



Heat stress develops with increased total-tract gut permeability, and dietary organic acid and pure botanical supplementation partly restores lactation performance in Holstein dairy cows

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ABSTRACT

To evaluate the effects of heat stress (HS) conditions and dietary organic acid and pure botanical (OA/PB) supplementation on gut permeability and milk production, we enrolled 46 multiparous Holstein cows [208 ± 4.65 dry matter intake (DMI; mean ± SD), 3.0 ± 0.42 lactation, 122 ± 4.92 d pregnant, and 39.2 ± 0.26 kg of milk yield] in a study with a completely randomized design. Cows were assigned to 1 of 4 groups: thermoneutral conditions (TN-Con, n = 12), HS conditions (HS-Con, n = 12), thermoneutral conditions pair-fed to HS-Con (TN-PF, n = 12), or HS supplemented with OA/PB [75 mg/kg of body weight (BW); 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride; HS-OAPB, n = 10]. Supplements were delivered twice daily by top-dress; all cows not supplemented with OA/PB received an equivalent amount of the triglyceride used for microencapsulation of the OA/PB supplement as a top-dress. Cows were maintained in thermoneutrality [temperature-humidity index (THI) = 68] during a 7-d acclimation and covariate period. Thereafter, cows remained in thermoneutral conditions or were moved to HS conditions (THI: diurnal change 74 to 82) for 14 d. Cows were milked twice daily. Clinical assessments and BW were recorded, blood was sampled, and gastrointestinal permeability measurements were repeatedly evaluated. The mixed model included fixed effects of treatment, time, and their interaction. Rectal and skin temperatures and respiration rates were greater in HS-Con and HS-OAPB relative to TN-Con. Dry matter intake, water intake, and yields of energy-corrected milk (ECM), protein, and lactose were lower in HS-Con relative to HS-OAPB.

Nitrogen efficiency was improved in HS-OAPB relative to HS-Con. Compared with TN-Con and TN-PF, milk yield and ECM were lower in HS-Con cows. Total-tract gastrointestinal permeability measured at d 3 of treatment was greater in HS-Con relative to TN-Con or TN-PF. Plasma total fatty acid concentrations were reduced, whereas insulin concentrations were increased in HS-Con relative to TN-PF. We conclude that exposure to a heat-stress environment increases total-tract gastrointestinal permeability. This study highlights important mechanisms that might account for milk production losses caused by heat stress, independent of changes in DMI. Our observations also suggest that dietary supplementation of OA/PB is a means to partly restore ECM production and improve nitrogen efficiency in dairy cattle experiencing heat stress.

Key words: heat stress, leaky gut, organic acid

INTRODUCTION

Heat stress negatively affects cow health, well-being, fertility, and milk production (Collier et al., 1982; Baumgard et al., 2017) and it is estimated to cost ~\$2.3 billion in annual losses to the US dairy industry (St-Pierre et al., 2003; Ferreira et al., 2016). Substantial effort has focused on defining the cow's mechanistic adaptations to heat stress and how these responses affect lactation (Baumgard and Rhoads, 2013) and gestation (Ouellet et al., 2020). It is estimated that heat stress reduces milk production in part by decreasing nutrient intake and shifting glucose utilization away from milk synthesis. This is achieved partly because blood supply is shunted from the visceral organs toward the body periphery (Kenney and Havenith, 1993). This redistribution of blood supply may compromise the integrity of the intestinal barrier (Hall et al., 2001) and contribute to systemic inflammation (Collier et al., 1982; Baumgard and Rhoads, 2013) in the cow. In turn, inflamma-

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tion may limit milk production by partitioning glucose toward an activated immune system (Kvidera et al., 2017). Although the clustering of immune cells in the small intestine of the heat-stressed cow (Koch et al., 2019) serves as supporting evidence for this hypothesis, whether increased gastrointestinal permeability occurs during exposure to heat stress in dairy cattle remains unclear.

Dietary supplementation of organic acids (**OA**; e.g., citric and sorbic acids) and pure botanicals (**PB**; e.g., thymol and vanillin) represents a promising strategy to support and reduce antibiotic usage in livestock production systems (Pearlin et al., 2020; Rossi et al., 2020). These natural compounds have unique antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory properties that, when combined, have the potential to improve gastrointestinal health by controlling bacterial pathogen growth and enhancing barrier function (Tugnoli et al., 2020). Organic acids are characterized as weak, short-chain acids that are widely distributed in nature. Although numerous OA and PB blends can be fed to livestock species, especially in swine and poultry (Hassan et al., 2010; Ma et al., 2021), the mode of action of these compounds is centered on acidification of the gastrointestinal tract. The OA undissociated form can penetrate bacterial cells (which possess a neutral pH) and dissociate, reducing intracellular pH and inhibiting enzymatic reactions and nutrient transport (Mani-López et al., 2012). By restricting the growth of pH-sensitive and pathogenic bacteria, supplementation of OA blends has been shown to improve weight gain and feed conversion ratio in broiler chicks (Hassan et al., 2010). Pure botanicals are single components of plant essential oils and oleoresins. These compounds are also reported to possess pH-reducing properties against bacteria and have anti-inflammatory, antioxidant, and immunomodulatory properties (Rossi et al., 2020). In vitro, thymol, a direct extract from thyme, has been shown to reduce the growth and expression of virulence genes in *Escherichia coli* K88 (Bonetti et al., 2020). Supplementation of OA/PB (25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 56% triglyceride matrix) in the diet of weaned pigs allowed for improved productive performance (i.e., increased BW gain) and, in vitro, restored intestinal barrier (i.e., increased transepithelial resistance) when supplemented at 0.2 g/L in cultured Caco-2 cells (Grilli et al., 2015b). More recently, dietary OA/PB supplementation was investigated in dairy calves experiencing moderate heat stress (Fontoura, 2022). It was observed that dietary OA/PB supplementation partly restored DMI, and the lower DMI was the main driver of reduced growth performance in grow-

ing calves during heat stress. It remained uncertain whether dietary OA/PB supplementation improves lactation performance and restores gastrointestinal barrier of the dairy cow experiencing heat stress.

Our current knowledge regarding physiological adaptations to heat stress suggests that heat exposure may result in compromised gastrointestinal integrity in lactating cows. Strategies aimed at improving gastrointestinal health, such as dietary OA/PB supplementation, may be a means to improve dairy cattle heat-stress resilience and milk production. Therefore, our objectives were to determine (1) whether heat stress increases gastrointestinal permeability in lactating dairy cows, and (2) whether dietary OA/PB supplementation serves as a nutritional strategy to enhance milk production in dairy cows experiencing heat stress.

MATERIALS AND METHODS

Experimental Design

All experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee (protocol #2018-0110). In 6 blocks of 8, 48 multiparous pregnant and lactating Holstein cows [208 ± 4.65 DIM (mean ± SD), 3.0 ± 0.42 lactations, 122 ± 4.92 days carrying calf, and 39.2 ± 0.26 kg of milk yield] were enrolled in a trial with a complete randomized design. Cows were transported from the Cornell University Dairy Research Center (Harford, NY) to the Cornell University Large Animal Research and Teaching Unit (Ithaca, NY). Animals were acclimated to the Large Animal Research and Teaching Unit in thermoneutrality [22.2 ± 0.25°C; 44.9 ± 0.05% relative humidity; 68 ± 0.32 temperature-humidity index (**THI**)] for 7 d. At the end of acclimation, cows blocked by lactation, days carrying calf, and milk yield were randomly allocated into 1 of 4 environmental conditions: thermoneutrality and unsupplemented (**TN-Con**; n = 12); heat-stressed and unsupplemented (**HS-Con**; n = 12); thermoneutrality and unsupplemented but pair-fed to match the feed intake of heat-stressed and unsupplemented cows (**TN-PF**; n = 12); and heat-stressed and supplemented with OA/PB (75 mg/kg of BW; **HS-OAPB**; n = 10) for 14 d. The OA/PB supplement was composed of 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride (AviPlus R; Vetagro S.p.A.) and delivered as a twice-daily top-dress in alignment with feed offering during the 14-d experimental period. All cows not treated with the OA/PB supplement received an equivalent amount of the triglyceride (vegetable lipid; Vetagro S.p.A.) that was used to encapsulate the OA/PB supplement for rumen protection.

Cows were housed in temperature-controlled rooms equipped with individual tiestalls (bedded with wood shavings) and provided fresh diets at 0730 and 1630 h daily. Diet was mainly composed of corn silage and haylage (Table 1). Cows had ad libitum access to water and feed and were fed to achieve 5% orts daily. For the pair-feeding regimen, TN-PF cows were fed based on the average percent decrease in feed intake of HS-Con cows. The daily decrease for the HS-Con cows was calculated relative to the last 4 d of the acclimation period. During the 14 d of the experiment, the daily percent decrease of HS-Con cows was applied to the TN-PF cows, relative to their baseline intake during acclimation. Cows were milked twice daily at 0600 and 1600 h. For thermoneutral conditions, daily ambient temperatures were kept at ~22 to 23°C. For heat-stress conditions, ambient temperatures increased at 0600 h from 27 to 37°C and decreased at 1800 h from 37 to 27°C. The goal was to maintain a THI of 68 in thermoneutrality and reaching but not exceeding a THI of 82 for heat-stress conditioning (Figure 1). Temperatures for each environment were monitored using HOBO loggers (model IMX2300; Onset Computer Corp.).

Data and Sample Collection

Diet ingredients were sampled weekly and composited for each block. Clinical assessments were performed thrice daily. During these assessments, rectal and skin temperatures, and respiration rates were recorded at 0700, 1200, and 1600 h. Rectal temperatures were measured using a large-animal digital rectal thermometer (model GLA M900; GLA Agricultural Electronics). Skin temperatures were measured using a noncontact infrared temperature gun (model 586; Fluke Corp.) on a shaved area of the left flank. Respiration rates were determined by counting flank movements for a 15-s period, then multiplying by 4 to obtain movements per minute. Baseline measurements for clinical assessments were calculated as an average during the last 4 d of the acclimation period. Feed, water intakes, and milk yields were recorded daily throughout the entire experiment. Body weights were recorded weekly. Baseline measurements for feed and water intakes were calculated as an average during the last 4 d of the acclimation period, and BW was recorded a day before the beginning of environmental conditioning. Milk was collected at 4 consecutive milkings, starting in the afternoon and ending on a morning, during the baseline period (last 2 d of acclimation, until the morning of heat-stress conditioning), at the beginning of heat stress conditioning (d 1 to 3), at the midpoint of heat-stress conditioning (d 6 to 8), and at the end of heat stress conditioning (d 12 to 14). Milk samples at each milking were collected in a

Table 1. Nutrient composition (% of DM unless otherwise noted) of experimental TMR fed to multiparous lactating Holstein cows¹

Item	Diet
Ingredient	
Corn silage	40.0
Concentrate mix ²	41.8
Haylage	15.6
Whey concentrate	2.60
Nutrient composition, %	
DM	43.9
CP	15.7
NDF	31.7
ADF	20.7
Crude fat (ether extract)	4.70
Mineral	
Ash	6.81
Calcium	0.85
Phosphorus	0.38
Magnesium	0.26
Potassium	1.56
Sodium	0.49
Energy, Mcal/kg of DM	
NE _L	1.70
NE _M	1.91
ME	2.86

¹Forty-six pregnant multiparous and lactating Holstein cows were randomly assigned to 1 of 4 treatments at enrollment: unsupplemented thermoneutral conditions (TN-Con, n = 12), heat stress with no supplementation (HS-Con, n = 12), thermoneutral conditions pair-fed to HS-Con (TN-PF, n = 12), and HS supplemented with organic acids and pure botanicals (OA/PB; 75 mg/kg of BW; AviPlus R; contains 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride; Vetagro S.p.A.; HS-OAPB, n = 10). Control cows (not supplemented with OA/PB) received a matching dose of the lipid matrix.

²Concentrate mix contained 43.9% corn meal, 14.1% canola meal, 10.2% soy hulls, 8.00% blood meal, 7.42% mineral premix, 4.30% amino acid blend (AminoPlus; Ag Processing Inc.), 3.30% corn gluten feed, 2.43% soybean meal, 1.40% salt carbonate, 1.30% calcium salts of fatty acids (Energizer Gold; IFFCO Animal Nutrition), 1.2% palm fat (BergaFat F-100; Berg + Schmidt GmbH & Co. KG), 1.04% white salt, 0.82% limestone, 0.32% Met (AminoShure-XM; Balchem Inc.), and 0.20% Lys (Smartamine ML; Adisseo USA Inc.).

sealed tube containing 2-bromo-2-nitropropane-1,3-diol and stored at 4°C for milk composition analysis within 3 d of collection.

Blood for plasma and serum separation was collected in the morning (preprandial) and afternoon (peak heat) on d -1 (baseline sample), 1, 2, 3, 7, and 14 by coccygeal venipuncture into an evacuated blood tube, which contained potassium EDTA as an anticoagulant when plasma was collected. Acute and chronic changes in intestinal barrier function were evaluated in vivo using the paracellular permeability marker chromium (Cr)-EDTA in a 180 mM solution on d 3 and 13, respectively, and as previously described (Wood et al., 2015; Horst et al., 2020). The Cr-EDTA solution was prepared in the laboratory within 5 d before use. Briefly, to make 1 L of Cr-EDTA solution, 47.7 g of chromium chloride hexahydrate (CAS#: 10060-12-5; Beantown Chemical Corp.) was dissolved in 300 mL of double-distilled wa-

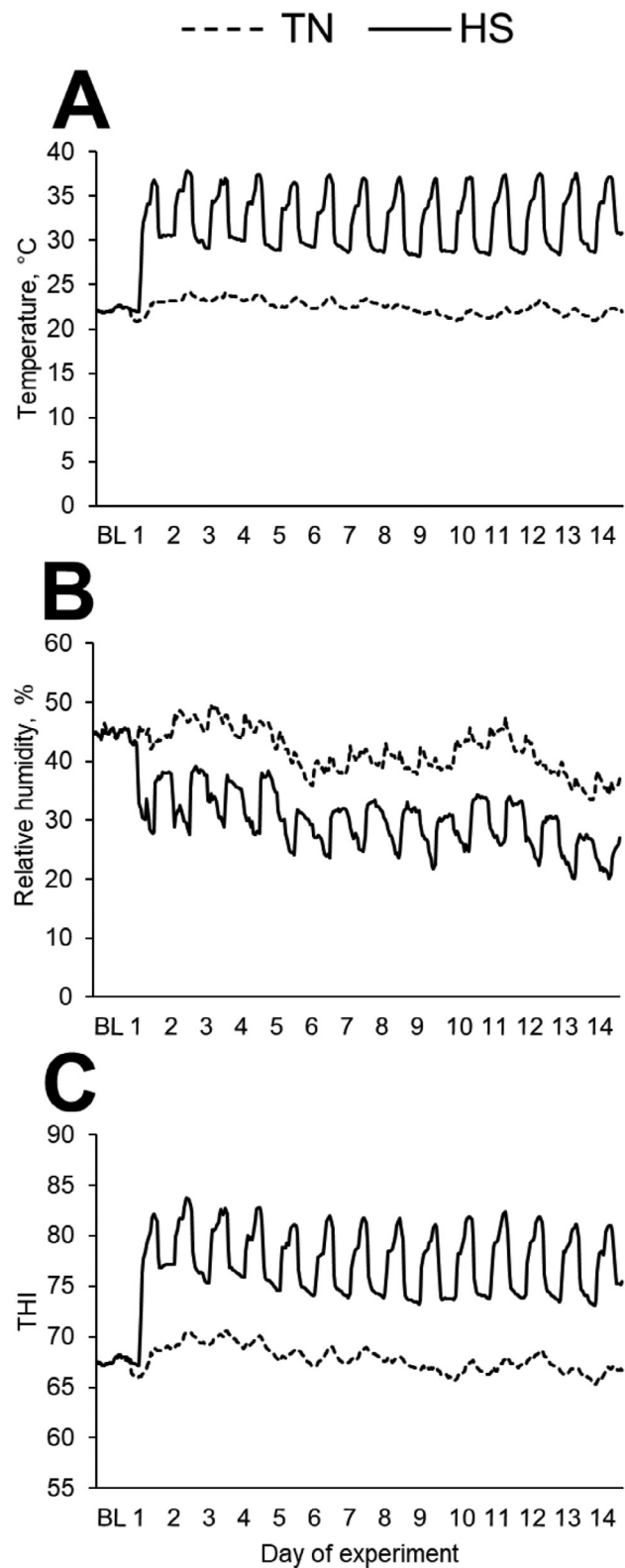


Figure 1. Average environmental conditions throughout the experiment. Data were averaged hourly throughout the experiment for (A) ambient temperature, (B) relative humidity, and (C) temperature-humidity index (THI). BL = baseline values for acclimation period; TN = thermoneutral conditions; HS = heat stress conditions.

ter. In a separate flask, 66.7 g of EDTA disodium salt dihydrate (CAS#: 30130; Chem-Impex International Inc.) was dissolved in 500 mL of double-distilled water using a plate warmer and stirrer. Once dissolved, both solutions were combined, and the flask was covered with foil and allowed to boil for 1 h. Thereafter, the solution was allowed to cool to room temperature and 1.937 g of calcium dichloride dihydrate (CAS#: 10035-04-8; MP Biomedicals LLC) was added. Dry sodium hydroxide pellets and 10 M sodium hydroxide were used to adjust the pH of the solution. Once pH reached 6.0, double-distilled water was added to reach 1 L. Beginning at 0700 h (after milking, before feeding), the 180 mM solution of Cr-EDTA (1.5 L) was pulse dosed into the rumen via the oro-esophageal route using a cattle pump system (Springer Magrath Co.). Blood samples were collected at 0, 1, 2, 4, 8, 12, 18, and 24 h relative to Cr-EDTA administration by coccygeal venipuncture into an evacuated tube containing dipotassium EDTA for total Cr determination using ^{53}Cr isotope analysis [i.e., ^{53}Cr represents 9.5% of total Cr (the sum of ^{50}Cr , ^{52}Cr , ^{53}Cr , and ^{54}Cr)]. Plasma and serum samples were separated using centrifugation ($3,100 \times g$ for 20 min at 4°C). Separated plasma or serum samples were initially stored at -20°C and then transferred to -80°C for long-term storage within 2 wk of collection.

Sample Analyses

Feed samples were analyzed for DM (AOAC International, 2000), CP (AOAC International, 2000), soluble protein (Krishnamoorthy et al., 1982), NDF (Van Soest et al., 1991), ADF (AOAC International, 2000), TDN (sum of digestible protein, digestible carbohydrate, and fat), ash (Thiex et al., 2012), ether extract (Thiex, 2009), and lignin (AOAC International, 2000) by Cumberland Valley Analytical Services Inc. (Cumberland, MD). Milk samples were analyzed for fat, true protein, lactose, and MUN concentrations using Fourier transform infrared spectroscopy and SCC by flow cytometry (Dairy One, Ithaca, NY). To measure changes in metabolic health markers, which are often responsive to heat stress (Garcia et al., 2015; Joo et al., 2021), plasma samples were analyzed for fatty acids (**FA**), glucose, total and free cholesterol, plasma urea-N (**PUN**), insulin, and LPS-binding protein (**LBP**). Serum samples were analyzed for triglyceride. Total FA, glucose, total and free cholesterol, and LBP concentrations were determined using enzymatic methods and commercially available kits (HR series NEFA-HR #999-34691, 995-34791, 991-34891, and 993-35191; Autokit Glucose #997-03001; Cholesterol E #999-02601, Cholesterol E free #993-02501; Wako Chemicals USA Inc.; and LBP #HK503; Hycult Biotech). A PUN analysis was per-

formed as described by Chaney and Marbach (1962) using an enzymatic colorimetric assay based on a purchased commercial kit (#640; Sigma-Aldrich). Plasma insulin concentrations were measured using RIA (#PI-12K Porcine Insulin RIA Kit; EMD Millipore Corp.) on an LKB-Wallac CliniGamma Counter (Beckman Coulter) as previously described (Krumm et al., 2019). Serum triglyceride concentrations (Triglyceride GPO liquid reagent set #T7532-500, Pointe Scientific) were measured following the manufacturer's instructions. All spectrophotometric measurements were conducted using a SpectraMax Plus 384 Microplate Reader (Molecular Devices). Intra- and interassay coefficients of variation were 7.21% and 8.85%, 4.72% and 3.31%, 4.88% and 12.3%, 3.97% and 6.97%, 5.39% and 6.56%, 7.80% and 7.40%, 1.57% and 2.36%, and 5.35% and 18.7% for FA, glucose, total and free cholesterol, PUN, insulin, triglyceride, and LBP, respectively.

Plasma samples collected during the Cr-EDTA challenge were submitted for analysis at the USDA-ARS Plant, Soil and Nutrition Research Laboratory (Ithaca, NY). Briefly, each sample was aliquoted into an individual glass tube containing 1 mL of plasma. Plasma samples were dried at 90°C for 12 h. Dried plasma samples were treated with 3 mL of 60:40 HNO₃:HClO₄ mixture and left overnight to destroy organic matter. The mixture was then slowly heated to 70 to 120°C for 2 h in a heating block. The temperature of the heating block was then raised to 145°C for 2 h. If necessary, more nitric acid (i.e., 1 to 2 mL) was added to remove the brownish color of organic matter. Then, the temperature of the heating block was raised to 190°C for 10 min and turned off. The cooled samples in the tubes were then diluted to 5 mL, vortexed, and transferred into autosampler tubes to run in inductively coupled plasma mass spectrometry (ICP-MS model: Agilent G3272A; Agilent Technologies, Inc.). Appropriate standards were prepared in 2% HClO₄ and measured isotopic ⁵³Cr.

Calculations and Statistical Analyses

The THI was calculated according to the equation reported by Kendall et al. (2008):

$$\text{THI} = (1.8 \times T + 32) - [(0.55 - 0.0055 \times \text{RH}) \times (1.8 \times T - 26)],$$

where T = air temperature (°C), and RH = relative humidity (%).

Total water intake was calculated as the sum of water contained in the feed and voluntary water intake. Yields of 3.5% FCM, ECM, and milk components were

calculated using milk yield and component concentrations for each milking, summed for a daily total, and averaged for each collection period. The efficiencies for milk yield, FCM (**FCME**) and ECM (**ECME**) production were calculated as the ratio of milk yield, FCM, or ECM in relation to DMI. Somatic cell score was calculated from SCC for statistical analysis using a logarithmic transformation: $\text{Log}_2(\text{SCC}/100,000) + 3$ (Ali and Shook, 1980). The concentrations of serum esterified cholesterol were calculated by subtracting concentrations of free cholesterol from total cholesterol. The concentration of Cr per unit of BW was obtained by dividing the amount of Cr at each time point by the regressed BW of the cows on the day of the Cr-EDTA challenge. These values were used to calculate the area under the curve (**AUC**) for each Cr-EDTA challenge. The AUC for Cr per unit of BW during Cr-EDTA challenge was calculated using the trapezoidal method as previously described by Pires et al. (2007).

Statistical analyses were carried out using the mixed model procedure of SAS (v9.4, SAS Institute Inc.) according to the following model:

$$Y_{ijklm} = \mu + C_i + T_j + B_k + D_l + Ti_m + T_j \times D_l + T_j \times Ti_m + \text{LACT} + \text{DCC} + \text{DIM} + p\text{Var} + e_{ijklm},$$

where Y_{ijklm} = dependent variable; μ = overall mean effect for the measure; C_i = random effect of cow ($i = 1$ to 45); T_j = fixed effect of treatment ($j = 1$ to 4); B_k = fixed effect of block ($k = 1$ to 6); D_l = fixed effect of day ($l = 1$ to 14); Ti_m = fixed effect of time ($m = 1$ to 3 for clinical parameters, and $m = 1$ to 8 for intestinal permeability), when appropriate to clinical and blood response variables; $T_j \times D_l$ = fixed effect of the interaction between treatment and day; $T_j \times Ti_m$ = the fixed effect of the interaction between treatment and time; LACT = number of lactations used as a covariate; DCC = days carrying calf used as a covariate; DIM = days in milk used as a covariate; $p\text{Var}$ = baseline measurement for each response variable used as a covariate; and e_{ijklm} = the residual error. The fixed effect of time and the interaction between treatment and time for blood metabolites were tested but removed from the model because $P > 0.25$. The covariance structures used to test fit statistics included variance components, compound symmetry, autoregressive one, unstructured, and ante-dependence one. Smaller fit values (Bayesian information criterion) were always selected. The model was used to evaluate production responses, clinical measurements, blood metabolites, and the in vivo intestinal permeability test.

Observations were deemed as outliers if Studentized residuals were > 3.0 or < -3.0 . Normality of

the residuals was checked with normal probability and box plots and homogeneity of variances with plots of residuals versus predicted values to ensure no violation of model assumptions. The least squares means comparisons were performed using preplanned nonorthogonal contrasts of interest (e.g., HS-Con vs. TN-Con, HS-Con vs. TN-PF, and HS-Con vs. HS-OAPB) using unadjusted *P*-values. Main effects were declared significant at $P \leq 0.05$ and trending toward significance at $0.05 < P \leq 0.15$. Interactions were declared significant at $P \leq 0.15$. Results are expressed as least squares means \pm standard errors of the means, unless otherwise noted.

RESULTS

Although 48 cows ($n = 12/\text{treatment}$) were initially enrolled in the experiment, during the experiment, 2 cows ($n = 2$ for HS-OAPB) had to be removed due to pre-existing health conditions discovered when exposed to the heat environment (i.e., stomach ulcer and clostridial infection) and 1 cow ($n = 1$ for HS-Con) had to be removed due to an abortion on d 12 of the study, which could be attributed to their exposure to a HS environment. Because the 2 cows in the HS-OAPB treatment group with pre-existing conditions had to be removed from the HS environment early in the experimental period (i.e., d 1 and 3), these cows were excluded from the final sample and data analyses, which is reflected in all tables and figures. The HS-Con cow that aborted on d 12 of the study was kept in the data set until d 12 because we cannot rule out the possibility that this outcome was caused by the treatment. Cows in the HS-Con and HS-OAPB groups had increased rectal and skin temperatures and respiration rates compared with cows maintained in thermoneutrality (Treatment, $P < 0.01$; Figure 1). The increase in rectal temperatures peaked at 2.1°C higher in HS-Con compared with TN-Con cows (Supplemental Table S1; <https://data.mendeley.com/datasets/6px2ns6spc/1>; Fontoura et al., 2022c). The peaks for rectal and skin temperatures were 40.7°C and 38.6°C during the afternoon clinical assessment. We also observed that the TN-PF group had lower rectal and skin temperatures and respiration rates relative to cows in the TN-Con group (Treatment, $P < 0.01$; Figure 2).

Exposure to heat stress resulted in lesser BW and greater BW loss for HS-Con, TN-PF, and HS-OAPB cows compared with TN-Con cows (Treatment \times Week, $P < 0.01$; Table 2 and Supplemental Figure S1A; <https://data.mendeley.com/datasets/j3sfwjhzxn/1>; Fontoura et al., 2022a). However, when compared with HS-Con, the OA/PB cows tended to have smaller BW loss (HS-Con vs. HS-OAPB, $P = 0.15$; Table 2 and Supplemen-

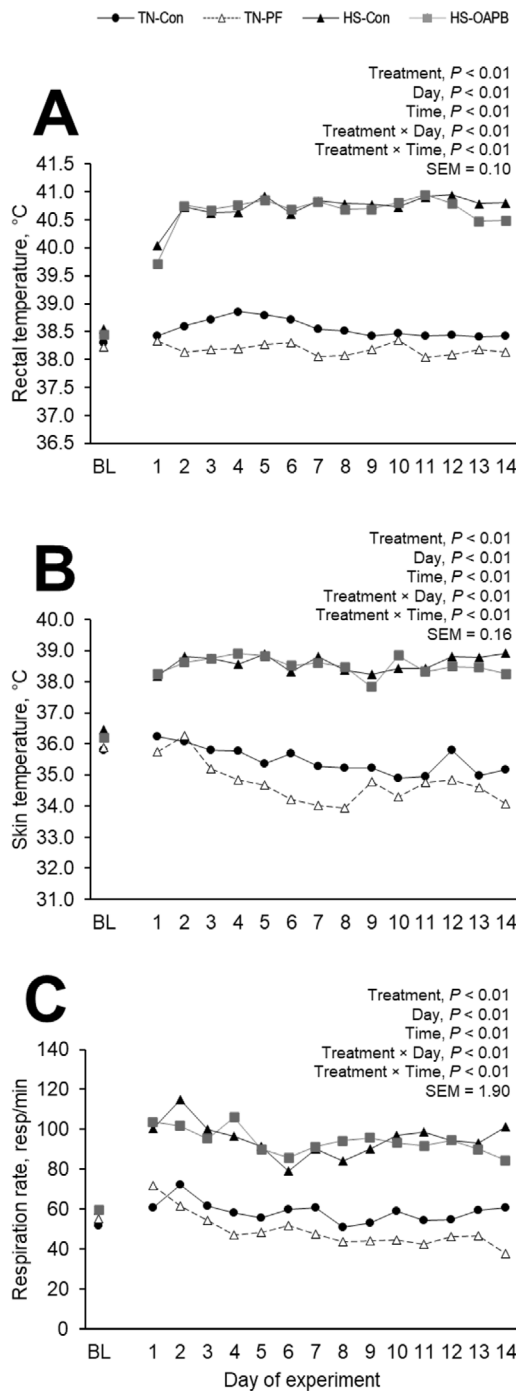


Figure 2. Effects of environmental conditioning and dietary organic acid and pure botanical supplementation on (A) rectal temperature, (B) skin temperature, and (C) respiration (resp) rate of pregnant multiparous lactating Holstein cows. Cows were randomly assigned to 1 of 4 treatments at enrollment: unsupplemented thermoneutral conditions (TN-Con, $n = 12$), heat stress with no supplementation (HS-Con, $n = 12$), thermoneutral conditions pair-fed to HS-Con (TN-PF, $n = 12$), and HS supplemented with organic acids and pure botanicals (OA/PB; 75 mg/kg of BW; AviPlus R; contains 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride; Vetagro S.p.A.; HS-OAPB, $n = 10$). Control cows (not supplemented with OA/PB) received a matching dose of the lipid matrix. BL = baseline values for acclimation period.

Table 2. Effects of heat stress and dietary organic acid and pure botanical supplementation on productive performance, milk composition, and feed efficiency of lactating multiparous Holstein cows

Variable	Treatment ¹				SEM	Treatment	P-value ²		
	TN-Con	TN-PF	HS-Con	HS-OAPB			HS-Con vs. TN-Con	HS-Con vs. TN-PF	HS-Con vs. HS-OAPB
Productive performance									
BW, kg	684	655	629	640	5.61	<0.01	<0.01	<0.01	0.17
Live weight variation, kg/d	0.23	-4.10	-5.22	-3.83	0.66	<0.01	<0.01	0.22	0.15
DMI, kg/d	24.8	12.8	13.4	14.8	0.65	<0.01	<0.01	0.54	0.14
Total water intake, ³ L/d	160	108	107	137	8.35	<0.01	<0.01	0.89	<0.01
Voluntary water intake, L/d	135	80.6	97.2	122	5.68	<0.01	<0.01	0.05	<0.01
Milk yield, kg/d	34.3	25.3	22.5	25.2	1.24	<0.01	<0.01	0.09	0.12
3.5% FCM, kg/d	42.4	33.3	29.0	31.5	1.08	<0.01	<0.01	0.01	0.10
ECM, kg/d	41.0	31.9	27.4	30.2	1.04	<0.01	<0.01	<0.01	0.05
Milk composition, %									
Fat	4.93	5.22	5.26	4.80	0.13	0.06	0.07	0.82	0.02
Protein	3.29	3.23	3.02	3.06	0.04	<0.01	<0.01	<0.01	0.53
Lactose	4.86	4.78	4.79	4.84	0.23	0.07	0.04	0.75	0.11
TS	14.1	14.1	14.0	13.6	0.13	0.08	0.64	0.52	0.08
SCS	2.01	2.14	2.32	1.92	0.17	0.42	0.20	0.44	0.12
MUN, mg/dL	11.5	11.7	15.9	13.5	0.44	<0.01	<0.01	<0.01	<0.01
Milk solids, kg/d									
Fat	1.66	1.35	1.18	1.24	0.05	<0.01	<0.01	0.01	0.34
Protein	1.13	0.86	0.69	0.80	0.03	<0.01	<0.01	<0.01	0.01
Lactose	1.67	1.28	1.08	1.26	0.05	<0.01	<0.01	<0.01	0.01
Feed efficiency ratio									
Milk ⁴	1.39	1.94	1.72	1.67	0.05	<0.01	<0.01	<0.01	0.51
3.5% FCME ⁵	2.67	2.36	1.85	1.76	0.20	0.02	0.02	0.09	0.65
ECME ⁶	2.53	2.24	1.76	1.69	0.23	0.01	0.01	0.08	0.81

¹Forty-six pregnant multiparous and lactating Holstein cows were randomly assigned to 1 of 4 treatments at enrollment: unsupplemented thermoneutral conditions (TN-Con, n = 12), heat stress with no supplementation (HS-Con, n = 12), thermoneutral conditions pair-fed to HS-Con (TN-PF, n = 12), and HS supplemented with organic acids and pure botanicals (OA/PB; 75 mg/kg of BW; AviPlus R; contains 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride; Vetagro S.p.A.; HS-OAPB, n = 10). Control cows (not supplemented with OA/PB) received a matching dose of the lipid matrix.

²Statistical significance was considered when $P \leq 0.05$. Trend toward significance was considered when $0.05 < P \leq 0.15$.

³Calculated as the sum of water in feed and voluntary water intake.

⁴Milk efficiency was calculated as the ratio of milk yield and DMI.

⁵ECM efficiency was calculated as the ratio of ECM and DMI.

⁶3.5% FCM efficiency was calculated as the ratio of 3.5% FCM and DMI.

tal Figure S1A). The DMI of HS-Con cows was reduced by approximately 44% compared with TN-Con cows (HS-Con vs. TN-Con, $P < 0.01$; Table 2; Figure 3A). As a result of the study design, TN-PF and HS-Con cows had similar DMI ($P = 0.54$; Table 2; Figure 3A). Supplementation with OA/PB tended to restore DMI by ~10% relative to HS-Con (HS-Con vs. HS-OAPB, $P = 0.14$; Table 2, Figure 3A). Voluntary water intake was decreased in HS-Con relative to TN-Con cows ($P < 0.01$; Table 2; Figure 3B), whereas supplementation with OA/PB increased voluntary water intake relative to HS-Con ($P < 0.01$; Table 2; Figure 3B). Importantly, these results were also observed when we summed total water intake (HS-Con vs. TN-Con, $P < 0.01$, and HS-Con vs. HS-OAPB, $P < 0.01$; Table 2 and Supplemental Figure S1C).

Heat-stress exposure reduced yields of milk, 3.5% FCM, and ECM (Treatment, $P < 0.01$; Table 2, Figure 3C and 3D). Both TN-PF and HS-OAPB groups

had significant or trended toward significantly superior performance (i.e., milk yield, FCM, ECM) compared with HS-Con cows (Table 2). The HS-Con cows also had reduced fat, protein, lactose, and TS relative to TN-Con cows ($P < 0.01$; Table 2; Figures 4A and 4C, Supplemental Figures S1E and S1G, respectively). Yield of ECM was also reduced in heat-stressed cows, especially when considering ECM efficiency (1.76 vs. 2.53, HS-Con vs. TN-Con, $P = 0.01$; Table 2 and Figure 3D). Although we did not observe a restorative effect of OA/PB for feed efficiency (HS-Con vs. HS-OAPB, $P \geq 0.51$; Table 2 and Supplemental Figure S1I), we did detect evidence for improved nitrogen efficiency that resulted in increased milk protein yield with OA/PB supplementation (HS-Con vs. HS-OAPB, $P = 0.01$; Figure 4B), reduced MUN (HS-Con vs. HS-OAPB, $P < 0.01$; Figure 4C), and trends toward a reduction in PUN (HS-Con vs. HS-OAPB, $P = 0.08$; Figure 4D) for HS-OAPB cows relative to their HS-Con counterparts.

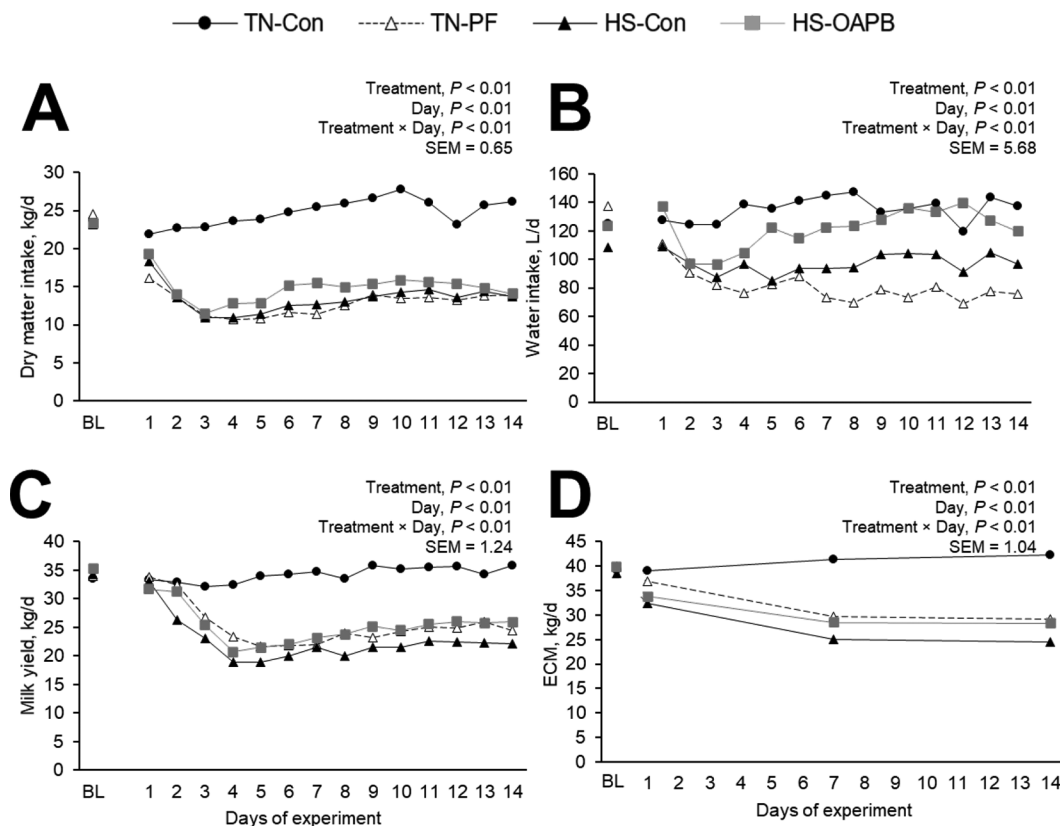


Figure 3. Effects of environmental conditioning and dietary organic acid and pure botanical supplementation on (A) DMI, (B) water intake, (C) milk yield, and (D) ECM of pregnant multiparous lactating Holstein cows. Cows were randomly assigned to 1 of 4 treatments at enrollment: unsupplemented thermoneutral conditions (TN-Con, $n = 12$), heat stress with no supplementation (HS-Con, $n = 12$), thermoneutral conditions pair-fed to HS-Con (TN-PF, $n = 12$), and HS supplemented with organic acids and pure botanicals (OA/PB; 75 mg/kg of BW; AviPlus R; contains 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride; Vetagro S.p.A.; HS-OAPB, $n = 10$). Control cows (not supplemented with OA/PB) received a matching dose of the lipid matrix. BL = baseline values for acclimation period.

Plasma total FA concentrations were increased in TN-PF relative to HS-Con (HS-Con vs. TN-PF, $P = 0.02$; Table 3, Figure 5A). Heat stress exposure in unsupplemented cows also resulted in decreased plasma glucose concentrations relative to TN-Con (HS-Con vs. TN-Con, $P = 0.02$; Table 3, Figure 5B), increased plasma insulin concentrations (HS-Con vs. TN-PF, $P = 0.03$; Table 3, Figure 5C) relative to TN-PF, and increased PUN levels ($P < 0.01$; Table 3, Figure 4D) relative to TN-Con and TN-PF. We also observed increased serum triglyceride concentrations in HS-Con relative to TN-Con (HS-Con vs. TN-Con, $P = 0.01$; Table 3, Figure 5D). We observed higher concentrations of plasma cholesterol in HS-Con relative to TN-Con (HS-Con vs. TN-Con, $P = 0.05$; Table 3, Figure 5E), with a reduction in esterified cholesterol concentrations in the HS-Con cows relative to TN-PF (HS-Con vs. TN-Con, $P = 0.01$; Figure 5F). Reflective of the esterified cholesterol patterns, plasma concentrations of total cholesterol were increased in TN-PF cows (HS-Con vs. TN-PF, $P = 0.03$; Table 3, Figure 5G). Heat-stress exposure

also elevated circulating plasma LBP concentrations relative to thermoneutrality and pair-feeding (HS-Con vs. TN-Con, $P < 0.01$; HS-Con vs. TN-PF, $P = 0.03$). Apart from the reduced PUN and LBP concentrations relative to HS-Con cows (HS-Con vs. HS-OAPB, $P = 0.08$ and $P = 0.02$, respectively), supplementation of OA/PB did not overtly modify circulating metabolites or insulin.

The results from the *in vivo* gastrointestinal permeability Cr-EDTA assay are presented in Figure 6 and Supplemental Figure S2 (<https://data.mendeley.com/datasets/xkr93dz6vr/1>; Fontoura et al., 2022b). During acute heat stress (i.e., d 3 of heat conditioning), we observed that HS-Con cows had increased plasma Cr concentrations from h 4 to 24 h compared with their TN-Con counterparts (Treatment \times Time, $P < 0.01$; e.g., TN-Con vs. HS-Con at h 12, $P < 0.01$; Figure 6A). Relative to the TN-PF cows, HS-Con cows also had increased plasma Cr AUC concentrations (HS-Con vs. TN-PF, $P = 0.04$; Figure 6B). The HS-Con cows also had increased apparent total-tract gastrointestinal

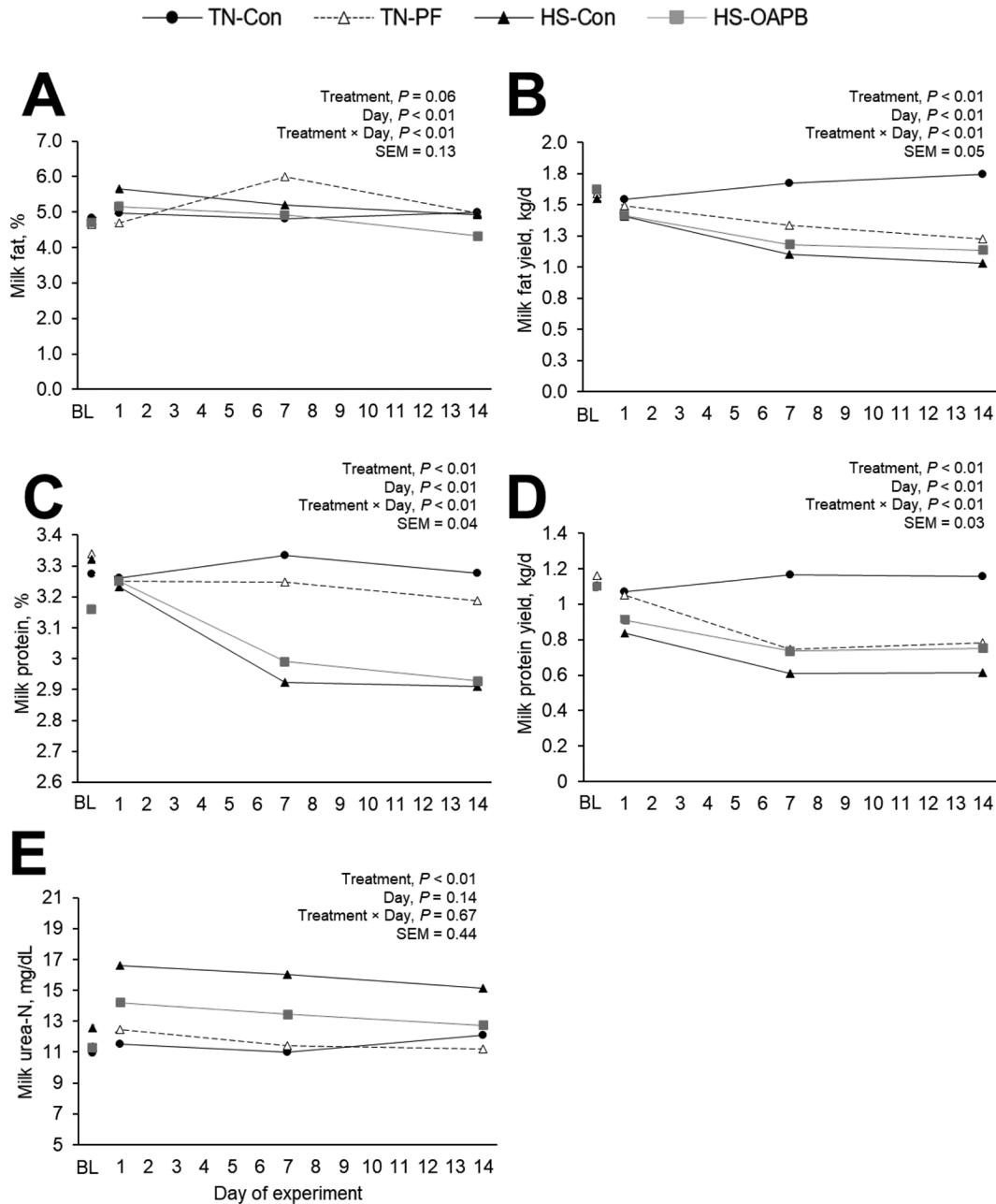


Figure 4. Effects of environmental conditioning and dietary organic acid and pure botanical supplementation on milk components and yields of pregnant multiparous lactating Holstein cows. (A) Milk fat percent, (B) fat yield, (C) protein percent, (D) protein yield, and (E) MUN. Cows were randomly assigned to 1 of 4 treatments at enrollment: unsupplemented thermoneutral conditions (TN-Con, $n = 12$), heat stress with no supplementation (HS-Con, $n = 12$), thermoneutral conditions pair-fed to HS-Con (TN-PF, $n = 12$), and HS supplemented with organic acids and pure botanicals (OA/PB; 75 mg/kg of BW; AviPlus R; contains 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride; Vetagro S.p.A.; HS-OAPB, $n = 10$). Control cows (not supplemented with OA/PB) received a matching dose of the lipid matrix. BL = baseline values for acclimation period.

permeability compared with their pair-fed counterparts (Treatment \times Time, $P < 0.01$; e.g., TN-PF vs. HS-Con at h 8 and 12, $P < 0.01$; Figure 6A). In addition, plasma Cr AUC for HS-Con cows were $\sim 35\%$ higher than that of TN-Con cows (HS-Con vs. TN-Con, $P = 0.05$; Figure 6B). Furthermore, we observed that OA/PB

supplementation lowered circulating Cr concentrations 12 h after Cr-EDTA pulse dose on d 3 relative to HS-Con cows (Treatment \times Time, $P < 0.01$, respectively; Figure 6A). However, plasma Cr AUC concentrations were not modified by HS-OAPB relative to HS-Con (Figure 6B). After chronic exposure (i.e., d 13 of en-

Table 3. Effects of heat stress and dietary organic acid and pure botanical supplementation on metabolic and inflammation markers and insulin concentrations of lactating multiparous Holstein cows

Variable	Treatment ¹					P-value ²			
	TN-Con	TN-PF	HS-Con	HS-OAPB	SEM	Treatment	HS-Con vs. TN-Con	HS-Con vs. TN-PF	HS-Con vs. HS-OAPB
Total fatty acids, $\mu\text{mol/L}$	147	459	349	325	33.3	<0.01	<0.01	0.02	0.62
Glucose, mg/dL	69.5	64.6	62.4	63.5	1.96	0.07	0.02	0.41	0.69
Insulin, ng/mL	1.03	0.59	0.86	0.97	0.09	<0.01	0.18	0.03	0.38
Plasma urea-N, mg/dL	9.4	10.1	12.8	11.8	0.42	<0.01	<0.01	<0.01	0.08
Triglycerides, mg/dL	11.0	12.0	13.0	12.0	0.50	0.07	0.01	0.17	0.19
Cholesterol, mg/dL	39.2	45.3	45.3	43.9	2.09	0.16	0.05	0.99	0.65
Esterified cholesterol, mg/dL	145	166	138	138	7.23	0.04	0.49	0.01	0.98
Total cholesterol, mg/dL	186	212	184	182	9.25	0.10	0.89	0.03	0.89
LPS-binding protein, $\mu\text{g/mL}$	3.45	4.09	5.57	3.91	0.52	0.02	<0.01	0.03	0.02

¹Forty-six pregnant multiparous and lactating Holstein cows were randomly assigned to 1 of 4 treatments at enrollment: unsupplemented thermoneutral conditions (TN-Con, $n = 12$), heat stress with no supplementation (HS-Con, $n = 12$), thermoneutral conditions pair-fed to HS-Con (TN-PF, $n = 12$), and HS supplemented with organic acids and pure botanicals (OA/PB; 75 mg/kg of BW; AviPlus R; contains 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride; Vetagro S.p.A.; HS-OAPB, $n = 10$). Control cows (not supplemented with OA/PB) received a matching dose of the lipid matrix.

²Statistical significance was considered when $P \leq 0.05$. Trend toward significance was considered when $0.05 < P \leq 0.15$.

environmental conditioning), we observed that HS-Con cows had increased plasma Cr concentrations relative to TN-Con cows from h 12 to 24 (Treatment \times Time, $P \leq 0.10$; e.g., HS-Con vs. TN-Con, $P = 0.05$ at h 18; Figure 6C). We also observed a tendency for higher plasma Cr AUC in HS-Con cows relative to TN-Con (HS-Con vs. TN-Con, $P = 0.14$; Figure 6D). However, when compared with TN-PF cows, HS-Con cows had similar plasma Cr concentrations (HS-Con vs. TN-PF, $P = 0.56$; Figure 6C) and similar Cr AUC (HS-Con vs. TN-PF, $P = 0.91$; Figure 6D). Furthermore, we observed increased plasma Cr concentrations in TN-PF cows relative to TN-Con cows from h 12 to 24 (Treatment \times Time, $P = 0.04$ for h 12, $P = 0.02$ for h 18, and $P = 0.10$ for h 24; Figure 6C). However, plasma Cr AUC was numerically but not significantly greater for TN-PF cows on d 13 relative to TN-Con cows.

DISCUSSION

As reviewed by Baumgard et al. (2017), heat stress is defined as a problem for dairy systems, and yet heat stress continues to jeopardize milk production and health in dairy cattle in the United States and beyond. The thermal comfort zone (TCZ) in dairy cattle can be assessed using a calculated index that incorporates both temperature and relative humidity of an environment (i.e., THI). The TCZ of dairy cows was considered when THI < 71 (Armstrong, 1994). However, recent research suggests that the TCZ for lactating cows is shifting to a lower THI threshold, with cows experiencing heat stress when THI exceeds 68 (Zimelman et al., 2009; Tao et al., 2018). This shift in THI can be attributed to the increased genetic potential for milk production of modern

dairy herds (Brito et al., 2021) and the inherent heat sensitivity of high-producing dairy cows (Aguilar et al., 2010). In our study, we were able to maintain a THI of 68 in thermoneutrality and a THI range from 74 to 82 during the day, maintaining a THI of 74 at night in heat-stress conditions. We observed the expected clinical responses, including higher rectal and skin temperatures and increased respiration rates, in heat-stressed cows relative to their TN counterparts. The observed increases in these parameters were within previously reported values observed for heat-stressed lactating cows (Becker et al., 2020). Another important observation was the reduced skin and rectal temperatures and respiration rates of TN-PF cows relative to TN-Con, which has been demonstrated previously (Rhoads et al., 2009; O'Brien et al., 2010). The decreased plane of nutrition by restricting the DMI of pair-fed cows most likely led to less metabolic heat generated by lower feed digestion (Lean, 2002).

Decreasing DMI is a hallmark adaptation that dairy cows undergo when exposed to environmental conditions that cause heat stress (West, 2003). It is not uncommon to see a >30% decrease in DMI in cows experiencing heat stress (Collier et al., 2017). This response is likely regulated by the central nervous system (Andersson and Larsson, 1961) and potentially mediated through the hypothalamic-pituitary-adrenal axis, as demonstrated in other farm animal species (e.g., poultry; Quinteiro-Filho et al., 2012; Song et al., 2012). In our study, we observed an overall ~45% reduction in DMI in cows exposed to high THI. By utilizing a pair-feeding regimen, the reduction in DMI alone explained 63 to 78% of the decrease in milk yield during wk 1 and 2, respectively. Similarly, the reduced DMI explained

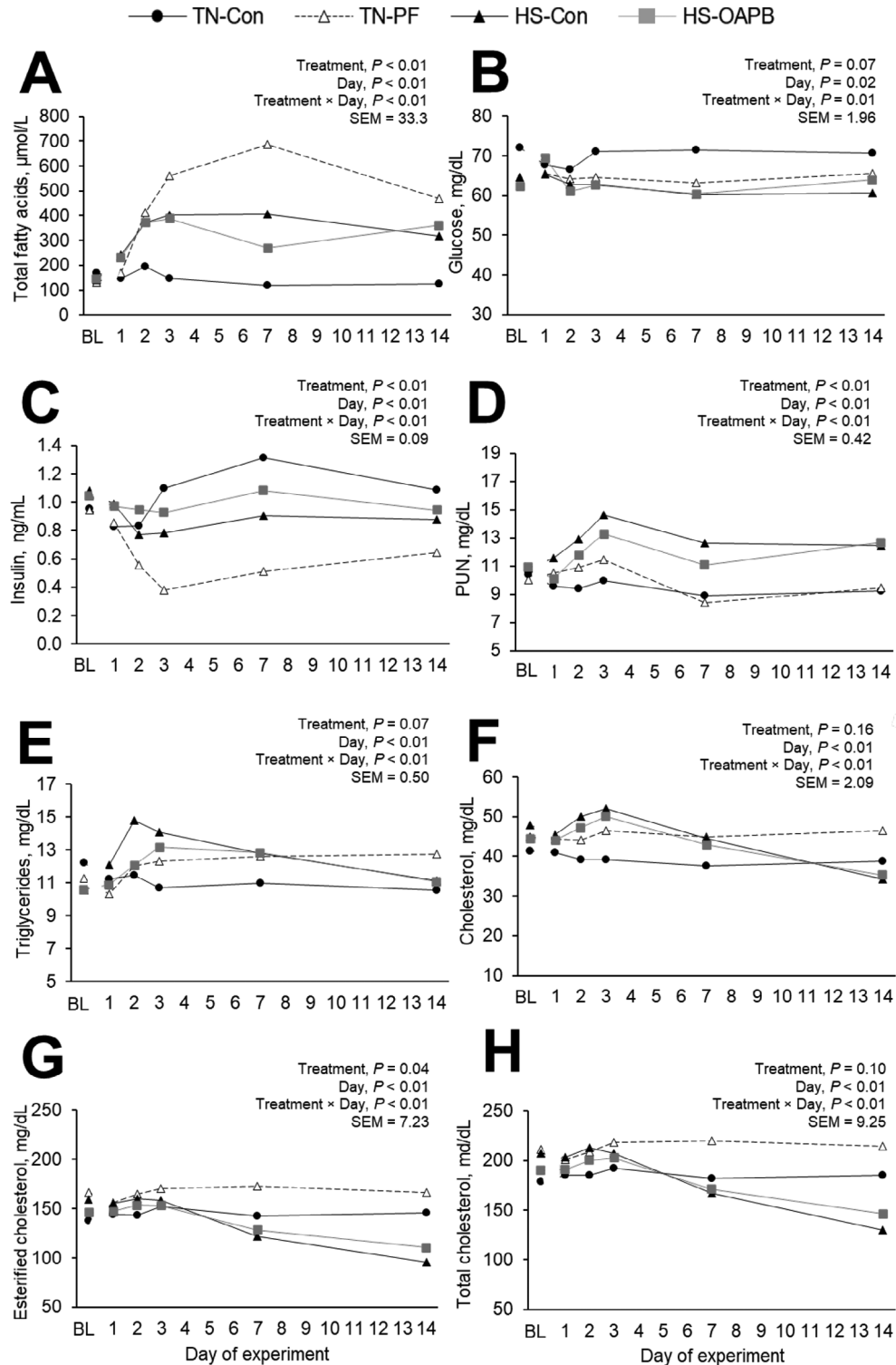


Figure 5. Effects of environmental conditioning and dietary organic acid and pure botanical supplementation on metabolic markers, and hormone concentrations of pregnant multiparous lactating Holstein cows. Circulating levels of (A) total fatty acids, (B) glucose, (C) insulin, (D) plasma urea-N (PUN), (E) triglycerides, (F) cholesterol, (G) esterified cholesterol, and (H) total cholesterol. Cows were randomly assigned to 1 of 4 treatments at enrollment: unsupplemented thermoneutral conditions (TN-Con, $n = 12$), heat stress with no supplementation (HS-Con, $n = 12$), thermoneutral conditions pair-fed to HS-Con (TN-PF, $n = 12$), and HS supplemented with organic acids and pure botanicals (OA/PB; 75 mg/kg of BW; AviPlus R; contains 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride; Vetagro S.p.A.; HS-OAPB, $n = 10$). Control cows (not supplemented with OA/PB) received a matching dose of the lipid matrix. BL = baseline values for acclimation period.

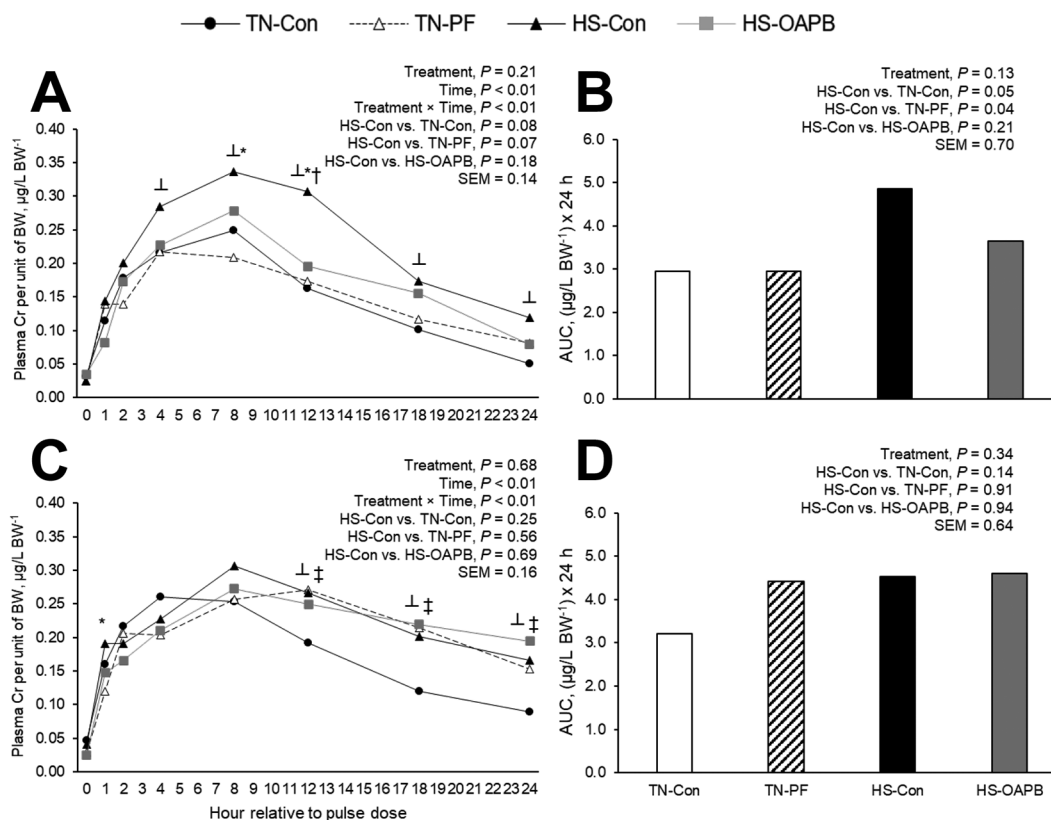


Figure 6. Effects of environmental conditioning and dietary organic acid and pure botanical supplementation on in vivo intestinal permeability measure by (A, C) chromium (Cr) concentrations in plasma per unit of BW, and (B, D) area under the curve (AUC) after a pulse dose of Cr-EDTA in pregnant multiparous lactating Holstein cows. Panels A and B are relative to Cr-EDTA challenge on d 3 of heat stress conditioning; panels C and D are relative to Cr-EDTA challenge on d 13 of heat stress conditioning. Cows were randomly assigned to 1 of 4 treatments at enrollment: unsupplemented thermoneutral conditions (TN-Con, $n = 12$), heat stress with no supplementation (HS-Con, $n = 12$), thermoneutral conditions pair-fed to HS-Con (TN-PF, $n = 12$), and HS supplemented with organic acids and pure botanicals (OA/PB; 75 mg/kg of BW; AviPlus R; contains 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride; Vetagro S.p.A.; HS-OAPB, $n = 10$). Control cows (not supplemented with OA/PB) received a matching dose of the lipid matrix. \perp HS-Con vs. TN-Con, Treatment \times Time, $P < 0.15$; $*$ HS-Con vs. TN-PF, Treatment \times Time, $P < 0.15$; \ddagger TN-PF vs. TN-Con, Treatment \times Time, $P < 0.15$; \dagger HS-Con vs. HS-OAPB, Treatment \times Time, $P < 0.15$.

34 to 74% and 40 to 75% of the decrease in ECM and FCM during wk 1 and 2, respectively. In relation to water intake, HS-Con cows consumed less water relative to TN-Con cows. In contrast to our hypothesis and common thinking, our findings differ from the notion that cows increase water intake during bouts of heat exposure (West, 2003; Hall et al., 2018). We attribute this divergence to the level of milk production of our study cows, as the reduction in water intake may be an adaptive response of high-producing cows (i.e., >30 kg/d in thermoneutrality) because of a reduced requirement for milk synthesis and demand for lower heat production associated with milk synthesis (Collier, et al., 2019). However, it is important to note that compared with TN-PF cows, HS-Con cows had higher water consumption (by ~ 17 L/d). Such reductions in TN-PF cows are reasonable, considering the strong positive correlation

between DMI and water intake (Holter and Urban, 1992; Dado and Allen, 1994).

Exposure to an environment with a high THI modifies milk fat and protein synthesis in lactating cows and, in turn, directly affects the synthesis of ECM. Indeed, we observed that HS-Con cows had reduced yields of milk, ECM, and milk solids compared with TN-Con and TN-PF cows. The reduction in milk fat yield of HS cows, relative to their TN-PF counterparts, is controversial in climate-controlled studies. Some studies do not observe alterations in milk fat percentage in cows exposed to elevated heat (Rhoads et al., 2009; Wheelock et al., 2010), whereas others detect reduced milk fat yield (Ouellet et al., 2019). Some argue that milk fat synthesis is sustained by an increase in the incorporation of preformed FA (i.e., $\geq C16$) into milk fat (Hammami et al., 2015; Liu et al., 2017). In the

present study, we did not detect a difference in milk fat percentage between HS-Con and TN-PF cows; however, the observed reduction in milk fat yield with heat stress is plausible considering several field observations of reduced milk fat during the warmer months in the United States and other temperate climates (Hammami et al., 2013; Bernabucci et al., 2014; Salfer et al., 2019). Previous reports indicate that exposure to a heat-stress environment decreases *de novo* FA synthesis (i.e., C4 to C14) in the mammary gland (Liu et al., 2017). This could stem from a shortage of acetate due to reduced DMI (Bauman et al., 1970; Urrutia et al., 2019). Exposure to a heat-stress environment also appears to inhibit milk protein synthesis. The observed decrease in milk protein concentrations in HS-Con cows, relative to TN-Con and TN-PF cows, agrees with previous reports (Cowley et al., 2015; Gao et al., 2017). Heat exposure does appear to affect the ability of the mammary gland to synthesize milk proteins by downregulating the expression of major milk protein genes such as β -casein and butyrophilin (Hu et al., 2016). Importantly, the transcriptional downregulation of milk protein genes, such as late endosomal/lysosomal adaptor, mitogen-activated protein kinase, and mammalian target of rapamycin activator 2, which are crucial for mammalian target of rapamycin activation and downstream protein synthesis (Gao et al., 2019), further suggests there is an intrinsic transcriptomic downregulation of milk protein synthesis in the mammary gland during heat exposure. Moreover, heat stress develops with oxidative stress and increased mitochondrial dysfunction in the mammary gland (Guo et al., 2021), which may further contribute to reductions in protein synthesis (Slimen et al., 2014).

Evaluating feed efficiency for dairy herds on the sole basis of milk volume per unit of feed consumed may be misleading (Britt et al., 2003). This is especially important for the heat-stressed dairy cow, as the metabolic changes in fat and protein metabolism (Baumgard and Rhoads, 2013) have the capacity to alter metabolism in the mammary gland and reduce milk component synthesis (Cowley et al., 2015; Ouellet et al., 2019). Thus, evaluating feed efficiency based on ECM is appropriate. We observed a decrease in milk efficiency (i.e., milk yield:DMI ratio) in HS-Con cows relative to TN-PF cows but not TN-Con cows. However, ECME and FCME values were lower for HS-Con cows than for TN-Con or TN-PF cows. Tao et al. (2018), while evaluating 3.5% FCME in early-lactation heat-stressed cows relative to actively cooled cows, observed no change in 3.5% FCME. The divergence across studies may be due to difference in environmental conditions (e.g., a climate-controlled study versus a heat-stress environment with or without cooling). Nonetheless, it is important for us to consider how heat stress affects feed efficiency

because economic loss involving inputs (e.g., feed costs and heat abatement using natural resources) and outputs (i.e., ECM, FCM) is a reality, and decreases in the efficiency of milk production can contribute to a greater carbon footprint by the dairy industry, assuming that the planet continues to warm, and Holsteins remain the dominant dairy breed in temperate climates.

We were able to confirm that heat-stressed cows mobilize body fat but to a lesser extent than TN-PF cows. This reduction in body fat mobilization is a classic feature of heat exposure in dairy cows (Baumgard and Rhoads, 2013). The observed higher plasma insulin concentrations of HS-Con, relative to TN-PF cows, is explanatory considering the strong antilipolytic action of insulin (Arner et al., 1981). The observed increase in circulating triglyceride concentrations of HS-Con relative to TN-Con cows is comparable to findings derived in nonruminants (Emami et al., 2020). A decrease in mammary gland uptake of triglyceride is one explanation. Alternatively, hepatic triglyceride export within very low density lipoproteins could be enhanced during heat stress; however, significant evidence suggests that a reduced capability of triglyceride export may develop during heat-stress conditioning due to downregulation of hepatic apolipoprotein B100 and a reduction in circulating apolipoproteins (Basiricò et al., 2009; Min et al., 2016). We also documented a temporal change in circulating cholesterol status with the heat-stress response. Specifically, circulating cholesterol concentrations were greater in TN-PF and HS-Con cows relative to the TN-Con cows. This could be due to an increase in hepatic acetate supply due to an increase in FA β -oxidation. In addition, the upregulation of hepatic 3-hydroxyl-3-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme of cholesterol synthesis, has been observed in response to elevated environmental temperatures (i.e., 37°C to 45°C) in rat hepatocyte cultures (Corton et al., 1994). Our observations are important to consider because cholesterol aids in the development of the heat shock response (Balogh et al., 2013). Cholesterol may also be esterified with fatty acyl chains. In our study, TN-PF cows had greater concentrations of esterified cholesterol than cows in the other treatment groups. This could be attributed to the heightened availability of plasma total FA in the pair-fed group. The potential implications of these findings on bovine biology are uncertain at the present time.

It is clear that other factors—beyond reductions in DMI—contribute to reduced milk synthesis in heat-stressed cows. The current hypothesis is that heat exposure increases gastrointestinal permeability and activates the immune system to shift nutrient partitioning away from the mammary gland (Kvidera et al., 2017; Koch et al., 2019). Our observation of higher plasma

Cr AUC of HS-Con cows, relative to TN-Con and TN-PF cows, during acute environmental conditioning (i.e., d 3) indicates that initial exposure to an elevated heat environment causes gastrointestinal permeability in lactating dairy cows. Our finding is in alignment with Koch et al. (2019), who observed the infiltration of macrophage-like cells in the mucosa and submucosal layer of the jejunum and reduced mRNA and protein abundance of paracellular tight junction protein zonula occludens in heat-stressed cows after 4 d exposure to moderate heat (i.e., THI = 76). Progressive exposure (i.e., 13 d) to a heat-stress environment was able to maintain heightened gastrointestinal permeability of HS-Con cows, relative to TN-Con or TN-PF cows, although the response was muted compared with that at d 3. It is important to highlight that we measured total-tract gastrointestinal permeability. Although the ruminal epithelial barrier may also experience disruptions caused by reduced pH and increased lactate levels after HS exposure (Steele et al., 2016; Zhao et al., 2019), postruminal gut permeability could be a greater contributor to impaired gut barrier because of the thinner and more delicate intestinal epithelium. Future work should determine whether heat stress increases postruminal gut permeability and whether this outcome persists with time. Nonetheless, the increased gastrointestinal permeability that we observed during acute and chronic heat exposure is an important observation related to the physiological adaptation to heat stress. Impairments in the gastrointestinal barrier are likely to result in increased transmigration of commensal and pathogenic bacteria and bacterial-derived metabolites, such as LPS. Our observations of increased circulating levels of LBP, a soluble acute-phase protein that binds to bacterial LPS and elicits an immune response (Kvidera et al., 2017), further supports this notion, because LPS has the potential to elicit a local inflammatory response in the gut and has been shown to lead to systemic inflammation in mouse models (Yue et al., 2012; Mohammad and Thiemermann, 2021). In the lactating dairy cow, this reprioritizes glucose utilization toward the immune system and away from milk synthesis (Kvidera et al., 2017).

Acute feed restriction did not impair total-tract gut permeability after 3 d. These findings contradict work by Horst et al. (2020). They observed that with a 40% feed restriction, which is similar to the feed reduction in TN-PF cows in our study, gastrointestinal permeability was enhanced in mid-lactation dairy cows housed in thermoneutrality. Our data suggest that heat-stress exposure is detrimental to the gastrointestinal tract, independent of changes in DMI during acute heat exposure. However, as nutrient restriction for TN-PF cows progressed (i.e., after 12 d), we observed an increase

in gastrointestinal permeability relative to TN-Con cows. Thus, it appears that chronic nutrient restriction can contribute to increased gut permeability. This has been shown in a swine model of heat stress, where after 7 d of heat exposure (i.e., ~35°C) or pair-feeding in growing pigs, intestinal permeability measured using Ussing chambers was similar between the HS and PF groups (Pearce et al., 2013). It is interesting to postulate whether chronic periods of nutrient deficit (e.g., during the postpartum period) would potentiate gut permeability following heat exposure. In our study, the contrasting response between acute heat exposure and pair-feeding on gut permeability also raises the question of how the mechanisms of impaired gut barrier are different or similar considering the delayed response to nutrient restriction. It is also important to note that even though we did not measure the isotopic composition of Cr in the diet, the fact that we still observed a significant increase in circulating ⁵³Cr concentrations in HS-Con cows with reduced DMI suggests that dietary Cr had a negligible effect on our results.

Although a common practice in poultry and swine production (Tugnoli et al., 2020), dietary OA/PB supplementation in ruminant species remains relatively unexplored. The large variety of OA and PB that can be blended makes it difficult to compare unique modes of action for each compound (Pearlin et al., 2020). However, we considered OA/PB as a dietary feed additive for heat-stressed cows because of its documented ability to improve growth performance in poultry species (Mohammadi Gheisar et al., 2015) and pigs experiencing weaning stress (Grilli et al., 2015b), and its potential to improve intestinal permeability demonstrated ex vivo (Grilli et al., 2015b; Toschi et al., 2020). Thus, the current OA/PB mixture (i.e., 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride matrix) has been shown to have bactericidal and anti-inflammatory properties (Toschi et al., 2020). Moreover, previous evidence supports the reduction of pathogenic bacteria (e.g., *Escherichia coli* and *Salmonella* spp.) in rumen fluid supplemented with water-solubilized OA/PB at 5% (vol/vol) (Grilli et al., 2015a); however, whether this effect occurs in the small intestine in response to feeding rumen-protected OA/PB is uncertain.

We were able to observe partial restoration of lactation performance of heat-stressed cows supplemented with OA/PB relative to their unsupplemented counterparts (i.e., HS-Con). The HS-OAPB cows had significantly greater yields of ECM, milk protein, and lactose; these increases were more pronounced after exposure to high THI for 7 d, remaining elevated until the end of the 14-d experimental period. Specifically, HS-OAPB cows produced 2.7 kg/d more milk than HS-Con cows.

Moreover, HS-OAPB cows tended to have higher yields of ECM and 3.5% FCM (i.e., +2.8 and +2.5 kg/d, respectively). The increase in ECM and milk solids could be partly explained by the increased DMI of HS-OAPB cows relative to HS-Con cows. Cows with HS-OAPB treatment consumed an additional ~1.4 kg of DM daily. Increased DMI with dietary OA/PB supplementation has also been observed in weaned dairy calves fed OA/PB at the same feeding level (i.e., 75 mg/kg of BW; Fontoura, 2022). Although the mechanistic reason for increased intake remains unclear, evidence supports sorbic acid action on the insulin growth factor (IGF) system. Previous work indicates that supplementation of sorbic acid also increases feed intake in swine and was demonstrated to increase circulating IGF-1 and mRNA expression of *IGF1R* in hepatocytes (Luo et al., 2011), which may stimulate the arcuate nucleus of the hypothalamus and thereby stimulate feed intake (Hong et al., 2017). In addition to increased DMI, dietary inclusion of citric acid could have served as a key substrate for ruminal acetate production (Wright, 1971). Although this requires confirmation in the dairy cow, dietary citric acid supplementation has been shown to increase ruminal acetate production in steers (Wang et al., 2009). The inclusion of thymol and vanillin might also have contributed to increased FCM and DMI, considering that Kung et al. (2008), when supplementing mid-lactation Holstein cows with a blend of PB that included thymol and vanillin (daily dose: 1.2 g/d for 9 wk), also observed substantial increases in DMI and 3.5% FCM in supplemented cows.

Optimizing nitrogen use efficiency (i.e., incorporation into milk while reducing excretion) is considered a key action to reduce the environmental impact of dairy systems (Calsamiglia et al., 2010). Importantly, during heat-stress exposure, lactating cattle go through metabolic adaptations to survive the increased heat load that results in increased use of glucose and AA in other body tissues rather than incorporation into milk (Baumgard and Rhoads, 2013), which leads to increased N excretion (Gao et al., 2017). Our observations of increased milk protein yields and decreased MUN and PUN in HS-OAPB cows, relative to unsupplemented HS-Con cows, suggests that OA/PB supplementation was able to improve nitrogen efficiency during exposure to heat stress. This improvement may have been achieved in part by the additional energy provided by higher DMI of HS-OAPB cows (i.e., ~4.0 Mcal of ME/d, ~2.4 Mcal of NE_L/d more relative to HS-Con) that allowed more energy to support protein synthesis in the mammary gland (Lobley, 2007). The elevated circulating insulin concentrations in heat-stressed cows could be another contributing factor that, combined with increased dietary energy intake with OA/PB feeding, could have

supported higher milk protein yields in HS-OAPB cows. It has been demonstrated previously that insulin is a potent regulator of milk protein synthesis by increasing the expression of genes directly involved in protein synthesis, casein synthesis, and AA uptake *ex vivo* (Menzies et al., 2009), as well as increased milk protein synthesis (by 15%) when administered during a hyperinsulinemic-euglycemic clamp in late-lactation cows (Mackle et al., 2000). The observed increased water intake in HS-OAPB cows, relative to HS-Con cows, could also be playing a role. It has been shown that a 50% decrease in water consumption increases nitrogen excretion in the urine and milk and increases circulating PUN (Steiger Burgos et al., 2001). In this scenario, it is likely that urea recycling in the kidneys plays an important role in water conservation and reduction of urinary volume and thus water loss (Maltz et al., 1981). However, whether a reduction of ~20% in water intake, which is closer to the reduction of HS-Con relative to HS-OAPB, can produce similar effects remains unknown.

In general, with the exception of circulating PUN levels, OA/PB supplementation did not greatly affect metabolic markers and insulin concentrations. The results obtained from the Cr-EDTA permeability test also displayed some degree of homogeneity, relative to HS-Con response, and although numerically lower, the plasma Cr AUC of HS-OAPB cows was not significantly different from that of HS-Con cows on d 3 or 13. However, dietary OA/PB feeding decreased circulating Cr concentrations following the Cr-EDTA challenge on d 3 (i.e., h 12). These findings suggest a modest but real improvement in gut barrier with OA/PB supplementation. This is in alignment with others that demonstrated improved intestinal barrier in response to OA/PB supplementation using *ex vivo* intestinal explants and Caco-2 cells (Grilli et al., 2015b; Toschi et al., 2020). In addition, the reduced concentration of LBP in HS-OAPB cows, relative to HS-Con cows, further supports an improvement in intestinal health of OA/PB supplemented cows. The hypothesized mode of action for OA/PB to improve the intestinal barrier likely involves reducing the presence of pathogenic bacteria and their direct anti-inflammatory properties (Bonetti et al., 2020; Tugnoli et al., 2020); however, this requires further study in ruminants. We are aware that components of the OA/PB supplement (e.g., citric acid) have chelating properties but we are uncertain whether this affected our Cr results; this finding deserves recognition and further attention. Regardless, the improvement of gastrointestinal barrier with OA/PB could explain the gains in ECM yields observed with this feeding strategy. Considering how changes in gut microbiota may influence intestinal permeabil-

ity and mucosal immunity (Bischoff et al., 2014) and the potential for OA/PB feeding to modulate the gut microbiome, future research should determine whether the ability of OA/PB supplementation to partly restore milk production is due to protective effects on the gut microbiome and barrier integrity.

CONCLUSIONS

In multiparous Holstein cows, we confirmed that repeated bouts of heat exposure (i.e., 14 d) impaired lactation performance (i.e., yields of milk, ECM, and 3.5% FCM, milk components and solids, and ECME and FCME) and developed a febrile response and increased total-tract gastrointestinal permeability. We were able to demonstrate that decreased milk production and increased apparent total-tract gastrointestinal permeability were not solely caused by a reduction in feed intake. However, our findings suggest that increases in apparent total-tract gastrointestinal permeability develop quickly with initial exposure to heat stress but reduce over time. The dietary supplementation of OA/PB in heat-stressed cows resulted in higher MY and ECM, which could have been partly explained by the increased DMI of HS-OAPB cows. The increased energy delivery provided by the higher DMI in HS-OAPB cows also contributed to improved nitrogen efficiency and responded with higher milk protein yield compared with their HS-Con counterparts. We conclude that dietary OA/PB supplementation is a means to partly restore milk production in lactating dairy cattle experiencing heat stress.

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