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Impact of heat stress length and dietary antioxidant supplementation on the nutrient digestibility, metabolism and immune response of fattening pigs

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ABSTRACT

Study aimed to investigate the impact of long-term high ambient temperature (HAT) exposure and dietary antioxidant supplementation (elevated levels and in combination) on nutrient digestibility, metabolism and immune response of pigs. A total of 36 Danbred barrows (65.1 ± 2.81 kg) were allotted to four treatments: Trm1: HAT (28.9 ± 0.9 °C) + control diet (CD); Trm2: HAT + elevated vitamin C and E and Se and Zn; Trm3: HAT + further elevated vitamin C and E and Se and Zn; and Trm4: thermo-neutral ambient temperature (19.5 ± 0.9 °C) + CD. Nutrients (DM, CP, Cfat, CF, CA, GE) and minerals (Ca, P, Na, Zn, Se) digestibility were determined. Plasma metabolite and cytokine concentrations were investigated from the blood samples collected on d 15 and 28. Heat shock proteins (*HSP 70 and 90*) and tumor necrosis factor-alpha (*TNF-α*) expressions were investigated from jejunum samples of pigs. HAT did not significantly affect pigs' nutrient digestibility and retention ($P > 0.05$). However, Trm2 pigs had greater digestibility in terms of DM, CF, Ca, Zn, Se and retention of Ca, Zn, Na, and Se than Trm1 and Trm4 groups. Trm3 pigs had significantly higher ($P < 0.05$) creatinine concentrations than Trm4 pigs. HAT did not significantly affect the expression of cytokines; however, vitamins and micro-minerals supplementation in the diet significantly ($P < 0.05$) improved interleukin (*IL*) 10 expression, reduced *TNF-α*, and *HSP70* expressions. Vitamin and micro-mineral fortified diet can improve pigs' nutrient and mineral digestibility and could alleviate inflammatory response in pigs exposed to HAT.

1. Introduction

Exposure of livestock to high ambient temperature (HAT) can lead to heat stress (HS), which is a global issue constraining animal agriculture productivity and is a severe threat to domesticated livestock species (Mayorga et al., 2018a; Thornton et al., 2021). Pigs are one of the most vulnerable livestock species to the adverse effects of HS, and many essential attributes of its productivity are negatively affected by this stressor (Babinszky et al., 2011; Babinszky et al., 2019; da Fonseca de Oliveira et al., 2019). In response to HS, pigs increase their peripheral blood flow to facilitate heat-dissipation. Consequently, it can lead to intestinal hypoxia and oxidative stress (OS) at the intestinal epithelium. Exposure of pigs to HAT damages the tips of their intestinal villi and causes shorter jejunum villi height and crypt depth, compromising intestinal integrity and function (Dong et al., 2012; Lambert, 2009; Lan and Kim, 2018; Pearce et al., 2012; Yan et al., 2006; Yu et al., 2010). The impairment of the pigs' gastrointestinal tract can allow the translocation of endotoxins into the blood, which in turn instigate an inflammatory response and production of cytokines (interleukin 1β (*IL-1β*)

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and tumor necrosis factor-alpha (*TNF-α*)) (Cui et al., 2019; Ganesan et al., 2016). Also, the pigs' capacity to digest and retain nutrients from the diet can be negatively affected. Studies have reported that pigs' exposure to HAT have led to an increase in protein excretion and decreased protein and mineral retention (Ferguson and Gous, 2002; Patience et al., 2005). Moreover, HAT is believed to induce-metabolic changes by altering the concentration of plasma metabolites, which is considered to take part in the altered carcass phenotype of heat-stressed pigs (Cui et al., 2019; Pearce et al., 2013).

Although, research reported that HAT can be detrimental to pig performance, several studies also reported that the duration of the pigs' exposure to HAT could influence the susceptibility and adaptability of pigs to HS consequences (Pearce et al., 2014; Renaudeau et al., 2008), and some dietary antioxidants (vitamins and micro-minerals) are capable of alleviating HS adverse effects. Vitamins and micro-minerals are potential nutritional tools to mitigate the HS adverse effects. These substances can neutralize the excess reactive oxygen species (ROS) produced during HS-induced OS and protect cells against the toxic effects of free radicals (Lobo et al., 2010; Pham-Huy et al., 2008). Several studies reported that vitamin E and micro-mineral Se and Zn supplementation could alleviate the HS adverse effects on pigs' intestinal integrity and function (Liu et al., 2016; Pearce et al., 2015a). Also, Vitamins E and C can suppress pro-inflammatory cytokines, and regulate inflammatory response (Lauridsen et al., 2021; Lewis et al., 2019). Moreover, improvements in the plasma metabolites of heat-stressed pigs were observed upon supplementation of micro-minerals (Se and Zn) (Liu et al., 2018; Pearce et al., 2015a). These researches shows the individual influence of the aforementioned vitamins and micro-minerals on specific parameters in heat-stressed pigs, hence, the combined supplementation of vitamins and micro-minerals might influence the over-all status of heat-stressed pigs. Limited information is available on the effect of the length of chronic HAT exposure on the nutrient digestibility and retention, immune response, and plasma metabolites of pigs, respectively, and whether elevated levels of vitamins (C and E) and micro-minerals (Se and Zn) in combination can improve the aforementioned parameters. Therefore, this research aims to study the impact of long-term and different duration of HAT exposure on the nutrient digestibility, metabolism, and immune response of fattening pigs and whether supplementing their diet with elevated levels of antioxidants in combination (vitamins C and E and micro-minerals Se and Zn) can help mitigate its adverse effects.

2. Materials and methods

2.1. Animal diet, arrangement, and management

Thirty-six Danbred hybrid barrows having an average weight of 65.1 ± 2.81 kg were used in the study conducted at the Institute for Agricultural Research and Educational Farm, Animal Husbandry Experimental Station, University of Debrecen, Kismacs, Debrecen, Hungary. The pigs were housed in groups of three on concrete floor pens with wood shavings as bedding (three pens per treatment (3 replication), a total of 36 animals). Throughout the trial, pigs were allowed ad libitum access to feed and water. Prior to the experimental period, all pigs experienced a seven-day adaptation period to their pens, fed ad libitum (with basal feed), and housed in a thermo-neutral ambient temperature (TNE) (19.3 ± 1 °C, RH- 93 ± 2.9 %). Afterward, the temperature of the thermo-neutral room, which housed nine pigs (three pens), was maintained at 19.5 ± 0.9 °C, RH- 85.9 ± 7.3 % with a heat stress index (HSI) (Mutua et al., 2020) of"normal" throughout the experiment. Meanwhile, the temperature of the HAT room was gradually raised to 30 °C for seven days (heat increment period, day 8–14, HI), and the main period of the experiment commenced, which lasted 14 days (15 to 28 days of the trial) with an average temperature of 28.9 ± 0.9 °C and RH of 60.4 ± 4.3 %, with a HSI of "danger to emergency" (Fig. 1).

The temperature and relative humidity were monitored daily using TESTO 174H (Testo SE & Co. KGaA, Lenzkirch, Germany) data logger. One data logger was used for every three pen (one in TN and three in HAT room). This equipment recorded the temperature and humidity in every minute 24 h.

A corn-soybean meal diet (basal feed) was formulated for 75 to 100 kg pigs having 155 g mean protein deposition per day (Table 1 and Table 2) in accordance to the National Research Council (NRC, 2012) recommendation. Two additional dietary treatments (elevated diet 1 (ED1) and elevated diet 2 (ED2)) were formulated by providing vitamins C and E, and micro-minerals Se and Zn (elevated levels), as

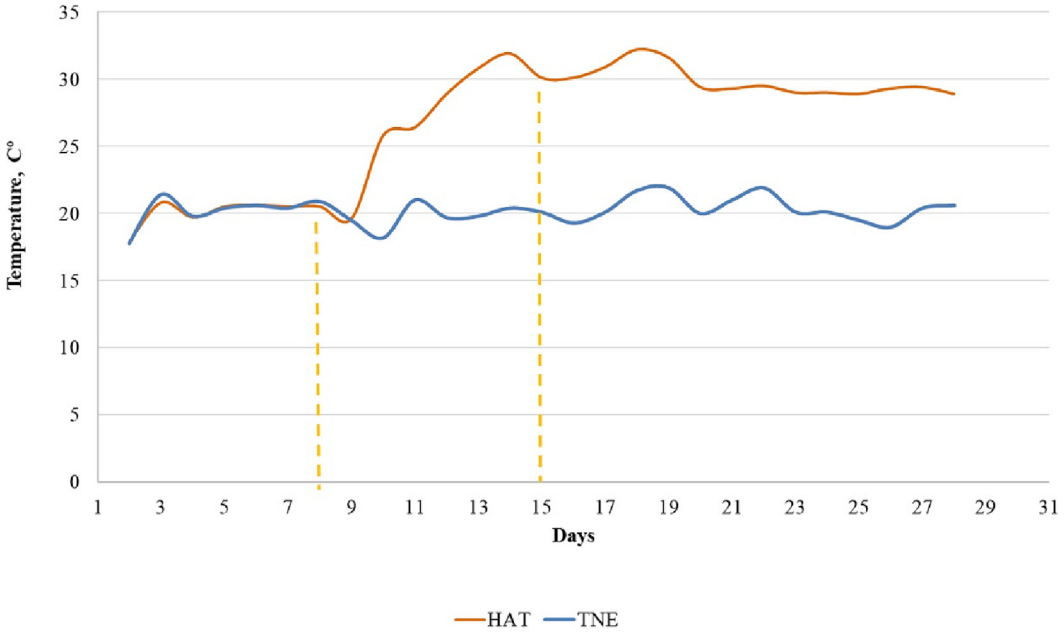

Fig. 1. Temperature of high ambient temperature (HAT) and thermo-neutral ambient temperature (TNE) rooms throughout the study. Day 1–7 Adaptation period; Day 8–14 Gradual temperature increase in HAT room and introduction to test diets; Day 15–28 Experimental period.

Table 1
Composition and calculated nutrient content of basal feed.[a]

Ingredients	Inclusion rate (%)	Energy and nutrients	Calculated value
Corn	78.68	Digestible energy, MJ/kg	14.24
Soybean meal	16.33	Crude protein[b], %	12.07
Plant oil	2.11	SID[c] Lys, %	0.78
Limestone	0.92	SID Met+Cys, %	0.45
MCP	0.80	SID Thr, %	0.49
L-Lysine	0.30	SID Trp, %	0.14
DL-Methionine	0.01	Ca[b], %	0.54
L-Tryptophan	0.03	digestible P, %	0.23
L-Threonine	0.06	Na[b], %	0.14
Salt	0.26		
Vit. and mineral premix	0.50		

[a] NRC (2012) recommendation for 75–100 kg live weight pigs having 155 g mean protein deposition per day.
[b] Analyzed values.
[c] Standardized ileal digestible.

shown in Table 3. The pigs were distributed among four treatment groups, which consisted of a combination of environmental and dietary treatments: Trm1: HAT + CD: high ambient temperature (28.9 ± 0.9 °C, RH- 60.4 ± 4.3 %) + control diet; Trm2: HAT + ED1 (elevated diet 1: elevated vitamin C and E and Se and Zn content), Trm3: HAT + ED2 (further elevated vitamin C and E and Se and Zn content), and Trm4: TNE+ CD: thermo-neutral ambient temperature (19.5 ± 0.9 °C, RH- 85.9 ± 7.3 %) and control diet.

2.2. Body temperature measurement

Skin and rectal temperature were measured with an infrared and digital thermometer, respectively, on the days of blood sampling (but before on resting animals). The former was measured on the following body parts: ear base, middle of the ear, and back of the pig.

2.3. Digestibility trial: sample collection, measurement and analysis

The evaluation was performed for two weeks during the main experimental period, with weekly changes of pigs in the digestibility cages (three for week one – one from each pen, and three for week two – another one from each pen). Both weeks consisted of two days of adaptation to the cage and five days of collection. Feed allowances were calculated based on the voluntary feed intake of the remaining counterparts in the pens, relative to their body weight. The feed allowance was divided into two equal portion and fed twice a day (8:00 and 16:00) with double amount of water. Feces, urine, and feed residue were collected daily from the metabolic cage collection tray and container and the feeding trough, pooled by cage, frozen at −20 °C, and sampled. Feed and feces samples were analyzed for dry matter (ISO 6496), crude ash (ISO 5984), crude protein (CP) by Kjeldahl method (ISO 5983-2), crude fat (Cfat) using petroleum ether extraction (ISO 6942), crude fiber (CF) with boiling samples alternating sulphuric acid and potassium hydroxide (ISO 6865). Calcium (Ca), phosphorus (P), sodium (Na), zinc (Zn), analysis were carried out after 1.0000 g samples were digested in a block digester (LABOR MIM, Budapest, Hungary) with 10 mL cc. nitric acid at 60 °C for 30 min and 3 mL of 30 % hydrogen peroxide alt. (Sigma-Aldrich, Saint Louis, MI, USA) at 90 min at 120 °C. For selenium (Se) analysis, 0.5000 g sample was measured into high pressure digestion bombs with 5 mL cc. nitric acid and 3 mL of 30 % hydrogen peroxide (Sigma-Aldrich, Saint Louis, MI, USA). The digestion was processed in microwave digester (ETHOS Plus, Milestone) applying digestion program suggested by the manufacturer (Application Note 076: 3 min at 85 °C; 9 min at 145 °C; 4 min at 200 °C; 14 min at 200 °C). All digested samples were filled to 50 mL with distilled water and filtered through MN640W (155 mm; Macherey-Nagel) filter paper. The analysis was carried out with the ICP-OES technique (iCAP 7000, Thermo Scientific Kandell, Germany). Multielement standard solution was applied from monoelement standards (for Ca, Na, P, Zn from VWR, Leuven, Belgium, for Se from Thermo Scientific, Kandell, Germany). The following wavelengths were tested and applied in the concentration measurement: Ca-393.366 nm; Na-589.592 nm; P-177.495 nm; Zn-202.548 nm; Se-196.090 nm.

Table 2
Guaranteed nutrient content of the pre-mixture (in 1 kg of premix).[a]

Nutrient	Inclusion rate	Composition
Zn	mg/kg	9999
Cu	mg/kg	1454
Fe	mg/kg	7281
Mn	mg/kg	9999
I	mg/kg	136
Se	mg/kg	32
Vitamin A	IU/kg	410,000
Vitamin D-3	IU/kg	82,000
Vitamin E	mg/kg	2205
Vitamin K-3	mg/kg	82
Vitamin B-1	mg/kg	62
Vitamin B-2	mg/kg	205
Ca-d-pantothenate	mg/kg	492
Vitamin B-6	mg/kg	164
Vitamin B-12	mg/kg	1
Biotin	mg/kg	5
Niacin	mg/kg	1026
Folate	mg/kg	25
Choline chloride	mg/kg	60,000

[a] At or above NRC (2012).

Table 3
Basal dietary treatments (supplementation mg/kg).

Nutrient	Control diet[a]	Elevated 1[b]	Elevated 2[c]
Vitamin C[d]	0	150	300
Vitamin E[e]	11	41	71
Zn[f]	50	100	150
Se[g]	0.16	0.21	0.26

[a] NRC (2012), supplemented to Trm1 and Trm4 treatment groups,
[b] Supplemented to Trm2 treatment group.
[c] Supplemented to Trm3 treatment group.
[d] L-ascorbic acid (ROVIMIX® Stay-C, DSM, Heerlen, Netherlands).
[e] α-Tocopheryl acetate (ROVIMIX® E50, DSM, Heerlen, Netherlands).
[f] Zinc chelate of amino acid hydrate (Vevomin Zn, DSM, Heerlen, Netherlands).
[g] L-selenomethionine (Excential Se, ORFFA, Werkendam, Netherlands).

2.4. Plasma biochemical parameter: blood collection and analysis

On the first and last day of the experimental period (15th and 28th days of the trial) blood samples were collected from the external jugular vein of the pigs into EDTA tubes. The collected blood samples were then centrifuged at 4 °C for 15 min at 3000 ×*g* after clotted for 20 min (Xin et al., 2018). The separated plasma samples were then stored at −80 °C, for later analysis. Plasma concentrations of glucose, uric acid, urea, and creatinine were analyzed through the photometric method using Lab-Analyse (Orvostechnika Ltd., Budapest, Hungary) half-automatic analyzer. The analysis of the plasma samples was performed in triplicate. Glucose and uric acid reagents and distilled water (urea and creatinine) were used as a blank before measuring every sample in the Lab-Analyse kit (Orvostechnika Ltd., Budapest, Hungary).

2.5. Cytokines and heat shock proteins expression

2.5.1. Sample collection

At the end of the trial, whole blood was collected from the external jugular vein of the pigs. White blood cells were separated from the whole blood (following the method of Sipos et al., 2004) to measure

interleukins (*IL-1β*, *IL-10*). On the first and second day after the experiment, six pigs from each treatment were slaughtered (three in one day from each treatment) after electrical stunning, and tissue samples of jejunum were collected. Jejunum samples were then cleaned in PBS, then snap-frozen in liquid nitrogen to measure heat shock proteins (*HSP 70* and *90*) and tumor necrosis factor-alpha (*TNF-α*).

2.5.2. RNA isolation and reverse transcription

At Addition of 500 μL TRI Reagent™ (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to white blood cell samples was done before RNA isolation which was carried out with Direct-zol™ RNA MiniPrep (Zymo Research, Orange, CA, USA) following the manufacturers' protocol and with the inclusion of DNase treatment. Total RNA from jejunum was extracted using peqGOLD Total RNA kit (C-line) (VWR International, LLC., Radnor, Pennsylvania, USA) following the manufacturers' protocol. RNA integrity was checked with 1 % agarose gel electrophoresis. RNA concentration and purity were determined by HTX Synergy Microplate Reader (Agilent BioTek, Agilent Technologies INC., Santa Clara, USA). RNA was reverse-transcribed into cDNA with qScript® cDNA SuperMix (Quantabio, Beverly, USA) in a 20 μL final volume containing 5× cDNA supermix (including MMLV-type reverse transcriptase, MgCl2, dNTPs, oligo (d)T primer, random primers, recombinant RNase inhibitor protein, stabilizers), 600 ng RNA template and distilled water. The conditions consisted of reverse transcription at 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min.

2.5.3. qPCR analysis of cytokines and heat shock proteins

Intron-spanning forward and reverse primers for pig *ACTB*, *HMBS*, *HPRT*, *HSP70*, *HSP90*, *IL-1β*, *IL-10*, and *TNF-α* were designed by Oligo 7 software and checked for target identity using National Center for Biotechnology Information (NCBI) Primer Blast (Ye et al., 2012). *ACTB*, *HMBS*, and 18S ribosomal RNA (*HPRT*) housekeeping genes were analyzed by three algorithms (ΔCt, BestKeeper, NormFinder). In white blood cells, *HPRT*, in jejunum *HMBS* were considered as the most stable reference genes for normalization. Quantitative PCR was performed by AriaMx Real-Time PCR System (Agilent Technologies INC., Santa Clara, USA), and reactions were run in triplicates using 96-well plates (4titude, Surrey, UK). Each reaction included a three ng cDNA template, 2× Xceed qPCR SG Hi-ROX Mix (Institute of Applied Biotechnologies, Prague, Czech Republic), 200 nM of each primer, and distilled water in 10 μL final volume. No template controls were included for each primer. Real-time PCR conditions were the following: initial denaturation at 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 5 s, and annealing/extension at 60 °C for 30 s.

Ct values and melting temperatures were collected with Aria Mx 1.7 software. Results were determined according to the method of Livak and Schmittgen (2001) by normalizing the expression of the target gene to a housekeeping gene. Results were determined as fold changes in the expression of the target gene in the experimental groups compared with the thermo-neutral control (Trm4).

2.6. Statistical analysis

All data were statistically analyzed using GraphPad Prism 8.4.3 software (GraphPad Software Incorporated, San Diego, CA, USA) with one-way analysis of variance (ANOVA) for digestibility, retention, cytokines and HSPs data and two way variance analyses for body temperatures data. Plasma biochemical parameters were analyzed with repeated measurements. Group mean differences were separated by Tukey test. Differences among the treatments were considered significant when $P < 0.05$.

3. Results

3.1. Rectal and skin temperature of pigs

Long-term exposure to HAT and high vitamin and micro-mineral supplementation did not significantly affect ($P > 0.05$) the rectal temperature of pigs (Fig. 2). However, significant difference ($P < 0.05$) was observed in their skin temperature (measured from the ear base, middle of the ear, and the back). All treatment groups exposed to HAT had higher temperature measurements on the ear base and middle than in the Trm4 group. In regards to the temperature measured on the pigs' back, Trm2 and Trm3 groups had a significantly better ($P < 0.05$) temperature measurement as compared to Trm1. Nevertheless, pigs in Trm4 had much better temperature measurements than those treatment groups exposed to HAT.

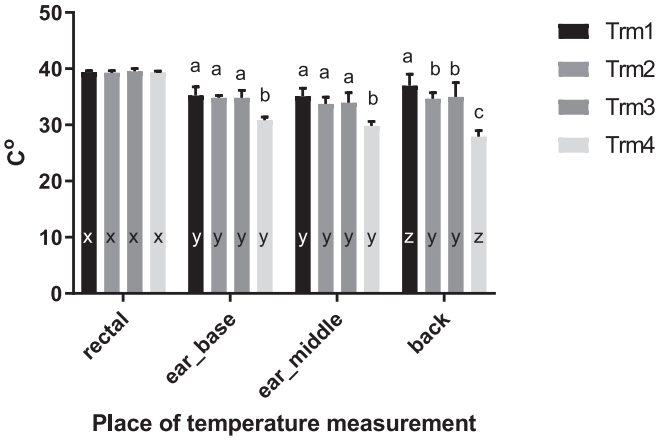

Fig. 2. Impact of high ambient temperature and dietary antioxidant supplementation on the rectal and skin temperature of fattening pigs after two weeks of heat exposure (n = 9/treatment/measurement place). Values are means, with their standard deviation represented by vertical bars;
[a,b,c] Means with the same letters within a measurement place between treatments are not significantly different ($P > 0.05$);
[x,y,z] Means with the same letters within a treatment between measurement places are not significantly different ($P > 0.05$). Trm1: HAT + CD: high ambient temperature + control diet; Trm2: HAT + ED1 (elevated vitamin C and E and Se and Zn content); Trm3: HAT + ED2 (further elevated vitamin C and E and Se and Zn content); and Trm4: TNE + CD: thermo-neutral ambient temperature + control diet.

3.2. Dry matter and nutrients digestibility

HAT did not significantly affect ($P > 0.05$) the dry matter and total tract digestibility of pigs. However, vitamin and micro-mineral supplementation improve pigs' DM and CP digestibility under HAT. Pigs in Trm2 had a significantly higher DM and CP digestibility ($P < 0.05$) as compared to pigs in Trm4 groups (Table 4). Moreover, the crude fiber and crude ash digestibility of heat-stressed pigs supplemented with vitamins and micro-minerals (Trm2 and Trm3) was significantly higher ($P < 0.05$) than those in the Trm4 and Trm1 group. Nevertheless, no significant differences were observed in the groups' digestibility of crude fat, energy, and crude ash ($P > 0.05$).

3.3. Macro and micro-mineral digestibility

Interestingly, pigs in Trm4 and Trm1 groups have similar mineral digestibility ($P > 0.05$). P and Na digestibility were not affected by HAT and supplementation of vitamins and micro-minerals at elevated levels. However, significantly higher digestibility of Ca ($P = 0.0166$), Zn, and Se ($P < 0.0001$) was observed in pigs fed elevated levels of vitamins and micro-minerals (Trm2 and Trm3) than in pigs fed a control diet (Trm1 and Trm4), despite being exposed to HAT (Table 4).

3.4. Nutrients and macro and micro-mineral digestibility and retention

CP retention of pigs was not significantly affected ($P > 0.05$) by HAT and vitamin and micro-mineral supplementation (Table 4). However, significant differences were observed in the retention of Ca, ($P < 0.0197$) Na ($P < 0.0063$), Zn and Se ($P < 0.0001$). Wherein pigs

Table 4
The impact of long-term heat exposure and high vitamin and micro-mineral supplementation in the total tract digestibility and retention coefficients of nutrients and minerals in fattening pigs ($n = 6$/treatment).

Parameters	Treatment				SEM	*P* value
	Trm1	Trm2	Trm3	Trm4		
Digestibility of nutrients, %						
Dry matter	91.43^{ab}	93.13^{a}	92.33^{ab}	91.02^{b}	0.30	0.0471
Crude protein	85.87^{ab}	89.38^{a}	87.27^{ab}	85.17^{b}	0.59	0.0442
Crude fat	92.22	92.55	92.25	91.17	0.25	0.2134
Crude fiber	63.77^{a}	74.98^{b}	74.72^{b}	61.52^{a}	1.80	0.0022
Crude ash	50.90	61.72	59.38	50.13	1.83	0.0394
Gross energy	91.38	93.03	92.60	90.88	0.32	0.0453
Digestibility of minerals, %						
Calcium	88.13^{ab}	91.40^{a}	89.57^{ab}	87.02^{b}	0.55	0.0166
Phosphorus	89.27	92.27	91.47	88.78	0.53	0.0435
Sodium	89.88	92.87	92.93	89.67	0.52	0.0173
Zinc	71.13^{a}	87.93^{b}	87.97^{b}	70.88^{a}	2.01	<0.0001
Selenium	63.77^{a}	79.80^{b}	84.98^{b}	61.57^{a}	2.39	<0.0001
Retention, %						
Crude protein	48.55	59.87	55.20	55.70	2.00	0.2577
Calcium	85.73^{a}	89.60^{b}	87.77^{ab}	85.30^{a}	0.59	0.0197
Phosphorus	76.05	82.53	81.98	79.20	0.98	0.0623
Sodium	80.17^{a}	85.55^{b}	85.43^{b}	81.57^{ab}	0.73	0.0063
Zinc	35.45^{a}	73.28^{b}	70.68^{b}	39.48^{a}	4.26	<0.0001
Selenium	37.22^{a}	63.95^{b}	72.13^{b}	37.37^{a}	3.63	<0.0001

Trm1: HAT + CD: high ambient temperature + control diet; Trm2: HAT + ED1 (elevated vitamin C and E and Se and Zn content); Trm3: HAT + ED2 (further elevated vitamin C and E and Se and Zn content); and Trm4: TNE + CD: thermo-neutral ambient temperature + control diet.
^{a,b} means in a row with the same superscripts do not differ ($P > 0.05$).

fed elevated levels of vitamins and micro-minerals (Trm2 and Trm3) showed significantly better performance. Among the various minerals, only P was not significantly affected.

3.5. Plasma concentration

The plasma concentration of glucose, uric acid, and urea was not significantly affected by HAT and vitamin and micro-mineral (vitamin E, C, Se, and Zn) supplementation. Nevertheless, the plasma concentration of creatinine in both time points was significantly ($P = 0.0092$) increased in pigs housed in HAT and fed with ED2 diet (Trm3) than in pigs in the Trm4 group. Furthermore, there was a significant day of sampling effect observed in the case of urea ($P = 0.0044$) and creatinine ($P = 0.0058$) (Table 5).

3.6. Cytokine and heat shock protein expression

HAT did not significantly affect ($P > 0.05$) the expression of cytokines and *HSP* in pigs (Fig. 3). However, supplementation of vitamins and micro-minerals at ED1 level significantly improves ($P = 0.0488$) the expression of *IL-10* and significantly reduces the expression of *TNF-α* in Trm2 ($P = 0.0345$) and Trm3 ($P = 0.0434$) pigs compared to Trm1 group. The mRNA expression of *HSP 70* was also significantly reduced ($P = 0.0487$) in Trm3 pigs compared to Trm4 pigs. Nevertheless, the expression of *IL-1β* and *HSP 90* in all groups was similar ($P > 0.05$).

4. Discussion

4.1. Effects of HAT and vitamin and micro-mineral supplementation on pigs' rectal and skin temperature

Pigs' exposure to high ambient temperature can cause an increase in body heat, as proven with elevated rectal and skin temperature (Cottrell et al., 2020; Qu et al., 2016; Xin et al., 2018). However, the results in our study about the rectal temperature of pigs suggest otherwise. Similar observation was also reported by Manno et al. (2006), were pigs exposed to chronic HS (32 °C for 35 days) had comparable rectal temperature with pigs under thermal comfort. In several studies involving HS in pigs, the average daily feed intake (ADFI) can be negatively affected as HAT exposure can force the animal to reduce ADFI to reduce metabolic heat production (Campos et al., 2014; Xia et al., 2022). However, we did not observe significant differences in this parameter. Nevertheless, among the treatment groups TN pigs had the highest live weight gain and feed intake, and the lowest feed conversion ratio. HS reduced feed intake by almost 300 g, but dietary treatments were not able to mitigate that effect. The reduction in daily gain and feed conversion ratio was partially mitigated by the dietary treatments (Ortega et al., 2022). HAT and vitamin and micro-mineral supplementation did not significantly affect the rectal temperature of pigs. The similar rectal temperature observed between pigs in Trm1 and Trm4 group might be due to the animals' acclimation to the said stressor. It was reported that pigs' can respond with gradual rectal temperature improvement upon

Table 5
Impact of heat exposure and vitamin and micro-mineral supplementation on the plasma biochemical parameters of fattening pigs (ls means, n = 9/treatment/different time points).

Parameters	Treatment				SEM	*P* values		
	Trm1	Trm2	Trm3	Trm4		Day	Treatment	D × T
Glucose, mmol/L						0.3995	0.3914	0.3081
15th day	4.76	5.71	5.60	6.26	0.27			
28th day	5.14	5.03	5.78	5.49	0.21			
Uric acid, μmol/L						0.7454	0.4888	0.3637
15th day	25.12	33.95	33.30	36.15	2.09			
28th day	34.79	27.79	33.77	36.22	2.29			
Urea, mmol/L						0.0044	0.4137	0.5667
15th day	4.36	4.91	4.81^{x}	4.84	0.17			
28th day	5.16	5.33	6.36^{y}	5.63	0.27			
Creatinine, μmol/L						0.0058	0.0092	0.4314
15th day	133.3$^{ab\ x}$	138.2^{ab}	158.2^{a}	123.6^{b}	4.08			
28th day	161.1$^{ab\ y}$	146.7^{ab}	165.6^{a}	136.2^{b}	4.43			

Trm1: HAT + CD: high ambient temperature + control diet; Trm2: HAT + ED1 (elevated vitamin C and E and Se and Zn content); Trm3: HAT + ED2 (further elevated vitamin C and E and Se and Zn content); and Trm4: TNE + CD: thermo-neutral ambient temperature + control diet.
^{a,b} means in a row with the same superscripts do not differ ($P > 0.05$).
^{x,y} means in a column with the same superscripts do not differ ($P > 0.05$).
15th day – last day of the heat increment period and the 1st day of the main experimental period.
28th day – last day of the main experimental period.
D × T – day of blood collection and treatment interaction.

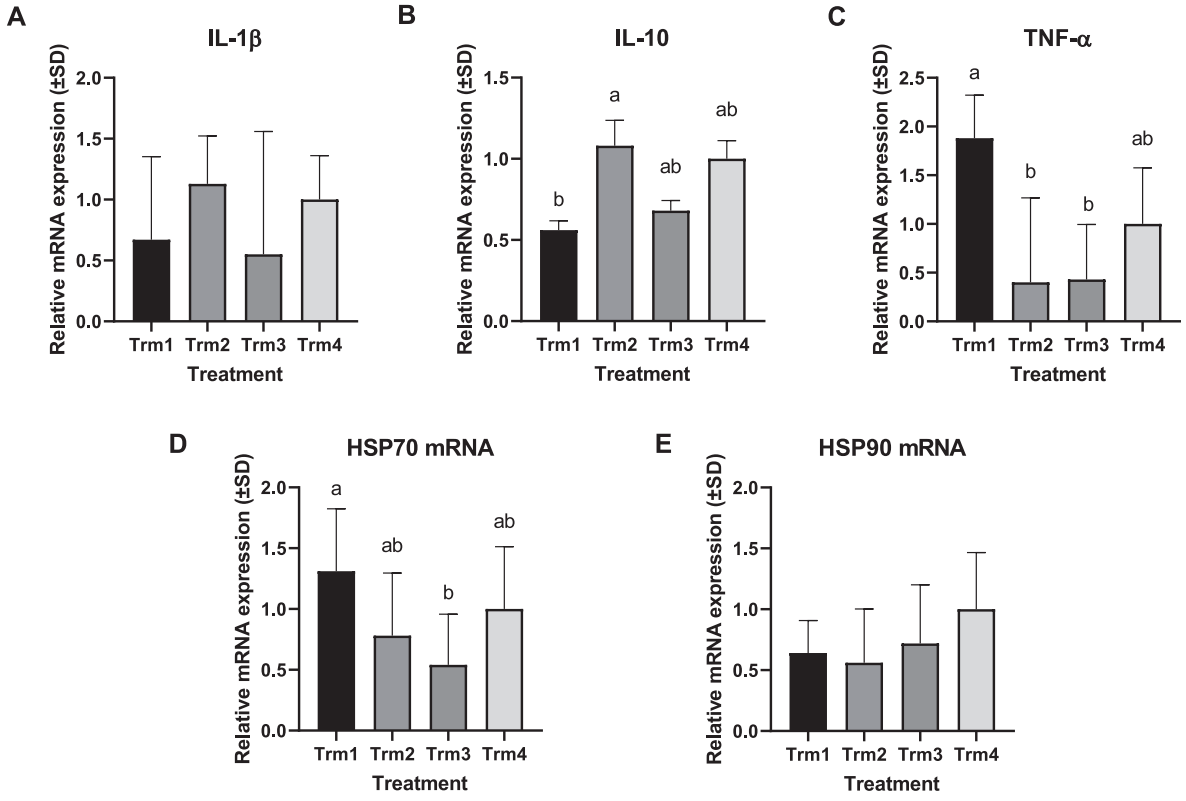

Fig. 3. The expressions of *IL-1β* (A), *IL-10* (B) in blood, *TNF-α* (C), *HSP 70* (D), and *HSP 90* (E) in the jejunum of pigs under thermo-neutral and high ambient temperature environment condition and supplemented with elevated levels of dietary antioxidants ($n = 6$/treatment). Values are means, with their standard deviation represented by vertical bars; [a,b] Means with the same letters do not differ ($P > 0.05$). Trm1: HAT + CD: high ambient temperature + control diet; Trm2: HAT + ED1 (elevated vitamin C and E and Se and Zn content); Trm3 HA: T + ED2 (further elevated vitamin C and E and Se and Zn content); and Trm4: TNE+ CD: thermo-neutral ambient temperature + control diet.

exposure to chronic HS (7 days) (Waltz et al., 2014). Nevertheless, HAT causes a significant increase ($P < 0.05$) in the pigs' skin temperature (measured from the base and middle of the ear and at the pigs' back). Such increase was alleviated by the supplementation of vitamin and micro-minerals (Trm2 and Trm3), which measurements are significantly lower ($P < 0.05$) than in the Trm1 group of pigs (measured at the pigs' back). Reduction of skin temperature in heat-stressed pigs upon supplementation of dietary antioxidant (zinc amino acid complex) was also reported by Mayorga et al. (2018b), suggesting the antioxidants' capability in lowering the body temperature of pigs exposed to HAT.

4.2. Nutrient and macro and micro-mineral digestibility and retention of pigs kept in HAT and supplemented with vitamins and micro-minerals

The nutrient and mineral digestibility and retention of pigs were not significantly affected ($P > 0.05$) by HAT. Our observation indicates that HAT conditions in this study have no significant impact on the gastrointestinal digestibility function of the pigs. Our findings contradict the observation of Brestensky et al. (2012), and Hao et al. (2014), which reported that digestibility of nitrogen, gross energy, DM, CP, and crude ash were reduced in heat-stressed pigs. However, it agrees with the report of Kim et al. (2020), wherein pigs reared under HS (33 °C) had comparable nutrient and mineral digestibility to pigs under thermal comfort (25 °C). Moreover, Renaudeau et al. (2008) observed a significant increase in DM digestibility of pigs over a long period of HS despite having a relatively lower feed intake. Although, it was reported that HS negatively affects the nutrient digestibility of pigs by impairing intestinal integrity and function (Pearce et al., 2015b). Most of these cases were observed after acute HS (24 h), where digestive capacity alteration and post-absorptive metabolism are prominent (Pearce et al., 2012). The comparable values observed in Trm1 and Trm4 groups might be due to the adaptability of pigs to HS when acclimatized. From a physiological standpoint, pigs' negative response to HS is more prominent during the first 2 to 3 days (Liu et al., 2009; Pearce et al., 2014; Yu et al., 2010) and might decrease as animals become acclimated over time. Renaudeau et al. (2010) reported that pigs under constant HS for 20 days had elevated rectal temperature in the first 24 h of exposure, but it was gradually reduced with the successive days of exposure. In addition, Vásquez et al. (2022) reported that the impairment of the pigs' intestinal integrity due to HS is realized upon 2 days of exposure and can gradually recover after the 7th day of exposure. This indicates that pigs can become partially acclimated within a week or weeks of exposure to HS (Morales et al., 2016a).

Morales et al. (2016b) reported that pigs under HS had lower CP digestibility than pigs in thermal comfort. In contrast, Renaudeau et al. (2008) and Campos et al. (2014) reported that pigs' exposure to HS (32 °C) showed an increase in N digestibility, despite having significantly lower average daily gain, and its digestibility is greater than in pigs under thermal comfort. Although, there were contradicting results from previous studies, our observation agrees with what was reported by Liao and Veum (1994), that faecal excretion and digestibility of proteins remained unchanged in pigs under constant high temperatures as opposed to pigs under TN conditions. Previous studies revealed that pigs' exposure to HAT (30–33 °C) could lead to low retention of N and protein (Brestensky et al., 2012; Renaudeau et al., 2013). However, results in our study revealed that both Trm1 and Trm4 groups have similar retention of protein and minerals. Our finding is similar to the observation of Patience et al. (2005), where they reported no difference in protein and mineral retention between pigs housed in diurnal HS (20–38 °C within 24 h). Similarly, no significant differences were observed in the mineral retention between Trm1 and Trm4 groups ($P > 0.05$).

Therefore, the experimental HAT had no significant impact on nutrient digestibility and retention in the pigs in this trial. Aside from the pigs' capability of acclimation to HAT, the pigs in our study did not respond with severe tissue damage as *TNF-α* expression of Trm1 and Trm4 pigs were comparable. Similar observation was reported by Wen et al. (2019) in pigs under long-term exposure to HS. While increased in the expression of *TNF-α* has been associated with damaged intestinal barrier functions (Abuajamieh et al., 2018), our observation indicates that there is only low incidence of intestinal barrier damage, supporting our results.

Liu et al. (2016) demonstrated that the combination of selenium and vitamin E at high levels reduced oxidative stress and intestinal leakiness, and Zn supplementation improved the intestinal integrity of pigs leading to better digestibility of nutrients under HS (Pearce et al., 2015a). It was hypothesized that supplementing pigs under HS with dietary antioxidants (vitamin E and C, Se and Zn) would mitigate against HS-induced OS. Our findings confirmed this hypothesis as our results indicate that despite being subjected to HS condition, pigs fed ED1 and ED2 diets (Trm2 and Trm3) have better mean performance in terms of DM, nutrients, and mineral digestibility and retention. The effects of supplementation with dietary antioxidants (vitamin and micro-minerals) can be observed in DM and CP digestibility between Trm2 and Trm4groups. DM and CP digestibility were significantly higher in Trm2 than in Trm4 ($P < 0.05$). Moreover, CF digestibility was significantly higher in Trm2 and Trm3 than in Trm1 and Trm4 groups. The digestibility and retention of minerals were also influenced by dietary antioxidants' supplementation and were observed in Ca, Zn, and Se (digestibility) and Ca, Na, Zn, and Se (retention). Dietary antioxidant supplementation at ED1 and ED2 levels significantly improved ($P < 0.05$) the digestibility and retention of these minerals, which can be supported by the fact that dietary antioxidants (vitamins C and E and minerals Se and Zn) can alleviate HS-induced damage in the intestinal epithelial cells (Tang et al., 2019) and can improve their intestinal barrier integrity and function (Liu et al., 2016; Pearce et al., 2015a; Sanz Fernandez et al., 2014).

4.3. Plasma concentration of pigs under HAT and vitamin and micro-mineral supplementation

HS experienced by pigs upon exposure to HAT can influence their metabolism as it can cause alteration in the plasma concentration of metabolic compounds (Sanz Fernandez et al., 2015). In our study, HAT and supplementation of vitamins (C and E) and micro-minerals (Se and Zn) above the recommended levels did not significantly affect the plasma concentration of glucose, uric acid, and urea of pigs. In previous related studies, Pearce et al. (2013); Cui et al. (2019) observed a high reduction of plasma glucose concentration after HS exposure of 7 and 21 days. While supplementation of ZnAA complex + zinc sulfate (60 + 60 mg/kg of diet) and high levels of selenium (1.0 ppm), respectively, had positively improved the plasma glucose concentration in finishing pigs (Liu et al., 2018; Pearce et al., 2015a). In our study, we observed similarly; however, the reduction and increase in the plasma glucose levels of pigs upon HS challenge and supplementation of dietary antioxidants, respectively, is not enough to reach a statistical level. Similar to our observation, the plasma concentration of urea (slight decrease in heat-stressed pigs) was also reported by Kim et al. (2020), Santos et al. (2018), and Shi et al. (2016), and. Although contradicting the reports of Mayorga et al. (2018b), Pearce et al. (2015a), and Xiong et al. (2020), where the said plasma concentrations were elevated upon HS challenge, our observed decrease was not at the level of significance. However, the plasma concentration of creatinine was significantly affected ($P < 0.05$) by HAT and supplementation of dietary antioxidants at ED2 levels observed in the 15th day of the trial. Trm3 pigs had higher plasma creatinine concentrations than Trm4 pigs. This observation is interesting as it was previously reported that HS influences the synthesis of creatinine and increases its plasma concentration in pigs (Pearce et al., 2013). Moreover, the level of plasma creatinine in pigs in all groups was slightly elevated at the 28th day of the trial. This might be associated with the age of the animals, as a similar observation was reported by Mendoza et al. (2017) upon measuring creatinine levels of pigs under thermal comfort and HS on days 3 and 28 of the experiment. Although the creatinine level was increased on day 28th, all treatment groups had similar concentrations, which agreed with the result observed by Oliveira et al. (2018) on finishing pigs.

4.4. Cytokine and heat shock protein expression

Chronic heat stress can have a detrimental impact on the animal immune system through cell mediated and humoral immune responses (Al-Zghoul et al., 2019; Bagath et al., 2019; Huo et al., 2019). Such exposure can result in severe immune disorders in pigs (Chauhan et al., 2021; Huo et al., 2019). These days, only a few studies focus on the immune responses of pigs, especially the cellular immune functions of the intestine (Huo et al., 2019). During cellular immune responses, cytokines act as an extracellular signal between cells (Kaiser and Staheli, 2008). *IL-1β* and *TNF-α* are involved in pro-inflammatory responses (Johnson et al., 2020). *IL-1β* has a role in inflammatory reactions and activates T-cells and macrophages (Corwin, 2000; Klasing, 1988; Lotz et al., 1988). In this study, the mRNA level of *IL-1β* was not changed among treatments. In contrast, Lan et al. (2019) reported increased *IL-1β* concentration in the liver of rats during cyclical heat stress (for 4 h per 7 days), while chitosan oligosaccharides decreased the level of the pro-inflammatory cytokine, so the inflammation was alleviated. *TNF-α* is a cytokine that inhibits activated immune cells during inflammation and can be applied as an indirect immune index for detecting immune functions (Kim et al., 2004). It also activates the NF-κB pathway, which is vital for regulating the inflammatory factors (Ghosh et al., 1998; Li et al., 2013). Gene expression level of *TNF-α* was not altered in the Trm1 group compared to the Trm4 pigs. Liu et al. (2016) measured the same when gene expression levels of *TNF-α* along with *IL-8* have not increased in heat-stressed (8 h for 2 days) pigs, and the authors discussed it might indicate that inflammation was not a significant factor. However, *TNF-α* mRNA expression level was higher in the Trm1 group than in the Trm2 and Trm3group in our study, suggesting that the applied treatments can decrease the level of *TNF-α* and so the inflammationduring heat stress conditions or HAT conditions. In contrast to this, the mRNA level of *TNF-α* was not altered when increased concentrations of Se and Vitamin-E were supplemented in heat-stressed pigs (Liu et al., 2016). *IL-10* is an anti-inflammatory cytokine and has a role in inhibiting apoptosis (Kaiser and Staheli, 2008; Hakimi et al., 2014; Kinzenbaw et al., 2013). Relative gene expression levels of *IL-10* were not altered among Trm4, Trm1, and Trm3 groups. Nevertheless, the mRNA level of the mentioned anti-inflammatory cytokine could increase upon the supplementation of ED1 diet (Trm2) which is significantly higher than Trm1 in this study. Similarly, the concentration of *IL-10* was higher in heat-stressed (for 4 h and 7 days) rats when chitosan oligosaccharides were applied as a treatment, and inflammation could be inhibited (Lan et al., 2019).

Besides cytokines, HSP are also remarkable indicators of alterations in immunity (Williams and Ireland, 2008). HSP are a family of proteins expressed in response to heat and other stressors and are significant for cell survival during stress conditions. Watanabe et al. (2004) said that, HSP can be classified into four families based on their molecular weight. Such classification includes: *small HSP*, *HSP60, HSP70,* and *HSP90*. Among them, *HSP70* protects the cells from stress damage and inhibits cellular proteins' irreversible aggregation (Gan et al., 2013). *HSP90* is vital to normal cellular functions and helps the cellular adaptation to stress (Zhang et al., 2011). HSP are part of the third of the three-level antioxidant defense system and have a role in repairing damaged macromolecular segments and restoring the structure of proteins with defective conformations (Irshad and Chaudhuri, 2002; Horváth and Babinszky, 2019). In this study, the gene expression level of *HSP70*

was not changed among the Trm4, Trm1, and Trm2 groups. However, the mRNA level of *HSP70* was elevated in the pigs housed in HAT (Trm1) compared to Trm3. As a result of Trm3 treatment, the mentioned heat shock protein gene expression level could decrease ($P = 0.0487$). Gan et al. (2013) defined the same, and different treatments (inorganic and selenium-enriched probiotics as organic selenium) could result in lower *HSP70* mRNA levels in the spleen, liver, and kidney of heat-stressed pigs (8 h for 42 days). Authors discussed this lower expression of the mentioned HSP may be due to the increased tissue Se level by the inorganic form, but organic Se was suggested to be even more beneficial during heat stress conditions. Yu et al. (2010) reported that the gene expression level of *HSP70* was significantly higher in pigs after heat exposure for 5 h during 10 days and discussed as heat stress conditions rapidly enhance the denaturation and mis-aggregation of proteins, which triggers *HSPs* expression as a response (Keller et al., 2008; Sonna et al., 2002; Young et al., 2009). In contrast to our results, Liu et al. (2016) defined a higher gene expression level of *HSP70* in heat-stressed pigs; however, antioxidant supplementation (elevated Se and Vitamin-E) did not result in the decreased level of the mentioned heat shock protein. In this study, none of the treatments influenced the mRNA level of *HSP90*. In contrast, Yu et al. (2010) defined upregulated gene expression level of *HSP90* after the heat exposure, and the authors explained that these changes in gene expression are a major part of the cellular mechanisms during heat stress, which can control protein translation and so cellular functions. Morales et al. (2014) also reported increased mRNA expression of *HSP90* in pigs exposed to heat stress for 20 days compared to the thermoneutral group. Cervantes et al. (2016) defined the same, and gene expression level of *HSP90* was higher in the duodenum and *longissimus dorsi* of 21-day-heat-stressed pigs compared to the thermoneutral ones, and authors discussed that *HSP90* might be involved in their long term acclimation mechanism.

5. Conclusions

The pigs used in this trial did not respond with impaired nutrient digestibility and retention, altered plasma metabolites, and cytokines and HSP expression to HAT exposure. However, increasing the dietary antioxidant level of feed resulted in higher digestibility and/or retention in DM, CP, CF, Ca, Na, Zn, and Se. The supplementation also increased the gene expression level of anti-inflammatory *IL-10* cytokine, and both of the supplemented diets (ED1 and ED2) could decrease the mRNA level of pro-inflammatory *TNF-α*, and the highest vitamin and mineral contents in the diet (ED2) lowered the mRNA expression of *HSP70*. From this the following conclusions can be drawn:

1. Pigs can be resilient to HAT adverse effects on nutrient and mineral digestibility, metabolism and expressions of cytokines and HSP;
2. High levels of vitamin and micro-mineral supplementation in the diet could improve the nutrient and mineral digestibility of pigs kept at HAT;
3. High levels of vitamin and micro-mineral supplementation may alleviate inflammatory response and could mitigate cell damage during pigs' exposure to HAT conditions;
4. Therefore, regulating the levels of dietary antioxidants in the diet of pigs upon HAT challenge is important.

Animal ethics

The University of Debrecen Animal Care Committee (Debrecen, Hungary – 9/2019/DEMÁB) reviewed and approved all the experimental procedures.

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CRediT authorship contribution statement

L.B. and C.S. prepared the research proposal to obtain the research funding, L.B., C.S. and A.D.S.V.O. designed the experiment and prepared the experimental protocol and the request for ethical approval; L.B., C.S. and J.O. was responsible to providing the technological background; A.D.S.V.O., X.E.O., O.H.O., L.C., J.O. and B.C. carried out the trial, the measurements and various samplings; A.D.S.V.O., X.E.O., L.C. and B.C. carried out the laboratory analyses; A.D.S.V.O. and O.H.O. prepared data tables and carried out the calculations; C.S. performed the statistical analyses, A.D.S.V.O. and C.S. prepared the first draft; review and editing were done by L.B., L.C., X.E.O., A.D.S.V.O. and C.S.. All authors have read and agreed to the final version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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