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Research paper

Effect of bovine genotype on innate immune response of heifers to repeated lipopolysaccharide (LPS) administration

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ABSTRACT

This pilot study provides a preliminary assessment of the impact of genotype on acute innate immune pro-inflammatory, metabolic and endocrine responses to repeated lipopolysaccharide (LPS) administered to growing heifers. Heifers (n = 4/genotype) were from unselected (stable milk yield since 1964, UH) or contemporary (CH) Holstein cows that differed in milk yield (6200 vs 11,100 kg milk/305 d) or from contemporary Black Angus (CA) cows bred to contemporary Red Angus bulls. Heifers were challenged with iv administration of 0.5 μg LPS/kg body weight on day 1 (Challenge 1) and d 5 (Challenge 2) of study to assess endotoxin tolerance. Plasma was collected at -1, -0.5, 0, 1, 2, 3, 4, 6, 8, and 24 h relative to each LPS administration. Rectal body temperature (BT) was measured before each blood sampling and at 5 and 7 h. Data were analyzed by repeated measures with sampling time as the repeated effect. Each genotype had at least one pro-inflammatory response that indicated it might have a more robust response than the other genotypes. The CH heifers had a greater TNF-α response, UH heifers had greater IL-6 and XO responses and CA heifers had greater BT and SAA response to LPS than the other genotypes. There was a genotype by time by interaction as cortisol peaked earlier in CH and UH than in CA heifers. Glucose response was less in CA and insulin response was greater in CH heifers. Endotoxin tolerance to LPS was evident as pro-inflammatory, cortisol, glucose and insulin responses were less during Challenge 2 than during Challenge 1. Differences among genotypes during Challenge 1 were eliminated during Challenge 2 except for the greater SAA response in CA heifers and indicate the potential for differential impacts of genotype on the development of endotoxin tolerance. Specific reasons for these effects of genotype are not clear from these data but the results support the hypothesis for differential innate immune signaling among these bovine genotypes.

1. Introduction

Selective breeding of cattle during the past several decades focused primarily on increased production because greater production per animal can increase productive efficiency and profitability. These production goals are known to reduce environmental impacts of the industry (Capper and Bauman, 2013). There is concern that insufficient selection for health traits has rendered the contemporary cow more susceptible to disease and metabolic disorders than her ancestors (Egger-Danner et al., 2015; Pritchard et al., 2013). Although beef and dairy cattle are selected for some common traits including increased milk yield (American Angus Association, 2019; Animal Improvement Program, 2019) specific selection criteria for beef and dairy cattle often differ so the impact on physiological functions could and likely does vary among cattle breeds. Indeed, differences in immune responses have been identified between beef breeds (Blecha et al., 1984; Carroll

Abbreviations: APP, acute phase proteins; BT, body temperature; C1, challenge 1 d1; C2, challenge 2 d5; CA, contemporary Red-Black Angus; CH, contemporary Holsteins; IGF, insulin-like growth factor; LPS, lipopolysaccharide; NEFA, non-esterified fatty acid; NOx, nitrate + nitrite; SAA, serum amyloid A; TLR4, Toll-like receptor 4; UH, unselected (1964 genetics) Holsteins; XO, xanthine oxidase

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et al., 2011) between dairy breeds (Bannerman et al., 2008) and between beef and dairy breeds (Benjamin et al., 2016).

Breed comparison studies have indicated differences in onset, cessation and duration of immune component responses to *E. coli* but no overall difference in ability of the genotypes to eliminate (clear) the infection or resolve the inflammation have been established (Bannerman et al., 2008). However, there is substantial evidence of differences in mastitis prevalence among breeds (Kelm et al., 2001). A more robust IL-8 response to LPS in fibroblasts isolated from Holstein than from Angus heifers was recently reported (Benjamin et al., 2016) and we have demonstrated that 50 years of selection has reduced the IL-6 and SAA response of Holsteins to LPS (Cousillas Boam, 2018). In addition, there is a growing body of evidence of genetic variants in immune related genes (Stojkovic et al., 2016). We have identified selection based genomic signatures associated with immunity (Ma et al., 2019) and selection practices continue to alter the genome and physiological functions in cattle. Thus, the impact of bovine genotype on immune response warrants further investigation.

The innate immune system is the first line of defense against infectious diseases. The acute phase response of the innate immune system aids in pathogen neutralization which helps prevent further invasion and minimize tissue damage. This response involves multiple physiological changes including fever, increased vascular permeability, hepatic production of acute phase proteins (APP) and alterations in circulating concentrations of metabolic, endocrine and immune components (Arango Duque and Descoteaux, 2014; Carroll et al., 2009). The immediate response is mediated largely by immune cells (primarily monocytes, macrophages, and neutrophils) that phagocytize and kill pathogens and simultaneously coordinate additional host responses by synthesizing a wide range of inflammatory mediators and cytokines (Arango Duque and Descoteaux, 2014). Gram-negative bacteria are responsible for many of the infections seen in cattle and clinical signs are mainly the result of host reactions to lipopolysaccharide (LPS) after it is released from the bacterial cell wall and recognized by toll-like receptor 4 (TLR4) on host immune cells (Bode et al., 2012; Netea et al., 2004).

Repeated or prolonged exposure of the host to LPS, either from pathogens or from administration of the isolated compound, results in endotoxin tolerance which is one of the primary mechanisms that regulate the magnitude and duration of the LPS-induced inflammatory response (Bode et al., 2012; López-Collazo and Del Fresno, 2013; Zaric et al., 2011). Although not able to reproduce all aspects of bacterial exposure, LPS administration can elicit an innate inflammatory immune response. The primary objective of this preliminary effort was to examine the impact of bovine genotype on acute innate immune pro-inflammatory response in growing heifers. A novel aspect is that we administered LPS to heifers from unique Holsteins that have not been subjected to selection since 1964 (unselected Holsteins, UH; Weber et al., 2007), to contemporary Holstein (CH) heifers, and to contemporary Red-Black Angus (CA) heifers. This study extends our initial evaluation of UH and CH heifers that received a single LPS challenge (Cousillas Boam, 2018) and incorporates a repeated LPS challenge model to also provide a preliminary assessment of the impact of genotype on development of a refractory state of endotoxin tolerance. This approach provides the opportunity to assess the impact of 50 plus years of selection that was primarily focused on production, on the immune response of Holsteins and to compare immune response in prevalent, contemporary beef and dairy breeds.

2. Materials and methods

2.1. Animals, experimental design, and treatments

Beef heifers were from contemporary Black Angus (CA) cows bred to contemporary Red Angus bulls at the University of Minnesota, North Central Research and Outreach Center in Grand Rapids, MN. Cows at this site are bred to bulls to produce offspring of moderate frame size with a selection emphasis on growth rate and carcass quality. The dairy heifers were from UH or CH cows (n = 4/genotype) housed at the University of Minnesota, West Central Research and Outreach Center in Morris, MN. Both of these sites utilize pasture-based, grazing management systems to raise their youngstock. Milk yield of the UH cows has been stable since it was established in 1964 (Weber et al., 2007; Young, 1977) and is more than 4500 kg/305 d lactation less than that of their CH herdmates (University of Minnesota herd records).

The 12 heifers (19.6 ± 0.19 months of age) were moved to the University of Minnesota, St. Paul campus and housed together for 47 d to adapt to facilities and handling procedures at the. Heifers were fed the same diet ad lib between 1500 and 1700 daily and handled (haltered, tethered, groomed) at least thrice weekly during the adaptation period. Heifers were pregnant (160–185 d of gestation on d 1 of study) except for 2 CH heifers that were treated (Bridges et al., 2008) to be at presumptive day 8 of their estrous cycle on d 1 of the study in order to have progesterone concentrations similar to those of their pregnant cohorts. Jugular catheters were implanted at least 24 h before LPS administration. Heifers received *iv* administration of 0.5 μg LPS (*Escherichia coli* O111:B4; Sigma-Aldrich L4391) per kg body weight between 0700 and 0800 on day 1 (challenge 1, **C1**) and d 5 (**C2**) of study. During each challenge, feed was withheld but heifers had ab libitum access to water. Blood samples (10 mL) were collected at -1, -0.5, 0, 1, 2, 3, 4, 6, 8, and 24 h relative to LPS administration. Rectal body temperatures (BT) were determined at these times and at 5 and 7 h. Blood was mixed with heparin (20 μL of 10,000 IU/mL), placed on ice and centrifuged (1500 x g, 15 min at 4 °C) within 30 min. Plasma was aliquoted and stored at −20 °C until analyzed. Heifers were observed daily throughout the study. All animal procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee.

2.2. Sample analyses

Plasma TNF-α concentrations were measured by a specific double antibody RIA (Kenison et al., 1990). The minimum detectable TNF-α concentration was 4.00 pg/assay tube. Samples were assayed in duplicate, in a single assay with an intra-assay coefficient of variation 7.8%. Plasma IL-6 concentrations were determined by a sandwich ELISA (Kandasamy and Kerr, 2012). The minimum detectable concentration was 0.1 ng/mL and the intraassay coefficient of variation was 7.5%. Plasma serum amyloid A (SAA) was determined in the 0, 4, 8 and 24 h samples by an ELISA (Phase™ Range, Tri-Delta Diagnostics, Inc., Morris Plains, NJ). Samples were analyzed in duplicate and according to the manufacturer's instructions. The ELISA was validated for recovery and parallelism with bovine plasma. The minimum detectable concentration of SAA was 1.50 μg/mL and the interassay coefficient of variation was 3.2%.

Plasma xanthine oxidase (XO) activity was determined using Amplex™ Red Xanthine/Xanthine Oxidase Assay Kit (A-22182, Molecular Probes, Eugene, OR; (Kahl and Elsasser, 2006). Samples were analyzed in a single assay. The minimum detectable concentration was 0.1 mU/mL and the intraassay coefficient of variation was < 5%. Plasma nitrate plus nitrite concentrations were used to measure nitric oxide (NOx) using the Griess reaction after enzymatic conversion of plasma NO_3^- to NO_2^- with nitrate reductase from Aspergillus species (Kahl et al., 1997). The minimum detectable concentration was 0.1 μM and the intra and interassay coefficients of variation were < 9.5%.

Plasma glucose (Autokit Glucose, Wako Life Sciences, Mountain View, CA) and non-esterified fatty acid (NEFA; NEFA-HR (2), Wako Life Sciences, Mountain View, CA) concentrations were determined by colorimetric assays with volumes modified for use in a 96-well plate. Samples were analyzed in duplicate. Intra and interassay coefficients of variation were 4.2 and 4.1% and 4.4 and 7.7% for glucose and NEFA, respectively. Plasma cortisol, insulin and progesterone concentrations were measured by RIA (ImmunoChem Cortisol, MP Biomedicals,

Orangeburg, NY; ImmunoChem Insulin, MP Biomedicals, Solon, OH; Coat-A-Count Progesterone, DPC, Los Angeles, CA). The assays were validated for recovery and linearity of dilution with bovine plasma. Samples were analyzed in duplicate. The minimal detectable concentration of cortisol was 0.17 μg/dL and the intra and interassay coefficients of variation were 8.4 and 6.2%, respectively. The minimal detectable concentration of insulin was 4.60 μIU/mL and the intra and interassay coefficients of variation were 9.1 and 6.9%, respectively. The minimal detectable concentration of progesterone was 0.02 ng/mL. All samples were assayed in one assay and the intraassay coefficient of variation was < 10%. Plasma insulin like growth factor-1 (IGF-1) concentrations were quantified with a validated double-antibody RIA (Weber et al., 2007). Samples were analyzed in triplicate. The minimal detectable concentration of IGF-1 was 0.20 ng/mL. All samples were assayed in one assay and the intraassay coefficient of variation was 5.5%.

2.3. Statistical analyses

Statistical power assessed before the study indicated a minimum of 4 heifers per genotype were sufficient to detect a serum IL-6 difference of 1.4 ng/mL as significant at $P < 0.05$ with 80% power. Ability to detect alterations in serum IL-6 concentration was used to assess power because IL-6 is the primary cytokine regulator of the acute phase protein response (Scheller et al., 2011). Data were analyzed using SAS version 9.3 (SAS/STAT, SAS Inst. Inc., Cary, NC) procedures. Some of the responses during C1 were expected to continue into C2 so the pre-challenge data were analyzed independently for C1 and C2. The model included genotype as a fixed effect and heifer as a random effect. Challenge data were analyzed by repeated measures using PROC MIXED with time as the repeated effect. The spatial power law for unequally spaced data was specified as the covariance structure. The model included genotype, time, challenge and their interactions as fixed effects and heifer as a random effect. Results are reported as least squares means. Means were considered to differ when $P \leq 0.05$ and trends identified when $0.05 < P \leq 0.10$.

3. Results

Heifers were well adapted to the facilities and handling procedures and no differences in temperament were observed. Administration of LPS resulted in transient characteristic signs of mild systemic innate immune responses during C1 and C2. Labored breathing accompanied by increased salivation, nasal discharge and coughing as well as mild diarrhea and lethargy were observed in all heifers within 1 h of LPS administration and were completely resolved within 6–7 h. On day 1, plasma progesterone concentration in each heifer exceeded 3.4 ng/mL. Pre-challenge BT and plasma components within challenge did not differ ($P > 0.19$) among genotypes except for NOx ($P = 0.07$) during C1 and NEFA ($P = 0.02$) during C2. All measured components were increased after LPS administration (Table 1) but there was no indication that the duration of response differed among the genotypes (Figs. 1–4).

Rectal BT was elevated through 7 h during C1 and beyond 8 h during C2 (Fig. 1). There was a genotype by challenge by time interaction ($P = 0.008$) for BT (Table 1) because BT increased earlier and peaked higher in CA than in UH or CH heifers during C1 but did not differ among genotypes during C2 when BT response in CA heifers remained similar to that during C1 while the BT increase in UH and CH heifers was greater during C2 than C1.

There was a genotype by challenge by time interaction ($P = 0.025$) for TNF-α as concentrations during C1 increased more in CH than in UH or CA heifers but were less and did not differ among genotypes during C2 (Table 1, Fig. 3A). During C1, concentrations of TNF-α peaked at 1 h in UH and CA (4.3 and 6.0 ± 0.6 ng/mL) and at 2 h in CH (8.9 ± 0.6 ng/mL). There was a genotype by challenge interaction ($P = 0.002$) for IL-6 as concentrations were greater in UH and CH heifers during C1 than C2 while response by CA heifers did not differ between challenges (Table 1, Fig. 3B). There was a genotype by time interaction ($P = 0.011$) for IL-6 as overall response was more persistent through 6 h in UH and CH than in CA heifers.

There was a genotype by time interaction ($P = 0.049$) for SAA as concentrations did not differ among genotypes until 24 h after LPS administration when they were greater in CA than in UH or CH (Table 1; Fig. 3C). There was a challenge by time interaction ($P < 0.001$) for SAA as concentrations during C1 were less than those in C2 through 8 h but greater at 24 h. There was a trend ($P = 0.098$) for a genotype by challenge interaction as XO concentration was less in CH and CA than UH (Table 1) during C1 but did not differ among genotypes during C2. There was a challenge by time interaction for XO ($P < 0.001$) as concentrations during C1 were greater at 8 and 24 h than the corresponding times in C2. Although XO concentrations decreased between 8 and 24 h, the 24 h concentration (2.2 ± 0.2 mU/mL) remained greater ($P < 0.001$) than the 0 h (1.5 ± 0.2 mU/mL) concentration. There was a trend for a challenge by time interaction ($P = 0.092$) for NOx as the greatest concentrations occurred at 8 h in C1 but at 4 h in C2. There was a trend ($P = 0.100$) for NOx concentrations to be greater in UH than in CA heifers.

Concentrations of IGF-1 (Table 1, Fig. 3D) tended ($P = 0.088$) to be greater in CH and UH than in CA heifers. There was a challenge by time interaction ($P < 0.001$) for IGF-1 as concentrations during C1 decreased through 24 h but concentrations during C2 decreased through 8 h and were greater than the 0 h values at 24 h (Fig. 3D). There was a challenge by time interaction ($P < 0.001$) for cortisol (Table 1, Fig. 2C) as concentrations after LPS administration were greater in C1 than C2 for all time points. There was a genotype by time interaction ($P < 0.001$) for cortisol (Table 1, Fig. 2C) as concentrations at 1 h were greater in UH and CH than in CA heifers. There was a genotype by challenge by time interaction ($P = 0.011$) for the biphasic glucose response because concentrations increased less at 1 and 2 h in CA than in UH or CH heifers during C1 but did not differ among genotypes during C2 when concentrations in CA heifers did not differ between challenges but were reduced in UH and CH heifers (Table 1, Fig. 2A). There was a genotype by challenge by time interaction ($P = 0.012$) for plasma insulin as peak concentration was greater in CH than in UH or CA heifers during C1 but there were no differences among genotypes during C2 (Table 1, Fig. 2B). There was a challenge by time interaction ($P < 0.001$) for NEFA as concentrations at 0, 1, 6, 8 and 24 h during C2 were greater than those from the corresponding times in C1 (Fig. 4D). There was a genotype by time interaction ($P < 0.001$) for NEFA as concentrations either did not differ ($P > 0.10$) among genotypes or were greater ($P < 0.05$) in CA than in CH heifers.

4. Discussion

Pathogen recognition activates macrophages, monocytes and neutrophils to synthesize and release pro-inflammatory cytokines, such as TNF-α and IL-6 which stimulate the APP response, inflammation and sickness behavior (Arango Duque and Descoteaux, 2014; Carroll et al., 2009). A robust, controlled and short-lasting pro-inflammatory response to endotoxin exposure is generally considered beneficial for early resolution of infection while a prolonged inflammatory response can increase the potential for tissue damage (Bode et al., 2012; Burvenich et al., 2003; Zaric et al., 2011). Endotoxin tolerance is considered a protective mechanism against excessive inflammation (Cavaillon et al., 2003; López-Collazo and Del Fresno, 2013) and, depending on the interval between challenges, can be assessed from activation through recovery by administration of repeated LPS challenges. We focused our efforts on evaluation of the acute phase of challenges separated by 4 d which was sufficient to demonstrate the refractory state of endotoxin tolerance in cattle (Kahl et al., 2011).

Our previous efforts demonstrated that 50 plus years of selection has decreased IL-6 response to LPS in CH heifers (Cousillas Boam, 2018)

Table 1
Effect of genotype (G), challenge (C), time (T) and their interactions on rectal body temperature (BT) and concentration of plasma components in heifers[a] challenged twice with lipopolysaccharide (LPS).[b]

Item	*P*-values						
	G	C	T	GC	GT	CT	GCT
BT[c], °C	0.120	< 0.001	< 0.001	0.045	< 0.001	0.002	0.008
TNF-α[d], ng/mL	0.153	< 0.001	< 0.001	0.024	0.107	< 0.001	0.025
IL-6[e], ng/mL	0.515	< 0.001	< 0.001	0.002	0.011	< 0.001	0.570
SAA[f], μg/mL	0.015	0.934	< 0.001	0.912	0.050	< 0.001	0.770
XO[g], mU/mL	0.145	< 0.001	< 0.001	0.098	0.683	< 0.001	0.137
NOx[f], μM	0.100	0.370	0.002	0.366	0.575	0.092	0.275
IGF-I[f], ng/mL	0.088	< 0.001	< 0.039	0.240	0.523	< 0.001	0.216
Cortisol[h], μg/dL	0.253	< 0.001	< 0.001	0.441	< 0.001	< 0.001	0.777
Glucose[h], mg/dL	0.021	0.116	< 0.001	0.397	0.002	< 0.001	0.011
Insulin[e], μIU/mL	0.348	0.041	< 0.001	0.084	0.327	0.022	0.012
NEFA[h], mEq/L	0.125	< 0.001	< 0.001	0.376	0.001	< 0.001	0.800

[a] Heifers (n = 4/genotype) from unselected (stable milk yield since 1964, UH) and contemporary (CH) Holsteins and contemporary Red-Black Angus (CA) cows.
[b] Identical LPS challenges (0.5 μg LPS/kg BW) were administered via jugular catheter on day 1 and 5.
[c] Measured at -1, 0, 1, 2, 3, 4, 5, 6, 7, 8 and 24 h relative to challenge.
[d] Measured at 0, 1, 2, 3, 4 and 6 h relative to challenge.
[e] Measured at -1, 0, 1, 2, 3, 4, 6, and 8 h relative to challenge.
[f] Measured at 0, 4, 8 and 24 h relative to challenge.
[g] Measured at 0, 8 and 24 h relative to challenge.
[h] Measured at -1, -0.5, 0, 1, 2, 3, 4, 6, 8 and 24 h relative to challenge.

and this was confirmed in the current study. Previous in vitro work indicated cultures of fibroblasts from CH heifers responded more robustly to LPS than fibroblasts from Angus heifers (Benjamin et al., 2016). Heifers used in the current study were from the same facilities as the heifers used in these 2 studies (Benjamin et al., 2016; Cousillas Boam, 2018). Genetic alteration associated with selection criteria clearly contribute to these differential immune responses and is likely the primary factor responsible for differences between the 3 genotypes used in this study. Epigenetic factors may also be involved. In-utero differences likely generated epigenetic effects that differed among individuals and contributed to variation in the within-genotype response to LPS. Influence of post-utero epigenetic effects on differences between UH and CH heifers in their response to LPS were likely minimal as the healthy heifers were housed at the same facility, subjected to the same age-appropriate management and exposed to the same environmental conditions from birth through the study. Even though the Angus and Holstein herds were both pasture-based, grazing systems and the study heifers were housed together for 6 weeks prior to the study, management and environmental factors differed between the Holstein and Angus herd sites and may be responsible for epigenetic effects that contribute to differences between breeds in their response to LPS.

Physiological differences among animals, including fluctuations in steroidal hormone concentrations, can affect the innate immune response (Kahl et al., 2011). The non-pregnant CH heifers were estrus-synchronized to minimize the potential influence of fluctuations in progesterone concentrations on immune response. There were no apparent differences between the pregnant (n = 2) and estrus-synchronized (n = 2) CH heifer response to LPS but the small number of observations makes this difficult to assess.

The dose of LPS used in this study was less (0.5 vs. 2.5 ug/kg BW) but increases in rectal BT were similar to those observed in other LPS challenge studies (Kahl et al., 2011, 2009). Akarsu and Mamuk (2007) demonstrated that the *E. coli* O111:B4 LPS serotype we used has a more potent hyperthermic activity than the *E. coli* O55:B5 serotype used by Kahl et al. (2009, 2011) which could contribute to the similar BT responses among these studies. In previous repeated LPS challenge studies, the increase in BT has either remained the same (Kahl et al., 2009) or has been reduced (Kahl et al., 2011) during the 2nd challenge. The reason for the greater increase in BT during C2 than C1 by the Holstein heifers in our study is not known and is inconsistent with their reduced immune component responses during C2. Rapid changes in immune, endocrine and metabolic components were induced in all three genotypes during both LPS challenges and the overall responses were generally similar to those observed with LPS treated growing calves (Kahl et al., 2011, 2009; Plessers et al., 2015; Steiger et al., 1999). The reduced responses of TNFα, IL-6 and cortisol during C2 indicate a period of immune tolerance persisted for at least 4 d after the initial exposure to LPS. This is supported by the reduced SAA concentrations at 24 h during C2 and by the XO concentrations that indicate a relative reduction in reactive oxygen species during C2 compared to C1. Although differences among genotypes were detected during C1, the immune tolerance response eliminated most of these effects of genotype during C2. These similar responses among genotypes during C2 could indicate there are no differences in development of tolerance, the 4 d interval

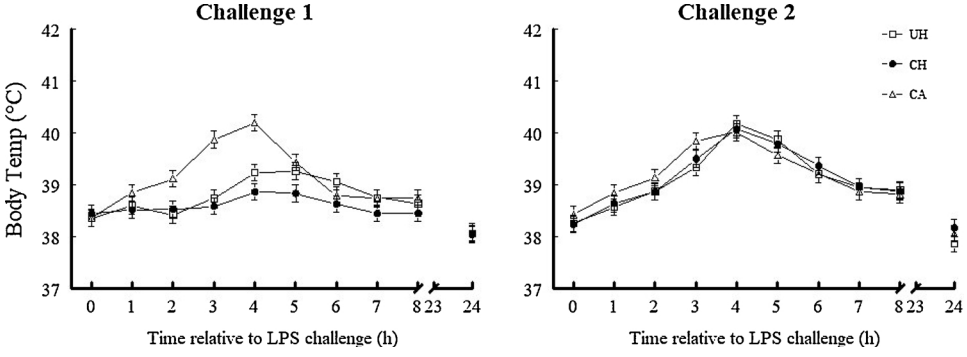

Fig. 1. Rectal body temperature (BT) of unselected Holsteins (stable milk yield since 1964, UH), contemporary Holsteins (CH) and contemporary Red-Black Angus (CA) heifers (n = 4/genotype) after LPS administration on day 1 (Challenge 1) and 5 (Challenge 2). Data represent least squares means ± SEM. There was a genotype by challenge by time interaction (P = 0.008).

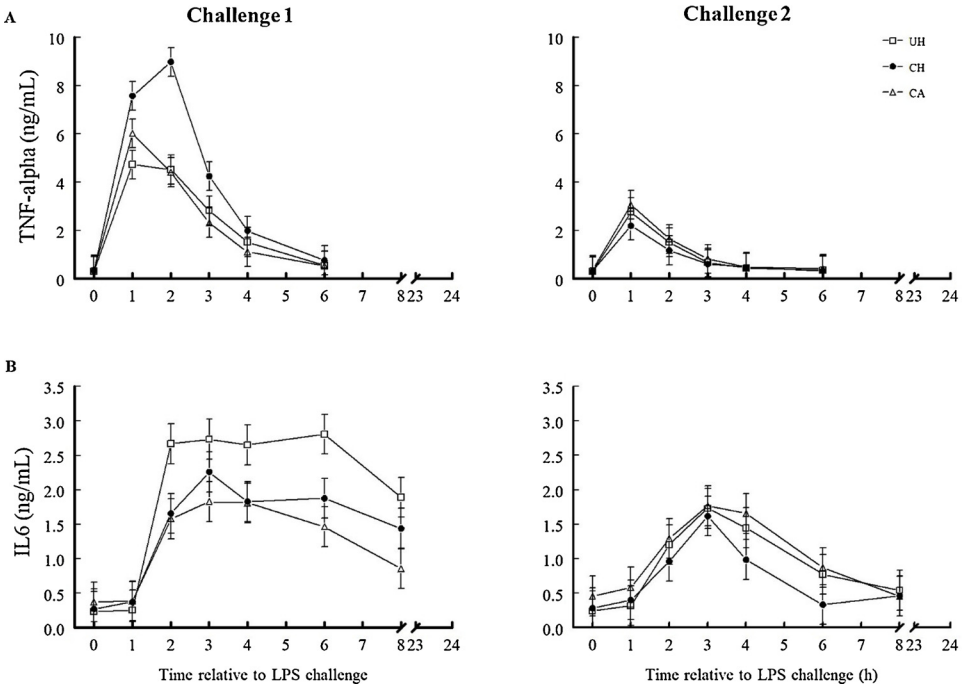

Fig. 2. Plasma TNF-α (A) and IL-6 (B) concentrations in unselected Holsteins (stable milk yield since 1964, UH), contemporary Holsteins (CH) and contemporary Red-Black Angus (CA) heifers (n = 4/genotype) after LPS administration on day 1 (Challenge 1) and 5 (Challenge 2). Data represent least squares means ± SEM. There was a genotype by challenge by time interaction ($P = 0.025$) for TNF-α. There were genotype by challenge, genotype by time, and challenge by time interactions ($P < 0.02$) for IL-6.

between challenges was too long to detect differences in impact of tolerance on these responses or the endotoxin tolerance mechanisms were not challenged sufficiently to elicit differences among the genotypes in expression of these immune components.

Pro-inflammatory cytokines are released by host immune cells when exposed to inflammatory stimuli and help regulate the APP response (Arango Duque and Descoteaux, 2014). The primary cytokine regulator of APP synthesis is IL-6 (Castell et al., 1989; Hagihara et al., 2004) which has both pro and anti-inflammatory functions that can affect immunity, tissue repair and metabolism (Arango Duque and Descoteaux, 2014; Scheller et al., 2011). We previously reported greater IL-6 and SAA responses to LPS in UH compared to CH heifers indicated a less robust immune response in CH heifers (Cousillas Boam, 2018). The greater IL-6 concentration profiles in UH heifers in the current study agree with our previous results but the similar SAA responses do not. In addition, TNFα concentrations were greater in these 19.6 month old CH than in UH heifers in the current study but did not differ between the 15-month old UH and CH heifers in our previous study (Cousillas Boam, 2018). Although IL-6 is the primary regulator of APP synthesis, TNFα also stimulates APP synthesis so the differential responses of greater IL-6 in UH and greater TNFα in CH heifers might minimize differences in SAA between the Holstein genotypes. This is supported in part by the greater SAA response in CA heifers despite having a reduced TNFα response relative to CH and a reduced IL-6 response relative to UH heifers.

Using another set of 19 month old CH and contemporary black Angus heifers, we demonstrated greater IL-8 responses to LPS and to IL-1β in fibroblasts from the CH heifers (Benjamin et al., 2016). An RNA-Seq analysis of these fibroblasts revealed that expression of TLR4, the pathogen recognition receptor for LPS, was greater in CH than in Angus heifers before and after LPS stimulation (Benjamin et al., 2016) and indicated CH heifers might be able to detect and induce a more vigorous inflammatory cascade in response to Gram-negative bacteria than contemporary black Angus heifers. Although the IL-6 response in the current study was only numerically greater in the CH heifers, the greater TNFα responses agree with our previous results that CH heifers respond more than CA heifers. In contrast, the CA heifers had a greater SAA response 24 h after LPS administration than either Holstein genotype despite not having greater responses in either of these pro-inflammatory cytokines. It is unclear from these results if the greater concentration of SAA in CA heifers is due to a less robust cytokine response which delayed the SAA response or if the SAA response to the combined stimulation from TNFα and IL-6 is more sensitive in CA than Holstein heifers. The individual cytokine results do not support a more robust immune response in CA heifers but it seems unlikely that SAA concentrations in the Holsteins peaked between the 8 and 24 h samples. Regardless, this greater SAA response could indicate the CA heifers might have a more rapid resolution of inflammation (Cheng et al., 2018) and an earlier return to homeostasis.

Glucocorticoids enhance the immune system response by up-regulation of cytokine receptors to increase cytokine binding and subsequent induction of APP production by the liver to regulate the inflammatory response to minimize tissue damage (Cheng et al., 2018; Wiegers and Reul, 1998). Although the increase in cortisol in C1 was less pronounced during the first hour after LPS administration in CA relative to UH and CH heifers, cortisol concentrations after 1 h did not differ among genotypes and there was no clear consistency in cytokine responses between the CA heifers and either the UH or CH heifers.

Glucocorticoids also stimulate mobilization of body reserves to provide energy to mount the acute response (Kvidera et al., 2017). Glucose is the preferred substrate for immune cell metabolism (Calder et al., 2007) and the initial stress response is characterized by increased plasma cortisol concentrations, a rapid increase in glucose to fuel the innate immune response, a concomitant increase in insulin, and a subsequent anorexic phase reflected by increased lipolysis (Steiger et al., 1999). The initial hyperglycemia is due to increased glycogenolysis and gluconeogenesis and this increase in glucose availability is supported by insulin resistance in peripheral tissues (Lang et al., 1990; Waldron et al., 2003). These typical changes in glucose and insulin after LPS administration occurred in all 3 genotypes in this study. Although glucose availability is increased, the caloric needs of the immune system are substantial. It is likely that this increased glucose metabolism and the counter regulatory aspects of the insulin surge contribute to the subsequent phase of hypoglycemia. The greater increase in insulin concentrations in CH than in CA heifers was likely due to the greater glucose response to LPS. In contrast, the small difference (20.4 mg/mL at 1 h) in glucose response between CH and UH heifers does not appear to be sufficient to explain their different insulin

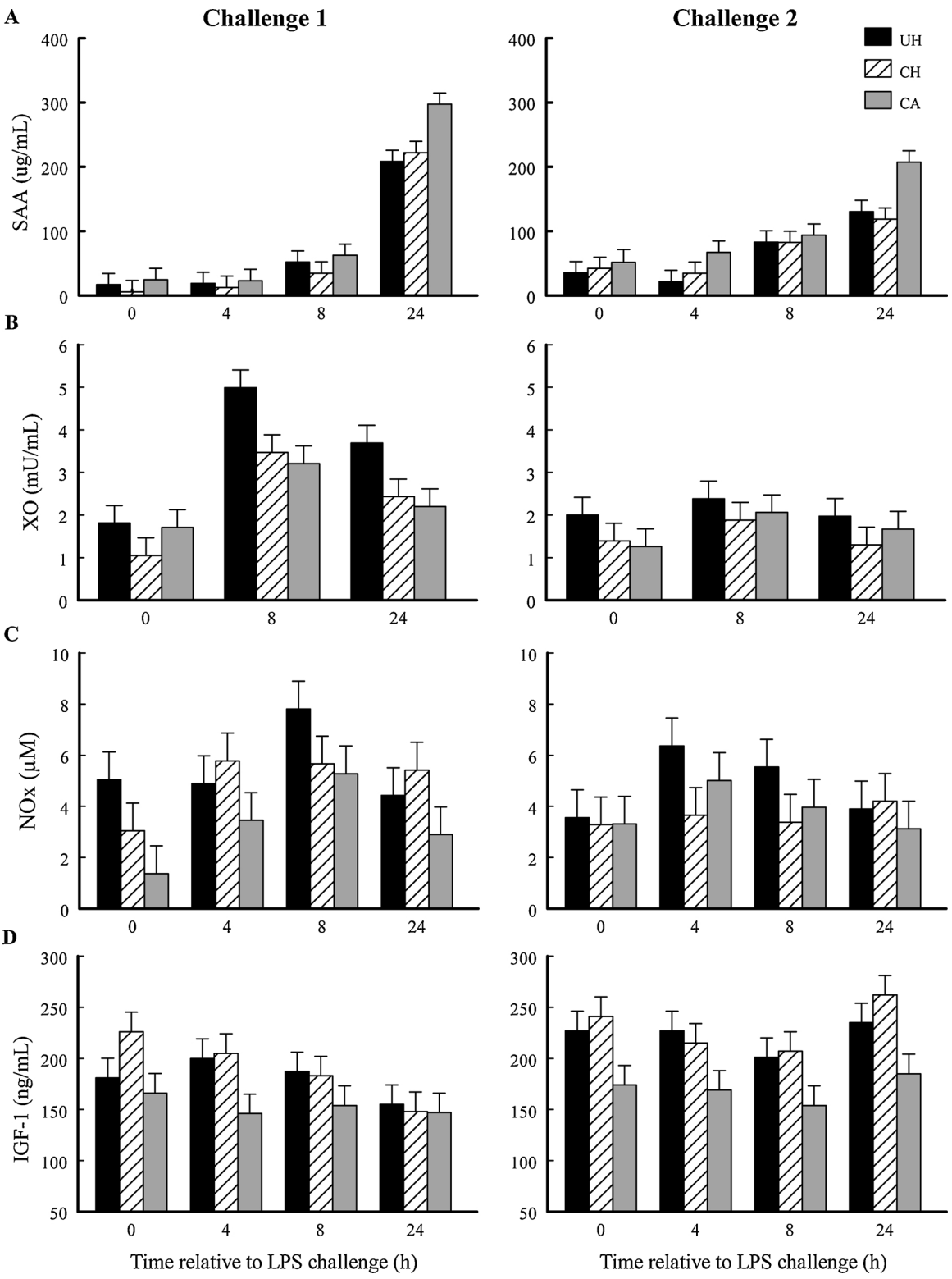

Fig. 3. Plasma serum amyloid A (SAA; A), xanthine oxidase (XO; B), nitric oxide (NOx, C) and insulin like growth factor 1 (IGF-1, D) concentrations in unselected Holsteins (stable milk yield since 1964, UH), contemporary Holsteins (CH) and contemporary Red-Black Angus (CA) heifers (n = 4/genotype) after LPS administration on day 1 (Challenge 1) and 5 (Challenge 2). Data represent least squares means ± SEM. There were interactions ($P < 0.05$) of genotype by time and challenge by time for SAA, challenge by time for XO, and challenge by time for IGF-1.

responses. Magnitude of the insulin resistance induced in peripheral tissue by LPS administration is associated with magnitude of the TNF-α response (Youd et al., 2000). The CH heifers had greater TNF-α and insulin responses and this might indicate selection has altered LPS-induced insulin resistance but there are no data currently available to support this.

It is estimated that a 1 °C increase in BT increases energy usage by 10–15% in animals (Kluger, 1989). Thus, the greater BT of CA heifers during C1 could reflect a more robust immune response that would lead to greater glucose utilization which might explain the reduced glucose

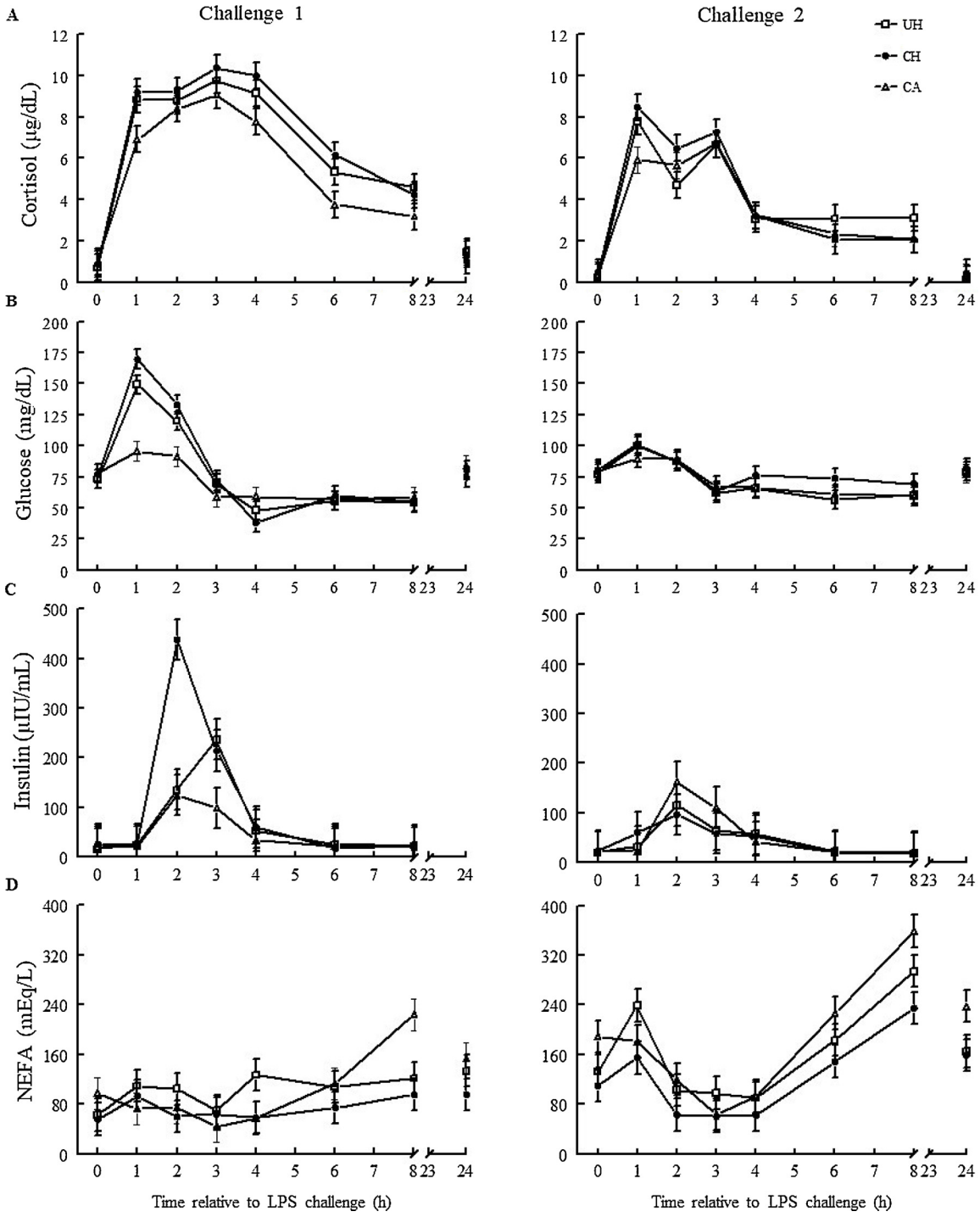

Fig. 4. Plasma cortisol (A), glucose (B), insulin (C) and non-esterified fatty acid (NEFA; D) concentrations in unselected Holsteins (stable milk yield since 1964, UH), contemporary Holsteins (CH) and contemporary Red-Black Angus (CA) heifers (n = 4/genotype) after LPS administration on day 1 (Challenge 1) and 5 (Challenge 2). Data represent least squares means ± SEM. There were interactions ($P < 0.05$) of genotype by time and challenge by time for cortisol and genotype by challenge by time for glucose, insulin and NEFA.

concentrations in CA heifers. However, the slower cortisol response and reduced concentrations of TNF-α and IL-6 compared to CH and UH heifers, are not consistent with a more robust response in CA heifers. In addition, there appear to be no data to indicate that Angus and Holstein heifers differ in their glycogenolytic and/or gluconeogenic capacities.

The plasma NEFA profiles are consistent with previous reports that NEFA increase 6–8 h after LPS administration (Lippolis et al., 2017) when glucose supply is diminished and intake is depressed (or withheld during the challenge as in our study). The greater increase in NEFA concentrations during C2 are consistent with tolerance decreasing the response to LPS which could reduce the duration of general lethargy and inappetence. Anticipation of the evening meal could contribute to the increase in plasma NEFA concentrations (Boisclair et al., 1997) between 6 and 8 h after LPS administration.

Consistent with our previous results (Baumgard et al., 2002; Weber et al., 2005), IGF-1 concentrations did not differ between the UH and CH heifers but they tended to be greater in UH and CH than in the CA heifers. The overall decrease in IGF-I concentrations through 24 h

during C1 and through 8 h during C2 agrees with previous reports that endotoxin-stimulated increases in pro-inflammatory cytokines decrease circulating IGF-1 concentrations, likely through reduced production of the IGF-I ternary complex and increased production of IGFBP-1 (Frost and Lang, 2004).

Plasma XO concentrations indicate the presence of cellular reactive oxygen species and XO is involved in the generation of NO (Kahl and Elsasser, 2004). The LPS-induced increase in XO tended to be greater in UH than in CH or CA heifers and the NO concentrations tended to be greater in CH than in CA heifers. These results support the potential for greater antimicrobial activity in the UH heifers. A greater ability of the UH heifers to neutralize invading pathogens is consistent with the recognition that the contemporary cow is more susceptible to disease than her ancestors (Egger-Danner et al., 2015; Pritchard et al., 2013).

Responses of all measured components returned to pre-LPS administration concentrations within 24 h except IL-6 and XO during C1 and SAA during both challenges. There was no indication of an impact on genotype on duration of the response for the other components, but determining the duration of the IL-6, XO and SAA responses by analysis of additional samples collected between C1 and C2, and at least for SAA past 24 h for C2, might provide additional insight on differences in the resolution of inflammation and development of endotoxin tolerance among these genotypes. Although 4 animals per genotype were sufficient to detect genotype differences in IL-6 and other immune component responses to LPS, greater power is needed for a more in-depth assessment of the impact of selection on factors that regulate innate immunity. Regardless, the results indicate components of the immune response differ between the Angus and Holstein heifers and that innate immune responses in Holsteins have been altered by the last 50 years of selective breeding.

5. Conclusion

Administration of LPS induced acute pro-inflammatory innate immune, metabolic and endocrine responses in all 3 genotypes and each genotype had at least one response that indicated it might have a more robust response than the other genotypes. The CH heifers had a greater TNF-α response, UH heifers had greater IL-6 and XO responses and CA heifers had greater BT and SAA response to LPS than the other genotypes. Although TNF-α, IL-6 and cortisol impact APP production, differences in SAA concentrations among genotypes were not consistent with differences in these components. Endotoxin tolerance to LPS was evident as pro-inflammatory, cortisol, glucose and insulin responses were less during the second than during the first challenge. Differences among genotypes during Challenge 1 were eliminated during Challenge 2 except for the greater SAA response in CA heifers and indicate the potential for differential impacts of genotype on the development of endotoxin tolerance. Specific reasons for these effects of genotype are not clear from these data and certainly other signals are also involved in the innate immune response to LPS. However, the results support the hypothesis for differential innate immune signaling among these bovine genotypes and additional examination is warranted. Results from studies that altered the intra-challenge interval, examined the impact of dose of LPS or expanded the analysis of components and included samples collected between the challenges could help elucidate the impact of genotype and selection on the innate immune response and development of endotoxin tolerance in cattle.

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