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PRECISIÓN DEL BHBCHECK EN LA MEDICIÓN DE β-HIDROXIBUTIRATO COMO HERRAMIENTA DE DIAGNÓSTICO DE HIPERCETONEMIA EN VACAS HOLSTEIN-FRIESIAN

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Tesis realizada por Gabriela Pérez Hernández bajo la supervisión del Comité Asesor indicado, aprobada por el mismo y aceptada como requisito parcial para obtener el grado de:

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DEDICATORIAS

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PRECISION OF THE BHBCHECK IN THE MEASUREMENT OF β-HYDROXYBUTYATE AS A DIAGNOSTIC TOOL OF HYPERKETONEMIA IN HOLSTEIN-FRIESIAN COWS

PRECISIÓN DEL BHBCHECK EN LA MEDICIÓN DE β-HIDROXIBUTIRATO COMO HERRAMIENTA DE DIAGNÓSTICO DE HIPERCETONEMIA EN VACAS HOLSTEIN-FRIESIAN

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ABSTRACT

The objectives were to evaluate the associations of concentrations of β-hydroxybutyrate acid (BHBA), calcium (Ca^{2+}) and non-esterified fatty acids (NEFA) in blood serum seven days prepartum with losses in milk yield (MY) and metabolic dysfunctions at seven and fourteen days of lactation, and to validate a hand-held electronic BHBA meter (BHBCheck) in the diagnosis of hyperketonemia, using a colorimetric laboratory assay as reference. One hundred and twelve Holstein-Friesian cows were sampled by coccygeal venipuncture in the peripartum period at -7, 7, and 14 d relative to calving date in a commercial herd in Northern Mexico. When BHBA levels were high seven days before parturition and were related to MY at day seven postpartum, it was observed that 11% of the cows lost 0.370 kg of milk. In contrast, no relationship was detected between BHBA prepartum and MY on day fourteen of lactation. Cows with NEFA blood serum concentrations ≥0.5 mmol L^{-1} on d 7 before calving were 7.6 more susceptible for lameness incidence ($P<0.01$), and when BHBA concentration were ≥0.8 mmol L^{-1}, cows were 2.4 times more likely to develop ketosis ($P<0.05$) in the first 60 days in milk. In the other hand, overall correlation coefficient (r) for the concentrations obtained with the BHBCheck and the laboratory assay was 0.86±0.03; when correlation was tested by day of sampling, the coefficients were 0.47±0.03, 0.80±0.03, and 0.92±0.04 for day -7, 7 and 14, respectively. In brief, data indicate that a high proportion of cows are above the thresholds of β-hydroxybutyrate and non-esterified fatty acids when determined one week before parturition. The risk thresholds for each metabolite were not associated with the amount of milk lost at day 14 after calving. Results indicate that the BHBCheck meter could be used as a reliable and rapid post-partum diagnostic test for hyperketonemia in dairy cows under field conditions.

Key words: cow-side diagnostic, biomarkers, transition cows, milk yield, metabolic dysfunctions.

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RESUMEN

Los objetivos fueron evaluar las asociaciones de la concentración de β-hidroxibutirato (BHBA), calcio (Ca^{2+}) y ácidos grasos no esterificados (AGNE) en suero sanguíneo siete días antes del parto con pérdidas en producción de leche (PL) y disfunciones metabólicas a los siete y catorce días de lactancia, y validar un medidor electrónico portátil de BHBA (BHBCheck) para el diagnóstico de hipercetonemia utilizando un método de laboratorio colorimétrico como referencia. Ciento doce vacas Holstein-Friesian fueron muestreadas mediante punción venosa coccígea en el período periparto a -7, 7 y 14 días con relación a la fecha de parto en un hato comercial en el norte de México. Cuando los niveles de BHBA fueron altos siete días antes del parto y se relacionaron con PL en el día siete después del parto, se observó que el 11% de las vacas perdieron 0.370 kg de leche. Por el contrario, no se detectó ninguna relación entre BHBA preparto y PL en el día 14 de la lactancia. Las vacas con concentraciones de AGNE en sangre ≥0.5 mmol L^{-1} 7 días antes del parto fueron 7.6 más susceptibles a la incidencia de laminitis ($P<0.01$), y cuando la concentración de BHBA≥0.8 mmol L^{-1} fueron 2.4 veces más propensas a desarrollar cetosis ($P<0.05$) en los primeros 60 días en leche. Por otro lado, el coeficiente de correlación general (r) para las concentraciones obtenidas con BHBCheck y el ensayo de laboratorio fue de 0.86±0.03; cuando la correlación se evaluó por día de muestreo, los coeficientes fueron 0.47±0.03, 0.80±0.03 y 0.92±0.04 para el día -7, 7 y 14, respectivamente. En resumen, los datos indican que una alta proporción de vacas está por encima de los umbrales de β-hidroxibutirato y ácidos grasos no esterificados cuando son determinados una semana antes del parto. Los umbrales de riesgo para cada metabolito no se asociaron con la cantidad de leche perdida en el día 14 después del parto. Los resultados indican que el medidor BHBCheck podría usarse como una prueba diagnóstica confiable y rápida para la detección de hipercetonemia en el posparto en vacas lecheras bajo condiciones de campo.

Palabras clave: prueba de diagnóstico, biomarcadores, vacas de transición, producción de leche, disfunciones metabólicas.

1 INTRODUCCIÓN GENERAL

El manejo nutricional del ganado lechero durante el período seco tiene un impacto determinante en la producción de leche (PL) y la salud al inicio de la lactancia. Este período es crítico y vulnerable para la vaca lechera porque se presenta un aumento conjunto de la demanda de energía causado por el desarrollo del feto, el crecimiento de la glándula mamaria, el parto y el inicio de la lactancia (Chapinal *et al.,* 2012). Este aumento en la demanda de energía se produce al mismo tiempo que la ingesta de materia seca (MS) no es suficiente para cubrir los requisitos energéticos de energía, lo que resulta en un desbalance energético generalmente grave en el animal.

Con el fin de preparar la lactancia, se produce un cambio metabólico conocido como control homeorrético que coordina los cambios en el metabolismo de los tejidos corporales para apoyar el estado fisiológico presente. Bauman y Currie (1980) sugieren que durante el período posparto existe una disminución de la absorción de nutrientes, que se traduce en cambios en la concentración de metabolitos sanguíneos. El calcio (Ca^{2+}) está particularmente involucrado en la función muscular y nerviosa, sin embargo, no sólo juega un papel esencial en las contracciones del músculo esquelético y liso, sino también en la función inmune de las vacas lecheras, ya que la demanda de Ca^{2+} durante el período periparto disminuye las reservas de Ca^{2+} intracelular en los leucocitos (Kimura, Reinhardt & Goff, 2006; Martínez et al., 2014). Algunos estudios han descrito la relación entre concentraciones de Ca^{2+} bajas en sangre y un efecto perjudicial sobre la salud posparto, específicamente reflejado en la incidencia de trastornos y un metabolismo lipídico alterado (Chamberlin, Middleton, Spain, Johnson, Ellersieck & Pithua, 2013; Martínez et al., 2012). Estos cambios resultan en una mayor captación de glucosa por el hígado y tejido adiposo. Durante este tiempo, las reservas de grasa corporal se movilizan al torrente sanguíneo en forma de ácidos grasos no esterificados (AGNE) y contribuyen a cubrir los requisitos generales de energía (Chapinal *et al*., 2012). Cuando existe un alto índice de oxidación de ácidos grasos, la cetogénesis aumenta en el tejido hepático provocando una

elevación en la concentración de cuerpos cetónicos [β-hidroxibutirato (BHBA), ácido acetoacético y acetona] en sangre, dando lugar a la presencia de cetosis, enfermedad metabólica prevalente en las vacas lecheras de alta producción que generalmente ocurre dentro de los primeros 60 días después del parto. Uno de los métodos más habituales para detectar cetosis en vacas lecheras es la concentración sanguínea de BHBA. Los valores de umbral para el diagnóstico de vacas con cetosis subclínica se han reportado en diferentes estudios entre 1.0 y 1.4 mmol L^{-1} de BHBA en sangre (Geishauser, Leslie, Tenhag & Bashiri, 2000), mientras que la cetosis clínica implica generalmente concentraciones de BHBA mucho más altas (≥ 3.0 mmol L^{-1}) de BHBA (Oetzel, 2004).

Las concentraciones elevadas de AGNE y BHBA, y la disminución de las concentraciones de Ca^{2+}, glucosa e insulina, son indicadores de la presencia de un balance energético negativo (BEN; van Knegsel, van den Brand, Dijkstra, Tamminga & Kemp, 2005). La asociación entre los niveles de BHBA antes del parto y su relación con producción de leche e incidencia de trastornos es prometedora debido a la disponibilidad de pruebas de campo para medir de forma inmediata este metabolito. Los objetivos de este estudio fueron comparar la concentración sanguínea de BHBA determinada con el BHBCheck y la concentración en suero obtenida con un lector de placas en el laboratorio, y establecer la relación entre las concentraciones sanguíneas de AGNE, BHBA y Ca^{2+} alrededor del parto para predecir la pérdida de leche y la presencia de trastornos metabólicos en la lactancia inmediata en vacas Holstein-Friesian.

2 REVISIÓN DE LITERATURA

Las funciones y disfunciones metabólicas impactan el rendimiento productivo de las vacas lecheras en los sistemas productivos intensivos. Como resultado de ambos aspectos, las vacas presentan trastornos metabólicos como cetosis, hipocalcemia y desplazamiento de abomaso, y otras enfermedades como mastitis y metritis. Dichos padecimientos, se presentan comúnmente alrededor del parto e inicios de la lactancia (Chapinal et al., 2012).

De los desórdenes más frecuentes e impactantes, posiblemente, cetosis sea el principal. Actualmente, se desconoce el origen del trastorno, sin embargo, se creé que la incidencia incrementa alrededor del parto debido al desbalance energético propiciado por la demanda de energía requerida por el parto y la lactancia, y la imposibilidad de la vaca para cubrir la deficiencia debido a la reducción natural en el consumo de alimento. El déficit se establece como balance energético negativo (BEN), impactando los sistemas inmune y endocrino, y la homeostasis de la vaca (Bradford, Yuan, Farley, Mamedova & Carpenter, 2015).

Debido a los efectos negativos de la cetosis, los ganaderos están interesados en su detección en campo para implementar manejo nutricional de prevención o su tratamiento temprano. Comúnmente, la presencia de cetosis se determina en función del nivel de β-hidroxibutirato (BHBA) en sangre (Pineda & Cardoso, 2015; Sailer et al., 2018). Sin embargo, la determinación de BHBA en campo, es un procedimiento complejo y tardado. Desde la colección de la muestra hasta el análisis en laboratorio transcurren varios días que pueden ser vitales en la prevención o el tratamiento del padecimiento.

En condiciones de campo, puede ocurrir que las vacas presenten concentraciones altas de BHBA (≥ 1.2 mmol L^{-1}) en sangre sin signos clínicos aparentes, lo que no permite su detección oportuna. Dicha falla en la detección puede permitir que las vacas pierdan el interés por el alimento y como consecuencia, producen un volumen menor de leche (Berge & Vertenten, 2014).

La rapidez en la detección de BHBA es una herramienta valiosa para tomar decisiones en el establo de manera rápida y efectiva. Rollin, Berghaus, Rapnicki, Godden y Overton (2010), sugirieron que 1.0 y 1.4 mmol L^{-1} de BHBA en sangre se refiere a cetosis subclínica, mientras que la clínica implica niveles más altos (≥ 3.0 mmol L^{-1}; McArt, Nydam, Ospina & Oetzel, 2011). A pesar de la falta de concordancia total en cuanto a los valores críticos que se pueden usar como diagnóstico, se pueden tomar decisiones a nivel de granja de forma inmediata si se tiene una herramienta efectiva que facilite el diagnostico de esta condición de forma confiable. En los últimos años, ha circulado un detector de BHBA conocido como medidor electrónico BHBCheck. Dicho aparato determina la concentración de BHBA en la sangre completa.

2.1 Origen de la cetosis

La cetosis es una enfermedad metabólica de origen nutricional que afecta a las vacas altas productoras en el inicio de la lactancia. La cetosis puede definirse como una irregularidad en la que las concentraciones de cuerpos cetónicos en los fluidos y tejidos corporales aumentan. Los cuerpos cetónicos incluyen al ácido acetoacético, acetona y β-hidroxibutirato. Kronfeld (1982) definió cuatro tipos de cetosis: 1) cetosis por subnutrición primaria, 2) subnutrición secundaria, 3) alimenticia, y 4) espontánea. La cetosis primaria se presenta cuando se restringe la alimentación de la vaca; la secundaria ocurre cuando el consumo voluntario de la vaca es afectado por alguna enfermedad. La cetosis alimenticia, también referida como cetosis cetogénica, resulta del consumo excesivo de alimentos cetogénicos. Finalmente, la cetosis espontánea se presenta cuando las vacas consumen en exceso carbohidratos y lípidos en la dieta.

Las investigaciones contemporáneas definen la cetosis como concentraciones de BHBA en sangre ≥ 3.0 mmol L^{-1}, y la cetosis subclínica con BHBA de 1.2 a 2.9 mmol L^{-1} en sangre (Süss et al., 2016). El origen, posiblemente, se debe al incremento de las respuestas lipolíticas en la etapa inicial de la lactancia, debido a una disponibilidad limitada de glucosa (Contreras, Strieder-Barboza & Raphael, 2017). La vigorización del déficit energético causa la activación del control

homeorrético, provocando alteraciones metabólicas, que residen en movilización de reservas lipídicas. Desde el punto de vista metabólico, cuando se señala la necesidad de energía, se movilizan las reservas de triacilgliceroles almacenados en el tejido adiposo para generar acetil-CoA a partir de la β-oxidación. El acetil-CoA producido durante la β-oxidación se convierte en cuerpos cetónicos, estos compuestos son absorbidos por otros tejidos, como el cerebro, el músculo o el corazón para servir como fuente de energía. Ya que este trastorno se relaciona con el balance energético, es posible que pueda prevenirse a través del manejo nutricional (Herdt, 2018).

2.1.1 Aspectos bioquímicos de la cetosis

La acetil-CoA se produce por oxidación de ácidos grasos en la mitocondria hepática para más tarde metabolizarse en el ciclo de los ácidos tricarboxílicos. Sin embargo, cuando hay exceso de acetil-CoA, el ciclo de los ácidos no lo metaboliza y tiende a enviarlo al hígado por un proceso conocido como cetogénesis (Botham & Mayes, 2015). En esto órgano la acetil-CoA es convertida a acetoacetato o β-hidroxibutirato. La formación del acetoacetato ocurre en tres reacciones: 1) Dos moléculas de acetyl-CoA son condensadas a acetoacetyl-CoA por una tiolasa que trabaja en forma reversible al paso final de la β-oxidación de las grasas; 2) Condensación del acetoacetyl-CoA por una tercera molécula de acetil-CoA para formar metilglutaril–coenzima A (HMG-CoA), y 3) La degradación de HMG-CoA a acetoacetato y acetil-CoA en un aldol mezclado. Finalmente, el acetoacetato es reducido a β-hidroxibutirato por una deshidrogenasa (Newman & Verdin, 2014).

2.2 Metabolitos - síntesis y actividad biológica

2.2.1 Ácidos grasos no esterificados

Las vacas en BEN inician lipólisis, una ruta hormonal y bioquímica que cataboliza triglicéridos (TG) almacenados en las células como gotas de lípidos (Eclinpath, 2018). Una lipasa sensitiva libera glicerol y AGNE a partir de los TG (Duncan, Ahmadian, Jaworski, Sarkadi-Nagy & Sul, 2007). Los TG son hidrolizados a formas más reducidas como diacilgliceroles y posteriormente, a monoacilgliceroles liberando ácidos grasos en cada paso. Los monoacilgliceroles son hidrolizados en glicerol y el último ácido graso. Los ácidos grasos son tomados por las células vía los transportadores específicos de ácidos grasos. La producción de AGNE en el tejido adiposo es estimulado por glucagón y epinefrina, y al mismo tiempo es inhibida por insulina (Laffel, 1999). Los ácidos grasos se usan como un combustible alternativo que ayuda a mantener la glucosa sanguínea (Herdt, 2000). Una vez que se liberan del tejido adiposo, los AGNE circulantes pueden ser secuestrados por la glándula mamaria y pueden ser usados hasta en 40% en la formación de grasa de leche en las primeras semanas de lactancia (Bell, 1995). Los AGNE decrecen cuando las concentraciones de glucosa se restablecen (Lucy, Escalante, Keisler, Lamberson & Mathew, 2013). Después de la liberación del tejido adiposo, los AGNE también pueden ser secuestrados por el hígado y convertidos en energía. Sin embargo, también pueden formar cuerpos cetónicos o pueden ser reesterificados a TG. Entonces estos últimos pueden ser secretados como lipoproteína de muy baja densidad.

La acumulación de TG es similar en todas las vacas. Sin embargo, las vacas con pérdida excesiva de condición corporal (CC) después del parto son especialmente problemáticas. Estas vacas presentan un consumo de alimento bajo después del parto en comparación con las vacas con un estado normal, por tanto, sufren un BEN posparto mayor (Grummer, 1995). La CC excesiva disminuye la circulación de los TG, debido a la disminución de la oxidación de los ácidos grasos hepáticos. La disminución en la oxidación de ácidos grasos puede contribuir a la acumulación de TG en el hígado de vacas lecheras posparto con

exceso de CC (Murondoti, Jorritsma, Beynen, Wensing & Geelen, 2004). El hígado graso puede preceder a las enfermedades metabólicas, como la cetosis, y se asocia con una reducción en el estado de salud y un rendimiento reproductivo reducido (Grummer, Winkler, Bertics & Studer, 1994; Veenhuizen et al., 1991).

Varios estudios (Chapinal et al., 2011; Ospina, Nydam, Stokol & Overton, 2010; Roberts, Ospina, Nydam, Stokol & Overton, 2012) han reportado que niveles de AGNE en sangre de 0.3 a 0.5 mmol L^{-1} en el periodo preparto y 0.7 a 1.0 mmol L^{-1} en el postparto son predictores de trastornos metabólicos. McCarthy, Mann, Nydam, Overton y McArt (2015) reportaron límites más flexibles, clasificando como bajas las concentraciones menores a 1.0 mmol L^{-1} entre tres y 14 días en leche. El balance energético se considera positivo cuando las concentraciones de AGNE son menores a 0.2 mmol L^{-1}.

2.2.2 β-hidroxibutirato

La concentración sérica de BHBA en la primera semana posparto es un biomarcador más sensible y específico de la presencia de BEN que la concentración de AGNE o Ca^{2+} (Seifi, LeBlanc, Leslie & Duffield, 2011). Chapinal et al. (2011) indicaron que niveles de BHBA de 0.8 mmol L^{-1} pre-parto se asociaron con problemas posparto, como la incidencia de cetosis. Los valores de umbral para el diagnóstico de vacas con cetosis se han reportado en diferentes estudios entre 1.0 y 1.4 mmol L^{-1} en sangre (Compton, McDougall, Young & Bryan, 2014; Garro, Mian & Cobos-Roldán, 2014; Vanholder, Papen, Bemers, Verenten & Berge, 2015;). McCarthy, Yasui, Ryan, Mechor y Overton (2015) clasificaron como positivas a cetosis o con hipercetonemia las vacas muestreadas entre los días tres y catorce después del parto con una concentración de BHBA ≥ 1.2 mmol L^{-1}.

La concentración de BHBA en suero ≥ 1.2 mmol L^{-1} (Kaufman, LeBlanc, McBride, Duffield & DeVries, 2016; Mahrt, Burfeind & Heuwieser, 2015; Süss et al., 2016) y ≥ 1.4 mmol L^{-1} (Abdelli et al., 2017; Gordon, LeBlanc & Duffield, 2013; Ospina,

McArt, Overton, Stokol & Nydam, 2013) es el valor usual de punto de partida de diagnóstico de cetosis subclínica o hipercetonemia. La cetosis clínica implica valores más altos (≥ 3.0 mmol L^{-1}) (McArt, Nydam & Oetzel, 2012; Vanholder et al., 2015).

2.2.3 Calcio

El Ca^{2+} se absorbe por transporte activo y por difusión pasiva a través de la mucosa intestinal. El transporte activo de Ca^{2+} depende de la acción del calcitriol y del receptor intestinal de vitamina D. Este mecanismo transcelular es activado por el calcitriol y representa la mayor parte de la absorción de Ca^{2+} a niveles de ingesta bajos y moderados (Ross, Whitaker & Reynolds, 2007).

Por otro lado, la vitamina D induce la producción de un grupo de proteínas conocido como calbindinas, las cuales facilitan el transporte de Ca^{2+} a través de la superficie luminal de las células epiteliales intestinales a través de los canales de Ca^{2+} y también puede aumentar la actividad de la bomba basolateral que transfiere el Ca^{2+} a la sangre de la célula intestinal (Nelson, 2016).

La hormona paratiroidea (PTH) promueve indirectamente la absorción de $Ca^{2+,}$ para convertir la vitamina D a su forma activa, calcitriol. La PTH estimula la resorción de Ca^{2+} y la excreción de fosfato, mientras que la vitamina D promueve la absorción de Ca^{2+} al aumentar los niveles de calbindina (Eclinpath, 2018). El Ca^{2+} está involucrado en la función muscular y nerviosa, sin embargo, no sólo juega un papel esencial en las contracciones del músculo esquelético y liso, sino también en la función inmune de las vacas lecheras. La demanda de Ca^{2+} durante el período periparto disminuye las reservas de Ca^{2+} intracelular en los leucocitos (Kimura et al., 2006; Martínez et al., 2014).

Algunos estudios (Rodríguez, Arís & Bach, 2017; Neves et al., 2018) han descrito la relación entre concentraciones de Ca^{2+} bajas en sangre y un efecto dañino en la salud posparto, específicamente, reflejado en la incidencia de trastornos incluyendo prolapso uterino, retención placentaria, endometritis, mastitis,

desplazamiento abomasal y el metabolismo lipídico trastornado (Chamberlin et al., 2013; Martínez et al., 2012; Roberts et al., 2012).

El umbral de Ca^{2+} óptimo al parto para predecir el riesgo de incidencia de enfermedades en los primeros 60 días de lactancia fue ≤ 2.3 mmol L^{-1}. Esta concentración también se asoció con posible riesgo de desecho. Antes del parto, las concentraciones de predicción fueron ≤ 2.2 y 2.3 mmol L^{-1} para la semana uno y dos, respectivamente (Roberts et al., 2012).

Aproximadamente, 69% de vacas lecheras experimenta niveles séricos bajos de Ca^{2+} en el periodo periparto (Martínez et al., 2016). La glándula mamaria y su lactogénesis correspondiente son responsables de las concentraciones de Ca^{2+} bajas en sangre (Goff, Kimura & Horst, 2002). Las demandas de Ca^{2+} para la producción de calostro y la síntesis de leche inducen una caída repentina en las concentraciones sanguíneas de Ca^{2+} de modo que algunas vacas desarrollan hipocalcemia clínica, también conocida como fiebre de leche (Goff, Liesegang, & Horst, 2014).

Las concentraciones normales de Ca^{2+} en sangre, generalmente, oscilan entre 2.2 y 2.7 mmol L^{-1}; sin embargo, el inicio de la lactancia resulta en retención de Ca^{2+} en la glándula mamaria antes del parto, seguido de la secreción de calostro, la cual representa de siete a diez veces la cantidad de Ca^{2+} presente en la sangre de la vaca (Horst, Goff & Reinhardt, 2005). Lo anterior da como resultado una disminución de Ca^{2+} en sangre a concentraciones menores a 2.2 mmol L^{-1}. La incapacidad de la vaca para restablecer rápidamente las concentraciones de Ca^{2+} en la sangre, debido a la absorción intestinal inadecuada, la resorción ósea o la reabsorción urinaria son los responsables del desarrollo de hipocalcemia en los primeros días de la lactancia (Vieira-Neto et al., 2017). Este desorden afecta aproximadamente del 65 al 69 % de las vacas multíparas con concentraciones de Ca^{2+} menores a 2.125 mmol L^{-1} posparto (Martínez et al., 2016).

2.3 Impacto en la producción lechera e incidencia de trastornos

Conocer qué metabolitos medir, en qué momento y establecer su relación con variables de interés como la producción e incidencia de trastornos, podría usarse como una herramienta potencial para predecir la salud, el estado metabólico y el desempeño animal en lactancias futuras. Durante el periodo de transición, el cambio de las demandas nutricionales requiere una coordinación precisa del metabolismo para cubrir los requisitos de energía. Algunos estudios han evaluado con anterioridad el comportamiento productivo, el estado metabólico y su relación con salud y producción (Chapinal et al., 2012; Roberts et al., 2012; Stangaferro, Wijma, Caixeta, Al-Abri & Giordano, 2016).

2.3.1 Producción de leche

Actualmente se desconoce la magnitud puntual del impacto de los trastornos metabólicos en la producción de leche (PL; Aleri et al., 2016). Más allá de la alteración del ciclo de la lactancia, la pérdida de leche y el costo de recuperación de la salud de la vaca, las vacas que presentan desórdenes metabólicos requieren un tiempo de adaptación para recuperar su curso normal en la línea de ordeña.

Chapinal et al. (2012), y Gordon et al. (2017) concluyeron que las concentraciones de BHBA alrededor del parto tuvieron efectos negativos en el volumen de leche cosechado, disminuyendo de 2.4 a 4.5 kg d^{-1}. Por otra parte, Rajala-Schultz, Gröhn y McCulloch (1999) reportaron que la presentación de cetosis en las vacas redujo de 3.0 a 5.3 kg d^{-1} el volumen de leche cosechado. Con la idea de superar el desconocimiento del impacto de la cetosis en la PL. McArt, Nydam y Oetzel (2012) propusieron una ecuación que se relaciona con el incremento en la concentración de BHBA (con tope máximo de 1.2 mmol L^{-1} en suero sanguíneo). Dicha ecuación estimó que por cada incremento de BHBA en 0.1 mmol L^{-1} la leche producida disminuirá en 0.5 kg d^{-1} durante los primeros 30 días de lactancia. Como consecuencia, las vacas positivas a cetosis con una concentración de 2.2 mmol L^{-1}, producirían 5.0 kg d^{-1} de leche menos

comparados con las vacas libres del padecimiento. Los niveles de pérdida en leche relacionados con diferentes concentraciones de BHBA se indican con mayor detalle en el Cuadro 1. Las pérdidas estimadas durante toda la lactancia (305 d) en vacas multíparas se han reportado desde 251 kg (Raboisson, Mounié & Maigné, 2014) hasta 388 kg por vaca (Ospina et al., 2010) cuando el nivel de BHBA supera concentraciones de 0.8 mmol L^{-1} (Cuadro 2).

Cuadro 1. Pérdida estimada de producción diaria de leche (PDL) relacionada con la concentración de β-hidroxibutirato (BHBA) sérico antes del parto (21-0 días) de bovinos lecheros en condiciones de confinamiento

PDL (Kg d^{-1})	Lactancia	BHBA (mmol L^{-1})	Referencia
1.22	≥1	≥1.2	Duffield et al. (2009)
1.30	1	≥1.2	Gordon et al. (2013)
1.60	≥2	≥1.2	Gordon et al. (2013)
1.71	≥1	≥1.6	Duffield et al. (2009)
1.88	≥1	≥1.4	Duffield et al. (2009)
2.10	≥1	≥1.2	McArt et al. (2013)
2.11	≥1	≥1.4	Edwards & Towser (2004)
2.20	≥1	≥1.2	McArt et al. (2012)
2.35	≥1	≥1.1	Suthar et al. (2013)
2.40	≥2	≥1.2	Gordon et al. (2017a)
3.8	≥1	≥0.8	Chapinal et al. (2012)
4.4	≥1	≥0.8	Chapinal et al. (2012)

Cuadro 2. Concentración de β-hidroxibutirato (BHBA) en suero sanguíneo antes del parto relacionada con la pérdida de leche (PL) estimada de vacas Holstein-Friesian durante una lactancia en condiciones de confinamiento

PL (Kg $lactancia^{-1}$)	Lactancia	BHBA (mmol L^{-1})	Referencia
240	≥1	≥1.4	Duffield et al. (2009)
251	≥1	≥1.4	Raboisson et al. (2014)
270	≥1	≥1.0	Duffield et al. (2009)
300	≥1	≥1.2	Duffield et al. (2009)
393	≥1	≥ 0.9	Ospina et al. (2010)
488	≥1	≥ 0.8	Ospina et al. (2010)

2.3.2 Trastornos metabólicos

Las primeras semanas de lactancia representan un período de alto riesgo para la salud en el ganado lechero. Aproximadamente, el 50% de las vacas padecen, al menos, un trastorno clínico o subclínico durante este período (Bradford et al., 2015). La incidencia de estos ha sido vinculada a la intensa lipogénesis y su relación con el estado inmunológico, una respuesta a esto es la inflamación crónica, que ha sido asociada con trastornos metabólicos (Hotamisligil, 2006). Las vacas experimentan algún grado de inflamación sistémica en los días posteriores al parto, donde hay mayor riesgo de desarrollar algún trastorno (Ringseis, Gessner & Eder, 2015).

Los trastornos que han sido asociados a esta etapa incluyen, retención placentaria, metritis, desplazamiento de abomaso, cetosis e hipocalcemia (Suthar, Canelas-Raposo, Deniz & Heuwieser, 2013; Roberts et al., 2012). Estos trastornos también se han vinculado con concentraciones elevadas de AGNE y BHBA, y bajas de Ca^{2+} sérico, y riesgo de desecho en los primeros 60 días de lactancia (Fetrow, Nordlund & Norman, 2006).

Los niveles en sangre de AGNE vinculados a incidencia de trastornos van desde ≤ 2.2 mmol L^{-1} en la primera semana y ≤ 2.3 mmol L^{-1} en la semana dos (Seifi et al., 2011), mientras que Roberts et al. (2012) mencionaron niveles poco restrictivos con concentraciones ≥ 0.8 mmol L^{-1} para la primera y segunda semana, en vacas de más de una lactancia. Por su parte, Ospina, Nydam, Stokol y Overton (2010) encontraron umbrales de ≥ 0.27 mEq L^{-1} para AGNE en el preparto y de 0.60 a 0.70 mEq L^{-1} en el posparto para predecir presencia de desplazamiento de abomaso, cetosis, metritis o retención de placenta. Además, LeBlanc, Leslie y Duffield (2005) encontraron que entre el día del parto y el día seis de lactancia, las vacas con una concentración de AGNE ≥0.5 mEq L^{-1} tuvieron 3.6 veces más probabilidades de desarrollar desplazamiento abomasal (DA) después del parto. Estos resultados encontrados demuestran que el incremento en el metabolismo de lípidos es un factor de riesgo determinante de la patogénesis del DA.

La incidencia de problemas uterinos como metritis y retención placentaria puede ser predichos cuando la concentración de AGNE es mayor que 0.4 mmol L^{-1} durante dos semanas antes del parto (Hammon, Evjen, Dhiman, Goff & Walters, 2006; LeBlanc, 2006; Quiroz-Rocha et al., 2010), aunque el umbral crítico de AGNE para predecir retención placentaria fue ≥ 0.3 mEq L^{-1}. Las vacas con concentraciones séricas de AGNE mayores que ≥ 0.3 mEq L^{-1} una semana anterior al parto tuvieron una probabilidad 1.8 veces mayor de retener la placenta después del parto, que las vacas con concentraciones más bajas (Chapinal et al., 2011).

El umbral crítico para BHBA durante la semana anterior al parto es de ≥ 0.7 a 1.0 mmol L^{-1} (Chapinal et al., 2011; Ospina et al., 2010), durante la semana posterior al parto fue 1.2 mmol L^{-1} (Roberts et al., 2012; Seifi et al., 2011) y en la semana dos ≥ 1.8 mmol L^{-1} (Duffield, Lissemore, McBride & Leslie, 2009) para predecir la incidencia de desplazamiento abomasal, cetosis, metritis y retención placentaria. Las vacas con concentraciones de BHBA ≥ 1.0 mmol L^{-1} tuvieron 13.6 veces más probabilidades de desarrollar DA y 4.7 veces de cetosis clínica (Seifi et al., 2011). Para el caso de Ca^{2+} el nivel ligado a la presencia de trastornos encontrado fue ≤ 2.2 mmol L^{-1} (Chapinal et al., 2011) en el preparto y ≤ 2.3 mmol L^{-1} posparto. Estas vacas tuvieron 5.1 veces más probabilidades de desarrollar desplazamiento de abomaso en la primera semana postparto (Seifi et al., 2011).

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3 COMPARISON OF ß-HYDROXYBUTYRIC ACID CONCENTRATION DETERMINED VIA AN ELECTRONIC METER AND A LABORATORY METHOD TO DIAGNOSE HYPERKETONEMIA IN DAIRY COWS IN A COMMERCIAL HERD IN NORTHERN MEXICO

3.1 ABSTRACT

Although precise and accurate laboratory methods are available for quantifying circulating β-hydroxybutyric acid (BHBA), dairy producers would benefit from having an accurate cow-side tool to quickly diagnose hyperketonemia. This study aimed to validate a hand-held electronic BHBA meter (BHBCheck; PortaCheck, Moorestown, NJ, USA) in the diagnosis of hyperketonemia using a colorimetric laboratory assay as reference (LiquiColor Assay; EKF Diagnostics-Stanbio, Boerne, TX). Blood samples were collected from multiparous Holstein cows (n=112) in the peripartum period at -7, 7, and 14 d relative to calving date in a commercial herd in Northern Mexico. Whole blood was obtained by venipuncture from the coccygeal vein/artery and BHBA concentration was directly measured with the BHBCheck meter. Blood samples were collected at the same time in sterile evacuated tubes with no coagulating agent, centrifuged to obtain serum, and frozen -20°C until further laboratory analysis. Data were analyzed using the UNIVARIATE, FREQ, and REG procedures of SAS for descriptive statistics, sensitivity and specificity, and regression analysis, respectively. Concentration of BHBA ranged from 0.30 to 5.20 mmol/L and averaged 0.87 ± 0.47 mmol/L (±SD) for the BHBCheck; whereas the laboratory assay ranged from 0.21 to 5.24 mmol/L with a mean of 0.79 ± 0.49 mmol/L. The overall correlation coefficient (r) for the concentrations obtained with the BHBCheck and the laboratory assay was 0.86 ± 0.03; when correlation was tested by day of sampling, the coefficients were 0.47 ± 0.03, 0.80 ± 0.03, and 0.92 ± 0.04 for d -7 (n=112), 7 (n=107) and 14 (n=106) respectively. The proportion of samples with BHBA, ≥ 1.2 mmol/L, was 12.0% for the electronic meter and 8.9% for the laboratory assay. The overall sensitivity and specificity obtained were 80.3 and 95.1%, respectively; when BHBA values were ≥ 1.2 mmol/L. The electronic meter BHBCheck could be used as a reliable and rapid post-partum diagnostic test for hyperketonemia in commercial dairy herds.

Key words: cow-side diagnostic, ketone bodies, transition cows.

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3.2 INTRODUCTION

Negative energy balance (NEB) usually occurs in dairy cattle in the peripartum period. It is caused by hormonal changes associated with parturition, onset of lactation and limited feed intake causing a deficit of energetic precursors that leads to low concentrations of glucose and insulin in blood. This NEB causes mobilization of body fat enhancing the lipolytic response at the onset of lactation (Contreras, Strieder-Barboza, & Raphael, 2017) contributing to an increase in circulating non-esterified fatty acids (NEFA) (Bradford, Yuan, Farley, Mamedova & Carpenter, 2015) and β-hydroxybutyrate (BHBA) in blood, detrimental to the health and productivity (Ospina, McArt, Overton, Stokol & Nydam, 2013; Van Saun & Sniffen, 2014). High concentrations of ketone bodies (≥ 1.2 mmol/L) are known as hyperketonemia (HYK; Kaufman, LeBlanc, McBride, Duffield & DeVries, 2016; Mahrt, Burfeind & Heuwieser, 2015; Süss et al., 2016). Hyperketonemia is an important disorder in high production dairy cows that occurs within 60 days after calving. Its prevalence in dairy herds in North America and Europe ranges from 8.9 to 43.2% (McArt, Nydam & Oetzel 2012, Suthar, Canelas-Raposo, Deniz, & Heuwieser 2013). However, in Mexico there is not information on HYK prevalence and detection.

The most recommended method to detect HYK, due to its stability, is the blood concentration of BHBA using a quantitative enzymatic procedure (Oetzel, 2004). Nevertheless, this method takes time, careful sample handling, special instrumentation, and skilled laboratory personnel. Therefore, it is neither convenient nor cost-effective to use it on a routinely basis as a cow-side diagnostic test for early detection of HYK (Voyvoda & Erdogan, 2010). Hyperketonemia shows no clinical signs and there may subtle signs that go unnoticed by farmers; therefore, its incidence is considered underdiagnosed (Berge & Vertenten, 2014). Consequently, the application of technologies such as an electronic handheld meter for BHBA is recommended for the diagnosis of HYK. Therefore, the objective of this study was to validate the concentrations of BHBA obtained with an electronic handheld meter (BHBCheck, Moorestown, NJ, USA)

in comparison with the laboratory determination (LiquiColor Assay; EKF Diagnostics-Stanbio, Boerne, TX, USA) in dairy cows in the peripartum period in northern Mexico. We hypothesized that the electronic handheld meter is a tool as effective and accurate as the laboratory method for hyperketonemia diagnosis.

3.3 MATERIALS AND METHODS

3.3.1 Study area and animals

One hundred and twelve pregnant Holstein cows from one commercial herd in the region known as “La Laguna”, in Northern Mexico were used in the study. Cows had two to five lactations, with milk yield (MY) averaging 38.3 kg/day during the study. The mean body condition score was 3.5 (Scale 1, thin, to 5, fat). Sampling was carried out during the winter period from November 2016 to March 2017.

3.3.2 Animal feeding and management

Animals used in the present study were pregnant (30 d from the expected calving date). Birth dates were obtained from the output generated by the software ‘AfiFarm’ (Ltd., Kibbutz Afikim, Israel). Each dairy cow group (pre and post-partum) were fed with the respective diet. In each diet was used the same ingredients during the experiment to avoid confusion in the interpretation of the BHBA concentrations. Diets (pre and post-partum diet) were formulated according to NRC recommendations (NRC, 2001).

3.3.3 Blood sampling

A total of 325 blood samples from 112 cows were collected over daily farm visits immediately following morning milking and before feeding on day seven (n = 112) before calving, and seven (n = 107), and fourteen days (n = 106) after calving. Approximately 10.0 mL of blood were collected from the coccygeal vein/artery per sampling in vacutainer tubes without anticoagulant (Becton-Dickinson, Franklin Lakes, NJ, USA). At the same time a whole-blood drop was exposed and assessed directly with the BHBCheck handheld meter. The system consists of a

hand-held meter and electrochemical test strips. After the test strip was inserted into the meter, blood (0.7 µL) was applied to the sample chamber directly from the coccygeal vein/artery. The BHBA in the sample is oxidized to acetoacetate in the presence of hydroxybutyrate-dehydrogenase with the concomitant reduction of NAD^+ to NADH. The NADH is re-oxidized to NAD^+ by a redox mediator (Iwersen, 2009). The current generated was directly proportional to the BHBA concentration in the sample. After 5 s, the concentration of BHBA was displayed on the meter (mmol/L) and registered. Blood samples were kept refrigerated until they were centrifuged at 1500 rpm for 25 minutes to separate the serum, and serum was aliquoted and stored in 2 mL microtubes at -20 °C until their analysis using the LiquiColor colorimetric assay (EKF Diagnostics-Stanbio, Boerne, TX, USA) according to the defined manufacturer's protocol. Samples were quantified in duplicate with an intra-assay coefficient of variation less than 10%. The BHBA concentration of sample obtained by the laboratory enzyme colorimetric assay was used as the standard BHBA concentration for statistical analysis.

3.3.4 Statistical analysis

Data analysis was carried on SAS 9.4 (SAS Institute Inc., Cary, NC). Regression and correlation analyses were carried out to estimate the association between BHBCheck and Plate reader determination. PROC REG was used with the colorimetric laboratory assay as dependent variable, and BHBCheck as the independent variable. Agreement between the BHBCheck meter test and the gold standard was analyzed graphically using the method of Bland and Altman (1986). For each sample, the differences between the two BHBA concentrations tests were calculated and plotted against their mean. Subsequently, a PROC MIXED analysis was carried out with a completely random design, with repeated measures, where cow identification was used as the subject and day of sampling as the repeated term. Sensitive was obtained by PROC FREQ as the proportion of samples with BHBA concentration greater than 1.2 mmol/L, correctly diagnosed as positive by BHBCheck. Specificity was estimated as the proportion of samples with BHBA concentration less than 1.2 mmol/L correctly diagnosed as negative

using the BHBCheck.

3.4 RESULTS AND DISCUSSION

In total, 325 samples were taken from 112 multiparous Holstein cows, with a daily average MY of 38.5 ± 0.5 kg (Table 3) in three different days in the peripartum period. Thirty-eight out of 325 samples HAD greater than 1.2 mmol/L BHBA in serum, resulting in 12.0% prevalence of HYK for the electronic meter and 8.9% for the laboratory assay during all experiment. These results are consistent with the reported prevalence rates range reported in previous studies (McArt et al., 2012; Suthar et al., 2013). However, these estimates should not be considered as conclusive because of the relatively small number of cows used in the experiment and only one location. Nevertheless, the management and nutritional program in this farm is very similar to other farms in the regions. Therefore, the disorder maybe a significant problem in dairy herds of the Northern Region of Mexico.

The mean ± standard deviation BHBA concentration determined by BHBCheck and laboratory determination was 0.87 ± 0.47 mmol/L and 0.796 ± 0.49 mmol/L, respectively. The concentration of BHBA ranged from 0.3 to 5.2 mmol/L when determined by BHBCheck and 0.2 to 5.2 mmol/L when determined by the colorimetric laboratory assay across all samples. Laboratory results and further descriptive statistical parameters for the BHBA concentrations measured using the two methodologies each day are presented in Table 4. In general, BHBCheck gave slightly greater readings than the serum BHBA estimated by laboratory assay. The preceding evaluation of BHBCheck (Sailer et al., 2018) showed the same trend, overestimation highlights the possibility of false-positive results when using a cow-side BHBA meter to diagnose HYK and would result in some over-treated cows with a BHBA less than 1.2 mmol/L. Descriptive statistical parameters of laboratory results obtained by PROC MIXED for the BHB concentrations measured using the two methodologies each day are presented in Table 5. The results obtained in day seven before calving showed a significant difference ($P < 0.0001$), nevertheless the results between the two methodologies are

comparable. While in day seven and 14 after calving were not difference. A significant difference between mean values from two methods can be clinically irrelevant. The significant difference between BHBA values in this study depended mainly on the paired test used for group comparisons, which emphasizes the presence of a general trend to increased (or decreased) values between the two groups rather than reflecting a true difference in mean values (Bland & Altman, 1986). Moreover, two different methods can show a perfect correlation coefficient (r = 1) but have a significant bias between them (Bland & Altman, 1986; Jensen & Kjelgaard-Hansen, 2006; Voyvoda & Erdogan, 2010).

Previously other handheld electronic meters have been evaluated for determination of blood BHBA concentrations, including BHBCheck (Pineda & Cardoso, 2015; Bach, Heuwieser & McArt, 2016; Sailer et al., 2018), using Pearson correlation coefficients for assessing the relationship between the two methods. The coefficient of correlation between BHBA concentration determined by BHBCheck and the commercial laboratory colorimetric assay in our study was 86.79 ($P < 0.0001$), without regarding day of sampling (Figure 1). Linear regression analysis of the BHBCheck compared with the lab essay resulted in a coefficient correlation and mean squared error of 0.47 ± 0.03 for cows sampled seven days) before expected calving date, 0.80 ± 0.03, and 0.92 ± 0.04 for samples seven, and fourteen days after calving, respectively (Figure 2).

To our knowledge, this is the first study validating BHBCheck ketone measuring test in specific days pre-and postpartum to identify hyperketonemia in dairy cows in Mexico. Sailer et al. (2018) reported strong correlation estimates between serum BHBA and whole blood concentrations measured with the BHBCheck. These authors found a correlation coefficient of 0.96. The correlation estimate (r = 0.86) between the two BHBA measurements in the present study supports the results of the above-mentioned study. Nevertheless, it is necessary to emphasize that results obtained before were slightly higher. This could be explained due to the limited number of animals used in the experiment. Additionally, it is known that the same method may show variability under different conditions. Another

possible cause may be directly related to the low number of positive cows, since in previous studies, the percentage of cows determined as positive to ketosis ranged from 21 to 50% evaluated using the colorimetric test (Sailer et al., 2018); whereas in our study it was 8.9%. On the other hand, the correlation estimate seven days before calving was the lowest, with 0.47 ± 0.03, affecting directly the global correlation, the low correlation on day -7 is explained due to the low concentrations of BHBA in the prepartum period, which were not detected due to the minimum reading level of BHBCheck.

The Bland–Altman difference plots are shown in Figure 3 for all sampling days and Figure 4, 5 and 6 for days -7, 7 and 14, respectively. They show the bias between the two methods by plotting, for each sampling day, the difference of results from the concentrations of BHBA obtained with the Lab assay subtracting the concentrations obtained with the BHBCheck (y-axis) compared with the mean of results (x-axis) (Voyvoda & Erdogan, 2010). The average differences of the general BHBCheck were -0.07 mmol/L. Mean differences on day seven after calving had the least bias compared to serum concentrations of BHBA with a mean difference of -0.03 mmol/L, with the most equidistant distribution of the points around the mean. For day fourteen and seven after calving, bias values were -0.06 and -0.13 mmol/L, respectively. Clinically, the observed bias at the cut-off point of 1.2 mmol/L is not important (Bach et al., 2016), therefore an average difference of 0.07 mmol/L would not affect the HYK diagnosis.

The overall sensitivity and specificity obtained were 80.3 and 95.1%, respectively, when BHBA values were ≥ 1.2 mmol/L. These results are close to those reported in the prior BHBCheck evaluation which were 91 an 93%. With thirty-eight HYK positive samples (≥ 1.2 mmol/L) from a total of 325, it is possible that we worked with a non-representative sample population that will not exceed the cut-off point; a more representative and uniform population through BHBA concentrations, including more cows positive to HYK would likely result in greater sensitivity. In other studies, (Voyvoda & Erdogan, 2010, Bach et al., 2016, Sailer et al., 2018) the sensitivity and specificity reported by electronic BHBA meters for cows ranged

from 85 to 100%, and from 74 to 94 %, correspondingly. The specificity of the BHBCheck was acceptable and can detect HYK in individual cows and the level of the herd. For a technology to be applied in field tests it is important to consider the prices. The cost of the device is close to $75 USD and the strips needed for its operation approximately $3 USD (total cost per test). These costs are usually less than the costs of sending samples to the laboratory and, they avoid the complicated logistics and the time to obtain diagnosis and treatment.

3.5 IMPLICATIONS

It is recommended that detection procedures on field provide fast and reliable results, and require minimal, robust and economical instrumentation. A blood measurement that immediately indicates BHBA concentration, would be helpful and practical to use in the field to diagnose HYK and male decisions to treat a cow immediately. BHBCheck fulfills the aforementioned conditions. Since, it requires only 0.7 µl of blood, it produces results in 5 s, and its handling is operator friendly. The results indicate that the BHBCheck meter can be used as a reliable and rapid postpartum diagnostic test to detect hyperketonemia in dairy cows under field conditions.

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Table 3. Means and standard errors cow variables during the study

Cows	112
Observations	325
Cow variables	
MY	38.3 ± 0.517
Lactation	2.6 ± 0.030
BCS	3.5 ± 0.008
Close-up, days	24.9 ± 0.180

Table 4. Descriptive statistical for BHBA concentration determined by BHBCheck hand held electronic meter and colorimetric laboratory determination in Holstein cows seven days before and seven and fourteen days after calving

	-7		+7		+14	
	BHBCheck[1]	Lab assay[2]	BHBCheck	Lab assay	BHBCheck	Lab assay
N (Measures)	224	224	213	213	212	212
Mean ± Std Dev	0.78±0.31	0.63±0.22	0.90±0.37	0.86±0.40	0.95±0.69	0.89±0.69
Minimum	0.3	0.2136	0.30	0.2611	0.5	0.2263
Maximum	4.2	1.5737	2.4	2.5725	5.2	5.241

[1]BHBCheck meter (PortaCheck, Moorestown, NJ).
[2]LiquiColor colorimetric assay (EKF Diagnostics-Stanbio, Boerne, TX).

Table 5. β -hydroxybutyrate concentration in whole blood quantified by BHBCheck and blood serum measured by a colorimetric determination in Holstein cows in confinement during the transition period

Item	7 d before calving	7 d after calving	14 d after calving	SE	*P*		
					Treatment (Trt)	Time	Trt*Time
Cows, no.	112	107	106				
BHBCheck, mmol/L	0.78[a]	0.89[a]	0.97[a]	0.040	0.01	0.0001	0.0001
Plate reader, mmol/L	0.65[b]	0.86[a]	0.89[a]	0.040			

*** Proc Mixed- repeated measures. The structure of covariance with the best adjustment was the Symmetric Compound = SC

[1]BHBCheck meter (PortaCheck, Moorestown, NJ).

[2]LiquiColor colorimetric assay (EKF Diagnostics-Stanbio, Boerne,TX).

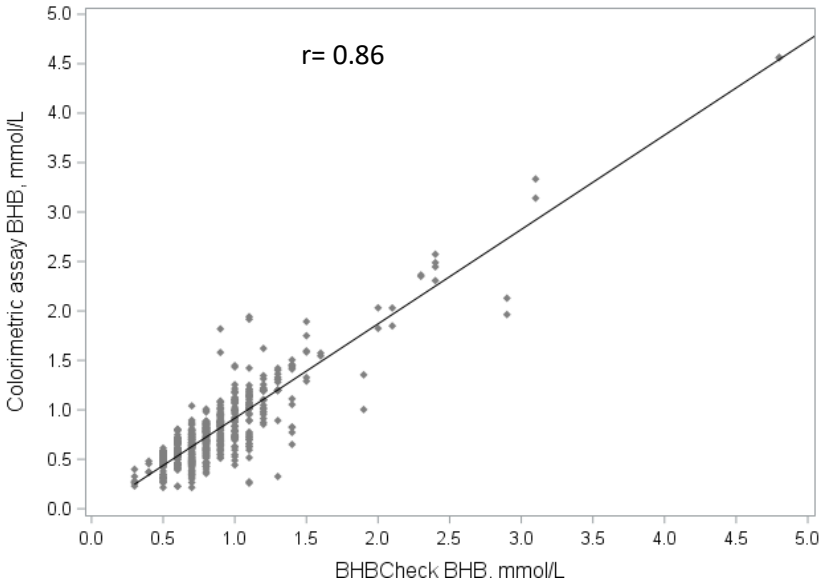

Figure 1. Linear regression of blood BHBA concentrations quantified by BHBCheck (PortaCheck, Moorestown, NJ; left panels) compared with a colorimetric laboratory assay (LiquiColor colorimetric assay; EKF Diagnostics-Stanbio, Boerne, TX) during all experiment.

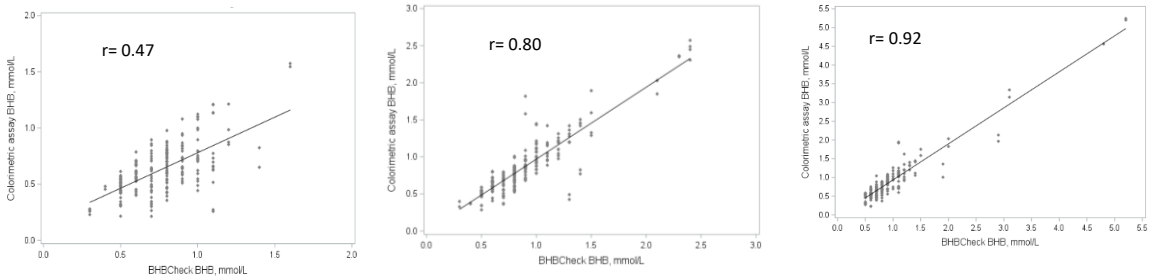

Figure 2. Linear regression of blood BHBA concentrations quantified by BHBCheck (PortaCheck, Moorestown, NJ; left panels) compared with a colorimetric laboratory assay (LiquiColor colorimetric assay; EKF Diagnostics-Stanbio, Boerne, TX) seven before, and seven and fourteen days after calving.

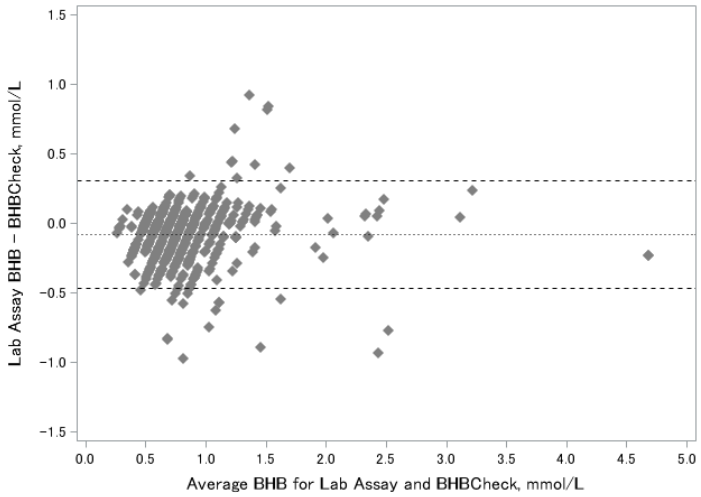

Figure 3. Difference of BHBA concentrations measured at the Lab as determined in 325 samples by a colorimetric laboratory assay (LiquiColor colorimetric assay; EKF Diagnostics-Stanbio, Boerne, TX) and BHBCheck (PortaCheck, Moorestown, NJ; left axis) plotted against their mean concentrations during all experiment. The dot line in the middle represents the mean; the upper and lower lines represent the mean ± 2 SD.

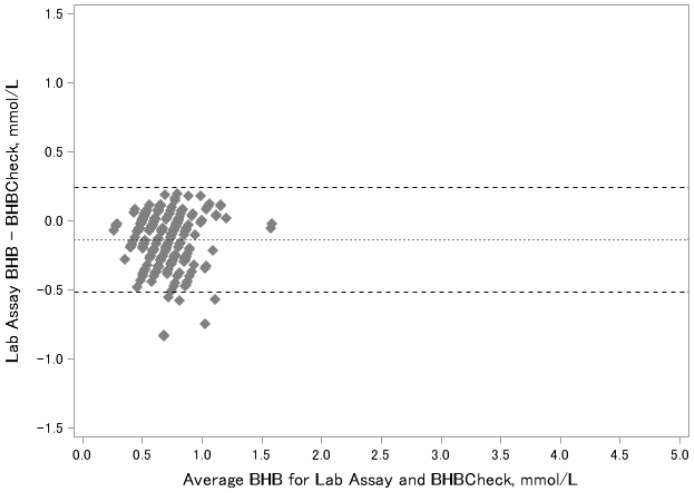

Figure 4. Difference of BHBA concentrations measured at the Lab determined by a colorimetric laboratory assay (LiquiColor colorimetric assay; EKF Diagnostics-Stanbio, Boerne, TX) and BHBCheck (PortaCheck, Moorestown, NJ) plotted against their mean concentrations seven days before calving. The dot line in the middle represents the mean; the upper and lower lines represent the mean ± 2 SD.

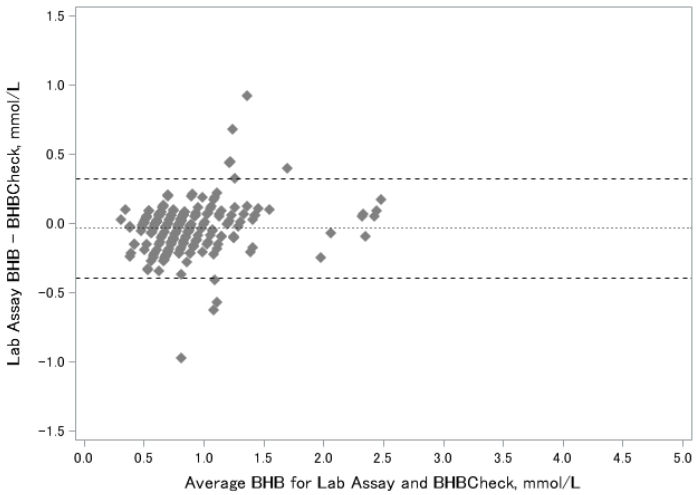

Figure 5. Difference of BHBA concentrations measured at the Lab determined by a colorimetric laboratory assay (LiquiColor colorimetric assay; EKF Diagnostics-Stanbio, Boerne, TX) and BHBCheck (PortaCheck, Moorestown, NJ) plotted against their mean concentrations seven days after calving. The dot line in the middle represents the mean; the upper and lower lines represent the mean ± 2 SD.

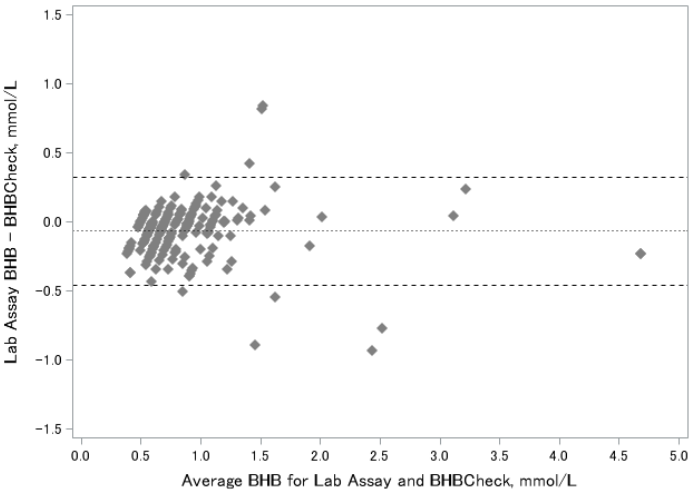

Figure 6. Difference of BHBA concentrations measured at the Lab determined by a colorimetric laboratory assay (LiquiColor colorimetric assay; EKF Diagnostics-Stanbio, Boerne, TX) and BHBCheck (PortaCheck, Moorestown, NJ) plotted against their mean concentrations fourteen days after calving. The dot line in the middle represents the mean; the upper and lower lines represent the mean ± 2 SD.

4 ß-HYDROXYBUTYRATE, CALCIUM AND NON-ESTERIFIED FATTY ACIDS IN BLOOD AND THEIR RELATIONSHIPS WITH MILK YIELD LOSSES AT EARLY LACTATION

4.1 ABSTRACT

The objectives were to study the associations of concentrations of β-hydroxybutyrate acid (BHBA), Calcium (Ca^{2+}) and non-esterified fatty acids (NEFA) in blood serum seven days prepartum with losses in milk yield (MY) and metabolic dysfunctions at seven and fourteen days of lactation. One hundred and twelve Holstein-Friesian (780 ± 36 kg BW; which had lactated more than twice) were sampled by coccygeal venipuncture. When BHBA levels were high seven days before parturition and were related to MY at day seven postpartum, it was observed that 11.00% of the cows lost 0.370 kg d^{-1} of milk. In contrast, no relationship was observed between BHBA prepartum and MY on day fourteen of lactation. Likewise, we did not observe any association between high NEFA and low calcium levels prepartum and MY postpartum. NEFA blood serum concentrations ≥ 0.5 mmol L^{-1} on d 7 before calving were 7.6 more susceptible for lameness incidence ($P < 0.01$), and when BHBA ≥ 0.8 mmol L^{-1} cows were 2.4 times more likely to develop ketosis ($P < 0.05$) in the first 60 days in milk. In brief, data indicate that a high proportion of cows are above the thresholds of β-hydroxybutyrate and non-esterified fatty acids, and are also deficient in calcium, when determined one week before parturition. The risk thresholds for each metabolite were not associated with the amount of milk lost at day fourteen after calving.

Key Words: negative energy balance, biomarkers, metabolites, milk yield, metabolic dysfunctions.

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4.2 INTRODUCTION

Animal health and herd productivity are the most difficult challenges that dairy producers facing on a regular basis. The period around calving is critical due to the reduction in dry matter intake (DMI), increases in the demand of nutrients, energy and calcium (Ca^{2+}) for the maintenance and synthesis of milk. Due to the reduction in DMI, the requirements of the animals cannot be met, and the deficit allows the animal to fall into negative energy balance (NEB).

At early lactation, the concentration of glucose in blood serum is low, with a parallel increase in the concentration of non-esterified fatty acids (NEFA) (Gross, van Dorland, Bruckmaier & Schwarz, 2011), and ketone bodies (Gross et al. 2011). The most prominent circulating ketone body in ruminants is β-hydroxybutyrate (BHBA), which is used as an energy source in body tissues such as brain, heart (Veech, 2004), kidney and skeletal muscle (Ruderman & Goodman, 1973). However, the increase of BHBA above 1.2 mmol L^{-1} is an indicator of subclinical ketosis in dairy cows (Ospina, Nydam, Stokol & Overton, 2010a). The increase in plasma BHBA reduces circulating glucose in blood (Schlumbohm & Harmeyer, 2004) and increases the risk of ketosis, hypocalcemia, abomasal displacement and metritis with the consequent reduction in production in dairy cows (Duffield, Lissemore, McBride & Leslie, 2009).

Measurements of BHBA, Ca^{2+}, and NEFA concentrations around calving may be potential indicators of the cow's ability to overcome metabolic challenges in the transition period, and possibly allow predicting some disease risks and possible milk yield (MY) losses at the start of lactation. Calcium concentrations demonstrate the ability of the cow to replace extracellular Ca^{2+} loss as a result of the milk production process, and the balance between bone, and the efficiency of absorption of insulin and Ca^{2+} (Horst, Goff & Reinhardt, 1994). Non-esterified fatty acids serve as an indicator of mobilization of body fat and reflect the particularity of the cow to adapt to the NEB.

At the level of the cow, reductions in serum Ca^{2+} concentrations, increases in NEFA and BHBA have been associated with an increased risk to contracting diseases (Seifi, LeBlanc, Leslie & Duffield, 2011) and milk loss (Chapinal et al. al., 2012). The cow-level thresholds of these metabolites have been used to identify individuals at risk of damaging their health and productivity. However, individual interventions to minimize the undesirable effects of NEB on hypocalcemia, ketosis or other metabolic disorders around parturition are difficult to achieve. Based on this premise, the objective of this study was to determine the serum concentrations of β-hydroxybutirate, calcium, and, non-esterified fatty acids, seven days before parturition, and the relationships between them and milk losses at seven and 14 days of lactation in Holstein-Friesian cows in confinement.

4.3 MATERIALS AND METHODS

4.3.1 Study area

The study was carried out in a commercial dairy farm located in the Comarca Lagunera Region, Northern, Mexico. The dairy farm was selected based on the accessibility of the manager to participate in the study; the farm met the criteria of having approximately 2,000 milking cows, and managing with two milkings per day and complete Sorghum-Soy diets. The dairy farm is located in San Pedro, Coahuila, at 1,100 m (25 ° 44' 36 "N and 103 ° 10' 22" W). Temperatures at animal pens were between 4 to 20°C during the study period. The climate of the region is desert. The precipitation is approximately 300 mm per year distributed mainly from July to September (Garcia, 2005).

4.3.2 Animal feeding and management

Animals used in the study were 112 Holstein-Friesian pregnant cows, approximately 30 days before the probable date of calving. The body weight was approximately 780 ± 36 kg, with a body condition score of 3.5 (scale 1, thin to 5, fat) and with more than two lactations. Based on previous reports, approximately

30 cows per herd were sufficient to detect the prevalence of high concentrations of metabolites in a confidence interval of 5 to 95% (Chapinal et al., 2011). The possible dates of calving were obtained from the lists generated by the AfiFarm' (Ltd., Kibbutz Afikim, Israel) software. Previous MY performance was used to select animals for the experiment. Selected animals were sampled at 7 days before, and at 7 and 14 days after calving (-7d, + 7d, + 14d). Each dairy cow group (pre and post-partum) were fed with the respective diet. In each diet was used the same ingredients during the experiment to avoid confusion in the interpretation of the BHBA concentrations. Cows were provided ad libitum access to fresh water and were fed a total mixed ration (TMR) daily that was designed to meet NRC recommendations (NRC, 2001) for close-up and fresh cows. The close-up diet (pre-partum) was based on oats hay, soymeal, and cracked corn, and the diet for fresh cows (post-partum) was based on alfalfa and soymeal (Table 6).

4.3.3 Serum analysis

In each sampling day, approximately 10.0 mL of blood from the coccygeal vein/artery was collected in vacutainer tubes without anticoagulant (Beckton-Dickinson, Franklin Lakes, NJ) immediately following morning milking and before feeding. The samples were kept under refrigeration and their coagulation was allowed. The blood was centrifuged at 1500 rpm for 25 minutes; serum was separated and stored at -20 °C within a period no longer than six hours of collection.

The samples were analyzed for NEFA and BHBA in the Universidad Autonoma Nacional de México at the laboratories. NEFA concentrations in blood serum were determined with an enzymatic colorimetric assay Half-micro test number *11 383 175 001* distributed by Sigma Aldrich laboratories (Roche, Diagnostics, Mannheim, Germany); whereas the BHBA was analyzed with an enzymatic colorimetric kit via plate reader. This kit is distributed by Stanbio Laboratories (EKF Diagnostics-Stanbio, Boerne, TX, USA). An atomic absorption spectrophotometer (AAnalyst 700, Perkin Elmer) was used to analyze calcium

concentrations in blood serum. Calcium concentrations were determined following procedures of the manufacturer (Perkin Elmer, 1996).

4.3.4 Recording diseases

The dairy herd was visited daily. Herd veterinarians recorded disease events nearly after morning milking with a standard sheet, that was handled in the systems area dairy farm by the AfiFarm software (Ltd., Kibbutz Afikim, Israel). After 60 days in milk, the final disorder incidence was obtained. Diseases such as acidosis, hypocalcemia, ketosis, and lameness were classified as clinical events. The veterinarians followed the protocols established by the dairy for the detection of diseases and disorders to standardize the information collected. The definitions of the diseases have already been described previously by Le Blanc et al. (2002).

4.3.5 Statistical analysis

Statistical analyses were performed using SAS software (version 9.4; SAS Inst. Inc., Cary, NC) with cow as the experimental unit. Descriptive statistics were obtained with the UNIVARIATE procedure; they are shown in Table 7. BHBA, Ca^{2+} and NEFA concentrations were analyzed as repeated measures using PROC MIXED. The model considered cow as a random effect, day of sampling as fixed effect, and parity (2-6), body condition score (3-5), MY (10.5-53 kg d^{-1}) and length of dry period (14-37 d) as covariables. The covariance structures selected were compound symmetry order one and autoregressive based on the lowest Akaike's information criterion.

Hierarchical Dummy variables were created to study the difference between thresholds, categorical values were 1.0 and 0, for cows considered in high and low risk, respectively, based on the serum concentrations of each metabolite in each sampling day thresholds used, were previously published by Chapinal et al. (2012). For day seven prepartum the thresholds considered at cow level were: BHBA ≥ 0.8 mmol L^{-1}, Ca^{2+} ≤ 2.3 mmol L^{-1}, and NEFA ≥ 0.05 mmol L^{-1}. While the thresholds used seven and 14 postpartum were, BHBA ≥ 1.4 mmol L^{-1}, Ca^{2+} ≤ 2.2

mmol L^{-1} and NEFA ≥ 0.7 mmol L^{-1}. BHBA, Ca^{2+} and NEFA levels in blood serum were dichotomized and evaluated individually against MY on days 7 and 14 after calving.

Dummy variables of MY and body condition, were defined following procedures published by López-Ordaz et al. (2017). For BCS, the cows were classified as moderate (2.25 to 3.25), and fat (≥ 3.5). Cows with BCS >3.5, were categorized with number one, and were considered as risk factor.

To predict MY lost or not harvested, and the possible relationship between metabolites and metabolic disorders, the categorization of low, medium and high was used; whereas MY was classified as low (≤ 18.20), medium (18.21 to 36.33), and high (≥ 36.33 kg d^{-1}) and where referred to as 0, -1 and 1, respectively, based on the discrete variables reported by López-Ordaz et al. (2017). Cows classified as 1 were those with the best MY, and therefore, the ones considered at greater risk factor.

For each concentration threshold, multivariate models were developed that were analyzed by conditional logistic regression including the dichotomized proportions of animals in the high-risk group. The results are presented as odd ratio and the confidence intervals between animals above and below the reference threshold. The odd ratio expresses the advantage or probability of experiencing an event (for example: lost or unharvest milk) for a high-risk group (above the threshold) when compared with a low risk group (below the threshold).

4.4 RESULTS

A total of 325 records were used to determine the relationship between serum concentrations of BHBA, Ca^{2+} and NEFA on seven days before, and seven and 14 days after calving with MY and incidence of metabolic dysfunctions in the first 60 days in milk. The mean MY on day seven after calving was 35.38 ± 0.55 kg d^{-1}, and for day 14 it was 40.12 ± 0.55 kg d^{-1}.

Figure 7 shows that serum metabolite concentrations were different across time evaluation. The mean concentration and standard error of BHBA was 0.424 ± 0.03, 0.704 ± 0.03 and 0.634 ± 0.03 mmol L^{-1} on day seven before, and seven and 14 days after calving, respectively. The mean Ca^{2+} concentration and their standard error on day seven prepartum was 2.577 ± 0.05, while on day seven and 14 postpartum were 2.510 ± 0.05 and 2.668 ± 0.05 mmol L^{-1}. The mean NEFA level on first sampling day (7 d before calving) was 0.335 ± 0.04, while in the second and third sampling (7 and 14 d after calving) the concentration means were 0.696 and 0.656 ± 0.04 mmol L^{-1}.

When BHBA ≥ 1.2 mmol L^{-1} it was considered that the cows presented hyperketonemia and subclinical hypocalcemia if Ca^{2+} ≤ 2.3 mmol L^{-1} in any of the 3 weekly samples. Overall, of the 112 cows used in the study, 40 developed at least one clinical or subclinical metabolic dysfunction in the postpartum period (60 d). The incidence of individual diseases was 2.68% for acidosis, 5.36% for hypocalcemia, 25.0% for ketosis, and 7.14% for lameness.

The thresholds associated with MY loss before calving were BHBA ≥ 0.8 mmol L^{-1}, Ca^{2+} ≤ 2.3 mmol L^{-1} and NEFA ≥ 0.05 mmol L^{-1}. While the thresholds used in week one and two after parturition were, BHBA ≥ 1.4 mmol L^{-1}, Ca^{2+} ≤ 2.2 mmol L^{-1} and NEFA ≥ 0.7 mmol L^{-1}. When serum concentrations of BHBA, Ca^{2+} and NEFA were determined on day 7 before calving and were related with MY on day 7 postpartum, BHBA showed an impact of milk lost or not harvested ($P < 0.05$) of 0.377 kg d^{-1} (95%, CI: 0.140 - 1.014). Relationships between metabolites and MY, and animal proportion at high-risk metabolites threshold are presented in Table 8.

The proportion of animals at high risk based on blood serum concentrations of BHBA are found on day 7 postpartum (11%), while the proportion of cows with lower Ca^{2+} concentrations in blood serum was 34% on day seven prepartum. Cows on day seven after parturition showed high risk with blood concentrations of NEFA (9%). The relationship of blood concentrations determined on day 7 before calving and disorders incidence in the first 60 days of lactation are presented in Table 9 and had a significant effect for the incidence of lameness (*P*

< 0.01), with an OR estimate of 7.6 times more likely to present this clinical disorder when NEFA levels ≥ 0.5 mmol L^{-1}.

Cows with blood concentrations of BHBA ≥ 0.8 mmol L^{-1} seven days after calving are 2.4 times more likely to suffer ketosis (P < 0.05) in the first 60 days in milk. While, acidosis and hypocalcemia did not show significant differences in incidence compared with serum levels of BHBA, Ca^{2+} and NEFA concentrations on day seven before and after calving (Table 10).

4.5 DISCUSSION

The objective of the present study was to relate the serum concentrations of BHBA, Ca^{2+} and NEFA in blood serum seven days before parturition with MY and metabolic dysfunctions at seven and 14 days of lactation in Holstein-Friesian cows. The main purpose was to find a value or range of metabolite values that allows predicting milk losses and metabolic disorders at the first 60 days in milk.

The serum concentrations of BHBA found in the present study were approximately, 0.73 mmol L^{-1}, with a range from 0.20 to 3.84 mmol L^{-1}. The mean concentration was 0.424 mmol L^{-1} on day seven prepartum. Subsequently, it increased to 0.704 mmol L^{-1} on day seven postpartum and 0.634 mmol L^{-1} on day 14 postpartum (Figure 7).

In the current study, the high-risk threshold of BHBA was ≥ 0.8 mmol L^{-1}. The amount of MY lost above the threshold was 0.37 kg cow^{-1} d^{-1} when BHBA was quantified seven days before parturition and was related to MY at seven days of lactation. This is because at the beginning of lactation the circulating blood glucose levels are low, and concomitantly, the levels of fatty acids and BHBA are high. From here, the BHBA can be used as energy source by several tissues. However, the increase promotes the reduction of a greater amount of glucose. In such situation, more Acetyl-CoA is generated that cannot be used, and in response increases the proportion of cows with ketosis. These results agreed with Suthar, Canelas-Raposo, Deniz, and Heuwieser (2013) who found that ketosis

incidence has been commonly related with BHBA ≥ 1.2 mmol L^{-1} in the first 15 DIM and were associated with odds ratios of 9.0 (6.1–13.5).

Chapinal et al. (2012) from a larger study in cows and locations than the present study but based on same NEFA, BHBA and Ca^{2+} thresholds concluded that concentrations of NEFA and BHBA low in weeks one and two were not associated with any milk yield loss. NEFA thresholds below 0.5 and 0.7 mmol L^{-1} in week one and two, respectively, and all BHBA thresholds in the same weeks were associated with higher MY (i.e., cows with NEFA and BHBA concentrations at the same level or above each threshold, they produced more milk than cows with concentrations below the same threshold).

In the first weeks of lactation, low concentrations of NEFA and BHBA that were associated with MY losses showed that a moderate degree of fat mobilization at the beginning of lactation could be critical to obtain high MY. Nevertheless, excessive mobilization of fat may cause suboptimal metabolic behaviors and probably serve as an indicator of a reduced adaptive response to NEB.

Though, there are still several predisposing factors to ketosis that are do not know yet. Conditions beyond NEFA and BHBA concentrations could be playing a role in the onset of ketosis in cows, as if the reduction of glucose occurs as a response by the increase in the concentration of BHBA or NEFA, or vice versa. Zarrin, Grossen-Rosti, Bruckmaier, and Gross (2017) observed that BHBA infusion reduces blood glucose concentrations in both pre- and postpartum periods of cows in transition. The excess of hypoglycemia due to the addition of exogenous BHBA after calving supposes an intrinsic regulatory ability in the metabolism of glucose. The same authors observed that the reduction of glucose during high concentrations of BHBA can be explained by a reduction in gluconeogenesis.

Another factor involved in the onset of ketosis is the milk production level of the cows. Harrison, Frod, Young, Conley, and Freeman (1990) indicated that higher NEFA concentrations were found in the first two weeks postpartum in high producing cows (approximately 10,800 kg per lactation, with NEFA values of 1.0

and 0.90 mEq L^{-1}, for weeks one and two, respectively); compared with low producers (6,900 kg with 0.7 and 0.5 mEq L^{-1} for weeks one and two postpartum).

In the current study, the possible association among metabolites found in the blood stream before parturition with onset of metabolic disorders at the beginning of lactation was evaluated by correlation analysis. BHBA concentration seven days before parturition was correlated with ketosis; but not with acidosis, hypocalcemia, and laminitis in the first days of lactation.

The results obtained in this study do not agree with those from other studies, Ospina, Nydam, Stokol, and Overton (2010b) found that NEFA concentrations greater than 0.3 mEq L^{-1} from day 14 to 2 prepartum, and NEFA greater than 0.6 and BHBA greater than 10 mg dL^{-1} for bovine from 3 to 14 days postpartum. Both prepartum and postpartum values above these thresholds were associated with increases in ketosis, metritis, abomasal displacement, and placental retention.

One of the most adverse disorders on MY is ketosis; this condition affects dairy cows by reducing the amount of MY harvested**.** Gordon et al. (2017) and McArt Nydam, and Oetzel (2013) reported 2.10 and 2.40 kg d^{-1} of milk not harvest, respectively, when BHBA concentrations were ≥ 1.2 mmol L^{-1} one week before calving in the transition period. Similarly, Duffield et al. (2009) indicated that blood BHBA above 1.4 mmol L^{-1} in the first two weeks postpartum resulted in substantial milk yield losses in early lactation (−1.88 kg d^{-1}). The origin of the sufferance is not clearly, what is observed is that the cow increases their body temperature and reduces DMI, concomitantly, it reduces MY. However, when talking about clinical ketosis, dairy producers claim that the subclinical is even worse. Subclinical ketosis also may be diagnosed when serum BHBA concentrations are above 1.2 mmol L^{-1}, while clinical ketosis is associated with BHBA concentrations above 2.6 mmol L^{-1} (Oetzel, 2004). González, Muiño, Pereira, Campos, and Benedito (2011) found that BHBA levels are a better sensitive metabolite for detecting subclinical stages of ketosis than NEFA in high-yielding dairy cows with a high degree of lipidic mobilization.

The serum Ca^{2+} concentrations found in the present study were approximately 2.46 mmol L^{-1}, with a range from 1.24 to 4.98 mmol L^{-1}, on day seven before calving the mean calcium concentration was 2.577 and, 2.510 and later, they increased to 2.668 mmol L^{-1} on day 14 postpartum (Figure 7). As indicated above, the optimal Ca^{2+} threshold was set at ≤ 2.3 mmol L^{-1}. The absorbed Ca^{2+} required per kilogram of MY is 0.22 g for Holstein-Friesian cows (NRC, 2001).

In the current study, 34.00 and 32.00% of the cows studied were at the high-risk threshold. However, there was no effect on MY lost or not harvested, when Ca^{2+} was quantified seven days before calving and was related to MY at day seven, and at day 14 after calving (Figure 7). These results did suggest that hyperketonemia in the prepartum period was not associated with clinical hypocalcemia or subclinical hypocalcemia at parturition, which is contradictory with several reports of the literature (Chapinal et al., 2012; Roberts et al., 2012).

However, low levels of BHBA and intermediate levels of NEFA indicate that there are a large number of animals with energy imbalance seven days before calving. These results are similar to those observed by Ospina et al. (2010ab). In contrast, Martinez et al. (2012) found that there is a significant difference between the concentrations of fatty acids between hypokalemic cows and non-hypokalemic seven days before calving and seven days in milk, although the BHBA was only different starting on day one in milk.

Neves et al. (2017) observed that prepartum cows with concentrations of Ca^{2+} ≤.2.4 mmol L^{-1}, one week before parturition, had higher risks of being classified as subclinical hypocalcemia at parturition. The authors indicated that the Ca^{2+} threshold (≤ 2.4 mmol L^{-1}) is required for the identification of animals in prepartum with a higher probability of presenting subclinical hypocalcemia. Herd-level studies using this cut-off point could establish objectives to measure the success of preventive strategies.

The results observed in the present study are contradictory to those observed by others. Chapinal et al. (2012) observed associations between Ca^{2+} milk yield

losses and incidence of metabolic disorders at the beginning of lactation. The threshold used by them is similar to the one used in this study, and similar to that of ≥ 2.0 mmol L^{-1} proposed by Oetzel (2004) and Reinhardt, Lippolis, McCluskey, Goff, and Horst (2011) in the definition of subclinical hypocalcemia, two days after parturition in Holstein-Frieisian cows under confinement conditions.

Chapinal et al. (2012) demonstrated that cows with serum Ca^{2+} concentrations ≤ 2.1 mmol L^{-1} at week 1, 2, and 3 postpartum produced 2.6, 4.8, and 7.1 kg less milk per day, respectively, when compared with normocalcemic animals; whereas, Martinez et al. (2012) categorized subclinical hypocalcemia based on the lowest Ca^{2+} concentration. They studied blood collected at parturition and during the following three days. In their study, they used a cut-point ≤ 2.15 mmol L^{-1} and found that subclinical hypocalcemic cows were more likely to develop metritis. Our results combined with those of previous studies suggest that both the timing of subclinical hypocalcemia diagnosis and the threshold used for subclinical hypocalcemia classification are important descriptors of the disease.

Approximately 34.0% of the cows of this study were at the high-risk threshold according with the Ca^{2+} blood concentration; however, it was not possible to establish predictive relationships of blood Ca^{2+} level with postpartum metabolic dysfunctions.

Additional studies are needed to determine the sampling times of blood around parturition, and to evaluate the relationship between fatty acids and Ca^{2+} in the prepartum. There was an interest in assessing the relationship between Ca^{2+} deficiency and energy imbalance in cows, and which one can support one or exacerbate another; and consequently, to better predict the negative results with the health and MY.

In the present study, the concentrations of Blood NEFA in the blood serum showed averaged 0.59 mmol L^{-1} with a wide range from 0.12 to 2.95 mmol L^{-1}, highest concentrations were found on days seven and 14 after calving averaging 0.696 and 0.656 mmol L^{-1}, respectively; lower concentrations were found seven

days before calving on the average 0.335 mmol L^{-1}. The threshold of NEFA defined as high risk was established in ≥ 0.5 mmol L^{-1}. The postpartum increase in NEFA levels (Figure 7) could be explained by the increase in nutritional requirements due to the particular needs of lactation. This effect is related to the fact that most cows are in NEB, and they need to mobilize their body reserves of lipids in order to cover their energy needs.

As shown in Table 8, the levels of NEFA on both dates (7 and 14 days after calving) did not influence the amount of milk produced. This is probably due to the fact that the levels of NEFA that were accompanied by increases in BHBA (Figure 7) were not sufficient to promote a greater reduction in blood glucose; and consequently, disruption of gluconeogenesis and the immune system did not reduce milk synthesis.

Results of the present study are contradictory with other reports in literature. Chapinal et al. (2012) found that approximately 36 of 55 farms in the United States and Canada were 30.0% above the risk of losing milk, when a threshold similar to that used in the present study was used. Milk loss was estimated at 3.00 kg per animal d^{-1}, when NEFA was determined one week before calving in conditions of high BHBA.

Similarly, in a study with of 91 freestall dairies in the northeastern United States, Ospina et al. (2010) indicated that dairy Holstein-Friesian cows with prepartum NEFA ≥ 0.27 mEq L^{-1}, postpartum NEFA ≥ 0.72 mEq L^{-1}, and postpartum BHBA ≥ 10 mg dL^{-1}. They observed a decreased MY in animals with prepartum NEFA concentrations ≥ 0.33 mEq L^{-1}. They also observed an increase in MY when postpartum NEFA was ≥ 0.57 mEq L^{-1} and BHBA ≥ 9 mg dL^{-1} in heifers sampled postpartum; however, in cows, MY decreased when postpartum NEFA and BHBA were ≥ 0.72 mEq L^{-1} and 10 mg dL^{-1}, respectively.

In this study, as the blood levels of NEFA increased (≥ 0.5 mmol L^{-1}) there was an increase of up to 7.6 times the chances of laminitis occurrence; this may be due to the fact that lameness alters resting and feeding behavior of the animals

(Gomez & Cook, 2010; Ito, von Keyerserlingk, LeBlanc & Weary, 2010). In addition, it could be possible that lameness in the early postpartum period may affect DMI at this crucial time and increase the NEFA concentrations. Collard, Boettcher, Dekkers, Petitclerc and Schaeffer (2000) indicated that plasma samples with NEFA concentrations of 0.6 to 0.8 mmol L^{-1} were associated with lameness presence around parturition in Holstein-Friesian dairy cows.

The results obtained in the present study in relation with the levels of NEFA and laminitis are contradictory with other reports in literature. Calderon and Cook (2011) reported no relationship between lameness and NEFA in Holstein-Friesian cows around parturition in a mattress-bedded commercial free stall facility. They also observed that lame cows had longer lying times throughout the transition period and notably for 3 d before and after calving. Lameness was also associated with a greater risk for ketosis in the study farm, as evidenced by the elevated BHBA concentration found in cows.

In this study, the proportion of animals at high-risk of blood serum NEFA was approximately 9%. Greater NEFA concentrations have, for instance, been associated with an increased lipid infiltration in the liver, possibly influencing the hepatic production of proteins involved in inflammatory and immune responses.

The highest concentration level is the best indicator of the state of health of the cow, higher concentrations of BHBA show that the cow mobilizes and uses energy derived from lipid metabolism as principal energy source, as a response to an energy gap, specifically in the absence of glucose. In the present study, day seven after parturition was considered as the best day of sampling, since it yielded the highest concentrations of NEFA, indicating that on that day of lactation the cow probably had a greater energy deficit.

The results obtained in the present study, even though they are different from those observed in the literature, offer the opportunity to examine the effects of pre and postpartum concentrations of NEFA and postpartum BHBA on MY at the cow level. The identification of these prepartum levels will allow the dairy producers to

improve their strategies of nutritional management in order to avoid losses in milk production or to avoid the presence of metabolic disorders such as laminitis. The results obtained also show how the metabolism state influence health cow.

4.6 CONCLUSION

High proportion of cows are above the thresholds of β-hydroxybutyrate and non-esterified fatty acids, and are also deficient in calcium, when determined one week before parturition. High β-hydroxybutyrate concentrations promotes losses in milk yield of 0.37 kg cow^{-1} d^{-1} seven days after calving in Holstein-Friesian cows. The risk thresholds for each metabolite were not associated with the amount of milk lost at day fourteen after calving in Holstein-Friesian cows.

4.7 AKNOWLEDGMENTS

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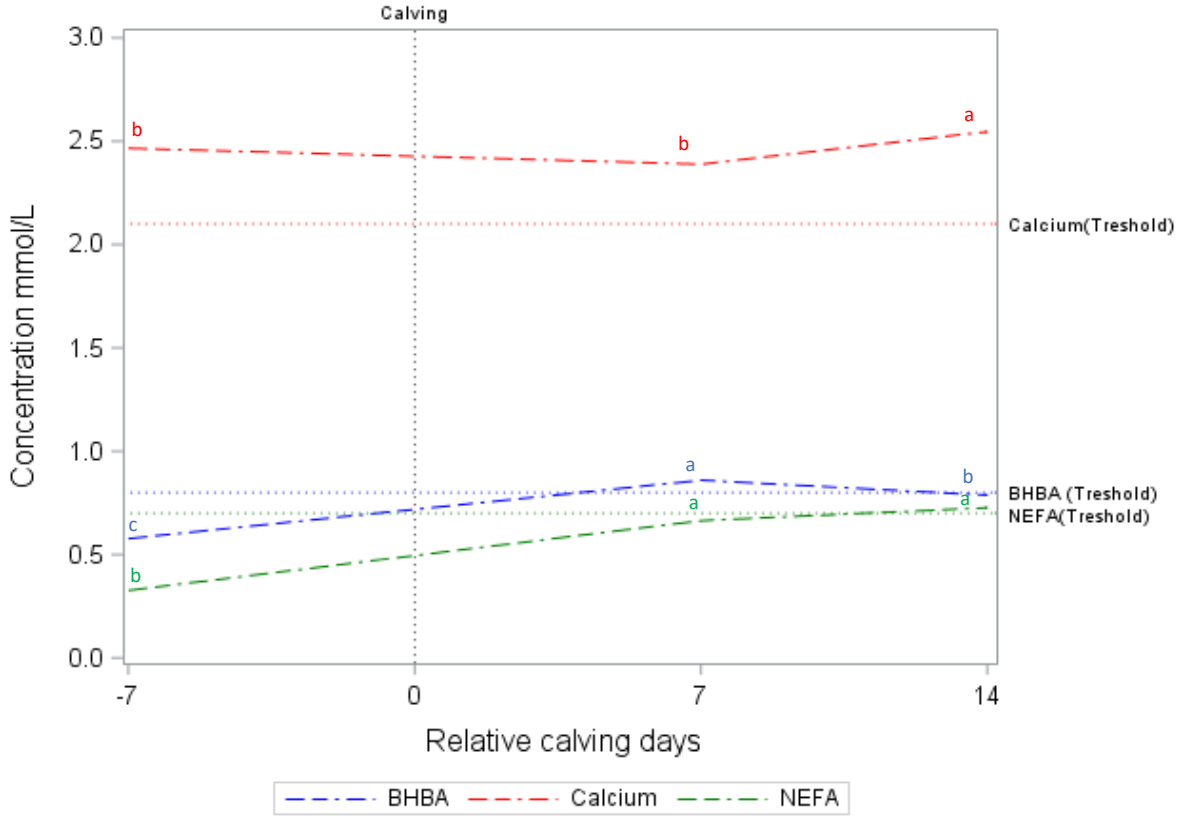

Figure 7. Blood serum concentrations of β-hydroxybutyrate (BHBA), calcium (Ca^{2+}) and non-esterified fatty acids (NEFA) sampled 7 days before relative calving date, and 7 and 14 days after relative calving date in Holstein-Friesian cows in a commercial dairy farm.

[a,b,c]Means in the same line with different superscript differ significantly ($P < 0.05$).

Table 6. Ingredients and chemical composition of the total mixed ration fed pre (close-up period), and post-partum (fresh period) to Holstein-Friesian cows in confinement conditions

Ingredients	**Close-up diet[1]**	**Fresh diet[2]**
Alfalfa hay, %	2.75	20.41
Salt	10.62	1.51
Corn silage		3.40
Sorghum grain steam-flake		4.75
Citrus pulp		12.76
Corn steam flake		3.43
Cotton seed		0.02
Distillery grain	4.22	1.47
Calcium soaps of fatty acids		5.08
Minerals,	9.24	0.45
Molasses	0.81	12.76
Oats hay	44.18	
Cracked corn	5.14	
Sodium bicarbonate	2.52	0.03
Soy bean	13.78	25.36
Soya hulls		
Dicalcium phosphate	1.38	1.45
Vitamin premix 1	5.36	0.30
Wheat bran		3.15
Vegetable oil		3.67
Chemical composition, % of DM		
ME	1.68	3.06
Net energy of lactation Mcal Kg^{-1}	1.06	2.04
Crude protein, %	12.60	22.00
Crude fiber (%)	25.60	41.90
Acid detergent fiber (%)	21.70	18.00
Neutral detergent fiber (%)	25.60	18.70
Calcium	3.60	1.70
Phosphorus	0.50	0.60

[1] Close-up period was from week 3 to 0 before calving.
[2] Fresh diet period was from calving to day 60 in milk.

Table 7. Descriptive statistics of samples from experiment used to evaluate the relation between blood serum metabolites concentrations and milk production and incidence disorder

Variable	Lectures	Mean	Standard Deviation	Minimum	Maximum
β-hydroxybutyrate mmol L^{-1}	1793	0.73	0.45	0.20	3.84
Calcium mmol L^{-1}	905	2.46	0.57	1.24	4.98
Non-esterified fatty acids mmol L^{-1}	800	0.59	0.46	0.12	2.95
Body condition score	1434	3.55	0.18	3	4
Lactation	1917	2.69	0.83	2	5
Close-up days	1911	24.86	4.49	14	37
MY kg cow^{-1} d^{-1}	1239	38.44	10.33	10.5	63.3

Table 8. Association between blood concentrations of BHBA, Ca^{2+} and NEFA sampled 7 days (n=112) prepartum with milk yield (kg d^{-1}) at 7 and 14 days (n=106) postpartum of Holstein-Friesian cows

Item	n	Animal proportion at high-risk metabolites threshold (%)[1]	Odds ratio	Wald confidence interval (95%)	*P*
7 d Pre vs 7 d Pospartum					
BHBA ≥ 0.8 mmol L^{-1}	100	11.00	0.37	0.14 - 1.01	0.05
Ca^{2+} ≤ 2.3 mmol L^{-1}		34.00	0.71	0.35 - 1.42	0.34
NEFA ≥ 0.5 mmol L^{-1}		9.00	0.43	0.14 - 1.25	0.12
7 d Pre vs 14 d Pospartum					
BHBA ≥ 0.8 mmol L^{-1}		11.65	0.86	0.27 - 2.78	0.81
Ca^{2+} ≤ 2.3 mmol L^{-1}	103	32.03	0.49	0.22 - 1.11	0.09
NEFA ≥ 0.5 mmol L^{-1}		8.73	0.57	0.17 - 1.93	0.37

Table 9. Association between blood concentrations of BHBA, Ca^{2+} and NEFA sampled 7 days (n = 112) prepartum with incidence disorders the first 60 days in milk in Holstein-Friesian cows

Item	Odds ratio	Wald confidence interval (95%)	*P*
Acidosis			
Ca^{2+} ≤ 2.3 mmol L^{-1}	1.03	0.09 - 11.77	0.98
Hypocalcemia			
Calcium ≤ 2.3 mmol L^{-1-1}	2.16	0.41 - 11.32	0.36
Ketosis			
BHBA ≥ 0.8 mmol L^{-1}	2.85	0.86 - 9.43	0.08
Ca^{2+} ≤ 2.3 mmol L^{-1}	1.61	0.65 - 4.00	0.30
NEFA ≥ 0.5 mmol L^{-1}	0.69	0.13 - 3.47	0.65
Lameness			
BHBA ≥ 0.8 mmol L^{-1}	2.57	0.46 - 14.37	0.28
Ca^{2+} ≤ 2.3 mmol L^{-1}	2.20	0.51 - 9.39	0.28
NEFA ≥ 0.5 mmol L^{-1}	7.62	1.50 - 38.74	0.01

Table 10. Association between blood concentrations of BHBA, Ca^{+2} and NEFA sampled 7 days (n = 107) after calving with incidence disorders the first 60 DIM in Holstein-Friesian cows

Item	Odds ratio	Wald confidence interval (95%)	*P*
Acidosis			
Calcium ≤ 2.3 mmol L^{-1}	0.88	0.07 - 10.09	0.91
NEFA ≥ 0.5 mmol L^{-1}	1.01	0.08 - 11.64	0.98
Ketosis			
BHBA ≥ 0.8 mmol L^{-1}	2.46	0.97 - 6.24	0.05
Calcium ≤ 2.3 mmol L^{-1}	1.16	0.21 - 6.42	0.86
NEFA ≥ 0.5 mmol L^{-1}	1.90	0.74 - 4.88	0.17
Lameness			
Calcium ≤ 2.3 mmol L^{-1}	1.06	0.24 - 4.77	0.93
NEFA ≥ 0.5 mmol L^{-1}	0.65	0.12 - 3.44	0.61