 $\pm$ 0.7
<i>Effects of:</i>				
	<i>P-value</i>	<i>LSD</i>	<i>P-value</i>	<i>LSD</i>
Heat stress $\times$ Diet $\times$ Day	0.085	0.784	0.001	0.362
Heat stress	0.110	0.402	0.001	0.192
Diet	0.194	0.405	0.007	0.196
Day	0.188	0.405	0.524	0.206
				0.516
				0.215
				0.038
				0.409

expression in the duodenum, but also when broilers were fed a supplemented diet under thermoneutral conditions. This is also in line with a previous study showing an increased SGLT mRNA expression in rats fed a diet rich in medium-chain triacylglycerols (Yasutake *et al.*, 1995). However, its interaction with MCFA remains to be understood.

In conclusion, our results indicate that duodenum, jejunum and ileum respond negatively to heat stress, but the jejunum remains the most sensitive intestinal section. Although morphological adaptation in the intestines is observed, a transient functional impairment and redox imbalance will certainly affect bird production and resilience. Feed management and the application of targeted feed additives appears to mitigate, or at least alleviate, functional alterations caused by heat stress. Feed enriched with a blend of MCFA, organic acids and a plant polyphenol has proven to be efficient in preventing heat stress-induced disturbances in redox balance, villus morphology and morphometry, as well as improving the integrity of tight junctions and nutrient transporters.

## ORCID

Regiane R. Santos  <http://orcid.org/0000-0001-7030-6097>

## Disclosure statement

Presan<sup>®</sup> is a product of Nutreco; AA, PR, TvK, and CS are or were employees of Nutreco; others report no conflict of interest.

## References

Aengwanich, W. & Suttajit, M. (2013). Effect of polyphenols extracted from tamarind (*Tamarindus indica* L) seed coat

- on pathophysiological changes and red blood cell glutathione peroxidase activity in heat stressed broilers. *International Journal of Biometeorology*, 57, 137–143.
- Akbarian, A., Michiels, J., Degroote, J., Majdeddin, M., Golian, A. & De Smet, S. (2016). Association between heat stress and oxidative stress in poultry; mitochondrial dysfunction and dietary interventions with phytochemicals. *Journal of Animal Science and Biotechnology*, 7, 37.
- Baltic, B., Starcevic, M., Dordevic, J., Mrdovic, B. & Markovic, R. (2017). Importance of medium chain fatty acids in animal nutrition. IOP Conf. Ser. In. *Earth Environmental Science*, 85, 012048.
- Burt, S.A., Adolfse, S.J.M., Ahad, D.S.A., Tersteeg-Zijderfeld, M.H.G., Post, J.A., Bruggemann, H. & Santos, R.R. (2016). Cinnamaldehyde, carvacrol and organic acids affect gene expression of selected oxidative stress and inflammation markers in IPEC-J2 cells exposed to *Salmonella typhimurium*. *Phytotherapy Research*, 30, 1988–2000.
- Carlson, S.J., Nandivada, P., Chang, M.I., Mitchell, P.D., O'Loughlin, A., Cowan, E., Gura, K.M., Nose, V., Bistriani, B.R. & Puder, M. (2015). The addition of medium-chain triglycerides to a purified fish oil-based diet alters inflammatory profiles in mice. *Metabolism*, 64, 274–282.
- Dokladny, K., Moseley, P.L. & Ma, T.Y. (2006). Physiologically relevant increase in temperature causes an increase in intestinal epithelial tight junction permeability. *American Journal of Physiology, Gastrointestinal, and Liver Physiology*, 290, G204–G212.
- Dong, S., Li, H., Gasco, L., Xiong, Y., Guo, K.J. & Zoccarato, I. (2015). Antioxidative activity of the polyphenols from the involucre of *Castanea mollissima* Blume and their mitigating effects on heat stress. *Poultry Science*, 94, 1096–1104.
- Du, Q., Min, S., Chen, L.Y., Ma, Y.D., Guo, X.L., Wang, Z. & Wang, Z.G. (2012). Major stress hormones suppress the response of macrophages through down-regulation of TLR2 and TLR4. *Journal of Surgical Research*, 173, 354–361.
- Ebrahimzadeh, S.K., Navidshad, B., Farhoomand, P. & Aghjehgheshlagh, F.M. (2018). Effects of grape pomace

- and vitamin E on performance, antioxidant status, immune response, gut morphology and histopathological responses in broiler chickens. *South African Journal of Animal Science*, 48, 324–336.
- Filosa, S., Fico, A., Paglialonga, F., Balestrieri, M., Crooke, A., Verde, P., Abrescia, P., Bautista, J.M. & Martini, G. (2003). Failure to increase glucose consumption through the pentose-phosphate pathway results in the death of glucose-6-phosphate dehydrogenase gene-deleted mouse embryonic stem cells subjected to oxidative stress. *Biochemistry Journal*, 370, 935–943.
- Garriga, C., Hunter, R.R., Amat, C., Planas, J.M., Mitchell, M.A. & Moretó, M. (2006). Heat stress increases apical glucose transport in the chicken jejunum. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 290, R195–R201.
- Gilbert, E.R., Li, H., Emmerson, D.A., Webb Jr K.E. & Wong, E.A. (2007). Developmental regulation of nutrient transporter and enzyme mRNA abundance in the small intestine of broilers. *Poultry Science*, 86, 1739–1753.
- Gu, X.H., Hao, Y. & Wang, X.L. (2012). Overexpression of heat shock protein 70 and its relationship to intestine under acute heat stress in broilers: 2. intestinal oxidative stress. *Poultry Science*, 91, 790–799.
- Havenstein, G.B., Ferket, P.R., & Qureshi, M.A. (2003). Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. *Poultry Science*, 82, 1500–1508.
- He, C.H., Gong, P., Hu, B., Stewart, D., Choi, M.E., Choi, A.M. & Alam, J. (2001). Identification of activating transcription factor 4 (ATF4) as a Nrf2 interacting protein: Implication for heme oxygenase-1 gene regulation. *Journal of Biology and Chemistry*, 276, 20858–20865.
- Incharoen, T. (2013). Histological adaptations of the gastrointestinal tract of broilers fed diets containing insoluble fiber from rice hull meal. *American Journal of Animal and Veterinary Sciences*, 8, 79–88.
- Kim, H.J., Yoon, H.J., Kim, S.Y. & Yoon, Y.R. (2014). A medium-chain fatty acid, capric acid, inhibits RANKL-induced osteoclast differentiation via the suppression of NFkB signaling and blocks cytoskeletal organization and survival in mature osteoclasts. *Molecular Cell*, 37, 598–604.
- Lara, L.J. & Rostagno M.H. (2013). Impact of heat stress on poultry production. *Animal*, 3, 356–369.
- Lorda-Diez, C.I., Montero, J.A., Garcia-Porrero, J.A. & Hurlle, J.M. (2010). Tgfb2 and 3 are coexpressed with their extracellular regulator *Ltbp1* in the early limb bud and modulate mesodermal outgrowth and BMP signaling in chicken embryos. *BMC Developmental Biology*, 10, 69.
- Madsen, S.L. & Wong, E.A. (2011). Expression of the chicken peptide transporter 1 and the peroxisome proliferator-activated receptor  $\alpha$  following feed restriction and subsequent refeeding. *Poultry Science*, 90, 2295–2300.
- Marder, J. & Arad, Z. (1989). Panting and acid-base regulation in heat stressed birds. *Comparative Biochemistry & Physiology Part A: Molecular and Integrative Physiology*, 94, 395–400.
- Mott, C.R., Siegel, P.B., Webb Jr K.E. & Wong, E.A. (2008). Gene expression of nutrient transporters in the small intestine of chickens from lines divergently selected for high or low juvenile body weight. *Poultry Science*, 87, 2215–2224.
- Nagai, M. & Iriki, M. (2001). Changes in immune activities by heat stress. *Thermotherapy for Neoplasia, Inflammation and Pain*, 1, 266–270.
- Nivon, M., Abou-Samra, M., Richet, E., Guyot, B., Arrigo, A.P. & Kretz-Remy, C. (2012). NF-kB regulates protein quality control after heat stress through modulation of the BAG3-HspB8 complex. *Journal of Cell Science*, 125, 1141–1151.
- Osselaere, A., Santos, R., Hautekiet, V., De Backer, P., Chiers, K., Ducatelle, R. & Croubels, S. (2013). Deoxynivalenol impairs hepatic and intestinal gene expression of selected oxidative stress, tight junction and inflammation proteins in broiler chickens, but addition of an adsorbing agent shifts the effects to the distal parts of the small intestine. *PLoS One*, 8, e69014.
- Parsell, D.A. & Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annual Review of Genetics*, 27, 437–496.
- Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, 29, e45.
- Quaedackers, J.S., Beuk, R.J., Bennet, L., Charlton, A., Oude Egbrink, M.G., Gunn, A.J. & Heineman, E. (2000). An evaluation of methods for grading histologic injury following ischemia/reperfusion of the small bowel. *Transplant Proceedings*, 32, 1307–1310.
- Rivero, R.M., Ruiz, J.M., Garcia, P.C., Lopez-Lefebvre, L.R., Sanchez, E. & Romero, L. (2001). Resistance to cold and heat stress: accumulation of phenolic compounds in tomato and watermelon plants. *Plant Science*, 160, 315–321.
- Ruijter, J.M., Ramakers, C., Hoogaars, W.M., Karlen, Y., Bakker, O., Van den Hoff, M.J. & Moorman, A.F. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative qPCR. *Nucleic Acids Research*, 37, e45.
- Sahin, K., Orhan, C., Tuzcu, M., Ali, S., Sahin, N. & Hayirli, A. (2010). Epigallocatechin-3-gallate prevents lipid peroxidation and enhances antioxidant defense system via modulating hepatic nuclear transcription factors in heat stressed quails. *Poultry Science*, 89, 2251–2258.
- Santos, R.R., Awati, A., Roubos-van den Hil, P., Tersteeg-Zijderveld, M.H.G., Koolmees, P.A. & Fink-Gremmels, J. (2015). Quantitative histo-morphometric analysis of heat stress related damage in the small intestines of broiler chickens. *Avian Pathology*, 44, 19–22.
- Takada, M., Otaka, M., Takahashi, T., Izumi, Y., Tamaki, K., Shibuya, T., Sakamoto, N., Osada, T., Yamamoto, S., Ishida, R., Odashima, M., Itoh, H. & Watanabe, S. (2010). Overexpression of a 60-kDa heat shock protein enhances cytoprotective function of small intestinal epithelial cells. *Life Science*, 86, 499–504.
- Tan, D.W. & Barker, N. (2014). Intestinal stem cells and their defining niche. *Current Topics in Developmental Biology*, 107, 77–107.
- Tanizawa, H., Shiraiishi, J., Tsudzuki, M. & Bungo, T. (2014). Effect of short-term thermal conditioning on physiological and behavioral responses to subsequent acute heat exposure in chicks. *Journal of Poultry Science*, 51, 80–86.
- Ulluwishewa, D., Anderson, R.C., McNabb, W.C., Moughan, P.J., Wells, J.M. & Roy, N.C. (2011). Regulation of tight junction permeability by intestinal bacteria and dietary components. *Journal of Nutrition*, 141, 769–776.
- Varasteh, S., Braber, S., Akbari, P., Garssen, J. & Fink-Gremmels, J. (2015). Differences in susceptibility to heat stress along the chicken intestine and the protective effects of galacto oligosaccharides. *PLoS One*, 10, e0138975.

- Xiaofang, H., Lu, Z., Ma, B., Zhang, L., Li, J., Jiang, Y., Zhou, G. & Gao, F. (2018). Chronic heat stress damages small intestinal epithelium associated with AMPK pathway in broilers. *Journal of Agriculture and Food Chemistry*, 66, 7301–7309.
- Yasutake, H., Goda, T. & Takase, S. (1995). Dietary regulation of sucrase-isomaltase gene expression in rat jejunum. *Biochimica et Biophysica Acta*, 1243, 270–276.
- Zachrisson, K., Neopikhanov, V., Wretling, B. & Uribe, A. (2001). Mitogenic action of tumour necrosis factor- $\alpha$  and interleukin-8 on explants of human duodenal mucosa. *Cytokine*, 15, 148–155.
- Zhang, J., Cao, M., Yang, W., Sun, F., Xu, C., Yin, L. & Pu, Y. (2016). Inhibition of glucose-6-phosphate dehydrogenase could enhance 1,4-benzoquinone-induced oxidative damage in K562 cells. *Oxidative Medicine and Cellular Longevity*, 2016, 3912515.