



Exploring the impact of high-energy diets on cattle: Insights into subacute rumen acidosis, insulin resistance, and hoof health

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ABSTRACT

Cattle lameness remains a significant concern, causing economic losses and compromising animal welfare. Claw horn lesions have been identified as a major cause of lameness in dairy cows, but their correlation with high-energy diets and ruminal acidosis remains unclear. Hence, the primary objective of this study was to assess the effects of a high-starch diet and a conventional diet on the rumen environment, acute-phase proteins, and metabolic alterations, with a particular focus on insulin resistance and the consequent implications for the histology of the hooves in Holstein steers. A total of 16 animals were divided into the high-starch (HS; 37% starch) and conventional (CON; 16.8% starch) groups. Glucose tolerance tests (GTT), blood analyses, rumen fluid analyses, and histological evaluations of the hoof tissue were conducted over a 102-d experimental period. The HS group showed a lower ruminal pH than the CON group, and with values indicating SARA. The plasma glucose and IGF-1 concentrations were higher in the HS group, suggesting an anabolic state. Both groups exhibited an increase in the insulin area under the curve (AUC) after the GTT on d 102. Histological analysis of the hooves showed a reduction in the length and width of the epidermal lamella in both groups. We found a significant negative correlation between the insulin AUC and the length and width of the epidermal lamella. Because both groups were similarly affected, the hypothesis that histological alterations were caused by the experimental diets still needs confirmation. Additionally, the development of SARA was not essential for the observed histological changes in the hoof. Further studies are warranted to thoroughly investigate the role of insulin and IGF-1 imbalances in claw health.

Key words: lameness, laminitis, claw horn lesions, subacute rumen acidosis, insulin sensitivity

INTRODUCTION

Energy-rich diets are widely used worldwide to enhance animal performance in beef and dairy cattle production. However, it is well described that these diets can lead to metabolic disorders in animals (Karikoski et al., 2015; Frutos et al., 2018; Ribeiro et al., 2020). In ruminants, energy-rich diets can induce SARA (Villot et al., 2018; Ferguson et al., 2022), resulting in insulin resistance, proinflammatory alterations, poor oocyte quality, and increased risk for mastitis and endometritis (Adamiak et al., 2005; Frutos et al., 2018; Bäßler et al., 2021; Fu et al., 2022). Furthermore, excessive consumption of energy-rich, low-fiber diets has been associated, to some extent, with an increased incidence of hoof lesions and lameness in cattle (Manson and Leaver, 1988; Shearer and van Amstel, 2017; Griffiths et al., 2018; Moreira et al., 2019; Bäßler et al., 2021). However, a lack of clarity remains regarding how and whether intensive feeding contributes to histological changes in hooves, thus influencing the occurrence of claw horn lesions (CHL) and lameness (Bicalho et al., 2009; Danscher et al., 2010; Passos et al., 2023).

Cattle lameness remains a significant problem in the dairy and beef industries, leading to substantial economic losses due to decreased productivity, treatment expenses, premature culling, and even animal mortality (Charfeddine and Pérez-Cabal, 2017; Puerto et al., 2021). Moreover, it is a pressing concern for animal welfare (Whay and Shearer, 2017). Lameness can result from various diseases, with CHL, encompassing noninfectious foot lesions such as sole ulcers, sole hemorrhage, and white line disease (or separation), emerging as a leading cause of lameness in dairy cows (Cramer et al., 2009; Solano et al., 2016; Browne et al., 2022).

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

Historically, CHL and other hoof alterations, such as deformations in shape, irregular growth, the development of ridges, and color alteration, have been associated with dietary factors such as elevated NFC levels and reduced fiber intake (Greenough, 2007; Passos et al., 2023). However, several studies have shown a stronger correlation between CHL and factors such as calving, housing conditions, and digital cushion thickness, with less emphasis on nutrition (Webster, 2001; Tarlton et al., 2002; Knott et al., 2007; Wilson et al., 2021; Passos et al., 2023). Nonetheless, an oligofructose overload protocol has successfully induced ruminal acidosis, resulting in lameness and vascular dysfunction of the hoof with a loss of structural integrity (Thoenes et al., 2005; Danscher et al., 2009; Ding et al., 2020). However, the connection between SARA and possible claw horn alterations and lesions is less clear (Nocek, 1997; Passos et al., 2023).

The theory has been suggested that increased LPS endotoxin production and absorption in the rumen during SARA would trigger a cascade of processes leading to circulatory alterations, inflammation, and activation of metalloproteinases in the dermal layers (Nocek, 1997). These alterations would weaken the structural integrity of the claw horn and suspensory apparatus of the pedal bone, thus leading to CHL and lameness (Nocek, 1997; Greenough, 2007; Danscher et al., 2010; Passos et al., 2023). However, these hypotheses lack conclusive evidence, and some results suggest otherwise (Danscher et al., 2010; Passos et al., 2023). Previous studies that examined the correlation between diet and hoof lesions found changes in histology but no impact on the resistance of the suspensory apparatus and no increased incidence of lesions (Webster, 2001; Tarlton et al., 2002; Knott et al., 2007; Danscher et al., 2010). Unfortunately, these studies provided limited information on the experimental diets and whether they induced SARA or not. Moreover, testing claw suspensory tissue is technically challenging and may not precisely replicate in vivo forces (Danscher et al., 2010).

Animals experiencing SARA normally present a low-grade inflammation, marked by higher concentrations of circulating LPS, acute-phase proteins (APP), and other inflammatory markers (Danscher et al., 2011; Bäbler et al., 2021; Fu et al., 2022). Several studies have demonstrated that haptoglobin, apolipoprotein A, and ceruloplasmin are relevant APP in cattle and can be altered in SARA or in lame animals (Danscher et al., 2011; Dong et al., 2015; Fu et al., 2022). However, although inflammation is hypothetically considered one of the components of the pathogenesis of CHL and other hoof alterations, limited evidence is available to confirm this hypothesis (Nocek, 1997; Greenough, 2007; Dong et al., 2015; Zhang et al., 2018; Ding et al., 2020).

Currently, in horses, strong evidence supports the notion that insulin dysregulation, linked to NFC-rich diets, can induce histological changes in the dermoepidermal junction by weakening the connection between the distal phalanx and the hoof capsule, as highlighted in Grenager (2021). This condition, commonly referred to as endocrinopathic laminitis in horses, is associated with episodes of hyperinsulinemia and increased IGF-1 concentration (Rahnama et al., 2020; Ribeiro et al., 2020). Insulin and IGF-1 act via the insulin-like growth factor receptor-1 in the lamina, triggering pathways that result in laminar tissue proliferation, stretching, and consequent failure of the suspensory apparatus of the distal phalanx (Rahnama et al., 2020; Grenager, 2021). Stretched white lines, bruised soles, divergent wall growth and rings, and recurrent abscesses are among the hoof lesions typically observed in horses with endocrinopathic laminitis (Karikoski et al., 2015; Cassimeris et al., 2019). Although the correlation between hyperinsulinemia and hoof health in cattle laminar tissue has not yet been described, this hypothesis has been raised, and some evidence suggests that this should be considered (Dong et al., 2015; Bäbler et al., 2021). Therefore, we hypothesize that the exposure of cattle to a high-starch diet could induce changes in insulin resistance and hoof histology. Accordingly, this study aimed to investigate and compare the effects of an energy-rich diet and a conventional diet on the rumen environment, inflammatory markers, metabolic changes, particularly insulin resistance, and their subsequent effects on hoof histology in Holstein steers.

MATERIALS AND METHODS

The project was approved by the Commission for Ethics in the Use of Animals of the Universidade Federal de Minas Gerais (Minas Gerais, Brazil) under protocol 192/19 and was in accordance with the provisions legislation and the rules issued by the National Council for the Control of Animal Experimentation (Brasília, Brazil).

Animals

A total of 16 healthy male Holstein steers, approximately 12 mo old and weighing (\pm SD) 250 ± 25.5 kg, were used in this study. Two experimental groups were established by uniformly pairing the animals according to their initial weight, then randomly assigning one to one group and the other to the other group. The high-starch group (HS; 37.0% starch) was fed a diet for an average daily gain of 1.5 kg. The control group was fed a conventional energy-rich diet (CON) containing 16.8% starch, with a predicted average daily gain of 0.7 kg. The diets were calculated according to NRC (2001). The

experiment lasted 102 d, after which the animals were slaughtered in a commercial slaughterhouse. The animals were weighed on a scale every 30 d to calculate daily weight gain. During the experimental period, the animals remained in a tiestall system in a closed barn with food, water troughs, and rubber mats. The barn had natural ventilation and fans in addition to exhaust fans on the roof.

Diet

At the beginning of the experiment (d 0), the animals in the HS group received 40% of the total concentrate programed, with an increase of approximately 8.5% every 4 d. The total period of adaptation to the diet was 28 d. During the experimental period, the CON group was fed a diet similar to that provided before the experiment. The amount of the diet offered was adjusted daily to ensure a feed residue in the trough of at least 5%.

The chemical compositions of the diets are shown in Table 1. The diets consisted of corn silage, ground corn, soy, and commercial mineral. The HS diet consisted of 19.6% roughage and 80.4% concentrate, whereas the CON diet consisted of 75.0% roughage and 25.0% concentrate in the DM. No additives were added to the diet.

The diets were distributed twice a day at 0800 and 1800 h, and food was turned over in the animals' troughs between meals. The food provided, as well as the leftovers, were weighed individually to calculate consumption.

Collection and Analysis of Rumen Fluid and Blood Samples

Rumen fluid was collected through esophageal tubing on d 0, 7, 28, 35, 63, and 91, immediately before and 4, 6, 8, and 10 h after the first diet offer. Samples of ruminal fluid (approximately 200 mL) were collected, and the pH was measured with a previously calibrated portable digital pH meter (Kasvi Digital pH Meter with KCL, Kasvi, Brazil).

Blood samples were collected by jugular venipuncture using dry vacuum tubes containing EDTA and another tube containing sodium fluoride at the same time as rumen fluid sampling (d 0, 7, 28, 35, 63, and 91). Serum and plasma were harvested and frozen at -20°C until analysis. Clinical biochemistry was performed via spectrophotometry using an automatic device (Cobas Mira Plus, Roche, Switzerland) and commercial kits (Biotécnica, Brazil). Insulin and IGF-1 concentrations were measured by chemiluminescence using an IMMULITE 2000 (Siemens, Germany) automatic analyzer and commercial kit (Sigma-Aldrich, St. Louis, MO).

We measured APP by electrophoresis on a polyacrylamide gel containing SDS-PAGE as described by Souto

Table 1. Dry matter and chemical composition (percentage of DM) of the diets used in the HS and CON groups

Item	HS	CON
DM (%)	59.5	34.1
CP	14.4	11.9
Available protein	13.9	11.0
ADF	15.0	29.4
NDF	26.8	45.2
Lipids	3.6	2.58
Ashes	5.8	10.5
Lignin	2.3	4.9
Starch	37.0	16.8
NFC	51.1	31.5
TDN	76.3	60.6

et al. (2019). Apolipoprotein A, albumin, haptoglobin, transferrin, α -2 macroglobulin, and ceruloplasmin were analyzed.

Glucose Tolerance Tests

Glucose tolerance tests (GTT) were performed on all animals on d 0, 39, and 102, according to Holtenius et al. (2003). The animals did not have access to feed one hour before and during the GTT. A catheter coated with sodium heparin was inserted into one of the jugular veins, and an infusion of 150 mg/kg glucose (50% wt/vol) was administered, which took approximately 8 min. Blood samples were collected at -15 , 0, 10, 20, 30, 45, 60, 90, and 120 min after infusion of the glucose solution. The GTT measurements included glucose and insulin curves and the area under the curve (AUC) of the insulin and glucose curves.

Histology Collection and Analysis

Samples of lamellar tissue from the wall of the lateral claw of the left hind limb were obtained 40 d before the beginning of the experiment using a previously established biopsy technique to obtain hoof lamellar tissue samples using a Falcão-Faleiros lamellotome (National Institute of Industrial Property, Brazil, BR102013018765-8; Mendes et al., 2018). The animals were sedated before the biopsy with xylazine (0.04 mg/kg) combined with acepromazine (0.04 mg/kg), and the limb was anesthetized with 2% lidocaine. After the animals were slaughtered (d 103), fragments from the wall of the lateral claw of the right hind limb (LCH) and the wall of the medial claw of the left forelimb (MCF) were sampled. All biopsies were obtained from the outer part of the abaxial wall, approximately 1 cm below the coronary band, which corresponds to zone 3 in the sole (Shearer et al., 2004). Histological samples of the rumen, liver, and lungs were collected after slaughter. All collected samples were immersed in 4% buffered formalin for 48 h, after which

they underwent histological processing and were embedded in paraffin. Histological sections (5 μ m thick) were obtained on slides and stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS).

Evaluations were performed using a Nikon Eclipse E 200 microscope coupled to a camera (Tucsen 2.0) with the aid of the Tucsen TC Capture software (V4.3.0.605) used for measurements after calibration, according to the manufacturer's instructions. Histological analysis was performed by 2 researchers who were blinded to the treatment. The length of the epidermal lamella (EL) was recorded in all lamellar tissue samples by measuring a straight line from the axial to the abaxial limits of the epidermal lamellae, as shown in Figure 1. Therefore, the width of the EL was determined and defined as the distance between the ends of the EL in the abaxial portion. We also measured the distance of the abnormal layer composed of nucleated cells between the lamellar tissue and the beginning of the horny layer (parakeratosis). The length and width of the EL were measured on slides stained with PAS, which facilitates the visualization of the basement membrane, whereas parakeratosis was measured on histological sections stained with HE, which facilitates the visualization of the stratum corneum.

To determine the required number of EL for measurement, 100 EL were initially measured for length and width across 3 slides. The mean and standard deviation were then calculated. To ensure accuracy, the number of measured EL was subsequently reduced, maintaining a maximum acceptable variation of 10% from the mean of the width and length measured across the initial 100 EL.

This criterion was satisfied by measuring 25 EL. Thereafter, the length and width of the 25 EL were measured for each histological section. EL was also classified according to its format in the axial region as standard, bifurcated, fringed, or sharp, as adapted from Ribeiro et al. (2020; Figure 2). The 25 EL for measurement were selected randomly, only avoiding areas that presented artifacts from histological techniques or other imperfections.

The presence of hemorrhage and inflammatory infiltrate in the dermis was evaluated on slides stained with HE as described by Boosman et al. (1989). Scores were graded from 1 to 4 for the presence of hemorrhage (1 = no hemorrhage; 2 = small hemorrhagic spots; 3 = fine columnar hemorrhage; and 4 = large irregular hemorrhage) and for the presence of inflammatory cells (1 = no leukocytes; 2 = some scattered leukocytes; 3 = some leukocytes with mild focal infiltration; and 4 = some scattered leukocytes with large focal infiltration).

Epidermal cell morphology and basement membrane integrity were evaluated on slides stained with PAS according to Mendes et al. (2013). Epidermal cells were examined, and a score from 1 to 4 was calculated (1 = predominance of epidermal cells with oval nuclei perpendicular to the basal membrane; 2 = presence of approximately 50% of epidermal cells with oval nuclei perpendicular to the basal membrane and 50% with round nuclei; 3 = predominance of epidermal cells with rounded nuclei; 4 = predominance of epidermal cells with elongated and flattened nuclei or absence of nuclei; Figure 3). The basal membrane was examined in its en-

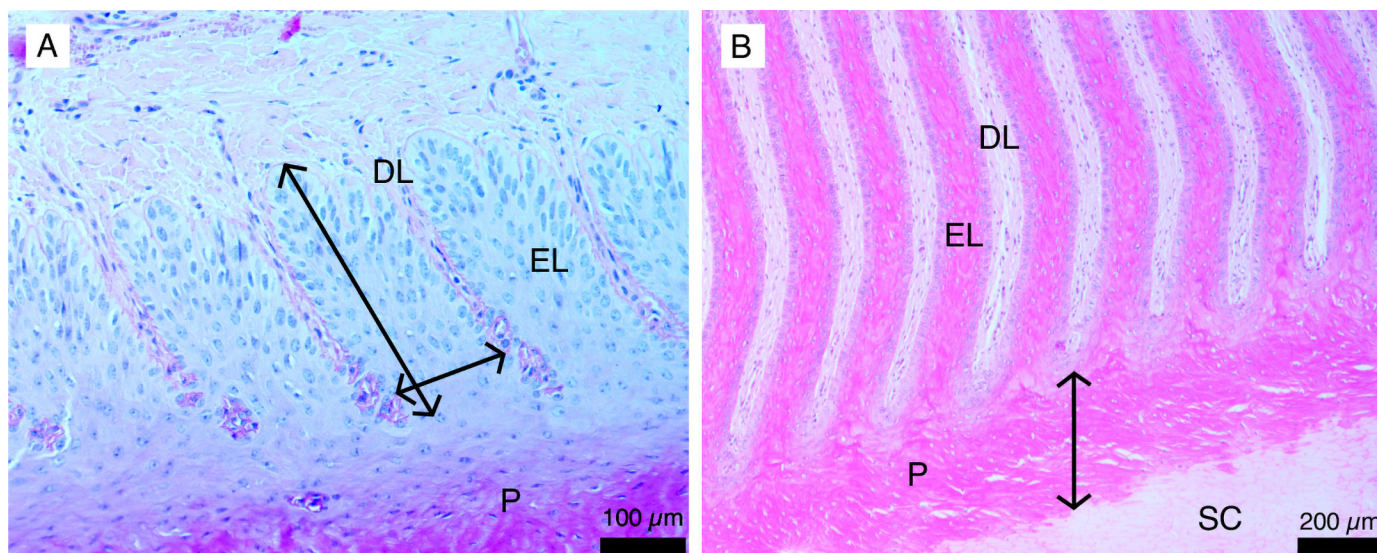


Figure 1. Photomicrograph of epidermal (EL) and dermal lamellae (DL) of bovine hooves using PAS dye (A) and hematoxylin and eosin (B). The lines with arrows show how we determined the length and width of the epidermal lamella (EL; A) and the thickness of the parakeratosis layer (P; B). The stratum corneum (SC) is also visible.

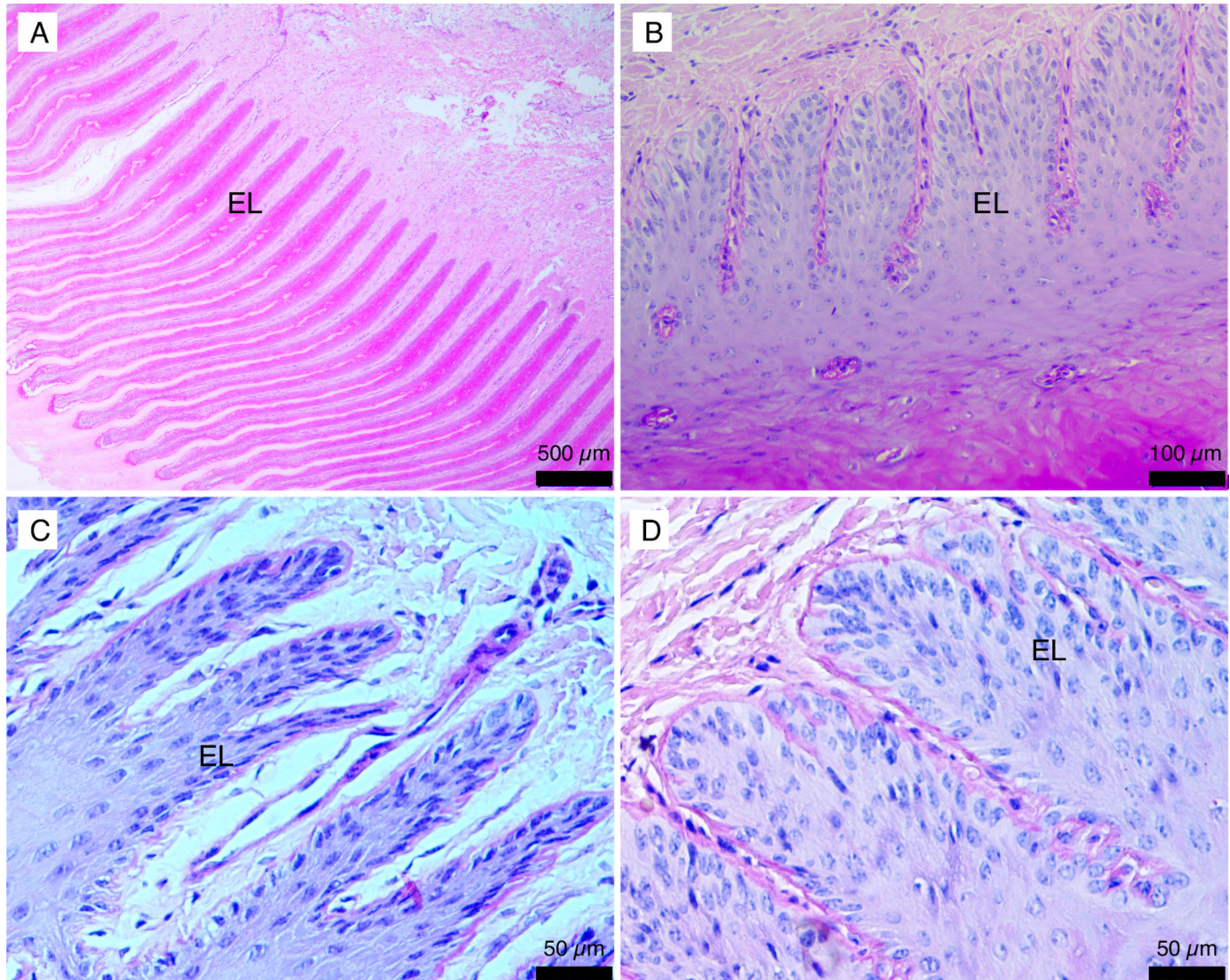


Figure 2. Photomicrograph of epidermal and dermal lamellae of bovine hooves demonstrating the criteria used for morphological analysis of the axial region of the EL: (A) standard; (B) fringed; (C) sharp; and (D) bifurcated.

tirety and graded from 1 to 4 according to the intensity of the irregularities (1 = absent or discrete; 2 = mild; 3 = moderate; and 4 = marked).

Ethogram

The ethograms were evaluated for 24 h in a row on d 80. To record the time spent on food and water consumption, rumination lying down and standing up, idleness lying down and standing up, and visual observation of the animals were performed every 5 min. During the nocturnal observations, the environment was maintained under dim artificial lighting. Observations were carried out by pairs of observers who were changed every 2 h, with no repetition of observers over the 24 h.

Statistical Analysis

The statistical analysis was conducted using GraphPad Prism 9.0.0 (GraphPad Software) for AUC during the GTT and histological morphometry. R software version 3.6.1 (R Core Team, 2019) was used for rumen pH and blood analyses. Normal distribution of data was assessed with the Shapiro-Wilk and Kolmogorov-Smirnov tests in both software packages. Generalized estimating equation models were applied to rumen pH and blood analyses, considering each response variable. The overall effects of hours, days, group, and their interactions were evaluated, and the mean values along with the 95% CI for each group at each hour and day were calculated. Group differences for each hour and day or differences between hours

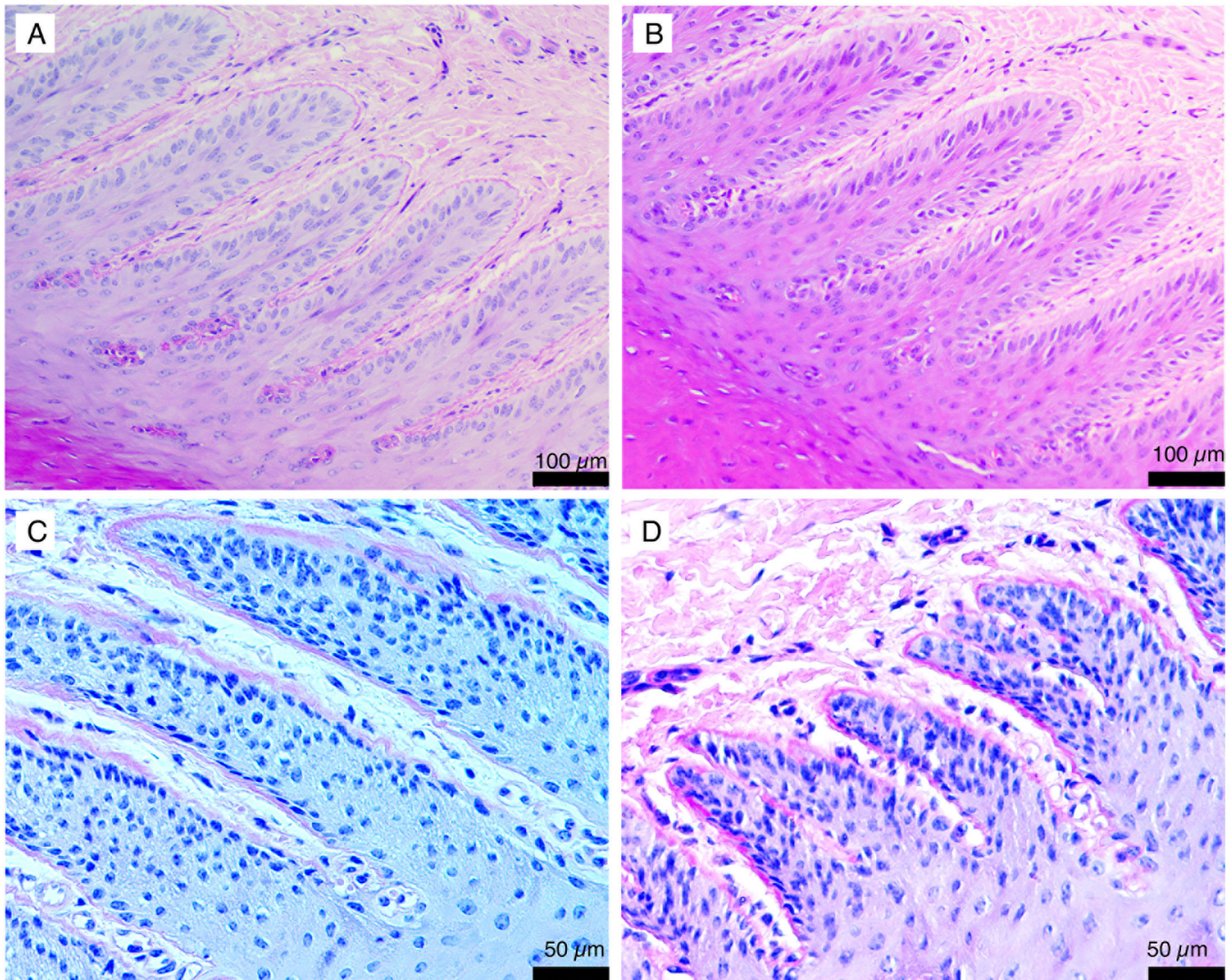


Figure 3. Photomicrograph of epidermal and dermal lamellae of bovine hooves demonstrating epidermal cell morphology scores. (A) Score 0, predominance of epidermal cells with oval nuclei perpendicular to the basal membrane; (B) score 1, presence of approximately 50% epidermal cells with oval nuclei perpendicular to the basal membrane and 50% with round nuclei; (C) score 2, predominance of epidermal cells with rounded nuclei; (D) score 3, predominance of epidermal cells with elongated and flattened nuclei or absence of nuclei.

and days for each group were determined using multiple pairwise comparison tests with Tukey's correction.

For data from the intravenous GTT, mixed-effects models were employed to assess the effects of time, group (diet type), and their interaction on blood insulin and glucose concentration. Means were compared using Dunnett's test. The same models were applied to AUC data to examine the effects of the day of exposure, time after glucose infusion, and their interaction. Histological data were analyzed with paired Student's *t*-tests or Mann-Whitney tests to compare values before and after the weight-gain period. Additionally, correlation with AUC values was performed using Spearman's test. A significance level of $P < 0.05$ was applied for all tests.

RESULTS

Weight Gain

The final average daily weight gains were 1.79 kg/d (95% CI = 1.62–1.95 kg/d) for the HS group and 0.89 kg/d (95% CI = 0.61–1.16 kg/d) for the CON group.

Ruminal Environment

Ruminal pH decreased in both groups from d 7 of the experiment, but the average pH of the ruminal fluid in the HS group was always lower than that in the CON group from d 7 onward ($P < 0.05$; Figure 4). The low-

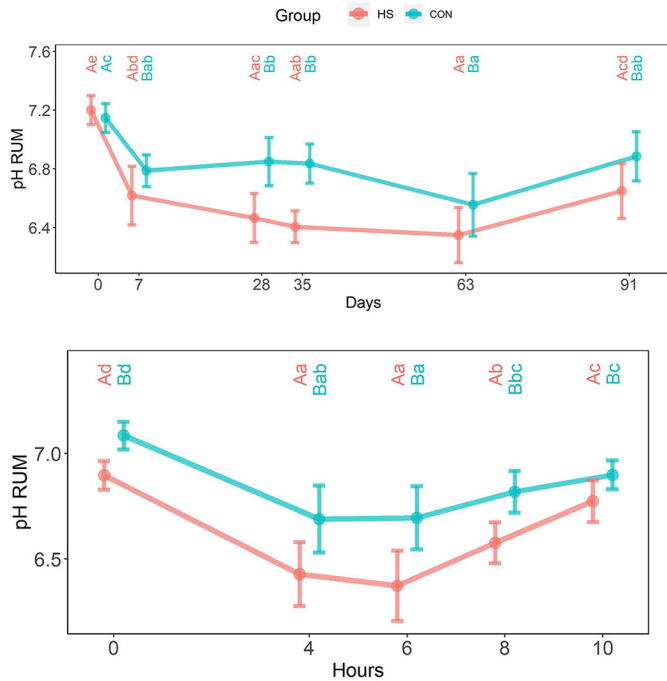


Figure 4. Mean ruminal fluid (RUM) pH values collected by tubing from confined Holstein steers and subjected to a high-starch diet (HS) and a conventional diet (CON) for 91 d and during the first 10 h after feeding. ^{a-c}Different lowercase letters indicate differences between times in the same group. ^{A,B}Different uppercase letters indicate statistical differences ($P < 0.05$) between groups. Error bars represent 95% CI.

est pH value normally occurred at 6 h after feeding. The lowest pH of the HS group at 6 h after feeding was 5.9 ± 0.3 and occurred on d 35, whereas in the CON group, the lowest mean pH was 6.6 ± 0.3 on d 35 and 63.

Ethogram

The time dedicated to food consumption was lower in the HS group than in the CON group, with average of 1.8 and 3.38 h/d, respectively ($P < 0.05$). The total daily ruminating time of the CON animals was greater (9.24 h) than that of the HS group (4.44 h; $P < 0.05$; Figure 5). The HS group spent an average of 17.54 h/d and the CON group 11.11 h/d in idleness ($P < 0.05$), and of this time, 3.31 h and 3.86 h were standing ($P > 0.05$), respectively. We found no difference in the time spent on drinking water between the groups (Figure 5). Animals in the HS group spent more time lying down ($P < 0.05$; Figure 5).

Glycemia, Insulin and IGF-1

From d 7 to d 91, the HS group had higher plasma concentrations of glucose and IGF-1 than the CON group ($P < 0.05$; Table 2). Insulin concentrations did not differ between the groups or over time at any point. In both groups, an increase in IGF-1 concentration was also observed on d 35 and d 91 compared with that on d 7 (Table 2).

Intravenous Glucose Tolerance Test

Figure 6 shows the insulin concentrations measured during the 3 GTT performed. No statistical differences were found between the groups on any day or at any time. Similarly, no statistical differences between the groups were found in the AUC of the insulin concentration (Figure 7). Looking at the different time points, the insulin concentrations at 10 and 20 min after glucose infusion

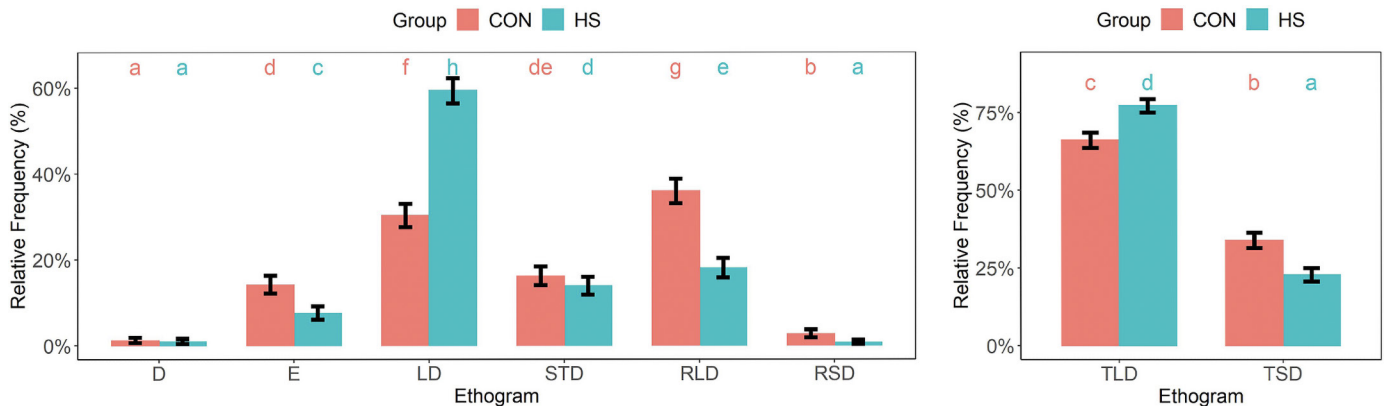


Figure 5. Ethogram of relative frequency (%) of time of Holstein steers fed a high-starch diet (HS) and a conventional diet (CON). ^{a-f}Values followed by different lowercase letters differ significantly from each other ($P < 0.05$). D = drinking water; E = eating; LD = lying down; STD = idle standing; RLD = ruminating while lying down; RSD = ruminating while standing; TLD = total time spent lying down; TSD = total time spent standing idle. Error bars represent 95% CI.

Table 2. Mean values of plasma concentration of glucose (mg/dL), insulin ($\mu\text{IU/mL}$), and IGF-1 (ng/mL) in Holstein steers fed a high-starch diet (HS) and conventional diet (CON)

Day	HS						CON					
	Glucose	SE	Insulin	SE	IGF-1	SE	Glucose	SE	Insulin	SE	IGF-1	SE
0	71.5 ^{a,A}	2.0					69.9 ^{bcd,A}	1.4				
7	77.9 ^{b,B}	1.6	19.7 ^{a,A}	8.4	248.5 ^{a,B}	18	66.6 ^{ab,A}	1.0	11.5 ^{a,A}	1.0	171.8 ^{a,A}	14.9
28	83.8 ^{c,B}	1.8					65.0 ^{a,A}	1.2				
35	84.4 ^{bc,B}	1.9	14.1 ^{a,A}	4.8	398.58 ^{b,B}	37.4	67.0 ^{abc,A}	1.8	9.9 ^{a,A}	1.3	222.3 ^{ab,A}	23.9
63	82.3 ^{bc,B}	1.1					70.3 ^{cd,A}	1.6				
91	86.9 ^{bc,B}	2.7	21.5 ^{a,A}	7.8	350.56 ^{b,B}	20.5	73.7 ^{d,A}	2.4	12.6 ^{a,A}	3.0	235.6 ^{b,A}	17.7

^{a-d}Values with lowercase letters in the same column differ between times ($P < 0.05$)

^{A,B}Values with uppercase letters in the same row differ between groups ($P < 0.05$).

on d 39 and at 10, 20, 30, and 45 min on d 102 were higher than those at baseline (time 0), whereas on d 0, we observed no increase in insulin concentrations. The bottom right graph in Figure 6 shows that the insulin concentration on d 102 was higher than that on d 0 at 10, 20, 30, and 45 min after glucose infusion. Furthermore, considering the data from both groups together, the AUC of insulin increased by more than 3-fold from d 0 to d 102 in both groups ($P = 0.0085$; Figure 7). The AUC of glucose concentration did not vary between the experimental days or between the groups.

Histology

In both groups, we observed a reduction in the length and width of the EL in the wall of the LCH ($P < 0.001$) compared with a biopsy performed on the lateral claw of the left hind limb at the beginning of the experiment (biopsy). The same effect was observed when we compared the pre-experiment biopsy with the analysis of the length and width of the EL of the MCF (Figure 8). When the groups were analyzed separately, the reduction in EL length in the LCH was 51% in the HS group ($P = 0.008$)

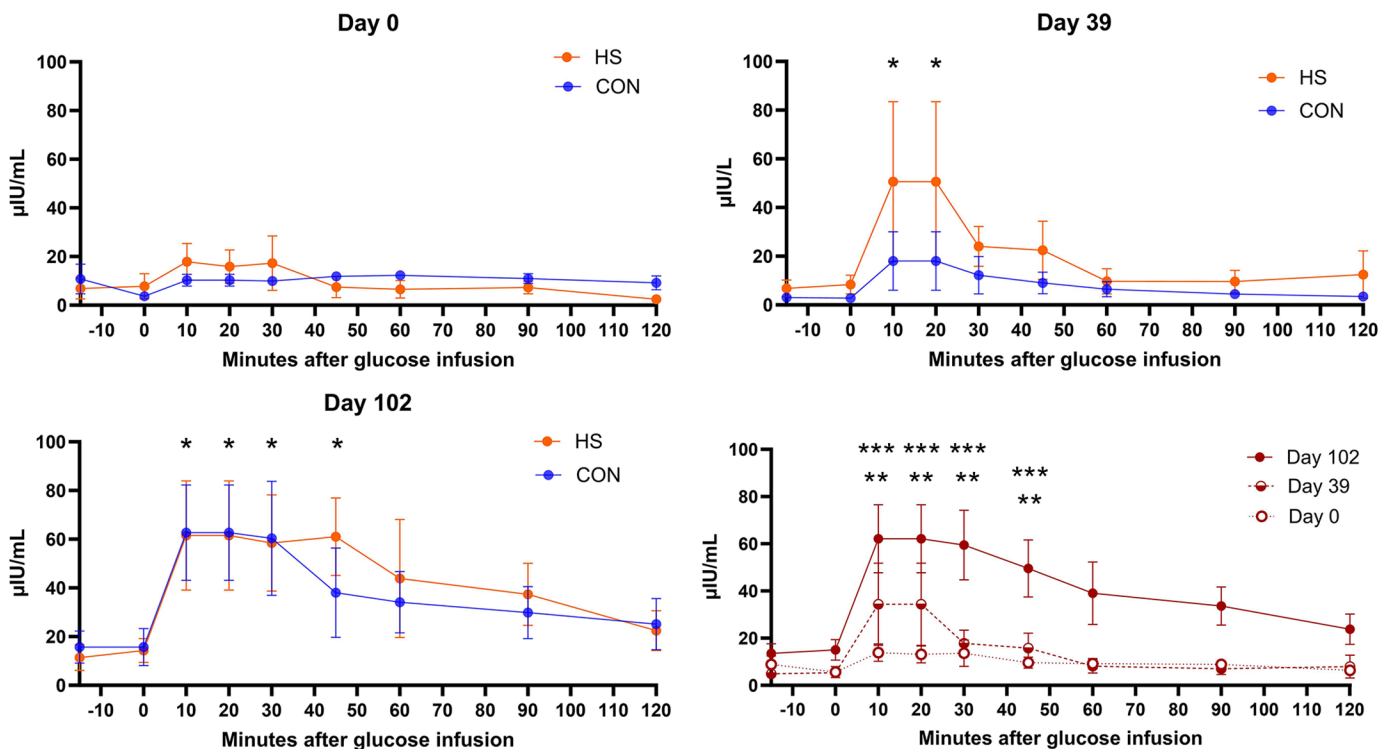


Figure 6. Mean (\pm SE) plasma insulin concentration ($\mu\text{IU/mL}$) during the intravenous GTT performed before (d 0) and after (d 39 and 102) the exposure of Holstein steers to a weight-gain diet with a high-starch content (HS) and a conventional diet (CON). *Time point values differ from the baseline (time = 0; $P < 0.01$) within each graph and without any group or interaction effects. **Time point values differ from baseline values (time = 0; $P < 0.0001$) in the d 102 group, considering group ($P = 0.001$) and interaction ($P < 0.0001$) effects. ***Day 102 values differed from d 0 values at the same time point, considering the group ($P = 0.001$) and interaction ($P < 0.0001$) effects.

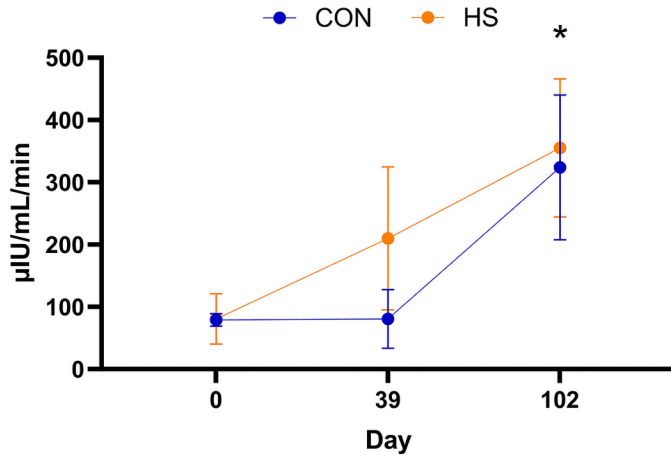


Figure 7. Means (\pm SE) of the AUC calculated from the plasma concentration of insulin during the intravenous GTT performed before (d 0) and after (d 39 and 102) the exposure of Holstein steers to a weight-gain diet with a high-starch content (HS) and a conventional diet (CON). *Time point values differed from the baseline values ($P = 0.02$) without any group or interaction effects.

and 36% in the CON group ($P = 0.02$), whereas the reduction in width was 37% in the HS group ($P < 0.001$) and 43% in the CON group ($P = 0.0001$). Regarding the MCF compared with the initial biopsy, the EL length was reduced by 40.2% in the HS group ($P = 0.005$), whereas the EL width was reduced by 38% in the CON group ($P = 0.001$) and by 42% in the HS group ($P = 0.0001$).

When analyzed together, the thickness of the parakeratosis layer increased in the LCH ($P = 0.0417$) and MCF ($P = 0.0097$) compared with that at the beginning of the experiment (biopsy). The thickness of parakeratosis was similar between the HS and CON groups, despite a statistical increase in the HS group ($P = 0.025$ for LCH and $P = 0.014$ for MCF).

In the histological analysis, several samples of the hooves of the animals showed loss of the keratinized axis of the EL and the presence of nucleated cells (parakeratosis) between the epidermal layers (internal layer) and beginning of the stratum corneum (middle layer) (Figures 1B and 2A). The EL shape exhibited no variation among the groups, time points, or claws ($P > 0.05$). Nonetheless, it is noteworthy that over half of the EL showed irregular shapes in both groups, with the fringed format being the most common. Furthermore, more than 50% of the EL from the pre-experiment sample deviated from what was considered typical (Table 3).

We found no difference in the histological assessments regarding basement membrane integrity, inflammatory infiltrate, and hemorrhage in the dermis between the groups or between time points ($P > 0.05$), except for a decrease in the basement membrane integrity score in the HS group compared with before and after diet exposure. However, even in the pre-experiment biopsy, high fre-

quencies of alterations in basement membrane integrity and hemorrhage were identified. We observed a difference in the morphology of the epidermal cells between the groups at d -40 ($P = 0.01$), with 100% of the animals in the CON group having a score 2 or lower and 25% of the HS group having a score of 2 (Table 4).

In rumen histology, 50% of the animals in the HS group presented abnormalities compatible with ruminal acidosis, with the rumen mucosal epithelium with discrete foci of vacuolation (discrete multifocal hydropic degeneration), sometimes associated with intact neutrophils and intraepithelial degeneration (intraepithelial microabscesses). Mild infiltration of neutrophils, lymphocytes, and histiocytes was observed in the underlying lamina propria. By contrast, no significant histological changes were observed in the CON group. Lung and liver histology revealed no significant changes in either of the groups.

Correlation Between Insulin AUC and Histology Measures

We observed a moderate and significant negative linear correlation between EL length ($r = -0.50$, $CI = -0.73$ to -0.17 ; $P = 0.004$) and width ($r = -0.58$, $CI = -0.78$ to -0.28 ; $P < 0.001$) and insulin AUC after GTT. The correlation between the thickness of this layer and the AUC of insulin was not significant ($r = 0.31$, $CI = -0.05$ to 0.60 ; $P = 0.095$; Figure 9).

Acute-Phase Proteins

The HS group presented higher concentrations than the CON group of transferrin on d 28 and d 84, apolipoprotein A1 on d 7 and d 84, albumin between d 35 and d 63, and α -2 macroglobulin on d 63 (Figure 10). The concentrations of ceruloplasmin and haptoglobin did not vary between groups at any time point.

Looking at how the APP varied over time, albumin concentrations in the CON group decreased between d 35 and d 63, returning to the initial values on d 84. In both groups, we observed a reduction in α -2 macroglobulin values on d 63 and d 84 compared with the initial values, whereas there was an increase in apolipoprotein A1 on d 35 onward in the CON group and on d 84 in the HS group compared with the initial values. Transferrin concentrations increased from d 28 to d 84 in the HS group, but increased from d 63 to d 84 in the CON group.

DISCUSSION

Over the past several years, the pathogenesis of CHL has shifted from acidosis and nutritional-base models to a biomechanical model focused on the digital cushion

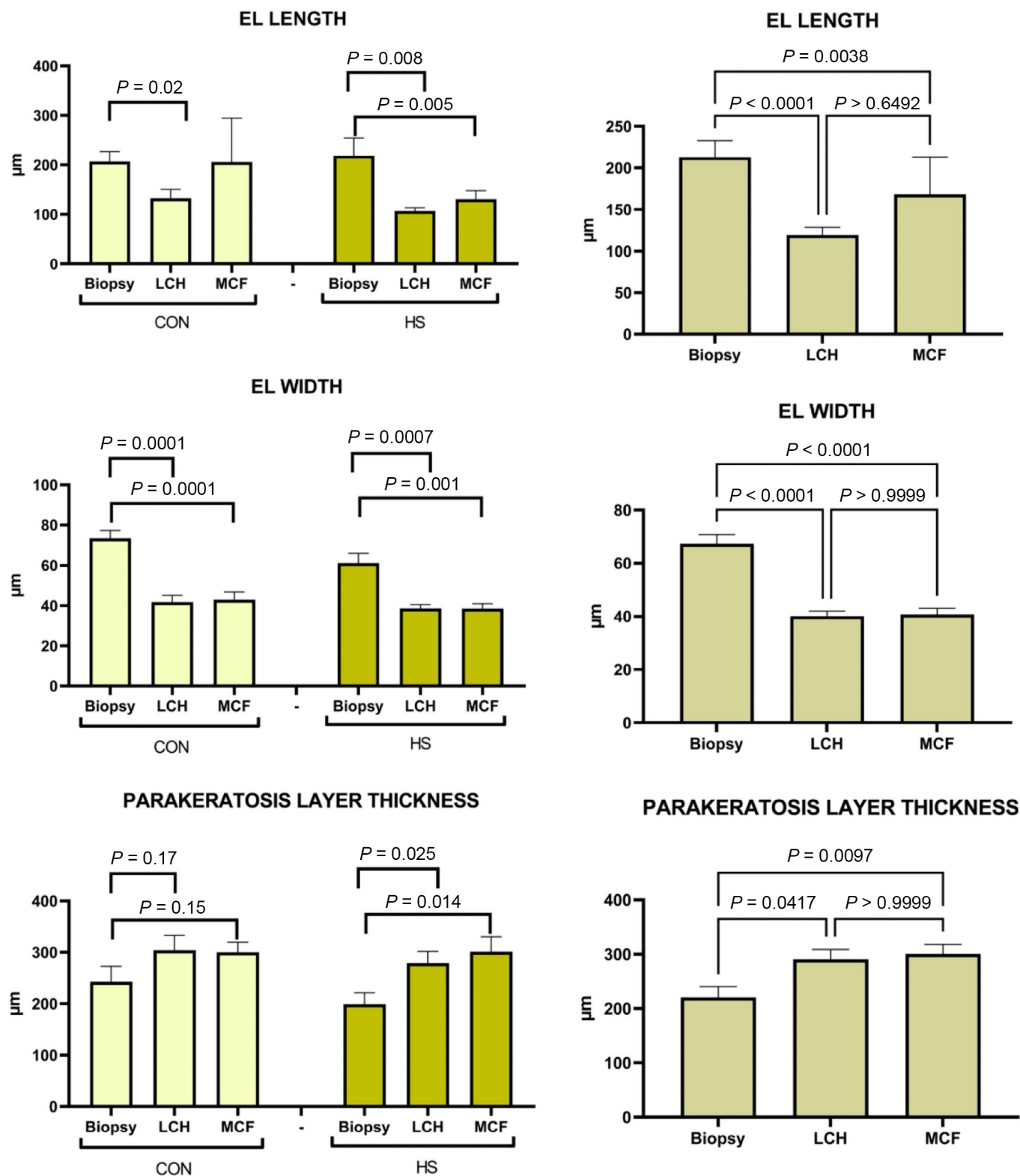


Figure 8. Comparison of the EL length, width, and the thickness of the parakeratosis layer measured by microscopy on histological sections of biopsies of the wall of the lateral claw of the left hind limb before exposure to the experimental diet (biopsy) and of the lateral claw of the right hind limb (LCH), and the medial claw of the left forelimb (MCF) after exposure to the experimental diet of Holstein steers fed a high-starch diet (HS) and a conventional diet (CON). The graphs on the left side are from analysis of the groups separately, and the graphs on the right side are from the analysis of the 2 groups together. Error bars represent SE.

Table 3. Frequency (%) of histological sections of Holstein steer claw walls subjected to a high-starch diet (HS; 37.0% starch) and a conventional diet (CON; 16.8% starch) showing different patterns of EL (standard, fringed, forked, or sharp)

Format ²	Biopsy ¹			LCH			MCF		
	HS	CON	<i>P</i> -value ³	HS	CON	<i>P</i> -value ³	HS	CON	<i>P</i> -value ³
Standard	43	46	0.613	42	49	0.613	54	53	0.939
Fringed	28	38	0.704	28	22	0.704	22	27	0.724
Bifurcated	24	15	0.788	17	21	0.788	18	18	0.893
Sharp	6	0	0.214	13	9	0.347	6	1	0.161

¹Biopsy of the lateral claw of the left hind limb before exposure to the experimental diet.²Epidermal lamellar shape³Comparison between groups.

thickness, metabolic alterations around calving, and housing conditions (Knott et al., 2007; Newsome et al., 2017; Wilson et al., 2021). However, the possibility that metabolism or inflammation may play a role should not be completely ruled out (Dong et al., 2015; Bäßler et al., 2021). Here, we investigate whether the metabolism (especially insulin resistance) may influence the histology

of bovine claws, which in turn could affect horn quality and the occurrence of future lesions.

In our study, the HS group had lower rumen fluid pH, reaching the lowest mean of 5.9, whereas that of the CON group was 6.6. The criteria for diagnosing SARA when using an esophageal probe proposed by Plaizier et al. (2008; i.e., rumen pH below 6.0, approximately 4

Table 4. Frequency (%) of epidermal cell morphology scores, basement membrane integrity, inflammatory cells, and hemorrhage from biopsies of the claw wall of Holstein steers subjected to a high-starch diet (HS; 37.0% starch) and a conventional diet (CON; 16.8% starch)

Claw	Group	Grade	Epidermal morphology	Basal membrane	Inflammatory cells	Hemorrhage
Biopsy ¹	HS	1	0.0	0.0	37.5	0.0
		2	25.0	37.5	62.5	0.0
		3	37.5	37.5	0.0	12.5
		4	25.0	25.0	0.0	87.5
	CON	1	12.5	37.5	62.5	25.0
		2	87.5	25.0	37.5	0.0
		3	0.0	25.0	0.0	25.0
		4	0.0	12.5	0.0	50.0
	<i>P</i> -value ²		0.011	0.173	0.364	0.108
	LCH	HS	1	12.5	25.0	50.0
2			25.0	50.0	37.5	0.0
3			62.5	25.0	12.5	50.0
4			0.0	0.0	0.0	25.0
CON		1	0.0	0.0	62.5	25.0
		2	57.1	42.9	37.5	12.5
		3	14.3	28.6	0.0	37.5
		4	28.6	14.3	0.0	25.0
<i>P</i> -value ²		0.901	0.085	0.669	0.867	
Biopsy vs. LCH ³		HS	p	0.589	0.041	0.765
	CON	p	0.256	0.414	1.00	0.678
MCF	HS	1	0.0	25.0	50.0	62.5
		2	25.0	25.0	50.0	0.0
		3	75.0	50.0	0.0	25.0
		4	0.0	0.0	0.0	12.5
	CON	1	12.5	28.6	50.0	37.5
		2	25.0	14.3	50.0	0.0
		3	37.5	28.6	0.0	37.5
		4	12.5	28.6	0.0	25.0
	<i>P</i> -value ²		0.953	0.673	0.669	0.360

¹Biopsy of the lateral claw of the left hind limb before exposure to the experimental diet.²Comparison between groups within the same time point.³Comparison between biopsy and LCH within the HS or CON groups.

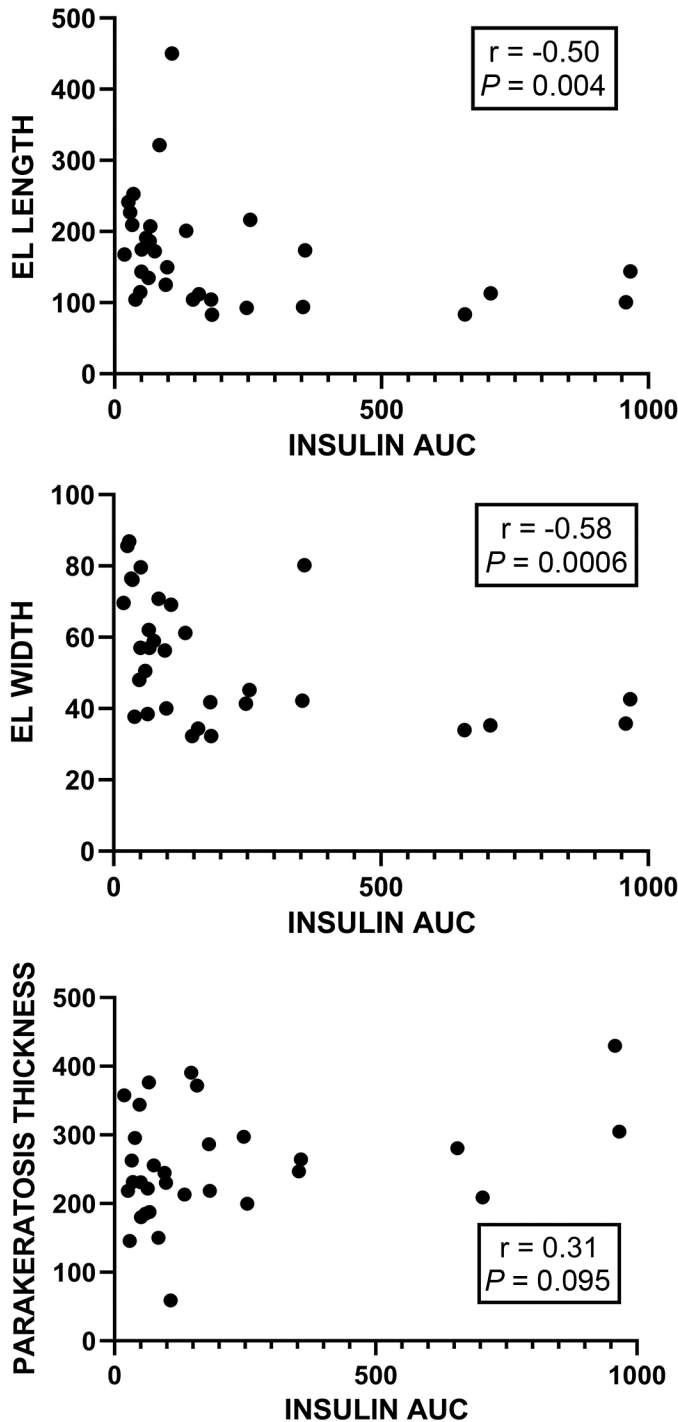


Figure 9. Correlations between the length of the EL, width of the EL, and thickness of parakeratosis with the AUC of insulin after GTT in Holstein steers.

h after feeding) or by Duffield et al. (2004; i.e., values below 5.9) indicate that the animals in the HS group had pH values indicative of SARA in limited moments, but the CON group did not. Alterations in rumen histology

associated with ruminal acidosis lesions, such as micro-abscess formation, cytoplasmic vacuolation of cells, and mild neutrophilic reactions (Ferguson et al., 2022), were observed in 50% of the animals in the HS group, indicating that the rumen environment was detrimental to rumen health.

Undoubtedly, changes in metabolic markers are interesting findings. Increases in plasma glucose concentrations were noted in the HS group starting at 7 d, with mean values above the reference values (45–75 mg/dL; Kaneko et al., 2008) and peaking at a concentration 40% greater than the baseline at the end of the experimental period (91 d). Insulin resistance was confirmed by analyses of data from both groups, which showed increased levels of circulating insulin after the GTT challenge at d 102 compared with basal values. According to Zachut et al. (2013), a higher AUC for insulin to eliminate the same dose of glucose indicates a degree of insulin resistance. The AUC for glucose did not differ between the HS and CON groups in the GTT. The glucose concentration during GTT depends on glucose consumption by peripheral tissues, endogenous glucose production, renal glucose excretion, and intestinal glucose absorption (Pires et al., 2007).

When analyzing claw histology, we identified the need to measure the distance of the layer between the lamellar tissue and the beginning of the stratum corneum, which comprises nucleated cells that supposedly should not exist (parakeratosis; Figure 10B). Typically, this distance is not measured, as the EL has a keratinized axis, and the stratum corneum immediately follows its end (Figure 10A; Danscher et al., 2010; Mendes et al., 2013). The length of the parakeratosis layer increased in the HS group after the experimental period, but we found no difference between groups at any moment.

The most significant changes in hoof histology were reductions in EL length and width, which is an interesting fact because, to our knowledge, no studies have compared EL length and width before and after animals were fed different diets. This type of comparison was only possible following Mendes et al. (2018). The width of the EL in the forelimb and hind limb was reduced compared with that before the experiment, indicating that the hooves were affected equally and systematically. The partial or total disappearance of the keratinized axis and the shortening of the EL have been described in acute laminitis (Greenough, 2007; Thoenes et al., 2005). The intercalation between dermal and epidermal lamellae is important for stabilizing and adhering to the claw horn (Thoenes et al., 2005; Danscher et al., 2010; Mendes et al., 2013), and the reduction in the length and width of the EL and the increase in parakeratosis layer may cause the laminar tissues of the animals to be less stable, probably implying lower tissue resistance.

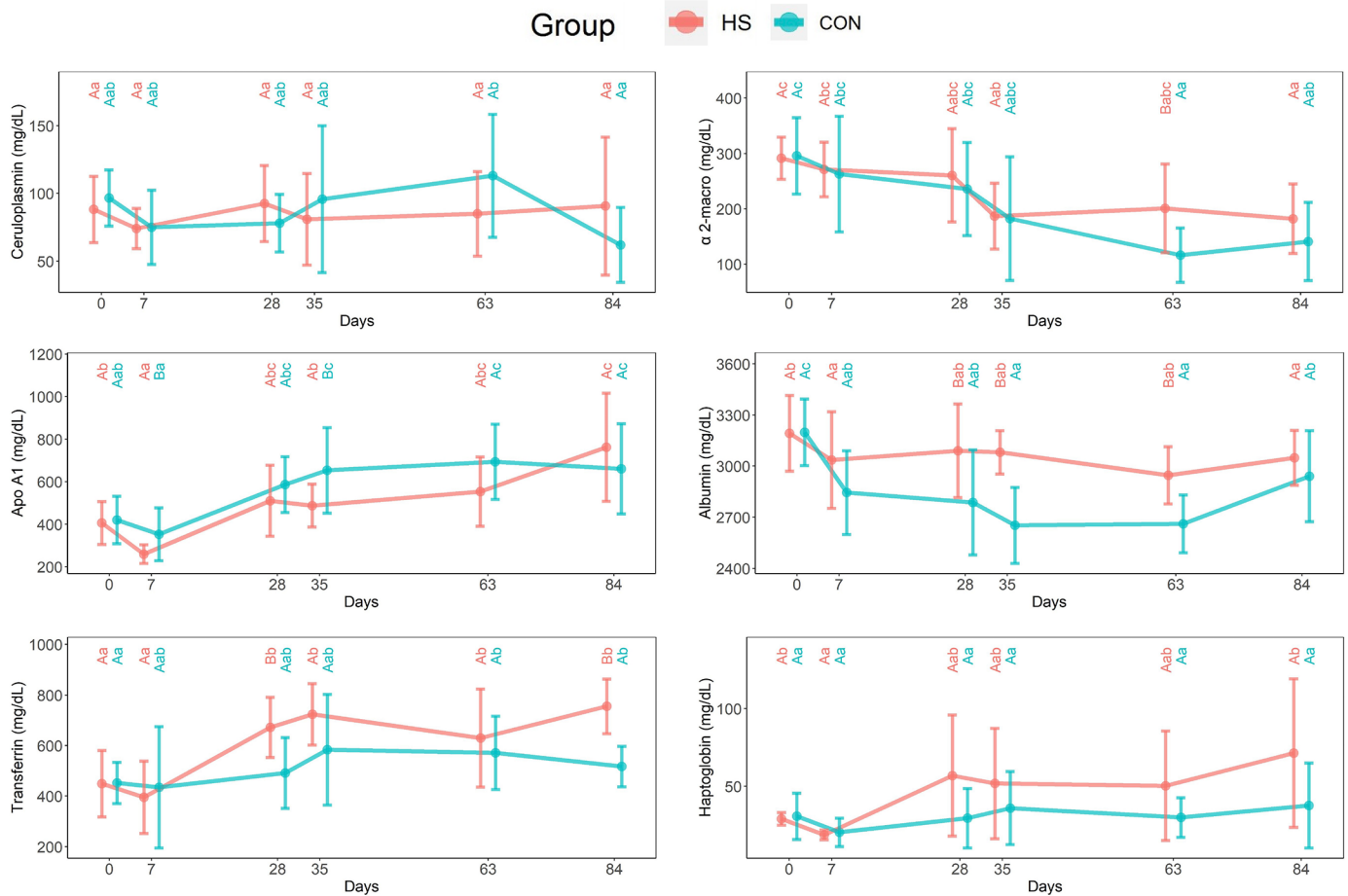


Figure 10. Plasma concentrations of APP (apolipoprotein A [Apo A], albumin, haptoglobin, transferrin, α-2 macroglobulin (α-2 macro), and ceruloplasmin) in Holstein steers fed a high-starch diet (HS) and a conventional diet (CON). Error bars represent 95% CI. ^{a-c}Different lowercase letters indicate differences between times within the same group. ^{A,B}Different uppercase letters indicate statistical differences ($P < 0.05$) between groups.

Similar to our study, Mendes et al. (2013) described no differences in hemorrhage and inflammatory infiltrate scores when evaluating the histology of animals with or without lameness and with or without CHL and hoof alterations. Although they did not differ, both groups had high scores for basement membrane irregularities, similar to Mendes et al. (2013), who described the presence of irregularities along the length of the basement membrane, and unlike Thoenes et al. (2005) and Danscher et al. (2010), who observed basement membrane collapse in cattle with induced acute laminitis.

Animals from both groups showed histological alterations in the claw at the beginning of the experiment, with more than half of the biopsies showing morphological changes. Both groups started the experiment with high scores for basement membrane irregularities and parakeratosis (i.e., the distance between the EL and the beginning of the high stratum corneum). Unlike animals raised on pastures with less dense diets, all animals were raised

from weaning in the tiestall and with diets based on corn silage and concentrate, which are starch-rich foods. Pre-exposure of the animals to these energy diets, housing conditions, or infectious diseases such as diarrhea or respiratory disease, may have caused these changes in lamellar tissue before the experiment.

Nocek (1997) hypothesized that rumen pH depression triggers the release of vasoactive substances, such as histamine and LPS of bacterial origin that damage the capillaries of the lamellae in the foot and cause hemorrhage, inflammation, and lameness. However, our findings did not support this hypothesis because the treatments did not interfere with the histological measurements and evaluation, even though the HS group presented a lower rumen pH and histological alterations associated with ruminal acidosis lesions. Thus, the results presented herein suggest that SARA may not necessarily be a prerequisite for histological alterations in the hoof. These findings were similar to other studies that reported limited impacts

of high-energy diets on hoof histology (Webster, 2001; Knott et al., 2007). Alternatively, the observed histological changes could be attributed to factors beyond diet.

Furthermore, considering the mild presence of inflammatory cells in the claw histology and the discrete changes in APP plasma concentrations during the experimental period, there was no obvious evidence that a systemic inflammatory response played a major role or even influenced the histological changes in the present experiment. Collectively, these findings indicate that inflammation is a secondary event in this process. This is similar to the results from Danscher et al. (2010) and Thoenes et al. (2005) that found limited inflammatory sign in the histological examination of claws from heifers subjected to a rumen acidosis protocol using oral oligofructose overload.

We observed a notable negative correlation between the length and width of the EL and the AUC of insulin (Figure 6), suggesting that the increase in the AUC and the reduction in the epidermal layers of the lamina tissue may be related. Although this correlation is intriguing, it is important to note that our study represents the first instance of such a finding in cattle. Further research in varied conditions is required to validate and explore this correlation comprehensively, especially considering that the initial expectation of intergroup differences was not realized. Another important point to note is that our study used Holstein steers in an anabolic state, which differs significantly from the metabolism of a dairy cow, particularly in the first months of lactation when most hoof lesions and lameness occur (Solano et al., 2016; Browne et al., 2022).

The animals exposed to both diets developed insulin resistance and exhibited reductions in the length and width of the EL, with no significant differences between the 2 dietary treatments. These results can be interpreted in 2 ways: either both diets similarly affected insulin sensitivity and negatively affected hoof histology, or the alterations were influenced by factors other than diet. In the experimental circumstances, these factors could be age and housing conditions. The movement restriction imposed by the tiestall system and floor type might have influenced claw structures. Furthermore, earlier studies have indicated that calving and housing exert a significant influence on hoof health and claw histology, whereas dietary factors exhibited minimal impacts (Webster, 2001; Tarlton et al., 2002; Knott et al., 2007). However, these studies often did not specify if they induced SARA or provided adequate information about dietary components and metabolic implications. Additionally, although housing conditions may affect hoof histology, they do not explain the observed increase in insulin resistance.

Given the well-documented capacity of high-energy diets to induce insulin resistance in cattle (McCarthy et al., 2015; Shi et al., 2020), the direct attribution of the insulin-resistant state observed in our study to di-

etary starch levels requires cautious interpretation. The identified negative correlation between insulin AUC and EL measurements, similar to findings in equine studies where hyperinsulinemia precipitated histological alterations in hoof structure (Ribeiro et al., 2020; Rahnama et al., 2020), tentatively supports the notion that insulin changes induced by diet may affect hoof health. However, the absence of significant differences in histological parameters and insulin AUC following GTT between groups introduces uncertainty into any definitive conclusions. Therefore, further investigations are necessary to elucidate these aspects.

Our CON diet, promoting an average daily weight gain of nearly 900 g, highlights a limitation in our methodology, which is the absence of a control group with a low-starch and a hay-based diet. Diets that are less energy-dense generally contribute to more stable ruminal environments and are associated with less severe metabolic disturbances, which could, in turn, lessen the impact on insulin sensitivity compared with more energy-dense, high-starch diets. Implementing these strategies could help determine if starch content or metabolized energy significantly affects metabolic health and ultimately affects hoof histology.

In our study, the experimental diets increased the serum concentration of IGF-1 and the AUC of insulin after the GTT. Apparently, in horses, high concentrations of insulin and IGF-1, through the activation of the insulin-like growth factor receptor-1 in the lamina, induce excessive proliferation and inadequate keratinization of the hoof, which can lead to morphological changes, clinical lesions, and lameness (Ribeiro et al., 2020; Rahnama et al., 2020). Rahnama et al. (2020) demonstrated that all insulin-treated horses developed laminitis within 30 h, whereas horses treated with an equinized version of a therapeutic anti-human IGF-1R monoclonal antibody showed less distal phalanx sinking and milder histological changes, with minor elongation at the tips of the secondary EL. In our study, there was no difference in parakeratosis layer thickness or the length and width of EL between groups, despite a higher IGF-1 concentration in the HS group than in the CON group. This raises the possibility that what is observed in horses might not necessarily be applicable to cattle, or it could be that our study did not capture the correlated changes in hoof measurements. Once again, including a control group with a low-starch and hay-based diet might have been important in providing further insights.

CONCLUSIONS

The HS diet induced mild SARA, as confirmed by changes in rumen pH, ethological characteristics, and histological lesions of the rumen wall. However, animals

on both experimental diets experienced a reduction in the length and width of the EL in claw histology, whereas the HS diet increased the thickness of the parakeratosis in the lamellar tissue. Additionally, animals in both groups developed a state of insulin resistance, as shown by an increased insulin AUC after the GTT, which was negatively correlated with EL measurements in histology. Because animals receiving both diets were affected similarly, it is not possible to confirm that the diets were responsible for the alterations seen in hoof histology. Furthermore, the occurrence of SARA was not a prerequisite for the histological alterations. Therefore, further studies are warranted to more extensively explore the role of insulin and IGF-1 and their imbalances as potential causative agents of the histological changes observed in this study, and their subsequent impact on claw health.

NOTES

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Abbreviations used: α -2 macro = α -2 macroglobulin; Apo A = apolipoprotein A; APP = acute-phase protein; AUC = area under the curve; CHL = claw horn lesion; CON = conventional diet; D = drinking water; DL = dermal lamella; E = eating; EL = epidermal lamella; GTT = glucose tolerance test; HE = hematoxylin and eosin; HS = high-starch diet; LCH = lateral claw of the right hind limb; LD = lying down; MCF = medial claw of the left forelimb; P = parakeratosis layer; PAS = periodic acid-Schiff; RLD = ruminating while lying down; RSD = ruminating while standing; RUM = ruminal fluid; SC = stratum corneum; STD = idle standing; TLD = total time spent lying down; TSD = total time spent standing idle.

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