

## Relationship between $\beta$ -hydroxybutyrate and the fat:protein ratio of milk during early lactation in dairy cows<sup>#</sup>

Relación entre el  $\beta$ -hidroxibutirato y la relación grasa:proteína de leche durante la lactancia temprana en vacas lecheras

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### RESUMEN

Durante la lactancia temprana en vacas lecheras, el balance energético negativo obliga a la vaca a utilizar su grasa y proteína corporal para cumplir con su producción láctea, incrementando los requerimientos de ácidos grasos libres y resultando en un aumento en la síntesis de grasa por la ubre. Al mismo tiempo, estos cambios pueden ocasionar una insuficiencia en la síntesis de proteínas por las bacterias ruminales, comprometiendo el flujo de aminoácidos a la ubre y disminuyendo el contenido de proteína láctea. Debido a que los componentes de la leche se analizan de forma habitual a nivel hato, el objetivo de este estudio fue utilizar los datos de la relación de grasa:proteína de leche reunidos rutinariamente en las granjas, para estimar la concentración sérica de  $\beta$ -hidroxibutirato, y como predictor de cetosis subclínica en hatos lecheros durante la lactancia temprana. Se analizaron los datos de 59 muestras, una para cada hato (media de 5 muestras individuales cada una) de leche de tanque y sangre de vacas Holstein-Friesian multíparas (entre 19 a 106 d en lactación). Se encontró una correlación positiva entre el  $\beta$ -hidroxibutirato sanguíneo y la relación grasa:proteína ( $R^2 = 0,42$ ;  $P < 0,001$ ). Para discriminar entre hatos sanos y hatos afectados por cetosis subclínica se emplearon diez puntos de corte de 0,9 a 1 mM. Desde el punto de corte de 0,96 mM y hasta el punto de corte crítico de 1 mM de  $\beta$ -hidroxibutirato, el modelo alcanzó 42,9% de sensibilidad, 98,1% de especificidad, un valor en la razón de probabilidades (*odds ratio*) de 6,19 ( $P < 0,001$ ), y valores bajos para falsos positivos (25%) y falsos negativos (7,3%), cuando se utilizó la relación grasa:proteína como indicador. Por tanto la relación grasa:proteína demostró ser útil como información de apoyo para la identificación de cetosis subclínica a nivel hato.

*Palabras clave:*  $\beta$ -hidroxibutirato, cetosis subclínica, relación grasa:proteína.

### SUMMARY

During early lactation in dairy cows, a negative energy balance compels the cow to use body fat and proteins to meet its milk producing requirements increasing the availability of free fatty acid, resulting in an increased milk fat synthesis by the udder. At the same time, these changes in energy balance may cause an insufficient protein synthesis by ruminal bacteria, compromising the flow of amino acids to the udder and decreasing the milk protein content. Since herd milk components are routinely analysed, the aim of this study was to use the data of the fat:protein ratio of milk routinely gathered at farms to estimate the serum concentrations of  $\beta$ -hydroxybutyrate, and as predictor of subclinical ketosis in dairy herds at early lactation. Data from 59 samples, one per herd (average of 5 individual samples each) of tank milk and blood of multiparous Holstein-Friesian cows (between 19 to 106 d of lactation) were analysed. A positive correlation of  $\beta$ -hydroxybutyrate levels with the fat:protein ratio ( $R^2 = 0.42$ ;  $P < 0.001$ ) was found. To discriminate between healthy herds and herds affected by subclinical ketosis, ten cutoff points from 0.9 to 1 mM were employed. From the cutoff point of 0.96 mM and up to the critical cutpoint of 1 mM of  $\beta$ -hydroxybutyrate, the model reached 42.9% sensitivity, 98.1% specificity, a value of odds ratio (OR) of 6.19 ( $P < 0.001$ ), low values of false positive (25%) and false negative (7.3%), when related to fat:protein ratio levels. Therefore, fat:protein ratio proved useful as supporting information for ketosis assessment at herd level.

*Key words:*  $\beta$ -hydroxybutyrate, subclinical ketosis, fat:protein ratio.

### INTRODUCTION

Increased milk yield due to genetic selection in dairy herds has enhanced the gap between energy expenditure and energy availability, especially during early lactation. Dairy cows have to fulfill this difference by an increased use of their body reserves (Friggens *et al* 2007). This mechanism

involves an incomplete oxidation of triglycerides, which originates subclinical ketosis (SCK) in cows by the biosynthesis of non-volatile fatty acids as  $\beta$ -hydroxybutyrate (BHBA) (Duffield *et al* 2003). Relatively low levels of ketone bodies in the systemic circulation can be used as an additional source of energy by body tissues to cover the shortage of glucose. However, when present in high concentrations, SCK occurs by decreasing the mobilization of fatty acids. As a result, the negative energy balance is maintained (Duffield *et al* 2009).

Duffield (2000) reported an average of 30% of dairy cows affected by SCK at least once during lactation. On a herd basis, SCK can result in a loss of \$ 78 per cow due to decreased milk yield (Duffield *et al* 2009), increased

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days open (Cook *et al* 2001), and increased risks of clinical ketosis (LeBlanc 2010) or displaced abomasums (Geishauser *et al* 1998). Bauman *et al* (2006) have described that negative energy balance in early lactation cows leads to increased lipolysis, increased H<sup>+</sup> in the extracellular fluid, and uptake of nonesterified fatty acids (NEFA) mobilized from body fat to be resynthesized in the udder. The increased availability of BHBA and NEFA due to the mobilization of endogenous lipids, leads to a decrease of glucose precursors and a slight decrease in the percentage of milk protein (Geishauser *et al* 1998, Buttchereit *et al* 2010) while fat levels tend to increase, hence increasing the fat:protein ratio of milk (FPR) (Toni *et al* 2011).

Current tests for SCK are based on measuring ketone levels, mainly BHBA, in the blood, milk, and urine. Blood BHBA concentrations are currently the gold standard, but these tests are costly compared with cow-side tests (Duffield 2000) and require time for laboratory analysis. For this reason it was tested if BHBA and SCK presence during early lactation of dairy herds can be estimated using FPR. Advantages of this model include the use of information routinely gathered at farms, assessment of BHBA levels by a non-intrusive technique, and flagging of SCK suspicious herds for further testing to prevent disease occurrence and milk losses.

## MATERIAL AND METHODS

All animals in this study were kept following the guidelines of the Canadian Council on Animal Care (1993). Data from 59 herds located in Quebec Canada, and registered at the Dairy Production Centre of Expertise for Quebec and the Atlantic (VALACTA), were used in this study. To increase the occurrence of negative energy balance among cows (Duffield *et al* 2009) only animals from herds with more than 85% of herd individuals being Holstein-Friesian, in early lactation with a milk yield over 8000 kg per cow on a 305 d basis, and within second or third calving were included.

From each herd, 5 individual milk samples (100 mL each) of early lactation cows (19 to 106 DIM) were collected during morning milking. All samples were stored at 4 °C with a preservative Bronopol (0.04 g/100 mL; Broad Spectrum Microtabs II; D&F Control Systems, Inc., Dublin, CA) until analyzed for fat and protein by Fourier transform infrared spectroscopy (MilkoScan 605; Foss Electric., Hillerod, DK). Average values of milk yield, milk fat, and milk protein per herd (59 herds), were obtained by pooling individual cow values of the aforementioned variables.

Blood samples were collected by venipuncture of the coccygeal vein using 10 mL vacuum tubes without anti-coagulant (BD Vacutainer 367820; Beckton-Dickinson Co., Franklin Lakes, NJ) at the end of the milking time. To obtain serum, aliquots of 10 mL were formed, cooled to 4 °C, and centrifugated at 1500 × g for 20 minutes (MicroCL 21 art. 75002465; Thermo Fisher Scientific., Northern Ireland, UK). Each sample was stored at -20 °C until analysis. BHBA was measured by enzymatic method

(BHBA 310-UV; Sigma Chemical Co., St. Louis, MO) with an autoanalyzer (Coulter Dacos Biochemistry Analyzer; Coulter, Hialeah, FL). Hemolysis of serum was recorded on a qualitative scale of 0 (none) to 3 (dark) (Duffield *et al* 1998). Significant interference of measurement of BHBA (hemolysis score 2 and above) was present in less than 3% of samples and was not a significant factor in any of the models presented; thus the influence of serum hemolysis was ignored. The average value of the serum aliquots was calculated in the same manner as milk average values.

## STATISTICAL ANALYSIS

A linear regression model (PROC REG, SAS, System, v. 8.2, Cary, NC) was employed to estimate whether the concentration of BHBA was associated to FPR. BHBA values were transformed to their natural logarithmic function ( $\ln$  of BHBA) to comply with residuals normality. The following regression model was tested:

$$\ln \text{ of BHBA} = \beta_0 + \beta_1 \text{FPR} + e_i$$

where:

$\beta_0$  = intercept coefficient;

$\beta_1$  = coefficient of estimate, and

$e_i$  = Standard Error of estimate

A diagnosis for outlier values was performed using robust multivariate outlier detection (OUTLIER) (SAS 2001). This macro calculates the robust Mahalanobis distance for each observation<sup>1</sup>. A diagnosis for regressions' main assumptions was performed (PROC UNIVARIATE) (SAS 2001). Linear functional form was visually checked by a normal plot. Shapiro-Wilk test was used to check normality of residuals. Homoscedasticity was checked by plotting residual versus predicted values, and the Durbin-Watson test was employed to check for error uncorrelation.

To discriminate between healthy herds and herds affected by SCK a critical cutpoint of 1 mM of BHBA was determined (Goldhawk *et al* 2009, Kinoshita *et al* 2010). Ten cutoff points between 0.9 to 1 mM were employed to identify the best values for sensitivity and specificity under the logistic regression model (PROC LOGISTIC; SAS, 2001). In the logistic regression model, the odds ratio function for a vector of explanatory variable is represented by the following equation:

$$[P/(1 - P)] = \exp(\beta_0 + \beta^T x)$$

where:

$P$  = probability that a herd with  $x$  covariate be classified with SCK;

$\beta_0$  = intercept coefficient;

<sup>1</sup> [www.datavis.ca/sas/mac/outlier.html](http://www.datavis.ca/sas/mac/outlier.html) Research date: January 07, 2011.

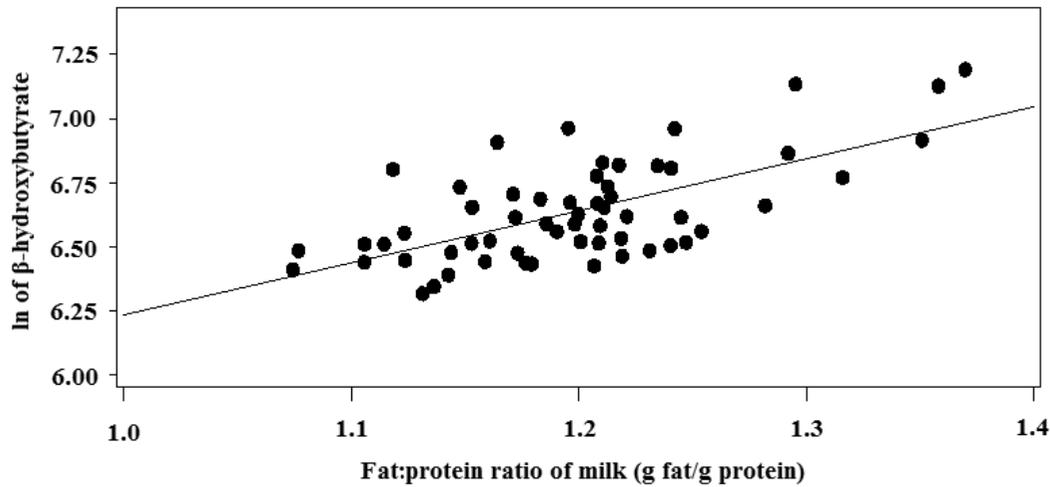
$\beta^T$  = vector of likelihood estimate for the covariate  $x$ , and  $x$  = covariate FPR.

A receiver operating characteristic (ROC) curve was used to examine changes in sensitivity and specificity at the ten cutoff points used. The sensitivity was computed as the proportion of truly herds affected by SCK for which the predicted probability of SCK exceeded the cutoff point. Similarly, specificity was computed as the truly healthy herds for which the predicted probability was below the cutoff point. The ROC was obtained from plotting the sensitivity against (1 - specificity). Since the likelihood is a very small number (between 0 and 1) the area under the ROC curve (AUC) was interpreted as the probability of the overall true diagnosis.

RESULTS

Average ( $\pm$  SD) milk yield of all herds were respectively  $33.21 \pm 4.04$  kg/d. Serum samples showed mean ( $\pm$  SD) values of  $778.51 \pm 1.69$   $\mu$ M for BHBA (range: 553 to 1329  $\mu$ M);  $1.25 \pm 0.15$  kg/d for fat production herd (range: 0.85 to 1.53 kg/d);  $1.05 \pm 0.12$  kg/d for protein production herd (range: 0.76 to 1.24 kg/d) and  $1.19 \pm 0.06$  g fat/g protein for FPR (range: 1.07 to 1.37 g fat/g protein). Results indicate that FPR was positively correlated with BHBA ( $r = 0.65$ ;  $P < 0.001$ ). Figure 1 shows the linear regression model for these variables ( $R^2 = 0.42$ ;  $P < 0.001$ ).

Table 1 shows sensitivity, specificity, odds ratio, false positive, and false negative values of cutpoints from 0.96 mM up to 1 mM of BHBA. The critical cutpoint of 1 mM



**Figure 1.** Relationship between  $\ln$  of serum  $\beta$ -hydroxybutyrate and fat:protein ratio of milk.  $\ln$  of serum  $\beta$ -hydroxybutyrate ( $\bullet$ ); predicted response ( $—$ ).  
Relación entre el  $\ln$  de  $\beta$ -hidroxibutirato sérico y la relación grasa:proteína de leche.  $\ln$  de  $\beta$ -hidroxibutirato ( $\bullet$ ); respuesta pronosticada ( $—$ ).

**Table 1.** Estimate, Odds ratio [exp (estimate)], sensitivity, specificity, false positive value (F<sup>+</sup>), and false negative value (F<sup>-</sup>) of sub-clinical ketosis through its association with fat:protein ratio of milk at different serum  $\beta$ -hydroxybutyrate cutoff points ( $n = 59$  samples).  
Estimador, razón de probabilidad [exp (estimador)], sensibilidad, especificidad, valores falsos positivos (F<sup>+</sup>), y valores falsos negativos (F<sup>-</sup>) de cetosis subclínica mediante su asociación con la relación grasa:proteína de leche en diferentes puntos de corte de  $\beta$ -hidroxibutirato sérico ( $n = 59$  muestras).

Cutoff point BHBA, mM	Estimate	Exp (Estimate)	Sensitivity	Specificity	F+	F-
				(%)		
0.91	24.55	4.61	45.5	95.8	28.6	11.5
0.92	25.71	1.46	33.3	98.0	25.0	10.9
0.93	28.05	1.52	37.5	98.0	25.0	9.1
0.94	28.05	1.52	37.5	98.0	25.0	9.1
0.95	28.05	1.52	37.5	98.0	25.0	9.1
0.96	24.84	6.19	42.9	98.1	25.0	7.3
0.97	24.84	6.19	42.9	98.1	25.0	7.3
0.98	24.84	6.19	42.9	98.1	25.0	7.3
0.99	24.84	6.19	42.9	98.1	25.0	7.3
1	24.84	6.19	42.9	98.1	25.0	7.3

of BHBA showed a likelihood estimate of 24.84 for FPR, which indicates that for each unit increase in FPR, a 24.84 change in log of odds is expected. Therefore, the odds ratio for presence of SCK versus absence of SCK is  $\text{Exp}(24.84) = 6.19$  ( $P < 0.001$ ), when presence/absence of SCK was related to FPR levels. Regression diagnostics showed that main assumptions were respected for each model.

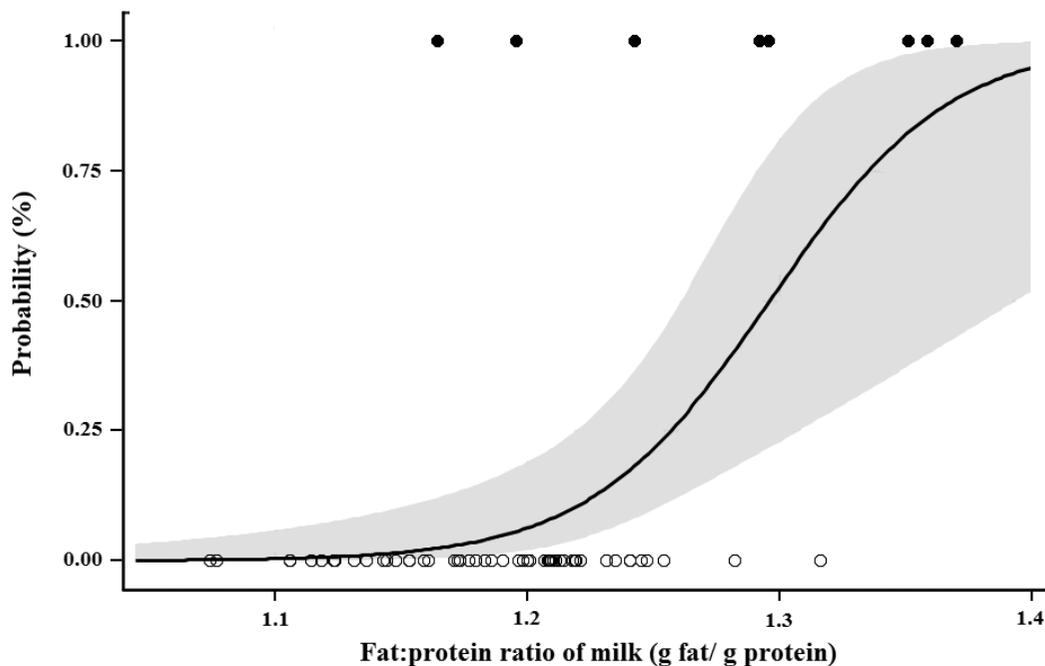
## DISCUSSION

The mammary gland (MG) uses energy-yielding nutrients to synthesize nonessential AA to cover the need for milk protein synthesis (Doepel and Lapierre 2010). However, during early lactation the NEFA uses glucose for their mitochondrial oxidation (Adewuyi *et al* 2005) generating a competition by glucose with MG. This would suggest a marked reduction in the milk protein synthesis in herds with SCK. However, the variation explained by the model was only 42% with FPR of 1.19. These results suggest that the MG alters differently its uptake of both glucose and AA in response to the amount and profile of nutrients presented to it. Hypothesis supported by Doepel and Lapierre (2010) and Lemosquet *et al* (2009) by establishing that when energy was limiting the MG gets part of its energy from nutrients other than glucose uptake mammary as Acetate, BHBA and essential AA. Therefore the proposed model reached only 42% because the observed variations of FPR are linked to metabolic

interchanges between several energetic nutrients at both the whole-body and mammary levels and are not explained only by the availability of glucose in the MG.

The proposed linear model suggests that during early lactation, increased BHBA levels are associated to FPR increased levels. Given that FPR reflects the energy balance status of a cow, this model could prove useful for multiple herd managers. Herds with very high lipo-mobilization, as shown through a high FPR during early lactation, may be flagged for additional attention to prevent disease occurrence and milk loss (Duffield *et al* 2009) or to make an early diagnose of postpartum problems. For herds with very low lipo-mobilization, (i.e. reduced FPR during early lactation) the information on the nutritional components of the diet is of the utmost importance. Diets with higher energy and nonfiber carbohydrate contents and a lower neutral detergent fiber content, are associated with decreased ruminal pH and increased d-lactate concentration (Bramley *et al* 2008), therefore, high milk production associated to low milk fat content, might lead to an underestimation of SCK risk. Unfortunately, the data base used did not include any information about dietary composition in each herd.

The logistic regression model proved useful to estimate the presence of SCK based on predicted probability from FPR values. According to results of the model, for a one unit increase in FPR, herds have 6.19 times more advantage over the likelihood to show SCK describing a sigmoid shape in the predicted response (figure 2).



**Figure 2.** Predicted probability of subclinical ketosis through its association with fat:protein ratio of milk. Confidence interval 95%; cutoff point at 1.0 mM of serum  $\beta$ -hydroxybutyrate; observed values: positive (●), negative (○); predicted response (—).

Probabilidad pronosticada de cetosis subclínica mediante su asociación con la relación grasa:proteína de leche. Intervalo de confianza del 95%, punto de corte en 1,0 mM de  $\beta$ -hidroxibutirato sérico, valores observados: positivo (●), negativo (○); respuesta pronosticada (—).

Čejna and Chládek (2005) established optimum FPR values of 1.2 g fat/g protein while FPR values higher than 1.5 g fat/g protein suggest a great energy deficiency and SCK if ketone bodies are present. Values registered in this study suggest that FPR of 1.3 indicate an increased likelihood of presence of SCK in several herds.

The AUC for logistic regression model showed a value of 0.81. Through this representation of the pairs (1-specificity, sensitivity) ROC curve provides a global representation of the diagnostic precision. The results indicate an 81% probability that the diagnosis made in a herd with SCK is correct. The logistic regression model showed specificity values of 98.1% but its sensitivity was lower than expected (42.9%). These values confirm that herds identified as probable SCK, must be monitored for additional testing, while herds with negative results can be discarded with a high degree of confidence.

This study was based on a relatively small number of herds, so we suggest further work should include an increased number of herd databases.

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