Effects of dairy products on intestinal integrity in heat-stressed pigs

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Heat stress compromises intestinal integrity which may partially explain its negative effects on animal health and productivity. Research suggests that challenged intestinal barrier function improves with dietary dairy products in various models. Thus, the study objective was to evaluate the effects of bovine milk whey protein (WP) and colostral whey protein (CWP) on intestinal integrity in heat-stressed pigs. Crossbred gilts (39 ± 3 kg body weight) were fed 1 of 4 diets (n = 8 pigs/diet): control (Ct), control diet containing an 80% WP and 20% CWP product (WP80), control diet containing a 98% WP and 2% CWP product (WP98), and control diet containing a 100% WP product (WP100). After 7d on experimental diets, pigs were exposed to constant heat stress conditions (32 °C) for 24h. There were no treatment differences in growth or body temperature indices prior to heat stress. During heat exposure, both rectal temperature and respiration rate increased (+0.85 °C and 3-fold, respectively; $P < 0.01$), and feed intake and body weight decreased (44% and -0.5kg, respectively; $P < 0.01$), but neither variable was affected by dietary treatments. Plasma L-lactate and D-lactate concentrations increased (36%; $P < 0.01$) and tended to increase (19%; $P = 0.09$) with heat stress. After 24h of heat exposure, WP100-fed pigs had lower plasma D-lactate relative to Ct-fed pigs. Ileal transepithelial electrical resistance was decreased (37%; $P = 0.02$) in WP80 pigs, compared with controls. No differences were detected in other intestinal integrity ex vivo measurements. These data demonstrate that dietary WP and CWP did not mitigate intestinal integrity dysfunction during severe heat stress.

Introduction

Heat stress negatively affects animal agriculture by reducing productivity and jeopardizing animal welfare. For the global swine industry, the effects of HS on growth, carcass quality, health and reproduction undermine efforts to improve efficiency and sustainability.[1] Further, the deleterious effects of HS will be aggravated if climate continues to warm as predicted.[2] In addition, genetic selection for rapid skeletal muscle growth might increase pigs' susceptibility to HS, as enhanced lean tissue accretion is accompanied by increased metabolic heat production.[3] Therefore, developing nutritional strategies to alleviate the effects of HS would be a key tool to maximize efficient animal protein production during the warm summer months.[4]

Heat stress compromises intestinal barrier function in a variety of species,[5] and this might partially explain its effect on animal production. In agreement, we have repeatedly demonstrated that heat-stressed growing pigs have reduced intestinal integrity and function.[6-8] This is caused by the re-distribution of blood flow to the periphery for increased heat dissipation, which reduces oxygen and nutrient supply to intestinal tissues, resulting in enterocyte damage and increased permeability to luminal content and pathogens.[9] The increased passage of bacterial components (e.g., LPS) and bacteria into portal and ultimately systemic circulation are partially responsible for the pathophysiology of heat related illnesses, as reducing intestinal bacterial load[10] or neutralizing plasma LPS[11] increase heat stroke survival. Hence, approaches attempting to preserve and restore intestinal barrier function might improve animal production and wellbeing during environmentally-induced hyperthermia.

Dietary dairy products have been demonstrated to improve gut health. For instance, milk, colostrum, and whey protein supplementation are beneficial in models of induced intestinal damage in both mice and humans,[12,13] as an ulcerative colitis treatment,[14] and in vitro in response to a tight junction disruptor.[15] Interestingly, dietary dairy products ameliorated the effects

Table 1. Test dairy products nutrient composition

	Test dairy products		
	WP80	**WP98**	**WP100**
Protein, %	78.44	79.70	79.85
Fat, %	5.76	6.27	6.33
Lactose, %	7.53	6.70	6.58
Ash, %	2.93	2.88	2.88
Moisture, %	4.32	4.36	4.36

WP80, test product containing 80% milk whey protein (WP) + 20% colostral whey protein (CWP); WP98, test product containing 98% WP + 2% CWP; WP100, test product containing 100% WP.

Table 2. Ingredients and formulated dietary nutrients

	Diet			
Parameter	**Ct**	**WP80**	**WP98**	**WP100**
Ingredients (%)				
Corn	73.51	84.27	84.27	84.27
Soybean meal (46.5)	22.30	5.75	5.75	5.75
Soybean oil	1.04	-	-	-
L-lysine HCl	0.3	0.2	0.2	0.2
DL-methionine	0.06	0.02	0.02	0.02
L-threonine	0.09	-	-	-
Monocalcium phosphate	1.14	1.16	1.16	1.16
Limestone	0.94	0.98	0.98	0.98
Salt	0.35	0.35	0.35	0.35
Vitamin Premix[1]	0.15	0.15	0.15	0.15
Trace Mineral Premix[2]	0.12	0.12	0.12	0.12
80% WP,[3] 20% CWP[4]	-	7	-	-
98% WP, 2% CWP	-	-	7	-
100% WP	-	-	-	7
Nutrients				
ME – kcal/kg	1530	1533	1533	1533
Crude Protein %	16.9	15.3	15.3	15.3
SID[5] Lys %	0.97	0.97	0.97	0.97
SID Met + Cys %	0.56	0.56	0.56	0.56
SID Thr %	0.61	0.61	0.61	0.61
SID Trp %	0.16	0.20	0.20	0.20
Calcium %	0.65	0.66	0.66	0.66
Phos. % - total	0.60	0.55	0.55	0.55
Phos. % - avilable	0.30	0.31	0.31	0.31
Sodium %	0.16	0.16	0.16	0.16
Chlorine %	0.25	0.25	0.25	0.25

[1]Provided the following per kg of diet: vitamin A, 7,656 IU; vitamin D, 875 IU; vitamin E, 62.5 IU; vitamin K, 3.75 mg; riboflavin, 13.75 mg; niacin, 70 mg; pantothenic acid, 33.75 mg; vitamin B12, 62.5 μg. [2]Provided the following per kg of diet: Fe, 121 mg as ferrous sulfate; Zn, 121 mg as zinc sulfate; Mn, 28.6 mg as manganese sulfate; Cu, 12.1 mg as copper sulfate; I, 0.22 mg as calcium iodate; Se, 0.22 mg as sodium. [3]Milk whey protein. [4]Colostral whey protein. [5]Standard ileal digestible.

of HS on intestinal barrier function in both mice[15] and a human colonic cell line.[16]

Thus, current study objectives were to determine the effects of dietary bovine WP concentrate and CWP concentrate on intestinal integrity parameters and blood biomarkers of 'leaky gut' in heat-stressed pigs. We hypothesized that feeding dairy products would prevent or at least ameliorate the deleterious effects of HS on gut permeability.

Materials and Methods

Animals and Experimental Design
Colostrum whey protein concentrate was obtained from Sterling Technologies and was blended with conventional WP concentrate (Main Street Ingredients). Iowa State University Institutional Animal Care and Use Committee approved all procedures involving animals. Thirty two crossbred gilts (39 ± 3 kg body weight) were stratified by body weight and then randomly assigned to 1 of 4 diets: 1) control (Ct), 2) the control diet containing an 80% WP and 20% CWP test product (WP80); 3) the control diet containing a 98% WP and 2% CWP test product (WP98); and 4) the control diet containing a 100% WP test product (WP100). Test dairy products were similar in nutrient composition (**Table 1**) and constituted 7% of the respective diets in order to provide 100 g/d of protein. Other than the added test dairy products, all diets were similar in ingredient and nutrient composition and were formulated to meet or exceed the estimated requirements[17] for essential amino acids, protein, minerals, and vitamins (**Table 2**). Pigs were ad libitum fed their respective diets and had free access to water throughout the entire experiment.

The study began after 3 d of acclimation to individual crates and was divided into two experimental periods. During period 1, pigs remained in constant thermoneutral conditions (19 °C; ~46% humidity; temperature-humidity index ≈63)[18] for 7 d. During period 2, pigs were exposed to constant HS conditions (32 °C; ~26% humidity; temperature-humidity index ≈76) for 24 h. At the end of period 2, pigs were sacrificed using the captive bolt technique followed by exsanguination. Throughout the experiment, ambient temperature was controlled but humidity was not governed, and both parameters were monitored and recorded every 30 min by data loggers (EL-USB-2-LCD, Lascar). Temperature-humidity index ranged between 58–65 and 75–79 during period 1 and 2, respectively.

During period 1, body temperature indices (respiration rate and rectal temperature) were obtained four times a day (0800, 1200, 1600 and 2000 h) and condensed into daily averages and period average. During period 2, temperature indices were obtained at 0, 4, 8, 12 and 24 h relative to the initiation of HS. Respiration rate was determined by counting flank movements and rectal temperature was measured using a digital thermometer (V901H, Vicks®). Individual feed intake was recorded daily as-fed throughout the experiment. Body weights were collected at the beginning of each period and immediately prior to sacrifice.

Blood was obtained (K_2EDTA blood tubes, BD vacutainers®, cat# 367861) on day 6 of period 1 (24 h prior to initiation of HS) and at sacrifice and kept in ice until processing. Plasma was

harvested by centrifugation at 1300 x *g* and stored at -80 °C for later analysis. Whole sections from both the proximal ileum (1.5 m proximal to the ileocecal junction) and distal colon (0.5 m proximal to the rectum) were harvested immediately following euthanasia. Intestinal segments were processed as previously described.[8] Due to logistical constraints in sample collection and analysis, groups of 4 pigs (1 pig per treatment) were sacrificed twice a day (8 pig/d) for 4 consecutive days. Each group of 4 pigs was considered a "set" for statistical purposes. The timing of each measured variable was similar among sets.

Ex vivo measures of intestinal integrity

Ileal and colonic segments of each animal were mounted into modified Ussing chambers (Physiological Instruments) for determination of intestinal integrity. The TER and FITC-Dextran APP (4.4 kDa; Sigma®, cat# FD4) were measured and calculated as previously described by Pearce et al.[7] Chamber slides had a surface area of 0.7 cm[2] and all readings were corrected to a 1 cm[2] surface.

Blood parameters analyses

Plasma L-lactate and D-lactate concentrations were measured enzymatically using commercially available kits (Biomedical Research Service Center). Plasma LBP concentrations were determined using an ELISA kit (Hycult® biotech, cat# HK503). The intra- and inter-assay coefficients of variation were 6.6 and 5.1% for L-lactate, 1.5 and 2.3% for D-lactate, and 8.7 and 22.2% for LBP.

Statistical analyses

Data are reported as LSmeans and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. Variables with single measurements were statistically analyzed using the PROC GLM procedure of SAS version 9.2 (SAS Inst. Inc.). The model included treatment and set as fixed effects. For a given variable, when an initial measurement (at the beginning of the period or during period 1) was available it was used as a covariate.

Variables with multiple measurements were analyzed using the PROC MIXED procedure of SAS. Each animal's respective parameter was analyzed using repeated measures with an auto regressive covariance structure. The repeated effect was hour after initiation of HS (rectal temperature and respiration rate) or period (blood parameters). The model included treatment, the repeated effect, set, and treatment by the repeated effect interaction as fixed effects; and covariate when available. For both procedures, set and the covariate were only kept in the model if their $P \leq 0.20$. Contrasts were performed to estimate differences between each dietary treatment and the Ct-fed pigs. For each
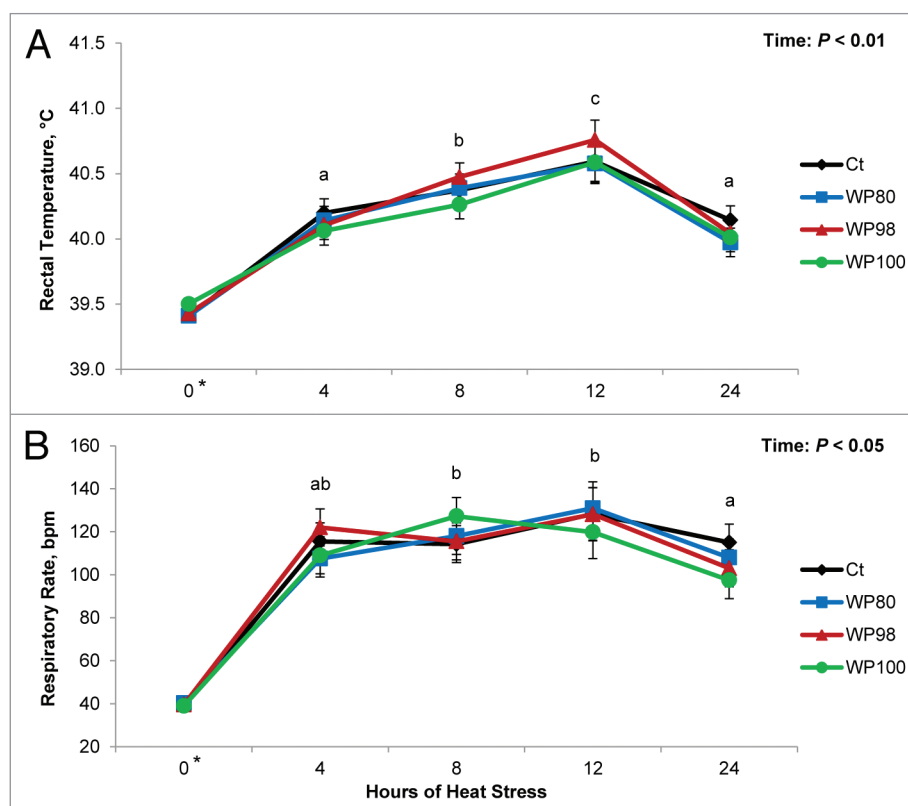

Figure 1. Effects of feeding diets containing no test product (Ct), or 80% milk whey protein (WP) + 20% colostral whey protein (CWP; WP80), 98% WP + 2% CWP (WP98), 100% WP (WP100) test products on (**A**) rectal temperature and (**B**) respiratory rate of pigs exposed to constant heat stress conditions (32 °C) for 24 h. [a,b,c] Represent differences between hours of heat stress ($P \leq 0.05$). *Represents the average values during period 1.

variable, residuals distribution was tested for normality and logarithmic transformation was performed when necessary.

Results

During period 1, there were no differences in body temperature indices (**Fig. 1**) among diets. As expected during period 2, rectal temperature and respiration rate markedly increased by 0.85 °C and ~3-fold, respectively ($P < 0.01$; **Fig. 1**). There was a time effect ($P \leq 0.05$; **Fig. 1**) for both indices as rectal temperature and respiration rate peaked at 12 and 4 h post-HS initiation, respectively; however, no dietary treatment differences in these variables were detected. Both rectal temperature and respiration rate remained markedly increased (0.6 °C and 3-fold, respectively) compared with period 1 (**Fig. 1A and B**).

During period 1, feed intake (2.1 kg/d), average daily gain (0.74 kg/d) and gain to feed ratio (0.35) were not different between diets (**Table 3**). During period 2 (after 24 h of HS), pigs in all treatments similarly reduced their intake and lost body weight (44% and -0.5 kg, respectively; **Table 3**).

There was a diet effect on ileal TER, as it was decreased ($P \leq 0.05$) and tended to be decreased ($P \leq 0.10$) in WP80 and WP100-fed pigs (37 and 27%, respectively; **Table 4**), compared with controls. Ileal TER did not differ between WP98 and Ct-fed

Table 3. Effects of dietary dairy products and environmental conditions on production parameters in pigs

	Trt					P	Contrast vs Ct		
Parameter	Ct	WP80	WP98	WP100	SEM	Trt	WP80	WP98	WP100
Period 1[1]									
ADG,[2] kg/d	0.76	0.73	0.71	0.74	0.04	0.73	0.48	0.29	0.69
FI,[3] kg/d	2.15	2.06	2.11	2.10	0.07	0.80	0.33	0.71	0.61
G:F[4]	0.36	0.35	0.34	0.35	0.02	0.73	0.61	0.27	0.51
Period 2[5]									
ΔBW,[6] kg	-0.64	-0.43	-0.52	-0.41	0.33	0.96	0.66	0.80	0.63
ΔFI,[7] kg	-0.90	-0.98	-0.93	-0.98	0.11	0.95	0.63	0.83	0.62

Ct, diet containing no test product; WP80, diet containing 80% milk whey protein (WP) + 20% colostral whey protein (CWP) test product; WP98, diet containing 98% WP + 2% CWP test product; WP100, diet containing 100% WP test product. [1]Thermoneutral conditions: 19 °C; ~46% humidity. [2]Average daily gain. [3]Feed intake. [4]Gain to feed ratio. [5]Heat stress conditions for 24 h: 32 °C; ~26% humidity. [6]Change in body weight (period 2 – period 1). [7]Change in feed intake (period 2 – period 1).

Table 4. Effects of dietary dairy products on intestinal permeability parameters in 24 h heat-stressed pigs

	Trt					P	Contrast vs Ct		
Parameter	Ct	WP80	WP98	WP100	SEM	Trt	WP80	WP98	WP100
Ileum									
TER[1]	142[bc]	90[a]	157[c]	104[ab]	16	0.02	0.03	0.51	0.10
FITC-Dextran APP[2]	17.2	20.5	15.2	23.2	8.5	0.91	0.77	0.84	0.63
Colon									
TER	90	89	89	100	7	0.66	0.87	0.94	0.34
FITC-Dextran APP	10.1	9.8	11.0	6.0	4.2	0.76	0.97	0.90	0.40

Ct, diet containing no test product; WP80, diet containing 80% milk whey protein (WP) + 20% colostral whey protein (CWP) test product; WP98, diet containing 98% WP + 2% CWP test product; WP100, diet containing 100% WP test product. [1] Transepithelial electrical resistance, Ω·cm². [2] Fluorescein isothiocyanate labeled dextran apparent permeability coefficient, μg/ml/min/cm².

pigs. There were no dietary treatment differences on ileal FITC-Dextran APP or any measure of colonic integrity (**Table 4**).

Both plasma L-lactate and D-lactate concentration increased ($P < 0.01$; 36%) and tended to increase ($P = 0.09$; 19%), respectively from period 1 to period 2, but the magnitude of the response was similar among dietary treatments (**Fig. 2A and B**). During period 2 and using period 1 as a covariate, plasma D-lactate was decreased in WP100-fed pigs ($P ≤ 0.05$; 29%; **Fig. 2C**), when compared with Ct-fed pigs. Neither dietary treatment nor period had an effect on plasma LBP concentration (**Fig. 2C**).

Discussion

Heat stress negatively affects animal agriculture by reducing growth and reproductive performance and jeopardizing animal welfare. Heat-stressed animals redistribute blood to the periphery in an attempt to maximize radiant heat dissipation.[5] Subsequent visceral vasoconstriction leads to intestinal hypoxia in addition to hyperthermia.[6,9] As demonstrated by the early upregulation of heat shock proteins during hyperthermia,[19] enterocytes are extremely sensitive to oxygen and nutrient restriction,[20] resulting in ATP depletion, and increased oxidative and nitrosative stress.[21] Ultimately, HS causes marked morphological changes, tight junction disruption, and reduced intestinal barrier

function.[5] Increased intestinal permeability during HS, elevates portal and systemic blood LPS concentration,[7,21] which might mediate some of the negative effects of HS on animal production.[22] Interestingly, dairy products have improved intestinal barrier function in a variety of models. Further, we have recently demonstrated that dietary supplementation with zinc partially ameliorated the effects of HS on intestinal permeability in pigs,[8] demonstrating that nutritional management is a feasible mitigation strategy for environmental hyperthermia. Thus, we hypothesized that dietary bovine WP and CWP would alleviate the decrease in intestinal integrity observed in pigs during HS.

Contrary to our hypothesis and in disagreement with the literature, feeding WP/CWP did not improve direct measures (i.e., TER and FITC-Dextran APP) of ileal or colonic permeability. Playford et al. demonstrated that dairy product supplementation ameliorates the effects of non-steroidal anti-inflammatory drugs on the gastro-intestinal tract in rodents (i.e., WP and CWP) and humans (i.e., bovine colostrum and milk).[12,13] In addition, local treatment with CWP improved symptoms and histological scores of ulcerative colitis patients.[14] In vitro studies have also shown the beneficial effects of bovine colostrum and goat milk on TER in response to a tight junction disruptor.[15] Finally, dairy products improved intestinal integrity in heat-stressed rats[15] and in an in vitro model of hyperthermia.[16] The mechanism by

which dairy products may protect intestinal health is not fully elucidated. Both bovine WP and CWP are rich in antimicrobial proteins (e.g., glucomacropeptides, lactoferrin), immunoglobulins, growth factors (transforming growth factor-β), and specific amino acids (glutamine, cysteine, and threonine).[23] However, the composition of these products is highly variable depending on the origin (e.g., breed, alimentation and health status), the time of collection, and the post-collection processing; making difficult to identify the bioactive constituents responsible for their positive effects.[24] With regard to intestinal health, several mechanisms of action have been attributed to dairy products, including upregulation of heat-shock proteins[16] and tight junction proteins,[25] or the increase in mucin production.[26] Consequently, there appear to be a variety of mechanisms by which dietary dairy products can reduce gut "leakiness."

Reasons for the lack of a protective response to our dietary treatments are not clear. In the present study, pigs were severely heat-stressed (constant 32 °C without a thermal recovery period during the night), as demonstrated by a marked increase in all the body temperature indices (rectal temperature = +0.85 °C, respiration rate = +76 breath/min) and sharp decrease in feed intake (44%). We hypothesize that the severe HS may have blunted the potential beneficial effects of WP and CWP on intestinal health. Whether or not mild and/or cyclical HS conditions (more similar to natural heat stress events), would allow for dairy products to express their improvement on intestinal permeability variables remains of interest. Not only were the products in the current study ineffective, feeding WP80 (the diet with the highest content in CWP: 20%) actually increased ileal permeability. This is not unprecedented as it agrees with a human report, where subjects supplemented with bovine colostrum had increased in intestinal permeability after a standardized exercise program compared with controls and individuals receiving WP.[27]

We recognize that our experimental design (with no thermoneutral control treatment) makes it difficult to directly demonstrate that the gut was actually compromised by HS. However, utilizing a similar heat load and type of pigs (i.e., genetics, body weight, gender) we have repeatedly reported that direct intestinal measurements of gut integrity deteriorated after 24 h of thermal exposure,[6,7] and circulating LPS increased.[28] In addition, in a similar experimental design we have reported an increase in oxidative stress parameters in skeletal muscle, ostensibly the consequence of heat-stressed induced endotoxemia.[29] In the current experiment we measured blood biomarkers of leaky gut (i.e., D- and L-lactate and LBP) in both period 1 and 2. D-lactate is a product of microbial metabolism, so its presence in blood likely indicates an increase in gut leakiness.[30,31] As expected, D-lactate increased from period 1 to period 2, confirming that the intestinal barrier function was compromised after 24 h of HS. Lipopolysaccharide binding protein is an acute phase protein that binds LPS and mediates its interaction with toll-like receptor 4,[32] resulting in the activation of the innate immune response. Interestingly, high circulating concentrations of LBP inhibit LPS-induced inflammation.[33] We have previously reported that plasma LBP decreases as HS progresses (1–12 h) and intestinal integrity deteriorates.[28] Reasons why we did not observe a period effect on plasma LBP
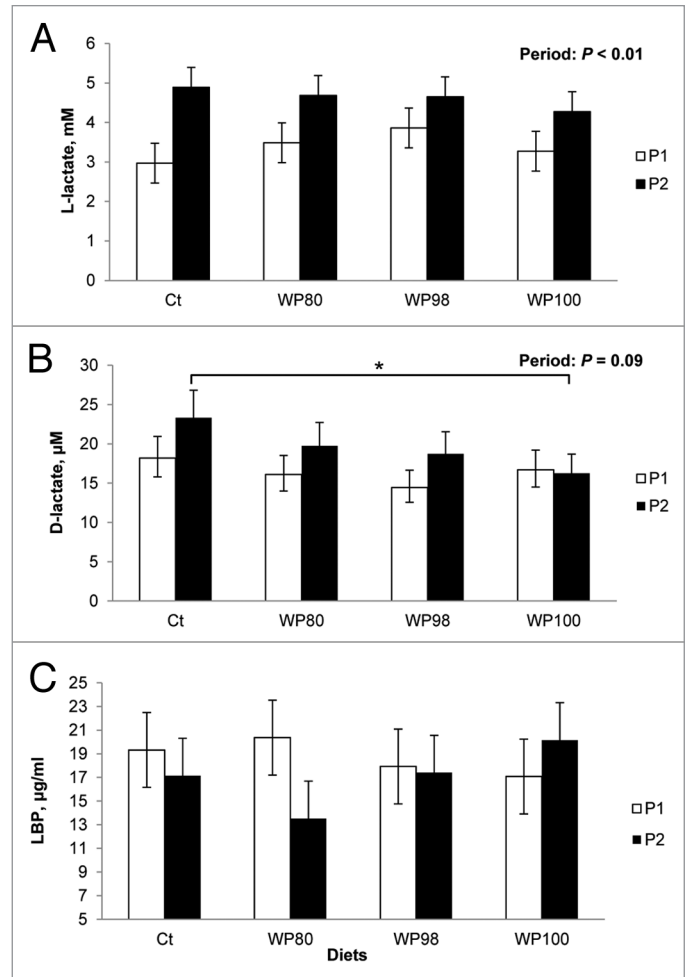

Figure 2. Effects of feeding diets containing no test product (Ct), or 80% milk whey protein (WP) + 20% colostral whey protein (CWP; WP80), 98% WP + 2% CWP (WP98), 100% WP (WP100) test products on plasma (**A**) L-lactate, (**B**) D-lactate, and (**C**) lipopolysaccharide binding protein (LBP) concentrations of pigs during period 1 (P1; thermoneutral conditions: 19 °C; ~46% humidity) and at the end of period 2 (P2; heat stress conditions for 24 h: 32 °C; ~26% humidity). *Represents differences between treatments during period 2 with period 1 as a covariate.

concentrations remain unknown. Noteworthy, at the end of period 2, WP100-fed pigs had lower plasma D-lactate concentration compared with Ct-fed pigs and a numerical increase in plasma LBP from period 1 to period 2, which has been previously associated with decreased circulating LPS.[28] In agreement, WP100-fed pigs had a numerical improvement in intestinal integrity (increased TER and decreased FITC-dextran APP) at the colon level, where most of the microflora is located, which might explain the aforementioned changes in plasma parameters.

L-lactate is the product of anaerobic glycolysis. The rationale to utilize this parameter as a biomarker of intestinal integrity is based on the release of intracellular L-lactate by damaged intestinal cells.[31] However, a similar phenomenon occurs in non-intestinal cells, which makes L-lactate a nonspecific measurement of cellular damage. Our baseline (period 1) L-lactate levels are higher than those previously reported,[31,34] however, our blood

collection method (jugular venipuncture), which requires physical constraint, could have triggered a stress response which is known to increase L-lactate levels.[35] Nevertheless, this procedure was consistently performed in both periods. Notably, the increase in plasma L-lactate has been repeatedly observed during HS.[22] Our recent data suggests that complete glucose oxidation in skeletal muscle is decreased during HS, thus L-lactate is produced via aerobic glycolysis.[22] Therefore, increased circulating L-lactate might be the result of metabolic adaptations to HS, resembling the Warburg effect employed by cancerous cells.[36] Whether the increase in circulating L-lactate from period 1 to period 2 is a result of cellular damage (presumably intestinal damage) or altered systemic glucose metabolism remains to be elucidated.

Heat stress is one of the largest impediments to efficient animal production. A hallmark of heat-stressed animals is a compromised intestinal integrity, and the subsequent endotoxemia might contribute to reduced animal productivity. We have herein demonstrated that feeding a combination of bovine WP and CWP in the tested proportions failed to ameliorate the effects of severe and constant HS on intestinal integrity of pigs. Whether milder and cyclical HS conditions (resembling those in commercial settings) and different levels of inclusion of the test dairy products would allow for improvement on intestinal health remains of interest.

Disclosure of Potential Conflicts of Interest

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