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The association of prepartum urine pH, plasma total calcium concentration at calving and postpartum diseases in Holstein dairy cattle

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ABSTRACT

The use of anionic salts to prevent milk fever in dairy cattle has been an effective nutritional strategy; however, the degree of acidification that determines the most acceptable productive responses and well-being of the cow is still a controversial topic. The objective of this study was to assess urine pH in prepartum Holstein cows fed anionic diets and determine its association with plasma total Ca, Mg, P, β-hydroxyl-butyrate (**BHB**) concentrations at parturition and the occurrence of peripartum disorders. This investigation consisted of 2 studies. Study 1 was conducted on a grazing dairy. Between February and May 2019, 60 prepartum multiparous cows were tested for urine pH and plasma metabolite concentration at parturition. Total Ca, P, Mg and BHB at day 1 in milk (**DIM**) were assessed and statistically analyzed by ANOVA (models for polynomial regression). Study 2 was conducted on a drylot dairy farm. Between July 2018 and January 2019, 203 cows were evaluated for urine pH and followed-up for 30 DIM to obtain the incidence of dystocia, stillbirths, milk fever, retained fetal membranes, metritis, clinical mastitis and ketosis. Cows were categorized based on their last urine pH as group 1: pH > 7.0 ($n = 135$); group 2: pH between 6.0 and 7.0 ($n = 46$) and group 3: pH < 6.0 ($n = 22$). A logistic regression model for each health event was conducted considering urine pH group as the main effect. Urine sample was collected at 2.71 ± 2.84 days before parturition. In study 1, there was a quadratic effect of urine pH on total Ca. Total Ca concentration was higher between urine pH 6.0 and 7.0, while decreasing below pH 6.0 and above pH 7.0. There was a trend ($P = 0.11$) for a quadratic effect of urine pH on the concentration of plasma BHB at parturition. β-Hydroxyl-butyrate was lower approximately between urine pH 6.5 and 7.5. In study 2, the odds for a stillborn in cows with urine pH < 6.0 was 2.39 (95% CI = 1.06–5.40) times the odds for a stillborn in cows with urine pH ≥ 7.0. There was no association between urine pH and the other diseases. In conclusion, cows with prepartum urine pH < 6.0 and >7.0 had lower concentration of plasma total Ca and tended to have a higher concentration of BHB. Cows with urine pH < 6.0 had a higher incidence of stillbirths than cows with urine pH > 7.0.

Implications

Anionic diets offered during the prepartum period assist in the prevention of hypocalcemia; however, targeted over-acidification of the body may be detrimental. Cows with a urine pH < 6.0 may decrease plasma Ca concentration and increase the incidence of stillbirths, suggesting that anionic diets should be offered with caution with a target urine pH between 6.0 and 7.0.

Introduction

Hypocalcemia is a common metabolic disorder affecting dairy cattle around parturition. Ten to 50% of cows may develop low blood Ca concentrations without evident clinical signs, a condition termed subclinical hypocalcemia, and is characterized by a blood total calcium concentration <2.15 mmol/l (<8.5 mg/dl; Caixeta et al., 2017; McArt and Neves, 2020). Other studies have defined a cut-off value for serum total calcium of 2 mmol/l (<8.0 mg/dl; Reinhardt et al., 2011). Concomitantly, hypocalcemia may affect organs with smooth musculature such as the uterus, rumen, abomasum and teat sphincter; consequently, subclinical hypocalcemia becomes a significant risk factor for dystocia, retained fetal membranes (**RFMs**), metritis, uterine prolapse, displacement of the abomasum (**DA**), mastitis, ketosis and fatty liver (Goff and

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Horst, 1997; Melendez and Risco, 2016). Clinical hypocalcemia (blood Ca < 1.25 mmol/l [<5 mg/dl]) is a more severe condition, becoming occasionally life-threatening.

A successful strategy to reduce the incidence of both clinical and subclinical hypocalcemia involves the use of anionic compounds, which alter the dietary cation–anion difference: (**DCAD**) = ([Na + K] − [S + Cl]) in mEq per kilogram of DM (Goff et al., 2004). Negative DCAD during the prepartum period can mitigate hypocalcemia via increased responsiveness of parathyroid hormone receptors at the level of the cell membrane, due to body acidification (Goff et al., 1991; Abu Damir et al., 1994); consequently, improved Ca absorption at the level of the digestive tract, through enhanced vitamin D activity, and better Ca resorption from the bone through improved osteoclast activity, may occur (Block, 1984; Goff et al., 2004; Goff, 2014).

As DCAD decreases, H^+ in the blood increases, HCO_3^- decreases and pH decreases. These changes are accompanied by a reduction in urinary Na and K excretion and a reduction in urinary pH as compensatory mechanisms. As a result, the strong negative relationship ($r^2 = 0.95$) (Vagnoni and Oetzel, 1998) between urinary pH and net acid excretion by cows fed anionic diets suggests the urinary pH assessment as a useful, quick and inexpensive tool to monitor the degree of metabolic acidification that is imposed by the anionic strategy, without the necessity to evaluate the mineral content of the prepartum diet offered to the animals (Block, 1984; Goff, 2014; Lean et al., 2019).

The subject of what urine pH is the ideal for the prevention of cases of milk fever has been very controversial. Urinary pH values below 6.0 may indicate a risk of overacidification and development of a potential uncompensated metabolic acidosis (Goff, 2014; Melendez and Poock, 2017). Urinary pH around 7.0 has been consistently related to a lower incidence of milk fever (Charbonneau et al., 2006). Consequently, it has been suggested the optimal urinary pH for prepartum dairy cows should be between 6.0 and 7.0 for Holstein cows (Goff, 2014). In the US dairy industry and other countries, some nutritional consultants still recommend reducing the urinary pH to <6.0. By reducing the pH from normal values of 8.5 to values close to 5.5 (3 pH units), an excessive load is imposed on the kidneys as they must excrete 1 000 times the additional H^+ produced by the body (Goff, 2014). Indeed, in a meta-analysis, it was consistently reported that the reduction of urine pH from 8.1 to 7.0 was sufficient to decrease significantly the incidence of clinical hypocalcemia, but not necessarily subclinical hypocalcemia (Charbonneau et al., 2006). In addition, the same study demonstrated that the lower the urine pH, the lower the feed intake, and the higher the risk for an uncompensated metabolic acidosis. Furthermore, in a recent study, cows fed anionic diets with an average urine pH of 5.9 had the same concentration of blood total calcium at parturition as cows with an average urine pH of 6.2 (Caixeta et al., 2020). Similar results were reported by Melendez and Poock (2017). In the work of Charbonneau et al. (2006), a DCAD of −100 mEq/kg DM resulted in urine pH close to 6.5 and blood tCa of nearly 8.5 mg/dl, the threshold for subclinical hypocalcemia. This suggests that a urine pH in the range 6.0 to 7.0, with an average of 6.5, is enough to prevent subclinical hypocalcemia. Accordingly, the hypothesis of this study was that cows with urine pH between 6 and 7 reach normal plasma tCa concentration at calving and cows with urine pH < 6.0 may develop a higher incidence of peripartum disorders, particularly considering fetal compromise, perhaps due to a higher risk of uncompensated metabolic acidosis. Therefore, the aim of the present study was to assess urine pH in prepartum Holstein dairy cows fed anionic diets and determine its association with plasma tCa, tMg, P, beta-hydroxy-butyrate (**BHB**) concentrations at parturition (study 1) and the occurrence of peripartum disorders (study 2).

Material and methods

This investigation consisted of 2 studies carried out in Chile and Argentina, respectively. Studies were approved by the Animal Care and Use Ethical Committee of the University of Chile and National University of La Pampa, Argentina, respectively.

Study 1

Farm

The study was conducted on a seasonal grazing dairy farm located in Chahuilco, Chile (Lat: −40.70; Lon: −73.27). The herd consisted of 1 200 lactating Holstein cows with two parturition seasons: 30% of animals calving in autumn and 70% in spring. Cows were milked twice a day. Partial mixed ration (**PMR**) for prepartum cows consisted of grass silage, grass hay, corn silage and concentrates. Pasture, PMR and concentrate in the parlor for lactating cows were combined to meet or exceed the nutritional requirements of the Cornell Net Carbohydrate and Protein System (**CNCPS**) 6.55 (Van Amburgh et al., 2015) (Tables 1 and 2).

Reproductive management consisted of timed artificial insemination following an intensive synchronization protocol during a period of 10 weeks in each season.

At dry-off, cows were maintained at pasture. At 21 ± 3 days before expected parturition (**BEP**), cows were moved to a prepartum lot receiving 95% of their DM as a PMR containing a commercial anionic product based on hydrochloric acid and ammonium chloride (Meganion, Origination O2D Inc., Maplewood, MN, USA) and 5% from pasture (DCAD −109 mEq/kg DM, using the equation [Na] + [K] − [Cl] + [S]). Both the pasture and the prepartum and postpartum PMR were nutritionally analyzed by wet chemistry by collecting representative samples at the beginning and in the middle of the study and submitted to the

Table 1
Ingredient and chemical composition by wet chemistry of prepartum dairy cow diets on a DM basis as kg/cow per day and as %, for Study 1 and 2.

Ingredient	Study 1		Study 2	
	kg/day	% of ration DM	kg/day	% of ration DM
Corn grain ground	0.43	3.11	0.51	3.84
Triticale grain ground	0.44	3.18	–	
Canola meal	0.45	3.25	1.65	12.42
Soybean meal	0.43	3.11	0.17	1.28
Gluten meal	0.18	1.30	1.79	13.48
Calcium carbonate	0.11	0.79	–	
Anionic premix	0.48	3.47	1.20	9.04
Mineral/vitamin premix	0.15	1.08	0.39	2.94
Ryegrass silage	1.20	8.67	–	–
Wheat straw	2.30	16.62	2.43	18.30
Corn silage	5.48	39.60	5.14	38.70
Ryegrass hay	1.50	10.84	–	–
Pasture (ryegrass)[1]	0.69	4.99	–	–
Total	13.84	100	13.28	100
Nutrient		% of ration DM		% of ration DM
DM, %		48.8		51.3
CP, %		14.10		14.5
NDF_{om}[2], %		40.55		38.47
Starch, %		18.42		18.81
Sugars, %		1.96		4.23
Fat (EE), %		2.97		3.24
Ash, %		8.53		11.93
Ca, %		0.94		1.02
P, %		0.30		0.40
Mg, %		0.44		0.48
K, %		1.10		1.14
S, %		0.31		0.25
Na, %		0.21		0.12
Cl, %		1.01		1.11
DCAD[3]		−109.0		−128.9

[1] Estimated consumption by difference from the total mixed ration for a total of 12 kg/cow per day DM, based on a cow of 630 kg BW and body condition score 3.5 (2.0% of BW, DM intake).
[2] Organic Matter.
[3] Dietary Cation–Anion Difference, Calculated as mEq/kg DM, (Na + K) − (Cl + S).

Table 2
Ingredient and chemical composition by wet chemistry of postpartum dairy cow diets on a DM basis as kg/cow per day and as %, for Study 1 and 2.

Ingredient	Study 1		Study 2	
	Kg/day	% of ration DM	Kg/day	% of ration DM
Corn grain ground	3.00	18.67	3.38	18.22
Triticale grain ground	1.26	7.84	–	–
Canola meal	0.43	2.68	0.51	2.75
Soybean meal	2.42	15.06	2.51	13.53
Soybean hulls	–	–	1.14	6.15
Gluten meal	0.15	0.93	–	–
Cotton Seed	–	–	1.61	8.68
Calcium carbonate	0.10	0.62	–	–
Mineral/vitamin premix	0.30	1.87	0.70	3.77
Ryegrass silage	3.44	21.41	2.55	13.75
Wheat straw	0.43	2.68	0.35	1.89
Corn silage	1.54	9.58	4.35	23.45
Alfalfa hay	–	–	1.45	7.82
Pasture (ryegrass)[1]	3.00	18.67	–	–
Total	16.07	100	18.55	100
Nutrient		% of ration DM		% of ration DM
DM, %		35.7		47.6
CP, %		17.98		16.58
NDF_{om}[2], %		28.9		30.5
Starch, %		22.3		23.5
Sugars, %		5.53		5.47
Fat (EE), %		3.93		3.75
Ash, %		8.44		7.95
Ca, %		0.78		0.81
P, %		0.39		0.40
Mg, %		0.29		0.25
K, %		1.54		1.20
S, %		0.21		0.17
Na, %		0.21		0.20
Cl, %		0.43		0.45
DCAD[3]		+ 232.1		+159.8

[1] Estimated consumption by difference from the total mixed ration for a total of 16 kg/cow per day DM, based on a cow of 630 kg BW and body condition score 3.5 (2.5% of BW, DM intake).
[2] Organic Matter.
[3] Dietary Cation–Anion Difference, Calculated as mEq/kg DM, (Na + K) − (Cl + S).

Cumberland Valley Analytical Service Laboratory (Waynesboro, PA 17268, USA). The PMR was offered once a day around 1800 hours, and the cows had continuous access to grazing. To provide only 5% of the DM intake as grazing, pastures were handled strategically with an electric fence system.

Parturition occurred in a barn next to the pasture. During the calving season, the prepartum group was monitored every 2 h for 24 h. Calves born without assistance were immediately separated from the dam. Cows needing assistance at parturition were handled in the barn with a special chute for animal processing.

After parturition, cows were moved to a postpartum lot for 30 days. Within the first 15 DIM, after the morning milking, the cows were separated in a treatment line to assess health parameters. The evaluation consisted on measuring rectal temperature, visualize uterine discharges for the diagnosis of metritis, rule out the presence of RFM, stripping the quarters to visualize the milk for the diagnosis of clinical mastitis, auscultate the left-side paralumbar fossa to assess rumen function and diagnose DA. Diseases were defined as Kelton et al. (1998). Treatment protocols established under standard operating procedures were applied by the farm veterinarian.

Study design

wThis research was a cross-sectional study where the potential association between prepartum urine pH and the concentration of blood metabolites at calving was evaluated. During autumn 2019 (from March 15 to June 15), dry cows were moved in a weekly basis at 21 ± 3 days before expected parturition to a prepartum lot. Throughout this 3-month period, a total of 345 prepartum multiparous cows were eligible for urine collection and pH assessment. The prepartum group was strategically managed in a corral next to the milking parlor to properly monitor the parturition. The dynamic of cows' movement and sampling was as follows: Every week (on Mondays), new cows were moved from the early dry cow group to the prepartum lot, while fresh cows were exiting the group daily, as they calved. Therefore, the prepartum lot maintained a daily average of about 90–100 cows. On Fridays, around 0800 hours, about 15 to 20 cows were randomly brought from the prepartum lot to the milking parlor to collect urine samples. A maximum of 20 cows was brought weekly because they were in an advanced state of pregnancy; therefore, any potential stress had to be avoided to prevent premature parturition. For inclusion sampling criteria, only cows residing in the prepartum group for more than 3 days were qualified to be tested because the effect of anionic diets on acid–base status takes about 48 to 72 h after feed consumption (Goff, 2014). Elapsing time from the urine collection and PMR feeding from the previous day was 14 h. A clean-caught, non-contaminated, mid-stream urine sample was obtained by gentle massage of the eschuteon area and placed in plastic containers for pH assessment using a portable electronic pH meter (Hanna Instruments, Ann Arbor, MI, USA). The meter was calibrated each morning using a 7.0 pH buffer solution and a 4.0 pH buffer solution (Hanna Instruments, Woonsocket, RI, USA), following the manufacturer's guidelines.

Sampled cows were monitored daily until calving. Several cows were resampled if they did not deliver and were randomly chosen again during the following week. In cows with more than one test (sampled a crossed multiple weeks), the urine sample closest to parturition was used for the statistical analysis. Assuming a pooled SD of 0.3 mmol/l for plasma tCa, detecting a true difference between a group of cows with a urine pH < 6.0 and a group of cows with urine pH ≥ 6.0 in plasma tCa mean of 0.25 mmol/l, with a power of 80% and a level of significance of 5% (one-sided test), this study required a total sample size of 66 cows. The 66 cows consisted of 11 cows (pH < 6.0) and 55 cows (pH ≥ 6.0), to ensure that the group with urine pH ≥ 6.0 is 5 times larger than the group with urine pH < 6.0 (Statistical Analysis Software (SAS), 2017).

Between 5 and 7 h post calving, a blood sample was obtained from the tail plexus with a vacutainer system. Samples were immediately centrifuged at 3 000 g × 10 min, and plasma was stored in plastic vials and frozen at −20° C until analysis. Samples were submitted to an accredited commercial laboratory (Coorpinsem, Osorno, Chile) for metabolite analyses.

Blood analyses

Plasma tCa and tMg concentrations were determined by atomic absorption spectrophotometry (Perkin-Elmer Corp., Waltham, Massachusetts, USA). Plasma P concentration was determined colorimetrically (Parekh and Jung, 1970). ß-hydroxy-butyrate was selected as an indicator of energy-related metabolite based on McArt et al. (2012) and was determined by an enzymatic-colorimetric method (Williamson and Mellanby, 1974), using a commercial kit (Sigma beta- BHA kit # 310 – A St. Louis, MO, USA). Intra- and inter-assay CVs for tCa, P, tMg and BHB were below 2.5% and 3.5%, respectively.

Statistical analysis

The outcome variables were the concentrations of blood metabolites at day 1 postpartum. The null hypothesis was that there is no association between urine pH and the concentration of blood metabolites.

Metabolites were analyzed by ANOVA using the PROC GLM for polynomial regression in SAS 9.4 (Statistical Analysis Software (SAS), 2017).

Models were defined as:

$$y_{ijkl} = \mu + UpH_i + (UpH*UpH)_j + Time_k + Par_l + e_{ijkl}$$

where
y_{ijkl} = Blood metabolite concentration (tCa, tMg, P, BHB).
μ = population mean.
UpH_i = linear effect of urine pH.
$(UpH^*UpH)_j$ = quadratic polynomial effect of urine pH.
$Time_k$ = effect of time in days from last urine pH to parturition.
Par_l = effect of parity.
e_{ijkl} = random error term.

Study 2

Farm

The second study was conducted on a drylot dairy farm located on the west border of the Province of Buenos Aires, Argentina (Lat: −35.55; Lon: −63.38). The herd consisted of 1 200 lactating Holstein cows, milked three times a day and parturitions all year around. Lactating cows received a total mixed ration (**TMR**) based on corn silage, wheat straw and concentrates. Prepartum and postpartum diets were formulated to meet or exceed the nutritional requirements of CNCPS 6.55 (Van Amburgh et al., 2015; Tables 1 and 2).

Reproductive management consisted of a 55-day voluntary waiting period and timed artificial insemination.

At dry-off, cows were maintained in a drylot with access to forage and minerals. At 30 days BEP, cows were moved to a prepartum lot receiving an anionic TMR containing a commercial anionic product based on hydrochloric acid (Soychlor, Landus Cooperative, Ralston, IA, USA) with a DCAD of −128.9 mEq/kg DM. Parturition occurred in a maternity pen next to the drylot. Calves delivered without dystocia were immediately separated from the dam. Cows needing assistance at parturition were put in a chute and handled according to standard procedures.

Study design

This second study was a prospective observational cohort investigation where a group of cows with prepartum urine pH < 6.0 were prospectively compared with a group of cows with prepartum urine pH between 6 and 7 and another group with prepartum urine pH > 7.0. This dairy farm was chosen because there were records of cows with pH < 6.0, which allowed having a subpopulation of cows with low urine pH (<6.0) to further compare the incidence of peripartum disorders prospectively with cows with urine pH > 6.0. The hypothesis of the study was that the incidence of peripartum disorders, especially stillbirths, is higher in cows with urine pH < 6.0 than in cows with urine pH ≥ 6.0. This dairy historically had low incidence of stillbirths, but during the last several months, there was a rise in the incidence of stillbirth. After detecting more cows with low urine pH, there was a perception that the higher incidence of stillbirth might be related to this lower pH; consequently, we used this judgment to calculate our sample size. Assuming that 5% of the cows with urine pH ≥ 6.0 would experience a stillborn and 25% of the cows with urine pH < 6.0 would experience the same outcome, after applying a continuity correction, the study required a sample size of 28 cows with urine pH < 6.0 and 140 cows with urine pH ≥ 6.0 to ensure that the reference group is five times larger than the test group, with a power of 80% and at a one-sided test *P*-value of 0.1 (Statistical Analysis Software (SAS), 2017).

Only multiparous cows (2 or more parturitions) that were managed in the same prepartum group and fed the same anionic diet were eligible to obtain a urine sample and assess their urinary pH. Sampling was conducted between July 2018 and January 2019. To avoid unnecessary stress and extra handling, on a weekly basis, about 20 cows were randomly brought to the milking parlor area and handled through a walking line to obtain the urine samples. Expecting to collect a representative sample from 15 out of the 20 cows brought to the management area each week, an amount of 240 urine samples was estimated for a period of 16 weeks. Sampling was similar as described in study 1. The assessment of the urine pH was carried out using a handheld electronic pH meter after a careful calibration of the device (Hanna Instruments, Ann Arbor, MI) as described previously. Cows were recorded and followed-up until parturition. After calving, cows were housed in the same early postpartum lot and handled similarly until 30 DIM. Within the first 10 DIM, cows were assessed every day for rectal temperature, presence of RFM and early diagnose of puerperal metritis, rumen activity and diagnosis for DA. Clinical mastitis was diagnosed every day during the milking routine. Cows with any health disorder were treated based upon standard operating procedures.

Health events considered as outcome variables for this study were: dystocia, stillborn, milk fever, RFM, metritis, clinical mastitis and ketosis. These disorders were recorded and diagnosed consistently until 30 DIM by the farm veterinarian. Case definitions for health events were as follows: dystocia was defined as any parturition with assistance by 2 or more people for more than 15 min of intervention (score > 2, scale 1–5; Lombard et al., 2007). The definition of a stillborn calf was dead at birth or within 24 h of birth after at least 260 days of gestation (Berglund et al., 2003; Lombard et al., 2007). Clinical hypocalcemia or milk fever was defined as any cow lying down within the first 3 DIM, inappetence, neurological signs, staggering, depression and good response to Ca salts injected intravenously (Goff, 2014). Retained fetal membranes were defined as either fetal membranes observed hanging out of the vulva or palpated by hand inside of the vagina or the uterus by gynecological examination beyond 24 h post calving. Metritis was defined as any foul-smelling uterine discharges obtained by massage of the uterus by rectal palpation. Clinical mastitis was defined as any visual alteration of the milk obtained by stripping the 4 quarters over a black color container, independent if the mammary gland was observed with signs of inflammation (hardness, redness and swelling) (Kelton et al., 1998). Ketosis was defined as a cow with a concentration of blood BHB ≥ 1.2 mmol/l, assessed at 5 DIM (McArt et al., 2012), using a handheld device (Precision Xtra® device, Abbott Diabetes Care Inc., Alameda, CA, USA), which has a reported sensitivity of 94.8% (CI 95%: 92.6–97.0) and a specificity of 97.5% (CI 95%: 96.9–98.1) (Tatone et al., 2016). All cows were scored for body condition (**BCS**) at parturition using the scale proposed by Ferguson et al. (1994).

After conducting urine sampling once a week of approximately 20% of the adult cows housed in the prepartum pen for a period of 8 weeks, a total of 203 cows were evaluated for urine pH and followed-up for 30 DIM. Cows were categorized based on their last urine pH before parturition as group 1: urine pH > 7.0 (*n* = 135); group 2: urine pH between 6.0 and 7.0 (*n* = 46) and group 3: urine pH < 6.0 (*n* = 22). A logistic regression model for each health event was conducted considering urine pH group as the main effect.

Logistic models were defined as:

$$\text{Logit}(\pi) = \alpha + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_6 X_6 + \beta_7 X_7$$

where
π = odds of the health event (yes, no).
α = intercept.
β_1 = parameter of X_1.
X_1 = effect of group (1, 2, 3).
β_2 = parameter of X_2.
X_2 = effect of parity.
β_3 = parameter of X_3.
X_3 = effect of month of urine pH.
β_4 = parameter of X_4.
X_4 = effect of days from urine pH to parturition.
β_5 = parameter of X_5.
X_5 = effect of BCS at calving.
β_6 = parameter of X_6.
X_6 = effect of gender of offspring.
β_7 = parameter of X_7.
X_7 = effect of dystocia (except for dystocia model).

A backward elimination procedure was carried out, considering potential interactions among variables. Groups were forced to remain in the models. Variables were removed from the model when *P* was ≥0.20. The best model was selected based on the deviance value of the model. Potential multicollinearity among variables was explored using the TOL, VIF and COLLIN statements of Statistical Analysis Software (SAS) (2017).

For all statistical analyses, the level of significance was established at *P*-value ≤ 0.05. Tendency was considered when the *P*-value was between 0.15 and 0.05. None of the data were deposited in an official repository (available upon request).

Results

Study 1

Based on the study protocol, 70 cows were sampled for urine pH and blood collection at parturition for plasma metabolite concentrations. Unfortunately, 10 blood samples were extremely hemolyzed, and consequently, only 60 samples were considered for blood metabolite concentration assessment and statistical analyses (Table 3). In Fig. 1, a curvilinear association between both variables was observed, where plasma tCa concentration was higher between urine pH 6.0 and 7.0, while it decreased below pH 6.0 and above pH 7.0. The polynomial function was: Plasma tCa (mmol/l) = −3.93 + 1.89*(UpH) − 0.14*(UpH)^2 − 0.01*(days to calving) − 0.03*(lactation). As observed in the equation, the model also included a significant effect of the days elapsed from urine sampling to parturition, and lactation number of the cow.

None of the variables were associated with the concentration of plasma tMg at parturition. However, for *P*, only lactation number was significant with a negative association between both variables ($P = 0.0057$) (Table 3).

Mean BHB concentration was 0.86 mmol/l. Only 1 cow out of 60 had a BHB concentration > 1.2 mmol/l (subclinical ketosis). Interestingly, there was a trend ($P = 0.11$) for a quadratic effect of urine pH on the concentration of plasma BHB (mmol/l) at parturition (Table 4). In Fig. 2, a pattern for a curvilinear association between both variables is observed, where there seems to be a higher concentration of BHB when urine pH is approximately below 6.5 and above 7.5.

Study 2

Models for dystocia, milk fever, RFM, metritis, mastitis and ketosis showed that the urine pH was not associated with the incidence of these disorders (Table 5). Although there were no cases of milk fever in the group with urine pH < 6.0, there were 2 cases in the group with urine pH 6.0–7.0 and there were 3 cases in the group with urine pH > 7.0. The differences were not statistically significant, perhaps for lack of statistical power (low sample size). Nevertheless, and the most remarkable finding of this study was that the model for the incidence of stillbirths was significant (Table 6). Variables that were dropped by the backward elimination procedure were: month of calving, days from urine pH to parturition, parity of the dam and BCS at calving ($P > 0.20$). Variables that were retained in the final model were gender of the calf, type of parturition and group. Although gender of the calf and type of parturition can be related to each other, there was no evidence of multicollinearity between both variables. Consequently, the final model revealed that the odds of having a stillborn in cows with urine pH < 6.0 was 2.39 (95% CI = 1.06–5.40) times the odds of having a stillborn in cows with urine pH > 7.0, adjusting for gender of the calf and type of parturition.

Table 3
Descriptive statistics for urine pH, plasma tCa[1], tMg[2], P[3] and BHB[4] at day of parturition for Holstein cows ($n = 60$) in Study 1.

Item	Mean	Median	SD	Range
Urine pH	7.25	7.50	0.76	5.5–8.3
tCa (mmol/l)	2.14	2.19	0.31	0.71–3.89
P (mmol/l)	1.88	1.80	0.60	0.60–3.40
tMg (mmol/l)	1.02	1.00	0.18	0.56–1.60
BHB (mmol/l)	0.86	0.76	0.44	0.10–2.88
Days pH-parturition[5]	2.71	1.50	2.84	0–10

SD = standard deviation.
[1] Plasma total Calcium.
[2] Plasma total Magnesium.
[3] Plasma Phosphorus.
[4] Plasma β-hydroxyl-butyrate.
[5] Days from urine pH evaluation to calving date for the closest sample relative to parturition.

Discussion

Some of the limitations derived from conducting our research on commercial operations included unreliable health records (study 1) and impossibility to collect postpartum blood samples (study 2). The overall hypothesis of the whole investigation proved to be partially true because the incidence of stillbirth and plasma tCa at calving differed depending upon the level of the urine pH measured in cows fed anionic diets during the prepartum period.

Study 1

Although the mean urine pH of evaluated cows ($n = 60$) was 7.25, the range was 5.5 to 8.3, which allowed enough variability to find differences in the concentration of blood metabolites. The variability was expected because the cows had partial access to grazing (approximately 5% of DM intake) on a pasture based on ryegrass. Although the urine pH did not reach consistently a value below 7.0, cows had a mean concentration of plasma tCa greater than the cut-off value for subclinical hypocalcemia in Holstein cows defined by McArt and Neves (2020).

In the present study, the anion inclusion rate to establish the theoretical DCAD (−100 mEq/kg DM) was not enough to acidify the urine of cows as was planned. Perhaps, grass intake was higher than predicted, and the PMR had a lower consumption than anticipated. It is acknowledged that anionic products are less palatable; therefore, lower DCAD diets may reduce the DM intake consistently (Glosson et al., 2020). In an earlier study, prepartum cows consuming a diet with even a higher DCAD than the present study (−53 mEq/kg DM) had also a urine pH between 6.0 and 7.5 and a normal plasma tCa concentration at parturition (Melendez and Poock, 2017). In addition, prepartum cows consuming 50% of their DM as a PMR and the other 50% as grazing, mean urine pH was between 7.3 and 7.7 and mean plasma tCa concentration was between 1.91 and 2.00 mmol/l; however, there were no cases of milk fever (Melendez et al., 2019). These studies suggest that the association between the DCAD and urine pH is neither linear nor always identical with similar DCAD rations. Indeed, in two recent studies, diets with a similar DCAD (approximately –230 mEq/kg DM) did show average urine pH between 5.5 and 6.5 (Caixeta et al., 2020; Glosson et al., 2020).

The most remarkable finding of study 1 was that there was a quadratic effect or association between urine pH and plasma tCa concentration. In addition, mean time from urine pH assessment to parturition was 2.71 days (range 0–10), which was enough to significantly affect the association between urine pH and plasma tCa. Urine pH decreases 48 h after cows started eating anionic diets (Glosson et al., 2020). Highest concentrations of plasma tCa were reached between urine pH 6.0 and 7.0 and were lower beyond these 2 extremes based upon the polynomial quadratic equation. Data from Melendez and Poock (2017) suggested that a moderate DCAD (−50 mEq/kg DM) and urine pH between 6.5 and 7.5 were sufficiently adequate to reach a normal plasma tCa concentration at parturition. All these findings are

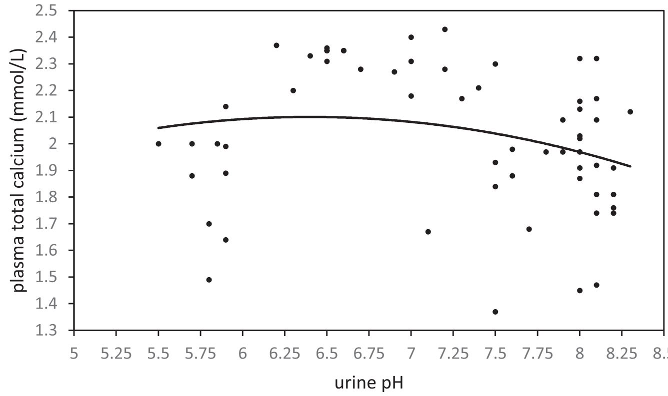

Fig. 1. Plasma total Calcium (tCa) concentration (mmol/l) in Holstein cows at day 1 postpartum as a function of urine pH (UpH). Polynomial quadratic effect equation: (R-Square: 0.155). tCa (mmol/l) = −3.93 + 1.89*(UpH) −0.14*(UpH)^2 –0.01*(days to calving) −0.03*(lactation).

Table 4
Parameter estimates for the general lineal model for plasma total Calcium and β-hydroxyl-butyrate (BHB) concentration (mmol/l) at parturition for Holstein cows ($n = 60$) in Study 1.

Parameter	Estimate	SEM	*P*-value
Model for plasma total Calcium (mmol/l)[1]			
Intercept	−3.93	1.89	0.0387
upH[2]	1.89	0.54	0.0005
upH*upH[3]	−0.14	0.038	0.0003
DP[4]	−0.01	0.006	0.0056
Lactation	−0.03	0.01	0.0029
Model for plasma BHB (mmol/l)[5]			
Intercept	5.33	2.76	0.0544
upH[2]	−1.25	0.78	0.11
upH*upH[3]	0.087	0.05	0.11

[1] $r^2 = 0.155$; CV = 13.61%; tCa mean = 2.149 mmol/l; Model *P*-value < 0.0001.
[2] Linear effect of urine pH.
[3] Quadratic effect of urine pH.
[4] Days from urine pH to parturition.
[5] $r^2 = 0.014$; CV = 52.2%; BHB mean = 0.86 mmol/l; Model *P*-value = 0.13.

comparable to the meta-analysis of Charbonneau et al. (2006), which found that the reduction of urine pH from 8.1 to 7.0 significantly decreased the incidence of clinical hypocalcemia. However, other studies, considering a lower DCAD (between −150 and −200 mEq/kg DM) and urine pH below 6.0 in Holstein cows, reported plasma tCa concentrations at parturition lower than 2.0 mmol/l (1.9 and 1.95 ± 0.037 mmol/l [7.6 and 7.8 ± 0.15 mg/dl]) (Ramos-Nieves et al., 2009; Wu et al., 2014), which are similar to our findings, with low plasma tCa concentrations when urine pH was <6.0. In the same line, the meta-analysis of Lean et al. (2019) showed that the effect of a lower DCAD on the concentration of tCa in blood at parturition was substantially heterogeneous. At least 5 studies showed lower Ca concentrations, while DCAD decreased. A potential explanation for the lower tCa concentrations with urine pH < 6.0 and a prepartum DCAD extremely negative might be the lower DM intake when DCAD is extremely reduced. This lower DM intake has been consistently reported (Charbonneau et al., 2006; Zimpel et al., 2018; Lean et al., 2019; Caixeta et al., 2020; Glosson et al., 2020). Taking into consideration all the reported studies, it is reasonable to suggest that either a very low (<6.0) or high urine pH (>7.5) are related to lower blood tCa concentration. Consequently, a desired prepartum DCAD should be conceived to reduce the incidence of milk fever, but not necessarily the subclinical hypocalcemia.

In the present study, the concentration of plasma tMg was within the normal ranges reported for postpartum dairy cows (1.02 ± 0.18 mmol/l) (Stämpfli and Oliver-Espinosa, 2015). None of the explanatory variables were associated with the concentration of plasma tMg at parturition. Dietary Mg was higher than the recommended levels for (National Research Council (NRC), 2001; 035–0.40%) for prepartum cows (0.44%). An important aspect of the management of Ca metabolism is to avoid hypomagnesemia, especially in cows under grazing conditions (Goff, 2014).

Regarding plasma P, the concentration of this macromineral (1.88 ± 0.6 mmol/l) was within normal ranges (1.38–2.58 mmol/l) and it was

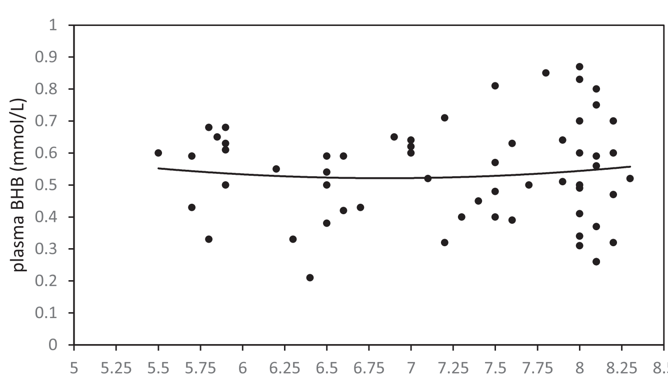

Fig. 2. Plasma β-hydroxyl-butyrate (BHB) concentration (mmol/l) in Holstein cows at day 1 postpartum as a function of urine pH (UpH). Polynomial quadratic effect equation: (R-Square 0.014). BHB (mmol/l) = 5.33 − 1.25*(UpH) + 0.087*(UpH)^2.

Table 5
Percentage and number of cases (n/total) of diseases in Holstein cows, adjusted odd ratio (AOR), 95% Confidence Intervals (95% CI) and *P*-values (Study 2).

Urine pH	Stillborn %	Milk fever %	Dystocia %	RFM[1] %	Metritis %	Ketosis %	Mastitis %
<6.0 ($n = 22$)	13.6[a] (3/22)	0.0 (0/0)	18.2 (4/22)	4.6 (1/22)	13.6 (3/22)	27.3 (6/22)	22.7 (5/22)
6.0–7.0 ($n = 46$)	8.7[ab] (4/46)	4.2 (2/46)	10.9 (5/46)	2.2 (1/46)	13.0 (6/46)	26.1 (12/46)	23.9 (11/46)
>7.0 ($n = 135$)	4.4[b] (6/135)	2.3 (3/135)	20.7 (28/135)	8.1 (11/135)	14.0 (19/135)	23.0 (31/135)	14.8 (20/135)
Comparison urine pH <6.0 vs >7.0							
AOR	2.39	0.68	0.89	0.65	1.05	1.16	1.09
(95% CI)	(1.06–5.40)	(0.15–3.17)	(0.49–1.63)	(0.34–1.27)	(0.58–1.92)	(0.71–1.89)	(0.67–1.74)
P-value	0.035	0.62	0.72	0.21	0.86	0.53	0.73

RFM = retained fetal membranes.
[a,b]Values within a column with different subscripts differ significantly at $P < 0.05$.
[1] Retained Fetal Membranes.

only affected by the parity number of the cow with lower concentration for older cows. This is in agreement with (Goff 2014 and 2018), stating also that blood *P* concentration is minimally affected by anionic salts.

The concentration of BHB (0.86 ± 0.44 mmol/l) was below the cut-off value used to define cows as subclinical ketosis (1.2 mmol/l) (McArt et al., 2012; Shin et al., 2015), with only one cow with a BHB > 1.2 mmol/l. This is particularly important especially since the highest incidence of ketosis may occur on days 3 to 5 postpartum in the modern Holstein dairy cow (McArt et al., 2012). Interestingly, there was a tendency ($P = 0.11$) for a quadratic effect of urine pH on the mean plasma BHB concentration. Again, higher BHB levels with lower urine pH's might be explained by potential lower DM intake that anionic salts may impose to prepartum dairy cows (Charbonneau et al., 2006; Lean et al., 2019). Subsequently, lower DM intake may trigger a higher fat mobilization and ketogenesis. Normal prepartum blood BHB levels are <0.8 mmol/l (Chapinal et al., 2011). In our study, more than 15% of cows had a plasma BHB concentration >0.8 mmol/l at parturition. On the other hand, high levels of BHB within cows with prepartum urine pH > 7.5 may be also explained by a lower DM intake, but now related to subclinical hypocalcemia, which also induces lower DM intake in dairy cows (Hansen et al., 2003).

Study 2

The incidence of dystocia, milk fever, RFM, metritis, ketosis and mastitis was within the typical ranges reported for dairy cows in the USA and Canada (Kelton et al., 1998; Liang et al., 2017). Our major hypothesis was that cows with prepartum urine pH < 6.0 were more likely to develop periparturient disorders. The hypothesis proved to be partially true because only the incidence of stillbirth was different between groups. Sample size was calculated based on the historical incidence of stillbirth and may have been too small to detect differences in low incidence diseases such as clinical hypocalcemia. Our study shows that prepartum cows experiencing a more aggressive metabolic acidosis (urine pH < 6.0) were more likely to have a stillborn than cows with urine pH > 6.0. A more severe metabolic acidosis imposed by very low negative DCAD diets may impact the fetal survival. It has been consistently demonstrated in humans that late-term pregnant women with metabolic ketoacidosis can have up to 35% of fetal demise (Dalfra et al., 2016; Jaber et al., 2019). Metabolic ketoacidosis reduces blood pH, similar to cows with lower urine pH that later developed left DA (Mecitoglu et al., 2016). Metabolic acidosis is associated with high fetal mortality rates, fetal hypoxia, lactic acidosis and impaired brain development (Kovács et al., 2017; Jaber et al., 2019). Unquestionably, prepartum cows exposed to metabolic stress adversely affected metabolic and inflammatory responses of the offspring that could influence disease susceptibility after birth (Ling et al., 2018).

Table 6
Adjusted odd ratio (AOR), 95% Confidence Intervals (95% CI) and *P*-values for the logistic regression model for stillbirths in Holstein cows (Study 2).

Effect	AOR	95% CI	*P*-value
Group pH < 6.0 vs pH ≥ 7.0	2.24	1.05–4.78	0.037
Gender of the calf Male vs Female	5.77	1.17–28.3	0.030
Dystocia Yes vs No	3.03	0.78–12.5	0.108

In one of the few studies associating prepartum urine pH and risk of postpartum diseases, the meta-analysis by Santos et al. (2019) shows a curvilinear relationship between risk of milk fever and prepartum urine pH, with little increase in risk between pH 5.5 and 6.5. Consequently, a urine pH between 6.0 and 6.5 is still beneficial, without the need to lower the pH to <6. Furthermore, they showed that the risk of DA decreased from 12% in primiparous cows fed a DCAD –200 mEq/kg (equivalent to a urine pH 5.7) to 6% when fed a DCAD of +200 mEq/kg (equivalent to a urine pH 8.0). Interestingly, the same conclusion was obtained in a Turkish study where cows with left DA had significantly lower prepartum urine pH (6.11 ± 0.2 vs 6.65 ± 0.1) and blood pH (7.27 ± 0.01 vs 7.32 ± 0.01) than healthy cows, respectively. Serum ionized calcium was not statistically different between both groups (Mecitoglu et al., 2016). Normal urine pH for ruminant species ranges from 7.5 to 8.5 (Parrah et al., 2013), which is highly influenced by diet, feeding time, bacterial infections, storage time, and metabolic and respiratory alkalosis (Piech and Wycislo, 2019). A mild metabolic acidosis improves the homeostasis of Ca metabolism around parturition in dairy cows (Charbonneau et al., 2006; Goff, 2014 and 2018; Lean et al., 2019); however, a more aggressive metabolic acidosis (urine pH < 6.0) might be potentially detrimental for the dairy cow.

In addition, insulin resistance in the periparturient dairy cow and its negative impact on calves has been recently reported (Hasegawa et al., 2019). Furthermore, a more negative prepartum DCAD ration reduced the gestation length of cows by 2 days (Lopera et al., 2018), a finding that deserves further attention. Undoubtedly, the excess of ketones, and state of insulin resistance, in conjunction with the more extreme metabolic acidosis imposed by the lower DCAD and lower DM intake is strong metabolic disturbances to induce stress on the pregnant cow, having detrimental effects on offspring survival (Ling et al., 2018). This prenatal stress may also induce dystocia with further implications for offspring welfare and performance. The most obvious detrimental offspring outcome of dystocia in cattle is stillbirths (Arnott et al., 2012). Consequently, these potential stressors for the maternal health status offer a rational explanation for the higher incidence of stillbirths in cows exposed to potential uncompensated metabolic acidosis reflected by low prepartum urine pH (<6.0).

Conclusion

Cows with prepartum urine pH < 6.0 had a lower concentration of plasma tCa, tended to have a higher concentration of BHB and had a higher incidence of stillbirths than cows with urine pH > 6.0. These results suggest that anionic salts should be fed with caution with a target urine pH between 6.0 and 7.0, and not overacidifying unnecessarily the prepartum dairy cow.

Ethics approval

This investigation protocol was approved by the Animal Care Committee of the NULP, Argentina, and University of Chile, Chile.

Data and model availability statement

Neither software nor data are deposited in an official repository.

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Author Contributions

Melendez: Conceptualization, Methodology, Formal analysis, Writing - Original Draft, Supervision; Bartolome: Conceptualization, Methodology; Roeschmann: Investigation, Data Curation; Soto: Investigation, Data Curation; Arevalo: Investigation, Data Curation; Möller: Investigation, Data Curation; Coarsey: Writing - Review & Editing.

Declaration of interest

We declare that there is no conflict of interest in the subject of this manuscript.

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