An Exploration of Diagnostic Patterns For Ketosis and an Innovative Management Approach to Ketosis Treatment in Lactating Dairy Cows

By

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ABSTRACT

AN EXPLORATION OF DIAGNOSTIC PATTERNS FOR KETOSIS AND AN INNOVATIVE MANAGEMENT APPROACH TO KETOSIS TREATMENT IN LACTATING DAIRY COWS

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This thesis was conducted to investigate the relationship between the concentrations of ketones in blood, milk, and urine during a case of ketosis; and to measure the effect of reducing milking frequency from 2 to 1 milking per day on ketosis resolution and milk production in ketotic dairy cows. A study at the University of Guelph dairy research facility measured concentrations of ketones in the blood, milk, and urine daily in fresh dairy cows and evaluated a 2 wk period of once-daily milking (ODM), in conjunction with 5 d of 300 mL propylene glycol, as a treatment for ketosis. Ketosis was detected ~2 d later using milk and urine tests, and data suggested differences in the concentrations of ketones in milk and urine relative to changes in blood. ODM cows were more likely to decrease blood BHB concentrations below 1.2 mmol/L, and produced less milk than twice-daily milked cows.
ACKNOWLEDGMENTS

Completing this Master’s degree has been an incredible learning experience that I am very grateful for. I would like to thank my advisor Dr. Todd Duffield for giving me this opportunity and for your ceaseless encouragement and support throughout this endeavour. Your unfaltering expertise, patience, and advice, throughout the data analyses (and re-analyses), and writing process have helped me develop my skills as a researcher beyond measure, and I am very thankful. I would also like to thank Dr. Brian McBride, Dr. Trevor DeVries, and Dr. Stephen LeBlanc for being part of my advisory committee. Your feedback, advice, and extensive expertise in dairy science have been irreplaceable and your unwavering assistance and support has been greatly appreciated. I would also like to thank Dr. Olaf Berke for going above and beyond in helping me analyze my time-series data. I am beyond grateful not only for the time you spent setting up, and analyzing that difficult dataset, but also for the time you spent teaching me about Cox regression and time-varying Cox regression analyses to ensure that I understood my data. Lastly, I would like to thank William Sears for helping me with my early data analyses and teaching me how to navigate through SAS. I feel very lucky to have been a part of this incredible team, and am very appreciative of the knowledge and experience I have gained in this degree due to your support.

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STATEMENT OF WORK

The funding for this research project was provided by Ontario Ministry of Agriculture, Food, and Rural Affairs (OMAFRA), and was obtained by Dr. Todd Duffield. The methodology of data collection was discussed and developed between Maggie Williamson and Dr. Todd Duffield. Data collection was conducted by Maggie Williamson with assistance from part-time hired assistants and a summer student. Data cleaning and analysis were conducted by Maggie Williamson with help from William Sears. More complex data manipulation was performed by Dr. Olaf Berke to create and effectively run the time-varying covariate Cox regression models and the Kaplan-Meier survival analyses in Chapter 2. The writing of this thesis was performed by Maggie Williamson with the guidance of Dr. Todd Duffield. Additional inputs and revisions were received from Dr. Olaf Berke, Dr. Brian McBride, Dr. Stephen LeBlanc, and Dr. Trevor DeVries.
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<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>305M</td>
<td>305-day milk</td>
</tr>
<tr>
<td>AcAc</td>
<td>Acetoacetate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>B+C</td>
<td>butaphosphan plus cyanocobalamin</td>
</tr>
<tr>
<td>BCS</td>
<td>Body condition score</td>
</tr>
<tr>
<td>BHB</td>
<td>β-hydroxybutyrate</td>
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<td>DIM</td>
<td>Days in milk</td>
</tr>
<tr>
<td>DMI</td>
<td>Dry matter intake</td>
</tr>
<tr>
<td>DOT</td>
<td>Day of trial</td>
</tr>
<tr>
<td>ECM</td>
<td>Energy-corrected milk</td>
</tr>
<tr>
<td>LDA</td>
<td>Left displaced abomasum</td>
</tr>
<tr>
<td>LS</td>
<td>Linear score</td>
</tr>
<tr>
<td>MF</td>
<td>Milking frequency</td>
</tr>
<tr>
<td>NEB</td>
<td>Negative energy balance</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
</tr>
<tr>
<td>ODM</td>
<td>Once-daily milking/milked</td>
</tr>
<tr>
<td>PG</td>
<td>Propylene glycol</td>
</tr>
<tr>
<td>PG + CM</td>
<td>Propylene glycol plus L-carnitine and methionine</td>
</tr>
<tr>
<td>SCC</td>
<td>Somatic cell count</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
<tr>
<td>TDM</td>
<td>Twice-daily milking/milked</td>
</tr>
<tr>
<td>TMR</td>
<td>Total mixed ration</td>
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<td>ThrDM</td>
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CHAPTER 1: LITERATURE REVIEW

Introduction

In early lactation essentially all dairy cows enter a period of negative energy balance (NEB), where the nutrient demands of milk production cannot be met through feed consumption alone (Baird, 1982; Herdt, 2000). Cows that are unable to adequately transition though this period of NEB are at a higher risk to develop subclinical ketosis, a metabolic disease that affects approximately 40% of dairy cows, and is defined by an excess of circulating ketone bodies (Herdt, 2000; McArt et al., 2012a). Ketosis has negative effects on cow health, increasing the risk of subsequent post-partum diseases (such as displaced abomasum and metritis) and also adversely affects milk production (Duffield et al., 2009; Ospina et al., 2010a; Ospina et al., 2010b). Furthermore, the costs involved with treating ketosis (as well as other possible diseases) and its effects on production can have a major impact on farm profitability and animal well-being (McArt et al., 2013).

Routine and accurate screening methods for ketosis are an important factor in managing ketosis in a dairy herd. There are currently a wide variety of cow-side tests available for producers to measure ketones through the blood, milk, or urine; and these tests help producers monitor the development of the disease in their transition herd, and more importantly, treat affected animals in order to more quickly resolve ketosis and decrease future financial losses. Convenient and accurate cow-side tests, and effective treatments are crucial towards the management and reduction of ketosis in dairy herds. This literature review will cover the development and impact of ketosis in dairy cows, as well as the detection, and treatment of ketosis. The effects of once-daily milking on cow productivity, metabolic health, and welfare will also be discussed.
**Ketosis**

The major challenge faced by transition dairy cows after calving is a drastic increase in nutrient demand at a time when dry matter intake (DMI) and therefore nutrient supply falls far behind these requirements (Baird, 1982; Drackley, 1999). This NEB in early lactation is coupled with reduced immune function and a period of insulin resistance to support the transition to producing milk (Baird, 1982; Herdt, 2000).

There is limited glucose storage in the cow’s body, so when energy demands exceed the glucose available, other forms of fuel are required to support lactation and maintenance. As a homeorhetic adaptation to lactation, carbohydrates are prioritized for lactogenesis before body maintenance (Bauman & Currie, 1980; Drackley, 1999; Herdt, 2000; Ospina et al., 2013). Milk production requires large amounts of glucose for lactose synthesis, but cows receive limited amounts of glucose through starch digestion due to the fermentative utilization of carbohydrates by microbes in the rumen (Baird, 1982; Herdt, 2000). Cows rely on the process of gluconeogenesis (synthesis of glucose) to meet the glucose demands of milk production not met through the limited carbohydrate absorption in the gut (Herdt, 2000). Propionic acid is one of the three main products of rumen fermentation, and is the major substrate that supports gluconeogenesis in dairy cows (Herdt, 2000). The liver regulates blood glucose concentration and glucose supply to the tissues; it is also the only site for gluconeogenesis (Herdt, 2000).

As a homeorhetic adaptation to NEB, muscles will use fat-derived fuels from the body (instead of glucose from feed consumption) for muscle metabolism in order to conserve glucose for milk production and meet their energy requirements (Drackley, 1999; Herdt, 2000). Triglycerides are broken down in adipose tissue in a process known as lipolysis, which produces non-esterified fatty acids (NEFA), which are mobilized from adipose tissue and circulated in the
blood (Bergman, 1971; Herdt, 2000; Schulz et al., 2014). As blood glucose concentrations diminish during periods of NEB, NEFA mobilization is stimulated (Herdt, 2000). While NEFA are circulated in the blood, they can be used as a good source of energy for other body tissues or used to synthesize milk fat (Herdt, 2000; Ospina et al., 2013). However, a large portion of NEFA are removed from circulation by the liver to either be converted to triglycerides (when glucose availability is adequate) or for β-oxidation to acetyl CoA and ATP through the tricarboxylic acid (TCA) cycle (when glucose availability is low) (Herdt, 2000; Drackley & Anderson, 2006; Ospina et al., 2013). β-hydroxybutyrate (BHB), acetone, and acetoacetate (AcAc) are the three main ketone bodies, which are a product of the incomplete oxidation of NEFA to acetyl CoA in the TCA cycle (Herdt, 2000; Ospina et al., 2013). Similar to NEFA, ketone bodies play an important role in providing an alternative fuel to body tissues when carbohydrate levels are low (Herdt, 2000; Duffield, 2000). Some elevation of ketones is normal during periods of NEB, but poor adaptation to NEB can result in intensified mobilization of NEFA to the liver, leading to an elevated rate of hepatic ketogenesis, and accumulation of ketone bodies, which may develop into hyperketonemia (or ketosis) if excessive (Baird 1982; Herdt, 2000; Duffield 2000; Schulz et al., 2014).

Ketosis is a metabolic disease defined by concentrations of circulating ketone bodies in the blood that are associated with an increased risk of undesirable outcomes, typically defined by blood BHB concentration ≥ 1.2mmol/L (Oetzel, 2004; Duffield et al., 2009). Cows can develop subclinical ketosis, where there are elevated ketone bodies in the blood milk, or urine, but an absence of clinical symptoms, or clinical ketosis. Cows with clinical ketosis commonly demonstrate a drop in feed intake, depressed attitude, and a noticeable decrease in both milk
production and body weight (Andersson, 1988; Duffield et al., 2009; Ospina et al., 2010a). However, there is no well-documented threshold at which clinical symptoms appear.

Subclinical ketosis is a common disease in dairy cattle, affecting approximately 40% of dairy cow lactations in North America, with extensive variation between farms ranging from 15 up to 80% (Duffield, 2000; Ospina et al., 2010b; McArt et al., 2012a; Gordon et al., 2013). The majority of subclinical ketosis appears within the first 10 d post-calving, with few new cases appearing after this period (McArt et al., 2012a). Older cows have greater risk of developing ketosis (McArt et al., 2012a, McArt et al., 2013; Vanholder et al., 2015).

Ketosis is typically negatively associated with milk production. McArt et al. (2013) reported subclinically ketotic cows lose 1.2 to 2.1 kg milk/d in their first 30 days in milk (DIM) compared to unaffected cows. In other studies, 305-day milk (305M) losses range from 130 kg to 680 kg in cows with subclinical ketosis (Duffield et al., 2009; Ospina et al., 2010a; Ospina et al., 2010b). When adjusted for periparturient diseases observed in each study above, Raboisson et al. (2014) reports the direct average 305M loss related to subclinical ketosis was 251 kg.

Ketosis can have many lasting negative impacts on the health, longevity, and welfare of dairy cows. Both clinical and subclinical ketosis are associated with an increased risk of periparurient diseases which may elevate the risk early culling from the herd (Duffield et al., 2009; Ospina et al., 2010a;).

In a study examining 1044 cattle in 20 herds, cows with serum BHB ≥ 1.2 mmol/L in the first wk postpartum were 8 times more likely to develop a left displaced abomasum (LDA) (LeBlanc et al., 2005). An increased risk of LDA in subclinically ketotic cows is also supported by McArt et al. (2012a) and Correa et al. (1993). McArt et al. (2012a) reported that every 0.1 mmol/L increase in blood BHB, increased the risk of the development of an LDA by a factor of
1.1. Additionally, Duffield et al. (2009) observed cows with serum BHB ≥1.2 mmol/L in wk 1 and 2 postpartum were 3 times more likely to develop an LDA, and 3 times more likely to develop metritis. Subclinical ketosis is also associated with 1.5 times greater risk for placental retention, 2 times greater risk for early culling or death, and increased duration and severity of mastitis (Suriyasathapom et al., 2000; Duffield et al., 2009; LeBlanc, 2010; McArt et al., 2012a; McArt et al., 2013; Raboisson et al., 2014).

The association between ketosis and reproductive performance has not been as extensively studied, but current research suggests that ketosis in early lactation negatively impacts reproductive performance months later in lactation. Two studies by Walsh et al. (2007a;b) examined the risk factors for postpartum anovulatory conditions and the effect of subclinical ketosis on reproductive performance. Cows with milk BHB ≥ 100 µmol/L between 2 and 8 DIM were 1.5 times more likely to be classified as anovular (Walsh et al., 2007a). Walsh et al. (2007b) observed that cows with increased circulating BHB concentrations were 0.7 times as likely to conceive at first insemination if BHB ≥ 1000 µmol/L in the first wk of lactation, and were 0.6 times as likely to conceive at first insemination if BHB ≥ 1400 µmol/L in the 2nd wk of lactation. Furthermore, experiencing either (or both) of those thresholds in their respective wk lead to a median time to time pregnancy 16 to 22 d longer relative to cows that never experienced elevated BHB concentrations (Walsh et al., 2007b). McArt et al. (2012a) also reported that cows with subclinical ketosis within the first 30 DIM were less likely to conceive at first service.

McArt et al. (2015) estimated that the average direct cost of one case of ketosis is $134 and $111 for primiparous and multiparous cows respectively, based on losses in milk production, treatment costs, reproductive losses, and culling. The estimated cost per case of ketosis sharply
increases to an average of $289 ($375 and $256 for primiparous and multiparous cows respectively) when accounting for the costs related to ketosis-attributable diseases (through the increase in disease risk associated with ketosis) (McArt et al., 2015). The large cost per case of ketosis, combined with the relatively high incidence of ketosis in North America poses a very large financial burden on producers. As most cases of ketosis are subclinical, many of these incurred costs are unnoticed by producers if they are not regularly screening their fresh cows for BHB (McArt et al., 2013; McArt et al., 2015). More emphasis needs to be placed not only on the prevention of ketosis, but also on early detection and fast and effective treatment to ensure cows return to a healthy state and minimize losses.

**Ketosis Detection**

Health monitoring programs for transition cows enable producers to more accurately and efficiently detect problems at a herd or cow level (LeBlanc, 2010). The aim of herd-level monitoring is to observe the efficacy of current management protocols with the goal of early detection of issues, while cow level monitoring is used to identify animals with, or at high risk for disease, with the objective of intervention to prevent or relieve disease (LeBlanc, 2010). The relatively high incidence of ketosis combined with its more common subclinical nature poses long-term consequences to the health and welfare of dairy cows and farm profitability if ketosis is not caught and managed quickly. Therefore, cow-level ketosis screening that is quick, convenient, and accurate is needed (Tatone et al., 2016b).

The gold standard for the diagnosis of ketosis is laboratory evaluation of serum BHB concentration (Duffield, 2000; Herdt, 2000). However, this method is not the most practical for daily use by producers, as it requires the centrifugation of samples, and additional time for
transportation and analysis at a laboratory (Iwersen et al., 2009). Many cow-side tests have been developed for a more rapid and convenient diagnosis of ketosis at a reduced cost compared to a laboratory but these tests may lack sensitivity or specificity compared to the gold-standard laboratory evaluation (Geishuauser et al., 2000; Oetzel, 2004; Iwersen et al., 2009). Ketones are most commonly measured by BHB in blood or milk samples, or acetoacetate in urine samples with cow-side tests (Duffield, 2000; Krogh et al., 2011).

The concentration of ketones in milk can be semi-quantitatively measured through cow-side tests by means of BHB test strips or nitroprusside powders (which measure acetoacetate) (Geishauuser et al., 2000; Oetzel, 2004; Krogh et al., 2011; Tatone et al., 2016b). Due to their poor sensitivity for ketosis detection compared to blood BHB tests and milk BHB strips, nitroprusside powders have limited value (Oetzel, 2004; Carrier et al., 2004). Keto-Test™ (Elanco, Greenfield, IN, USA) is the most commonly used and tested commercially available BHB milk strip, and it has sensitivity of 69 to 91% and specificity of 52 to 96% at the manufacturer recommended cut point of $\geq 100 \, \mu\text{mol/L}$ relative to reference thresholds of 1.2 and 1.4 mmol/L in serum (Geishauuser et al., 2000; Carrier et al., 2004; Oetzel, 2004; Iwersen et al., 2009; Samiei et al., 2010; Tatone et al. 2016b). Cow-side milk tests are more convenient for producers to screen for ketosis compared to both blood and urine tests, due to the ease of collection of milk samples while cows are being milked, and the certainty of obtaining a sample, which is not the case when testing with urine (Oetzel, 2004).

The presence of ketones in urine can be semi-quantitatively measured by two different cow-side tests; Ketostix® test strips (Bayer, Germany) and Acetest™ tablets (Bayer, Germany), both of which change in colour based on the concentration of AcAc and acetone in urine (Carrier et al., 2004; Tatone et al., 2016b). The Ketostix® test is reported to have 59 to 78% sensitivity
and 95 to 97% specificity at the test-designated ketone cut point of 15 mg/dL of AcAc (Carrier et al., 2004; Oetzel, 2004; Iwersen et al., 2009; Tatone et al. 2016b) while the Acetest™ reportedly has very high sensitivity (100%), but poor specificity (59%) both with reference positive thresholds of 1.2 and 1.4 mmol/L in serum (Nielen et al., 1994; Tatone et al., 2016b). The Ketostix® test is the best test for cow-side urine evaluation due to its high specificity and good sensitivity, and has been more widely examined in the literature than Acetest™ tablets (Oetzel, 2004; Tatone et al., 2016b). The urine Ketostix® tests are generally more sensitive than cow-side milk strip tests in detecting ketosis, but some cows may fail to urinate during testing (Oetzel 2004; Carrier et al., 2004; Tatone et al., 2016b).

The Precision Xtra® meter (Abbott Laboratories, Abbott Park, IL, USA) is a handheld blood ketone meter originally developed for human use that has been applied as a quantitative tool for diagnosing ketosis in dairy cows using minimally invasive blood samples to determine circulating BHB concentrations (Iwersen et al., 2009; Bach et al., 2016). The Precision Xtra® meter has been extensively validated against analysis of serum in a diagnostic laboratory for quantifying blood BHB concentrations in dairy cows, boasting sensitivity of 85 to 100% and specificity of 94 to 100% at test thresholds from 1.2 to 1.4 mmol/L (Iwersen et al., 2009; Voyvoda and Erdogan, 2010; Oetzel et al., 2010; Tatone et al., 2016b). The Precision Xtra® meter is a fully quantitative test and provides less risk for misclassification compared to the semi-quantitative milk and urine strip tests, which are subject to individual interpretations of the colour and depend on the amount of time from application of the sample to interpretation (Carrier et al., 2004; Krogh et al., 2011; Tatone et al., 2016b). The Precision Xtra® meter is more sensitive and specific than cow-side milk and urine strip tests at diagnosing ketosis, and has shown very little variation in sensitivity and specificity regardless of the thresholds evaluated in
In the last few years, several additional blood ketone meters have been developed and evaluated for potential use in ketosis diagnostics including the BHBCheck (PortaCheck, Moorestown, NJ) and TaiDoc (Pharmadoc, Lüdersdorf, Germany) (Iwersen et al., 2013; Bach et al., 2016; Sailer et al., 2018). The preliminary examinations of six of these handheld devices report that four of these meters produce similar sensitivity and specificity to the Precision Xtra® meter at a cut point of 1.2 mmol/L, and may provide valuable alternatives to the Precision Xtra® meter, but additional research using larger sample sizes is recommended to better understand the performance of these new meters (Iwersen et al., 2013; Bach et al., 2016; Sailer et al., 2018).

The test accuracy, cost per test, ease of use, and time required are all factors that are considered when producers are determining which cow-side test is best suited for them. Tatone et al. (2016b) recently performed a systematic review and meta-analysis (n=18 studies) of the diagnostic accuracy of cow-side tests for the detection of ketosis. The Precision Xtra® handheld blood BHB meter, the Keto-Test™ BHB milk strips, and the KetoStix® acetoacetate urine strips were evaluated in the meta-analysis (Tatone et al., 2016b). The Precision Xtra® had a summary sensitivity of 94.8% and summary specificity of 97.5% in whole blood at a cut point of 1.2 mmol/L. The Keto-Test™ semi-quantitative milk strips had a summary sensitivity and specificity of 81.5% and 81.9%, respectively, at the 100 µmol/L cut point. The KetoStix® semi-quantitative urine strips had summary sensitivities of 87.6% and 70.5% and summary specificities of 89.2% and 96.2% for the 500 µmol/L and 1500 µmol/L cut points respectively, indicating that as the positivity threshold for ketosis increases, specificity increases but sensitivity more strongly decreases. The results for the KetoStix® test were based on only three studies at each threshold (500 µmol/L and 1500 µmol/L), and should be interpreted with more
caution (Takwoingi et al., 2015; Tatone et al., 2016b). The meta-analysis reported the best thresholds for the KetoStix® and Keto-Test™ strips to be at 500 µmol/L and 100 µmol/L respectively (Tatone et al., 2016b). Tatone et al. (2016b) found that the Precision Xtra® considerably out-performed the KetoStix® and Keto-Test™ strips, and is the best option for cow-level diagnosis of ketosis. Nonetheless, they state that the KetoStix® and Keto-Test™ strips still have reasonable accuracy and are valid, less-invasive options for producers who may not be comfortable taking blood samples.

Despite over 50 years of research on ketones, there is little understanding on the physiological paths of ketone production and elimination in the blood, milk, and urine in cows with ketosis, and how they change over time. The concentration of BHB in milk is approximately one-eighth of the concentration in blood, and concentrations of ketone bodies in the urine are reported to be approximately 4 times higher than in blood (Shultz, 1971; Enjalbert et al., 2001). But it is not completely understood whether changes in ketone concentrations in the milk and urine mirror those in the blood during the course of ketosis or if these changes in ketone concentrations in the milk and urine are delayed, or occur earlier than in blood. Multiple researchers have compared samples of blood, milk, or urine in cows with ketosis on the same day, but these tests have not been compared over time (more specifically, over the course of the disease). While test accuracy compared to the laboratory gold standard method and misclassification bias due to test limitations (e.g. semi-quantitative cow-side test) may explain some of the discrepancies between blood and milk or urine tests, it is possible that the biology of ketosis itself could be a factor.
Ketosis Treatment

The general goal of ketosis treatment is to provide precursors for glucose, stimulate gluconeogenesis, and decrease lipolysis, thereby reducing ketogenesis (Herdt & Emery, 1992; Gordon et al., 2013). There has been little advancement in research regarding ketosis treatment over the past twenty years leading up to 2012, despite the high prevalence of ketosis in North America (Gordon et al., 2013). A large number of studies have been undertaken to identify an effective treatment, and virtually all of this research on ketosis treatments has focused on supplementing cows with different forms of glucose precursors or with products that may support gluconeogenesis and/or the TCA cycle. Dextrose, propylene glycol (PG), butaphosphan, cyanocobalamin, glucocorticoids, glycerol, insulin, recombinant bovine somatotropin, and combined therapies are some of the treatments that have been examined, yet most been have relatively unsuccessful or satisfactory at best in treating ketosis (Gordon et al., 2013).

As early as 1954, PG was described as a treatment for ketosis (Gordon et al., 2013). PG is either converted to propionate (a major glucogenic precursor) in the rumen, or directly absorbed in the rumen and enters the TCA cycle through pyruvate for complete oxidation, stimulating gluconeogenesis (Herdt, 2000; Nielsen & Ingvartsen, 2004; Gordon et al., 2013).

A study by McArt et al. (2011) helped lead to the current base recommendation for treating ketosis by Gordon et al. (2013), which suggests providing cows with 300 mL of PG administered orally for 3 to 5 d (Nielsen & Ingvartsen, 2004; McArt et al., 2011). Cows were drenched with 300 mL of PG once daily (or given no treatment) from the day they were detected with BHB levels of 1.2 to 2.9 mmol/L until they tested < 1.2 mmol/L or > 3.0mmol/L, or reached 17 DIM (McArt et al., 2011). Cows treated daily with a 300 mL PG oral drench were 1.5 times more likely to resolve subclinical ketosis and 0.5 times as likely to develop clinical ketosis.
compared to non-treated cows (McArt et al., 2011). By the fifth day of PG treatment, approximately 50% of the cases of subclinical ketosis had resolved (McArt et al., 2011). Excluding animals with initial blood BHB concentrations > 3.0 mmol/L from treatment was a limitation as it is unknown how these cows with BHB > 3.0 mmol/L would have affected the overall cure rate. Gordon et al. (2017b) found the PG treatment length of 3 versus 5 d to have no effect on ketosis resolution in cows with blood BHB 1.2 to 2.4 mmol/L, but cows with BHB > 2.4 mmol/L at detection were 1.7 times more likely to resolve ketosis and had a greater reduction in blood BHB levels one wk after enrolment if treated for 5 d compared to 3.

Generally, treating cows with PG increases plasma glucose and insulin concentrations, while decreasing plasma BHB and NEFA concentrations (Nielsen & Ingvartsen 2004; McArt et al., 2011). Supplementing with PG may also decrease triglyceride accumulation in the liver, and has shown a tendency to increase milk yields in early lactation compared to untreated ketotic cows (Pickett et al., 2003; Nielsen & Ingvartsen 2004; McArt et al., 2011; Piantoni & Allen, 2015). However, resolution of ketosis (BHB < 1.2 mmol/L within one wk after diagnosis) is only 30 to 50% with 5 d of PG treatment (Picket et al., 2003; Nielsen & Ingvartsen 2004; McArt et al., 2011; Tatone et al., 2016a; Mann et al., 2017; Jeong et al., 2018). Nevertheless, McArt et al. (2012b) showed that ketotic cows treated with PG were 40% less likely to develop a displaced abomasum, half as likely to die or be culled compared, and 1.3 times more likely to conceive at first insemination compared to untreated controls.

Due to the proven (but limited) efficacy of oral PG in treating ketosis, recent researchers have investigated a variety of combination therapies in which treatments are used in conjunction with PG. Piantoni & Allen (2015) assessed a combination of 300 mL PG and 300 mL glycerol but found no differences compared to treating with 300 ml PG alone. Jeong et al. (2018)
examined a combination treatment of L-carnitine and methionine in conjunction with PG for 3 d (or 5 d if ketosis was not resolved on d 3) in ketotic cows. There were 3 treatment groups in the trial, which were the control group (no treatment), PG only, and PG plus L-carnitine and methionine (PG+CM). Cows treated with PG+CM had a higher probability of resolving ketosis (48% resolved from PG+CM vs. 26% resolved in control, by d 5) and lower BHB concentrations than cows in the untreated control group (Jeong et al. 2018). The PG+CM treatment appears to be more successful than treating with only PG, but Jeong et al. (2018) did not contrast the ketosis resolution or BHB concentration between the PG and PG+CM treatment groups, so we cannot be certain of the magnitude of the improvement, or whether the difference between treatments is significant. A single injection of dexamethasone was examined as an adjunct treatment for ketosis with daily oral PG by Tatone et al. (2016a). The addition of dexamethasone was found to decrease the odds of being of ketotic in the 2nd wk post-treatment. However, the dexamethasone treatment increased the odds of ketosis in cows with initial blood BHB > 3.2mmol/L in the first wk post-treatment. Additionally, it decreased the odds of ketosis in the first wk post-treatment in cows that were treated with blood BHB between 1.2 and 1.5 mmol/L, but the dexamethasone treatment also decreased the odds of pregnancy at first insemination for this same group (Tatone et al. 2016a). Due to the marginal and conditional benefits observed, Tatone et al. (2016a) did not recommend the use of dexamethasone with PG to treat ketosis. Mann et al. (2017) investigated the effect of a 500 mL 50% dextrose solution given intravenously in combination with a 300 mL PG oral drench, both treated once-daily for 3 d on ketotic cows in a 2x2 randomized controlled trial. In previous studies dextrose alone as a treatment for ketosis was reported to decrease BHB concentrations, but only for < 24 h (Wagner & Schimek 2010; Gordon et al. 2013). Mann et al. (2017) found the combination treatment led to a greater magnitude and
more prolonged decrease in BHB levels compared to treating with PG alone. Mann et al. (2017) failed to observe differences in other secondary outcomes such as NEFA, insulin, and blood glucose concentrations but they hypothesized that this was due to the smaller sample sizes. These results support a possibly beneficial treatment combination, but further research should compare this treatment with PG for 3 to 5 d, examine the longevity of the effect, and should also employ a larger study population to better observe possible treatment effects, especially economically important effects on health, reproductive performance, and milk yield.

A combination of vitamin B12 (cyanocobalamin) and a source of organic phosphorous (butaphosphan) has been examined as a preventative as well as a treatment for ketosis. It has been hypothesized that cyanocobalamin may encourage the production of key enzymes needed in the TCA cycle, and butaphosphan may contribute to gluconeogenesis because phosphorylation is necessary in many stages of gluconeogenic pathway (Rollin et al., 2010; Gordon et al., 2017a). Gordon et al. (2017a) treated cattle with a combination of cyanocobalamin and butaphosphan (B+C), and/or insulin and found that B+C treatment did not have an effect on animals with blood BHB 1.2 to 2.4 mmol/L, and the probability of cure was decreased and post treatment BHB levels were increased for older animals and animals with high blood BHB at enrolment. But they did find that the B+C treatment might be beneficial in resolving ketosis in ketotic mature animals with low blood glucose concentrations at the time of ketosis diagnosis. Additionally, insulin alone had no effect on ketosis resolution (Gordon et al., 2017a). Gordon et al. (2017b) re-examined the butaphosphan-cyanocobalamin (B+C) combination with a PG treatment of either 3 or 5 d in a 2x2 study design. Animals with BHB > 2.4mmol/L were 1.7x more likely to resolve ketosis from 5 d of PG compared to 3 d. The B+C treatment had no effect on cure risk or blood BHB concentrations when used in conjunction with PG. Interestingly, in both studies, animals
with blood glucose concentrations \( \leq 2.2 \text{ mmol/L} \) produced more milk (2.8 to 3.4 kg/d) if they were given a treatment with B+C, milk production also slightly increased if the PG treatment was 5 d instead of 3 (Gordon et al., 2017a; Gordon et al., 2017b).

In summary, different combinations of supplementary treatments may improve ketosis recovery, but the cure rates remain disappointing. Perhaps one reason for this lack of efficacy of treatment relates back to the biology of the disease. Ketosis is ultimately caused by an imbalance of energy input and energy output due to the demands of milk production in early lactation. Perhaps a temporary reduction in energy output could reduce the metabolic challenge and more effectively resolve ketosis. Could a reduction in metabolic challenge be mediated by temporarily reducing the frequency of daily milking, and could this more effectively treat ketosis?

**Once-Daily Milking**

Dating back to the 1960’s, the dairy science community has extensively researched, tested, and examined reducing milking frequency (MF) in dairy cattle. This research explored the effects of reducing MF on many aspects of management, from milk production, to animal health and reproduction, to welfare and behaviour. Once-daily milking (ODM) is more commonly performed in pasture-based systems, where less emphasis is placed on milk production per cow (Stelwagen et al., 2013). In pasture-based systems, producers can maintain dairy herds on land that is not suitable for crop production and can increase herd size more easily and affordably compared to intensive farming systems (Bewsell et al., 2008). ODM in pasture-based systems reduces labour requirements, allowing producers to spend less time in the parlour and more time managing the pasture and animal needs (Bewsell et al., 2008, Stelwagen et al., 2013). ODM is especially beneficial in these systems during seasonal calving periods when there
is a massive increase in fresh cows on pasture which is likely to put stress on feed availability and cow management, or in the later part of the summer when pasture availability/quality is low, or when milk price is low (Bewsell et al., 2008, Phyn et al., 2014).

While there have not been any studies that have examined reduced MF as a treatment for ketosis, many studies have successfully shown the effects of reducing MF (or incompletely milking) on cow energy balance and metabolic health. In many cases, it has been tested as a measure to prevent metabolic challenges in early lactation. Once-daily milking can be particularly helpful for dairy cows during periods of metabolic pressure. Reducing energy demand through ODM reduces overall milk output and subsequently improves energy balance in dairy cows (Rémond et al., 2002; McNamara et al., 2008; Kay et al., 2013).

Trials that have examined short periods of ODM (1 to 4 wk) in early lactation consistently report 30 to 55% lower plasma NEFA and BHB concentrations and higher plasma glucose concentrations in ODM cows compared to twice-daily milked (TDM) cows (Rémond et al., 1999; McNamara et al., 2008; Loiselle et al., 2009; Schlamberger et al., 2010; Kay et al., 2013; Phyn et al., 2014). Short-term periods of ODM may also have a residual effect on energy balance once cows are returned to TDM. Loiselle et al. (2009) reported lower plasma BHB concentrations in ODM cows for an additional wk post-treatment, but most studies examining this have not observed this effect (McNamara et al., 2008; Schlamberger et al., 2010; Kay et al., 2013; Phyn et al., 2014). Plasma NEFA concentrations have remained lower in ODM cows than TDM cows for 1 to 4 wk post-treatment (Loiselle et al., 2009; Schlamberger et al., 2010; Phyn et al., 2014), and higher plasma glucose levels were shown to persist in ODM cows for 2 to almost 8 wk post treatment compared to TDM cows (Loiselle et al., 2009; Schlamberger et al., 2010; Kay et al., 2013; Phyn et al., 2014).
Body condition score (BCS), DMI, body weight, and blood metabolites such as BHB help to serve as indicators of the nutritional status of a dairy cow, and whether or not an animal is struggling to meet their energy requirements. During an entire lactation of ODM, ODM cows are reported to have a higher BCS than TDM cows, and will gain weight over their lactation, while TDM cows are more likely to lose weight (Holmes et al., 1992; Rémond et al., 2004; Clark et al., 2006). Short-term early lactation studies have observed similar results, reporting that ODM cows lose less BCS than TDM cows in early lactation (Rémond et al., 2002; Patton et al., 2006; McNamara et al., 2008; Schlamberger et al., 2010). This information indicates that ODM enables cows to reduce lipolysis of body tissue reserves and partition more available energy into body reserves, resulting in improved energy balance.

There is not much information on ODM’s effects on DMI, mainly because ODM trials are usually conducted in pasture-based dairying systems where it is difficult to measure intake (Stelwagen et al., 2013). In full-lactation ODM studies, Holmes et al. (1992) found no differences in DMI between TDM and ODM. O’Driscoll et al. (2010) reported no difference in daily grazing time (but different grazing patterns) between ODM and TDM cows. In agreement with O’Driscoll et al. (2010), Tucker et al. (2007) observed no difference in grazing time, only differences in grazing patterns between ODM and TDM cows at peak lactation, and when cows initially milked 2x were transitioned to 1x, they immediately changed their grazing pattern. Rémond et al. (2004) also examined full-lactation ODM, and found no difference in DMI between ODM and TDM cows for the first 6 wk of lactation, however, from wk 7 to 14 of lactation there was a tendency for ODM cows consume less feed (~1 to 2 kg DM/d) than TDM cows. It should be noted that cows in this study spent the winter in stables, and the spring and summer on pasture, but feed intake was only measured while animals were in the stables, and
there were only 16 cows (which calved out separately over 10 months). Loiselle et al. (2009) reported no difference in DMI between ODM and TDM cows during the ODM treatment which occurred for the first wk of lactation; the only difference in DMI occurred between wk 1 and 2 of trial (when ODM cows began TDM), where the increase in DMI was greater for ODM cows. Patton et al. (2006) did not find a difference in DMI between ODM and thrice-daily milked (ThrDM) cows during the first 4 wk of lactation. However, McNamara et al. (2008) reported that ODM cows had lower DMI than TDM cows during the first 4 wk of lactation. One would assume that due to the decrease in milk production associated with ODM, cows would not need to consume as much feed as TDM cows. These findings, along with the reported effects of ODM on blood concentrations of BHB, NEFA, and glucose, suggest that a reduced DMI is not occurring because the same amount of feed is required to meet their metabolic demands. The TDM cows are not meeting their energy requirements as well as the ODM cows are in these trials, and these DMI data support that cows cannot consume enough feed to meet the metabolic demands of lactation and body maintenance in early lactation (Herdt, 2000).

While ODM has metabolic benefits there are risks for cow productivity. Regardless of the duration, an immediate decrease in milk yield is the most predictable outcome in almost all studies where cows are milked once per day compared to being milked twice per day (Davis et al., 1999; Stelwagen et al., 2013). Early lactation, short-term studies have reported milk yield losses from ODM ranging from 13 to 40% while MF was reduced, compared to TDM, with the majority of milk yield losses reported around 20 to 25% (Stelwagen & Knight, 1997; Rémond et al., 1999; Rémond et al., 2002; Patton et al., 2006; Rémond & Pomiès, 2007; Loiselle et al., 2009; Schlamberger et al., 2010; O’Driscoll et al., 2012; Kay et al., 2013; Stelwagen et al., 2013; Phyn et al., 2014). Factors such as stage of lactation, duration of ODM, parity, type of
management (pasture vs. indoors), and breed all affect the magnitude of production losses (Stelwagen et al., 2013). In long-term studies where ODM has been examined over an entire lactation, ODM cows were observed to produce 29 to 35% less milk over an entire lactation compared to TDM cows (Holmes et al., 1992; Rémond et al., 2004; Hickson et al., 2006; Clark et al., 2006). When ODM was examined over an entire lactation, Hickson et al., (2006) observed that TDM cows displayed better lactation persistency compared to ODM cows, further supporting the evidence of large milk yield losses when cows are milked once-daily for an entire lactation. Interestingly, in studies where MF has been examined alongside reducing feed allowance, ODM cows displayed a smaller difference (and sometimes no difference) in milk yield between feeding levels (ad lib fed vs. underfed) compared to TDM cows which experienced more dramatic changes in milk yield when feed was limited (O’Driscoll et al., 2012; Kay et al., 2013).

Along with milk production losses at the time of ODM, there is also a risk for long-term milk yield loss as a residual effect from short-term reductions in MF. The details on the length of ODM associated with long-term effects on milk production, and the magnitude of these effects are still unclear. This can be attributed to the lack of studies examining the long-term impact of short-term milk reduction, the differences in experimental conditions (pasture vs. TMR, trial duration, etc.) among this small group of studies, and small sample sizes. In this review, studies that tested ODM for 4 wk or less will be considered “short term”. Schlamberger et al. (2010) observed a lactational milk yield loss of 15.7% from a 4 wk period of ODM vs. TDM in early lactation (n=12 cows/group) in TMR-fed cows. McNamara et al. (2008) observed a residual negative effect on milk production in ODM cows which was present up to wk 15 of lactation from a period of ODM lasting 4 wk in early lactation in pasture-fed cows (n=21 cows/group).
Meanwhile, Patton et al. (2006) compared ODM and ThrDM over the first 4 wk of lactation, where all cows were milked twice daily for the rest of lactation (n=22 cows/group). Milk yield remained ~2 kg/d lower for ODM cows than ThrDM cows over the first 20 wk of lactation, resulting in a cumulative milk yield reduction of 11.3% at wk 20. Patton et al. (2006) did not include a control (TDM) group, which makes it challenging to evaluate this study next to other ODM research. Phyn et al. (2014) examined the carryover effect of ODM on pasture-grazed cows milked once per day for the first 3 or 6 wk of lactation compared to cows milked twice per day for their entire lactation (n=150). Phyn et al. (2014) detected a cumulative milk yield reduction of 6% and an energy-corrected milk (ECM) yield reduction of 8% during wk 8 to 32 of lactation; the duration of ODM did not affect the yield reduction in this study. Rémond et al. (2002) tested a 3 wk period of ODM at the beginning of lactation and followed cows for 30 wk (n=24). Contradictory to Phyn et al. (2014), differences in milk yield between the ODM and TDM groups were no longer significant as early as the first wk that TDM was resumed for the ODM group (up to the last wk of trial), but this could be attributed to a lack of power. Kay et al. (2013) observed an 5% ECM yield loss from a 3 wk period of ODM compared to TDM (n=120), but cows did not begin the trial until 34 ± 6 DIM. Loiselle et al. (2009) observed the effect of ODM when cows were milked either once or twice daily for the first wk post-partum, and then twice-daily for the remainder of lactation (n=22). Over the following 13 wk of lactation, they reported that ODM cows produced 8% less milk than TDM cows, but when milk yield was adjusted for components, the ECM yields were similar for ODM and TDM cows. Rémond & Pomiès (2007) implemented three separate 1 wk periods of ODM over 1 lactation in 9 dairy cows, while 9 other cows were milked twice daily throughout the entire study period as a control. The first 1 wk period of ODM began in the 3rd wk post-partum (~15 DIM), with all cows having
been milked twice daily for the first 2 wk of lactation. While there were (expected) milk yield losses during ODM, Rémond & Pomiès (2007) reported that implementing a 1 wk period of ODM did not exhibit any carry-over effects once TDM resumed. The differences in study designs between these papers help explain the variability in their results, but the small sample sizes in some of these studies (specifically Rémond et al., 2002; Rémond & Pomiès 2007; Loiselle et al., 2009; and Schlamberger et al., 2010) make it difficult to draw concrete conclusions on the long-term effects of short-term ODM. From this literature, one can assume that periods of ODM lasting 3 wk or greater will have a negative impact on lactational milk yield. However, the duration of ODM that avoids the negative carryover effect on milk production requires further examination.

The effects of ODM on milk components vary between studies. Since component yields rely on milk yield, and the negative effect of ODM on yield has already been discussed, the following will focus on component percentages rather than yields of fat and protein. ODM is usually observed to either increase or have no effect on milk fat percentage both during ODM and post-treatment compared to TDM (Stelwagen et al., 1994; Rémond et al., 1999; Rémond et al., 2002; Clark et al., 2006; Rémond & Pomiès 2007; McNamara et al., 2008; Loiselle et al., 2009; Schlamberger et al., 2010; Kay et al., 2013; Phyn et al., 2014). In most cases, milk protein percentage is greater in ODM cows than TDM cows both during and after treatment (Clark et al., 2006; Loiselle et al., 2009; Schlamberger et al., 2010; Kay et al., 2013; Phyn et al., 2014). If an increase in protein percentage isn’t seen, no differences between groups are observed (Rémond et al., 2002; Rémond & Pomiès 2007; Loiselle et al., 2009). While there may be increases in the fat and protein content in milk, these increases do not compensate for the milk losses attributed to ODM (Clark et al., 2006; Stelwagen et al., 2013). A reduction in lactose yield is characteristic
of ODM (Davis et al., 1999; Clark et al., 2006; Phyn et al., 2014), which in some cases, returned to similar levels as TDM cows post-treatment (Rémond & Pomiès 2007; Kay et al., 2013).

There is obvious concern for udder health when milking once per day, as high-producing cows milked once daily are more likely to leak milk outside of the parlour (Rémond et al., 2002, Gleeson et al., 2007, Tucker et al., 2007), which makes the teat canal more susceptible to bacterial pathogen invasion. When cows were transitioned from TDM to ODM in mid-lactation in a grazing study, transitioned cows were twice as likely to leak milk when entering the parlour compared to cows milked ODM or TDM from calving in the 7 d following the transition (Tucker et al., 2007). Many researchers report higher somatic cell count (SCC) or linear score (LS) for ODM cows during the ODM period, ranging from 2 to 18% greater SCC than TDM cows (Clark et al. 2006; Rémond & Pomiès 2007; Schlamberger et al., 2010; O’Driscoll et al., 2012; Kay et al., 2013). In some cases, SCC does not differ between ODM and TDM cows during ODM (Rémond et al., 1999; Rémond et al., 2002; Loiselle et al., 2009; Phyn et al., 2014). Notably, this increase in SCC does not seem to be correlated with an increase in intramammary infections or with the prevalence of major mastitis-causing pathogens in the milk of ODM cows (Holmes et al., 1992; Lacy-Hulbert et al., 2005; Rémond & Pomiès 2005; Clark et al., 2006; Schlamberger et al., 2010). Additionally, short-term ODM does not seem to have a residual effect on SCC, as the SCC in the milk of ODM cows returns to similar levels as TDM cows post-treatment (Rémond et al., 1999; Rémond & Pomiès 2007; Loiselle et al., 2009; Schlamberger et al., 2010; Kay et al., 2013; Phyn et al., 2014). The increase in SCC due to ODM may be caused by a mild inflammatory response (possibly due to udder distension) resulting in an increase in neutrophils in the milk and the activation of the mammary immune system (Stelwagen and Lacy-Hulbert, 1996; Stelwagen et al., 2011).
There are concerns that ODM during early to peak lactation may have negative effects on the welfare of dairy cows due to discomfort caused by udder distension, which can develop due to the prolonged milking interval of ODM. Gleeson et al. (2007) investigated the effect of MF (ODM vs. TDM) and differing nutritional levels (high and low) on aspects of dairy cow health and welfare. There was no effect of MF alone on udder firmness. However, ODM cows on the high nutritional level had greater udder firmness scores than cows in the other MF and feeding level groups (Gleeson et al., 2007). Additionally, ODM cows had worse locomotion scores, lower blood lymphocyte counts at peak lactation, and higher neutrophil and monocyte counts at peak lactation compared to TDM cows which suggested that ODM cows experienced stress and discomfort at peak lactation due to udder distension (Gleeson et al., 2007). Gleeson et al. (2007) hypothesized that reducing the concentrate input for ODM cows may ameliorate the negative effects. Tucker et al. (2007) evaluated the effects of once daily milking on cow behaviour and udder firmness in two experiments. The first examined the effect of ODM at peak yield (52 DIM) in cows milked ODM or TDM from calving, and the second examined the effect of ODM at mid-lactation where cows were transitioned from TDM to ODM, both studies were performed in grazing conditions. Supporting the hypothesis made by Gleeson et al. (2007), ODM cows on pasture had similar udder firmness and stepping and kicking behaviour in the parlour to TDM cows at peak lactation. There were also no differences in the lying time or lying postures in the four hours before the morning milking, and no differences in stride length between ODM and TDM cows (Tucker et al., 2007). ODM cows were also more likely to lie with their hind legs touching their body, and spent more time lying down in general. When cows were transitioned from TDM to ODM in mid lactation, transitioned cows had increased udder firmness compared to cows milked TDM or ODM from calving in the 7 d following transition (Tucker et al., 2007).
Despite the increase in udder firmness, there were no differences in lying times, lying postures, and vocalizations between groups in the 7 d following transition (Tucker et al., 2007). This suggests cows transitioned from TDM to ODM were able to adapt within the first wk of ODM, and were not adversely affected by the change in mid-lactation. The lying behaviours and times both indicate that the ODM and transitioned cows did not need to alter their lying behaviour or posture to reduce mammary pressure, suggesting that the increased pressure did not create discomfort. In contrast, Rémond et al. (2002) found the number of vocalizations/hour and the number of cows vocalizing were higher in the ODM group than the TDM group during the evening milkings (from which ODM cows were omitted) over 3 wk of ODM in early lactation. The authors hypothesized that the vocalizations were not due to udder pain/discomfort, but instead due to the social stress of being separated from part of the group during milking, as vocalizations sharply decreased in the minutes after the TDM cows returned from the parlour (Rémond et al., 2002). Lying time and position, rumination, and standing behaviour were not different in the 30 minutes before and after each milking (Rémond et al., 2002). Kohler et al. (2016) investigated the effect of a change to a 24-hour milking interval for 1 d on signs of health and well-being in early to mid-lactation dairy cows (~89 DIM). In the last 6 h of the 24 h interval Kohler et al. (2016) observed cows to have decreased eating time and increased ruminating time, udder firmness, and hind limb abduction while walking and standing, compared to the cows in the last 6 hours of a 12-hour milking interval. There were no differences in lying time, limb movements, or stride length between the groups. Measuring behaviour of only the last 6 hours of the milking interval instead of a full day may have biased some of the observations. As shown by Tucker et al. (2007), cows immediately changed their feeding patterns when MF was reduced to ODM. This may have affected the eating patterns Kohler et al. (2016) observed
in the 6 h periods, but their observation could also be attributed to discomfort due to udder pressure. Cumulatively, these results suggest that cows likely experience stress in the first 24 h after MF has been reduced, but these adverse symptoms appear to decrease fairly quickly, and ODM does not appear to adversely affect the welfare or behaviour of cows further into their lactation. There has not been much research specifically examining the welfare impacts of ODM in early lactation aside from Rémond et al. (2002), but it would be beneficial to determine if cows can adapt to the effects of ODM as easily as in mid-lactation, and whether behaviour and cow comfort are affected differently earlier in lactation.

Once-daily milking has not been associated with any negative effects on reproductive performance. Long-term and lactational ODM studies have indicated areas of improved reproductive performance with ODM, such as higher 3-wk submission rates (Clarke et al., 2006), higher 3-wk pregnancy rates (Clarke et al., 2006), reduced time from calving to conception (Clarke et al., 2006), and higher final pregnancy rates (Rémond et al. 2004). However, Clark et al. (2006) reported no difference in final pregnancy rates between ODM and TDM cows over 4 consecutive lactations. Early lactation, short-duration ODM studies by McNamara et al. (2008) and Phyn et al. (2014) reported no differences in fertility or reproductive performance between ODM and TDM cows; but Phyn et al. (2014) did find that ODM cows had a higher pregnancy rate compared to ThrDM cows. Patton et al. (2006) found that cows milked once-daily for the first 4 wk of lactation resumed estrus cycling 10 d earlier than ThrDM cows, but they did not observe differences between MF in any other reproductive outcomes such as conception rate and pregnancy rate. This research indicates that longer periods of ODM may be required to be associated with a possible improvement in reproductive performance. It should be emphasized that none of these studies were specifically designed to measure reproductive performance and
all had relatively small sample sizes. As there has not been research explicitly targeted to examining the effects of reducing MF on fertility and reproductive performance, we cannot conclude whether or not ODM affects reproduction.

Incompletely milking cows is an alternative approach to reducing energy demand that has recently been evaluated as a preventative measure for ketosis. A randomized controlled trial performed by Carbonneau et al. (2012) examined incompletely milking cows for the first 5 DIM (n=31), and Krug et al. (2018) and Morin et al. (2018) recently evaluated the same method in a commercial setting (n=838). Carbonneau et al. (2012) collected a maximum of 6, 8, 10, 12, and 14 kg milk/d from d 1 to 5 respectively, and blood samples were collected at 2, 3, 4, 5, 14, 21, and 28 DIM. Blood glucose concentrations were greater, and NEFA and BHB concentrations were lower for incompletely milked cows compared to the conventionally milked cows during the treatment period (d 2 to 5) and persisted until d 21 (Carbonneau et al., 2012). In the randomized controlled trial performed by Krug et al. (2018) and Morin et al. (2018), a maximum of 10, 12, and 14 kg milk/d were collected on d 1-3, 4, and 5 respectively, and blood samples were collected 3 times from each cow in weekly intervals. Partially milked cows were less likely to develop ketosis (defined by blood BHB ≥ 1.4 mmol/L) than conventionally milked cows within the 4 to 7 DIM and 8 to 17 DIM periods (Morin et al., 2018). While milk production was lower during the 5 d treatment period, Carbonneau et al. (2012) found that incomplete milking did not have a residual effect on milk production during the first 9 wk of lactation and observed a tendency for lower fat percentage in the partially milked group. Additionally, Krug et al. (2018) observed no differences in milk production, fat percentage, or protein percentage from 2 to 44 wk of lactation between the treatment groups. This research supports the ODM research that has shown positive effects on energy balance due to a reduction in energy demand, however this
research also has shown a much smaller negative effect on productivity compared most of the ODM research.

Once-daily milking improves energy-balance during early lactation and periods of metabolic stress without negatively affecting animal welfare, but at the cost of a 13 to 40% reduction in milk production, which may continue to negatively affect milk yields after TDM has resumed. More research is required to better determine the duration of ODM that minimizes the residual negative effect on milk production from short term, early lactation ODM. Ketosis is caused by an imbalance of energy input and output, and ODM presents a practical tool, which may more effectively treat ketosis by reducing metabolic challenge and supporting better energy balance, compared to current treatment options which focus only on providing cows with more energy.

**Conclusion**

Ketosis remains a huge (and sometimes hidden) cost for producers this is due to its negative impact on the health and productivity of dairy cattle. It is also due to the typically subclinical nature of the disease, which may result in ketotic cows going unnoticed and untreated in herds. Early and accurate detection followed by rapid and effective treatment are crucial factors in successfully managing and resolving cases of ketosis on dairy farms.

While there is a large body of published research on the detection and treatment of ketosis, there are still gaps in the literature that leave many questions unanswered. The physiological path of ketones in blood, milk, and urine, as well as the dynamics of these ketone concentrations in the ketotic cow are relatively unknown, and may be affecting our current interpretation of diagnostic tests. Research is needed to follow cows in early lactation over the
course of a case of ketosis and monitor the changes in blood, milk, and urine ketones, to develop a better understanding of the circulation of ketones and provide a more accurate assessment of diagnostic test performance.

Virtually all ketosis treatments that have been suggested and researched have focused on providing ketotic cows with more energy. At best, these treatments are only effective at diminishing the duration or resolving ketosis for 50-60% of affected animals. Research is needed to evaluate treatments that temporarily reduce energy output as a means of decreasing metabolic challenge instead of only increasing energy input. Prior research has shown that once-daily milking can improve energy balance in healthy cows in early lactation and during periods of metabolic stress. Once-daily milking presents a unique possibility to more effectively resolve ketosis by reducing energy demand.
Research Objectives

The objectives of this thesis were to explore the diagnostic trends of ketosis, and identify opportunities to control energy balance in ketotic cows. More specifically the objectives were to:

1. Investigate the relationship between the concentrations of ketones in the blood, milk, and urine leading-to and over the course treatment for ketosis in Holstein cows in early lactation by:
   a. Comparing the test performance of the milk-based cow-side BHB test (Keto-Test™) and the urine-based cow-side AcAc test (KetoStix®) against blood BHB measurements over time
   b. Identifying changes and measuring the magnitude of the relationship between ketones in the blood, milk, and urine over time in cows diagnosed with ketosis

2. Evaluate the effects of reducing milking frequency from two milkings per day to one milking per day for 2 wk (in conjunction with 5 d of oral PG oral drench) in ketotic dairy cows on ketosis resolution and milk production.
REFERENCES


CHAPTER 2: AN EXPLORATION OF DIAGNOSTIC PATTERNS FOR KETOSIS IN THE BLOOD, MILK, AND URINE IN LACTATING DAIRY COWS

INTRODUCTION

Ketosis is a metabolic disease commonly experienced by dairy cows, affecting approximately 40% of dairy cow lactations in North America, with extensive variation of on-farm incidence ranging from 30% up to 80% (Duffield, 2000; McArt et al., 2012; Gordon et al., 2013). Ketosis develops during the period of negative energy balance (NEB) experienced by cows in early lactation when they are unable to meet the metabolic demands for milk production through feed intake alone (Herdt, 2000; Duffield, 2000). It is characterized by an excessive concentration of circulating ketone bodies and is diagnosed when blood/serum β-hydroxybutyrate (BHB) levels are ≥ 1.2 mmol/L (Duffield, 2000; Duffield et al., 2009). Some elevation of ketones is normal during periods of NEB, but poor adaptation to NEB can result in intensified mobilization of non-esterified fatty acids (NEFA) to the liver, overwhelming the liver and leading to the accumulation of ketone bodies, which may develop into ketosis if uncontrolled (Baird 1982; Herdt, 2000; Duffield 2000). Cows with ketosis experience decreases in milk production, and have an increased risk of displaced abomasum, metritis, and removal from the herd (due to culling or death). The relatively high incidence of ketosis combined with its more common subclinical nature poses long-term consequences to the health and welfare of dairy cows and the profitability of a farm if ketosis is not diagnosed and managed quickly.

Screening for ketosis at the cow-level helps producers diagnose individual cases of ketosis earlier in the course of the disease, allowing for more rapid treatment and the possible reduction/prevention of further economic losses due to ketosis (Enjalbert et al., 2001; Tatone et al., 2016). The main ketones that circulate in the body of the cow are BHB, acetone, and
Acetoacetate (AcAc); and all three may be used for the detection of ketosis (Duffield et al., 2009). The gold standard for the diagnosis of ketosis is through laboratory evaluation of serum or whole blood BHB concentration, but this method is not practical as an on-farm test if immediate results are required (Duffield 2000; Herdt, 2000).

A number of cow-side tests have been developed for a more rapid and convenient diagnosis of ketosis status through the blood, milk, or urine at a reduced cost compared to a laboratory; but these tests may lack in sensitivity and/or specificity compared to the gold-standard laboratory evaluation (Geishauser et al., 2000; Oetzel 2004; Iwersen et al., 2009). The cow-side tests that are the most commonly used on farm, and have been examined the most within the literature are the Precision Xtra® blood BHB meter (Abbott Laboratories, Abbott Park, IL), the Keto-Test™ milk BHB test strip (Elanco, Greenfield, IN), and the KetoStix® urine AcAc and acetone test strip (Bayer Corporation, Elkhart, IN).

The Precision Xtra® meter quantitatively measures the concentration of BHB in small samples of blood and has been extensively validated against the gold standard laboratory evaluation (Iwersen et al., 2009; Voyvoda & Erdogan, 2010). A meta-analysis performed by Tatone et al. (2016) reports the Precision Xtra® having a summary sensitivity of 94.8% and specificity of 97.5% in diagnosing ketosis at BHB concentrations ≥ 1.2 mmol/L against the gold-standard test. The Keto-Test™ and KetoStix® test strips semi-quantitatively measure ketones by changing colour based on the concentration of ketones in the milk or urine sample provided. The Keto-Test™ BHB milk test and KetoStix® AcAc urine test have only moderate test accuracy when compared with the blood BHB laboratory gold standard, but provide a less-invasive option for ketone-monitoring. The meta-analysis by Tatone et al. (2016) reports the Keto-Test has a summary sensitivity of 81.5% and specificity of 81.9% at the BHB cut point of 100 μmol/L in
milk; and the KetoStix® test has summary sensitivities of 87.6 and 70.5% and specificities of 89.2 and 96.2% at the AcAc cut points of 5 and 15 mg/dL, respectively.

Despite over fifty years of research on ketones in dairy cows, there is little understanding on the physiological paths of ketone production and elimination in the blood, milk, and urine in cows with ketosis, and how these concentrations are affected over time. The concentration of BHB in milk is approximately one-eighth of the concentration in blood, and concentrations of ketone bodies in the urine are reported to be approximately 4 times higher than in blood (Shultz, 1971; Enjalbert et al., 2001). However, is not well understood whether fluctuations in ketone concentrations in the milk and urine mirror the changes in ketone concentrations in the blood during the course of ketosis, or if these changes in ketone concentrations in the milk and urine are delayed, or occur earlier than in blood. Samiei et al. (2010) aimed to investigate the relationship between the concentrations of BHB in the blood and milk by sampling cows (n=50) 8 times between 3 and 28 days in milk (DIM). Samiei et al. (2010) found the blood and milk BHB tests to have high correlation based on the Spearman’s correlation at the milk cut point of 200 µmol/L and blood cut point of 1.4 mmol/L, but did not expand on the changes in the milk test performance over time. Multiple researchers have compared samples of blood, milk, and/or urine in cows with ketosis on the same sample day, but beyond the research done by Samiei et al. (2010) the tests have not been compared over time or over the course of the disease (Carrier et al., 2004; Iwersen et al., 2009; Samiei et al., 2010). While test accuracy compared to the laboratory gold standard method and misclassification bias due to test limitations may explain some of the discrepancies observed in the diagnostic accuracy of milk and urine ketosis tests; it is possible that the biology of ketosis itself could be a major factor in the diagnostic accuracy of milk and urine ketosis tests. In order to determine whether or not there are differences in when
concentrations of ketones in the blood, milk, and urine change leading to and during a ketosis event, the test performance of the milk and urine tests need to be compared to blood BHB measurements over a period of time during a case of ketosis.

The objective of this study was to investigate the relationship between the concentrations of ketones in the blood milk and urine in leading-to, and during a case of ketosis in Holstein cows in early lactation.

MATERIALS AND METHODS

Study Population

Early lactation Holstein cows of all parities at the University of Guelph Livestock Research Innovation Centre – Dairy Facility were utilized for this prospective cohort from November 2016 to September 2017. To be eligible for observation, fresh cows needed to be in good clinical health post-calving. Cows that experienced milk fever, displaced abomasum, or any other serious health issue within their first 3 d postpartum or that received Caesarean-sections were excluded from the study.

The research methods and study protocol were approved by the University of Guelph Animal Care Committee (AUP#3617), and were followed by the researchers and the dairy facility staff.

Data Collection and Study Design

In the days prior to calving, close-up dry cows at the dairy facility were moved to separate box stall pens bedded with straw, to allow for closer monitoring by the facility staff. Post-calving, fresh cows remained in their box stalls, and were milked via pipeline in the stall
until the barn staff deemed them sufficiently healthy to enter the herd; wherein they were moved to a free-stall pen with a milking robot (typically around 4 to 6 DIM). The free-stall robot pen had a two-row design with a DeLaval VMS™ robot located at one end. The stalls had mattress-padded beds topped with 5 to 8 cm of chopped straw, cleaned daily, and re-bedded once/wk. All cows in the robot pen were milked by one DeLaval VMS™ robot equipped with Herd Navigator™. Cows in the box stall pens were fed once daily between 0800 h and 1000 h. Cows in the robot pen were fed once-daily between 0900 h and 1100 h, and consumed their total mixed ration (TMR) from Insentec (Hokofarm Group, Marknesse, Netherlands) feed bins. Cows within the robot pen also received 3 kg of grain pellets from the robot every day. Diets for the fresh pens and robot area are described in Table 3.1, 3.2, and 3.3.

Cows initially had blood, milk, and urine samples collected daily to test for ketosis, beginning at 3 DIM up to a possible 16 DIM. Ketosis was defined by a blood BHB concentration \( \geq 1.2 \text{ mmol/L} \) (Oetzel, 2004; Duffield et al., 2009). If cows did not test \( \geq 1.2 \text{ mmol/L} \) on blood BHB by 16 DIM, they completed the trial. Following a blood BHB test \( \geq 1.2 \text{ mmol/L} \), cows were enrolled in a separate trial examining ketosis treatment. For the treatment trial, cows were treated with 300 mL of oral propylene glycol once per day for 5 d and the sampling schedule changed slightly. After ketosis diagnosis, cows were sampled daily for the first 3 d, then sampled every 3rd day for a 21 d period (see Table 2.1).

All cows were sampled in the morning between 0700 h and 1000 h, cows in the individual box stall pens were always sampled first. Cows were held in headlocks during sample collection. Blood, milk, and urine samples were collected from the trial cows on each sampling day and were used to measure the ketones from each respective medium through three different diagnostic tests. The Precision Xtra® meter was used to measure blood BHB concentrations,
Keto-Test™ test strips were used to measure milk BHB concentrations, and Ketostix® test strips were used to measure the concentration of acetoacetate and acetone in urine. The Precision Xtra® meter was used as the “gold standard” and reference test in this study, as its diagnostic test accuracy has been validated many times in the literature, and it provides information more rapidly than the laboratory test (Iwersen et al., 2009; Tatone et al., 2016).

Blood samples were collected through coccygeal venipuncture using a 20-gauge needle attached to a 1 mL syringe; approximately 0.1 to 0.5 mL of blood was collected for each sample. Blood samples were immediately tested with the Precision Xtra® meter and BHB test strip, a handheld point of care meter that quantitatively measures the concentration of BHB in blood. To perform this measurement, a blank ketone strip was inserted into the Precision Xtra® meter and a fresh droplet of blood was placed onto the ketone strip’s test cavity to be analyzed following a prompt to “add blood”. After ten seconds, a BHB blood concentration in mmol/L was displayed. A blood BHB reading of \( \geq 1.2 \) mmol/L was considered a ketotic test (Oetzel, 2004; Duffield et al., 2009).

Milk ketone monitoring was performed using the Keto-Test™ strips, which semi-quantitatively measure ketones by changing in colour based on the concentration of BHB in the milk sample. The colour scale shows six different colour blocks that correspond to concentrations of BHB, reported in \( \mu \text{mol/L} \), (Table 2.2). Milk samples were not collected during the milking period. Instead, milk samples were collected in the headlocks, at the same time the urine and blood samples were collected. The udder was stimulated and stripped, then an unused Keto-Test™ strip was placed into a stream of milk for approximately 3 seconds, ensuring the strip’s test square was fully wetted. The strip was compared to the colour chart on the side of the Keto-Test™ bottle 30 to 60 seconds after contact with milk, and a measurement was recorded.
Elanco indicates that a test of $\geq 100 \, \mu\text{mol/L}$ signifies a ketotic test result, and a meta-analysis performed by Tatone et al. (2016) supports this cut point. Therefore, milk BHB $\geq 100 \, \mu\text{mol/L}$ was used for determining a ketotic milk test in this study. The Keto-Test™ strips were kept in a refrigerator when not in use, and were given 15 to 30 min to reach room temperature in the mornings before use. In some cases milk samples could not be obtained due to the temperament of the cow, particularly in irritated primiparous cows that were uncomfortable with, and reacted aggressively to udder contact.

Urine ketone monitoring was performed using Ketostix® strips which semi-quantitatively measure ketones by changing in colour based on the concentration of acetoacetate in the urine sample. Similar to Keto-Test™, the KetoStix® colour scale shows six different colour blocks that correspond to concentrations of acetoacetate reported mg/dL, (Table 2.3). Urine samples were collected by placing an unused Ketostix® urine strip into the stream of urine (after massage of the region underneath the vulva) for 3 seconds, ensuring that the reagent area was completely covered in urine. The result was interpreted according to the colour scale on the side of the Ketostix® bottle 10 to 15 seconds after contact, and a measurement was quickly recorded. Based on the meta-analysis performed by Tatone et al. (2016), KetoStix® test results of 5 mg/dL and 15 mg/dL may both be valid options as thresholds for ketosis, but differ greatly in sensitivity and specificity. Based on this knowledge, both cut points were initially evaluated for their sensitivity and specificity based on all tests collected, and the cut point with the greatest/highest diagnostic accuracy was chosen to use for the remainder of the analyses.

Urination required stimulation for most cows. If a cow failed to urinate after a prolonged period of stimulation, researchers would leave the cow locked in the headlocks and attempt to collect a
urine sample again after a few minutes. Samples were not collected if cows failed to urinate within a 10 to 15 minute time period.

The Precision Xtra® meter, Keto-Test™, and Ketostix® will herein be called the blood, milk, and urine tests respectively.

Statistical Analyses

All data were recorded in Microsoft Excel (Microsoft Corp., Redmond WA). Statistical analyses for the test characteristics were performed in Stata (StataCorp, College Station, TX), and statistical analyses using survival analyses and Cox regression models were performed in R (R Core Team, Vienna, Austria).

Test characteristics

The sensitivities and specificities of the milk and urine cow-side tests were calculated for the cut-off points of interest using the blood BHB value supplied by the blood test as the gold standard for the diagnosis of ketosis. The initial analysis of each tests’ performance characteristics (sensitivity and specificity) used all of the observations collected for each test and were calculated for the cows in our study population. In cases where one of the two test results was missing for a specific cow for a test day, the comparison between that missing test and blood was not made. For example, if a cow had blood and milk tests collected at 5 DIM, but was missing a urine test, the milk+blood comparison would still be analyzed, but the urine+blood comparison would be omitted. This approach is called complete case analysis and it relies only on observed data, instead of imputing missing values or observations with a predicted observation (Dohoo et al., 2003). Complete case analysis was used because we were specifically
examining test performance, and imputing missing data points would bias those results. Sensitivity was estimated as the proportion of cows with blood BHB $\geq 1.2$ mmol/L that tested positive for ketosis based on the cut point of the urine or milk test in the study population. Specificity was estimated as the proportion of cows with blood BHB $< 1.2$ mmol/L that tested negative for ketosis based on the cut point of the urine or milk test in the study population. Confidence intervals were estimated using the DIAGTI function in Stata. Based on the resulting sensitivity and specificity the most suitable cut point for the urine test was selected for use in the rest of this study.

Separate sensitivity and specificity analyses of the milk and urine tests were also performed using data from the day of a cow’s first ketotic blood test, along with the three consecutive days after the first ketotic blood test, to determine any differences or patterns in the test performance over time, during a ketosis event. Since all cows on trial began sampling at 3 DIM, we cannot be certain that cows that tested blood-positive for ketosis at 3 DIM actually became ketotic at 3 DIM, as there is a possibility they were ketotic before 3 DIM. To account for possible bias these analyses were performed twice; once with all cows that had a ketotic blood test and once only with cows that had their first ketotic blood test after 3 DIM.

*Time-to-Ketosis Analysis by Different Diagnostic Methods*

The time from calving to detection of ketosis was analyzed at herd level using survival analysis methods. The analysis was performed comparatively for the three diagnostics: blood, milk, and urine tests, with analysis restricted to complete cases. At first, descriptive methods were applied, and the crude median survival and 95% confidence interval were estimated. Survival curves (and 95% confidence bands) were estimated for each diagnostic method using
the Kaplan-Meier estimator. In cases where the upper confidence interval for the median survival time is infinite, the confidence interval extends beyond the study period. Cows were aggregated into three lactation groups (1st lactation, 2nd lactation, and 3rd and greater lactations) and additional Kaplan-Meier survival curves were created for each lactation group in order to gain more insight into the time-to-ketosis for each test within the herd. Log-rank tests compared the survival curves between lactation groups. Furthermore, the survival data for ketosis was fit with Cox regression models that were adjusted for lactation in order to measure the risk of ketosis associated with lactation for each diagnostic method. Again, analysis was restricted to complete cases.

Strength of the Relationship Between the Blood Test and the Milk or Urine Test - Hazard Ratio

A time-varying covariate Cox regression model (Collett, 2003) was applied to measure the strength of the association between the blood test as an indicator for ketosis and the milk or urine tests as time varying covariates or predictors for time to ketosis. Furthermore, the time-varying Cox model can be adjusted for lactation beside the milk and urine diagnostic results to generate an expected survival curve for serological ketosis. Again, model fit was restricted to a complete case analysis. As a result, some cows were completely excluded from analysis because their first ketotic blood test occurred on a test day where a milk test was missing and their next ketotic blood test did not occur until after 16 DIM.

The modeling processes started with a fit of an ordinary Cox regression model (without extension for time-varying-covariates) to the blood test data, indicating time to ketosis and lactation as the only covariate. Similar models were fit with lactation and the milk or urine test as
covariates/predictor variables. The hazard ratios for the milk or urine tests were fit with cows in first lactation as the referent group.

RESULTS AND DISCUSSION

Sample Description

Between November 2016 and October 2017, a total of 1729 blood samples were collected from 148 cows (74 primiparous and 74 multiparous), 14 blood observations were missing. The incidence of ketosis over the trial duration was 74.3%, meaning that 110 cows (43 primiparous, 67 multiparous) tested positive for ketosis based on a blood BHB concentration of $\geq 1.2$ mmol/L on the blood test. The overall proportion of blood BHB levels of 1.2 mmol/L and higher was 23.9%. The distribution of blood BHB concentrations is shown in Figure 2.1. Some cow side milk and urine tests were missing. Approximately 86% of the 1743 possible urine samples and 94% of the 1743 possible milk samples were obtained.

Comparison of Test Characteristics for all Samples for each Test

Sensitivity and specificity of all milk and urine tests against the blood test are reported in Table 2.4 with 95% confidence intervals. The 95% confidence intervals provide an estimate for future populations at the research farm. The 95% confidence intervals should be used with some caution when/if applied to estimates for standard farms because this sample was taken from a research farm, and is not representative of a typical farm in Ontario. The milk test used with the cut-off point of 100 $\mu$mol/L was highly specific (91.4%) but was only adequately sensitive (60.8%). These results differ from the meta-analysis on the Keto-Test™ performed by Tatone et al. (2016), which found a summary sensitivity of 81.5% and summary specificity of 81.9% when
using the 100µmol/L cut point. In addition, these results differ from most previous studies, which used the same milk cut point (Jorritsma et al., 1998; Geishauser et al., 2000; Enjalbert et al., 2001; Iwersen et al., 2009). The Keto-Test™ in the current study had a much lower sensitivity (61 vs. 79, 80, 90, and 96%, respectively in the cited studies), and either a much higher, or similar specificity (91 vs. 85, 76, 94, and 64%, respectively). Enjalbert et al. (2001) was the only study of the four above that used the same blood BHB threshold of 1.2 mmol/L for defining ketosis, the remainder used 1.4 mmol/L which may partially explain the observed differences. Studies by Carrier et al. (2004) and Shire et al. (2013) reported Keto-Test™ results the most similar to what was observed in this study, finding sensitivities of 69 and 72% and specificities of 84 and 96% respectively. While both of these studies used a different blood BHB threshold for ketosis (1.4 mmol/L), both also sampled their cows within time periods post-calving that were very similar to those found in the current study (2 to 15 and 5 to 17 DIM respectively, ~80% of our samples were collected between 3 to 16 DIM), which may explain the similarities observed in test performance.

The sensitivity and specificity of two different ketosis cut-points were examined for the urine test, 5 mg/dL (trace), and 15 mg/dL (small). The cut-off point of 5 mg/dL had a sensitivity of 94.1% and specificity of 70.7%, while the threshold of 15 mg/dL had a lower sensitivity of 77.3% and a high specificity of 93.5%. The decrease in sensitivity and increase in specificity when the cut point was increased from 5 mg/dL to 15 mg/dL is typical when the threshold of a diagnostic test is increased (Dohoo et al., 2003). These results slightly differ from previous reports that have examined the KetoStix® cut points (Carrier et al., 2004; Iwersen et al., 2009; Galvaõ et al., 2013). The KetoStix® at the 5 mg/dL cut point in our study had a higher sensitivity (94 vs. 91, 88, and 78% respectively in the cited studies) and a much lower specificity
(71 vs. 86, 93, and 92% respectively). The test performance characteristics for the urine test at the 15 mg/dL cut point were more similar to the previous studies compared to the 5 mg/dL cut point, having a similar or higher sensitivity (77 vs. 78, 59, and 67% respectively in the cited studies) and a similar specificity (94 vs. 96, 95, and 96% respectively). All three studies used a blood BHB threshold of 1.4 mmol/L for ketosis (while our study used 1.2 mmol/L), which may explain some of the differences in test performance. Since the “small” cut point at 15 mg/dL had a higher combined sensitivity and specificity than 5 mg/dL, this threshold was used as the ketosis cut-point for the KetoStix® for the remainder of the analyses in the current study.

The exact reasons for the differences in the Keto-Test™ and the KetoStix® test performance compared to (and within) the literature are not fully understood. Factors such as the differences in reference positive thresholds, choice of reference test (Precision Xtra® vs. laboratory analysis), DIM at testing, number of tests per animal, prevalence of ketosis, providing a propylene glycol treatment to ketotic cattle, exclusion of cattle with additional illnesses, and variations in interpretation of test strips among studies, may all contribute to the reasons why these tests differ so much from study to study.

Through analysis of all of the tests performed, the urine strips had a much higher sensitivity and slightly higher specificity than the milk strips when compared against the blood test. While the urine strips diagnostically out-performed the milk strips, the greater convenience and likelihood of collecting milk samples compared to urine samples should be considered.

**Comparison of Test Characteristics During a Ketotic Event**

When the milk and urine tests were analyzed on the day of the detection of ketosis (in blood) and the 3 d following detection, the milk test had much lower sensitivity and slightly
higher specificity compared to the urine test. The sensitivities and specificities of the milk and urine tests from the day of serological ketosis detection, and the 3 d following detection are shown in Tables 2.5, 2.6.

The milk test had the greatest test sensitivity on d 1 for all cows, and cows that were ketotic after 3 DIM (55.8% and 50%, respectively) and the greatest test specificity on d 2 (91.9% and 93.6%, respectively). The urine test had the highest test sensitivity on d 3 for all cows and d 1 for cows that were ketotic after 3 DIM (74.2% and 70.8%, respectively), and had the highest specificity on d 2 for all cows, and cows that were ketotic after 3 DIM (86.7% and 91.3%, respectively). The daily sensitivities and specificities from the first 4 d of ketosis were typically lower than the results from all of the samples taken for the milk and urine tests. This is most likely attributable to the much smaller sample sizes per day.

The milk and urine tests in Tables 2.5 and 2.6 both displayed a similar trend where sensitivity appeared to increase from d 0 to d 1, dropped on d 2, and then another increase from d 2 to d 3.

The milk test experienced a fairly large increase in sensitivity from the day of ketosis detection (d 0) to d 1 in both scenarios, increasing by 34% for all ketotic cows and 37% for cows that developed ketosis after 3 DIM. This large increase in sensitivity between the day of detection of ketosis and 1 d after blood-defined ketosis detection may suggest that the increase in concentration of BHB in milk is delayed 1 d or less compared to the increase in blood BHB concentrations.

The large decrease in sensitivity from d 1 to d 2 for both the milk and urine tests may have a correlation to the natural path of the ketones in the milk and urine in ketotic cows treated with propylene glycol. These results may indicate that the concentrations of ketones decrease
below the threshold for ketosis in the milk and urine before they do in the blood, which in turn, would cause those milk and urine tests to appear falsely negative against the blood test. The delay may be more pronounced in cows that had higher blood BHB concentrations upon ketosis detection compared to the other cows (e.g. 2.0 mmol/L vs. 1.2 mmol/L) and may explain why this drop wasn’t also observed on d 1 when ~1/2 of the originally ketotic cows had decreased blood BHB < 1.2 mmol/L. The increase in sensitivity from d 2 to d 3 may support the hypothesis above that the ketone concentrations may decrease below the threshold for ketosis in the milk and urine before blood.

It is also probable that these changes in sensitivity occurred due to the small sample size, or simply occurred due to lack in accuracy of the milk and urine tests compared to blood, but either way, this pattern should be examined further.

Test specificity did not change as much as sensitivity over the 3 d following ketosis diagnosis. However, there was a noticeable increase in specificity for both the milk and urine tests from d 1 to d 2, indicating fewer false positive milk and urine tests. This supports our hypothesis that the propylene glycol treatment may decrease ketone concentrations in the milk and urine before those in the blood.

These observations have provided a starting point towards developing a better understanding of the physiological paths of ketone production and elimination in the blood, milk, and urine. They suggest that the timing of changes in ketone concentrations may differ in the blood, milk, and urine of the ketotic dairy cow over time, and some of the diagnostic inaccuracies of the milk and urine tests compared to the blood test may be partially attributed to these differences in concentrations. The trends in the test performance during ketosis indicate that milk and urine ketone concentrations may experience a delayed increase compared to blood,
and may also be reduced below ketosis test cut points before blood ketone concentrations. It would be valuable to sample a much larger cohort of dairy cows daily in early lactation for ketosis and follow them daily over a case of ketosis without treatment intervention.

Comparison of Time-to-Ketosis at the Herd Level Based on Different Diagnostic Methods

Kaplan-Meier estimated survival curves for the blood, milk, and urine tests are presented in Figures 2.2, 2.3, and 2.4 respectively. The blood test had the shortest median time-to-ketosis of 5 d, and detected the most cases of ketosis (110/148 cows) compared to the milk test and the urine test (Table 2.7). The milk and urine tests both had a median time-to-ketosis of 7 d, and the milk test detected more cases of ketosis than the urine test (101 vs. 96 respectively).

To gain more insight into the time-to ketosis within the herd, the Kaplan-Meier estimated survival curves stratified by lactation are presented with their log-rank p-values for the blood, milk, and urine tests in Figures 2.5, 2.6, and 2.7 respectively. The survival curves of the blood and urine test show a difference in time-to-ketosis between the lactation groups. The respective log-rank test supports this finding at the 5% significance level ($P<0.001$, and $P=0.037$ respectively, Table 2.8). The log-rank test did not indicate differences between lactation groups for the milk test at the 5% significance level ($P=0.25$). The median time-to-ketosis according to the blood test results were 7.5 d for 1\textsuperscript{st} lactation cows, 5 d for 2\textsuperscript{nd} lactation cows, and 3.5 d for 3\textsuperscript{rd}+ lactation cows. Interestingly, 1\textsuperscript{st} lactation cows had a shorter time-to-ketosis than 2\textsuperscript{nd} lactation cows according to the urine test and this could be a function of the urine test’s lower specificity (which is expected to result in more false positives) compared to the blood test.

Table 2.9 presents the Hazard Ratios resulting from Cox regression modeling of the time-to-ketosis by lactation separately for the blood, milk, and urine tests. Lactation 1 cows formed
the referent population in these models. The hazard ratio is interpreted as the relative speed that an event (here, a positive ketosis test result) will occur, or what the relative risk is for an event to occur instantaneously among the still non-ketotic cows at any given time during the study period.

The hazard ratios for 2nd and greater lactation cows were larger according to the blood test compared to the milk and urine tests. According to the blood test, 2nd and 3rd+ lactation cows would become ketotic 2.3 and 2.5 times (respectively) faster, or were 2.3 and 2.5 times more likely to become ketotic than 1st lactation cows ($P<0.001$). This supports more recent research by Vanholder et al. (2015) that found that cows with greater parities have a greater risk of developing ketosis in early lactation. According to the milk test, there was not enough evidence to show a difference in the hazard ratio of ketosis between lactations 2 and 3+ compared to lactation 1 ($P=0.87, 0.09$ respectively). There was not enough evidence to show a difference in the hazard ratio for ketosis between 1st and 2nd lactation cows using the urine test ($p=0.32$); but 3rd and greater lactation cows became ketotic 1.8 times more quickly compared to the 1st lactation cows.

Based on these results from the survival analyses, if relying on the milk or urine tests alone, on average ketotic cows will be treated two d later than cows in herds managed by blood testing. This delay could be explained by the diagnostic inaccuracies of the milk and urine tests compared to the blood test. In addition, it could also be explained by a delayed increase in the concentration of ketones in the milk and urine compared to blood, or a combination of these two factors. It is possible that the milk and urine tests would have appeared positive sooner if the ketotic cows on this trial were not treated for ketosis immediately. Regardless, there is a delayed detection of ketosis in the herd when comparing the median survival time of the milk and urine tests to the blood test, and further research should more thoroughly examine this finding.
Comparison of the Strength of the Relationship Between the Blood Test and the Milk or Urine Test

The “time-varying covariate Cox regression” model analyzed the time to ketosis as indicated by the blood test, using the milk and urine test results as predictors. The goal was to determine whether or not the milk and/or urine tests are associated with the outcome of the blood result. Strong associations would identify milk or urine tests as good predictors for ketosis. In the model, the milk and urine tests were both identified as strong predictors for ketosis diagnosis using the blood test. The detailed hazard ratios estimated by the model are presented in Table 2.10.

The hazard ratios for the time-varying covariate Cox regression model is an extension of the ordinary Cox model. The extension being the inclusion of time-varying covariates to adjust for time-dependent hazard ratios. Therefore, the proportional hazard ratio interpretation applies in this extended Cox model as well (see Hosmer, Lemeshow, & May, 2008, p.216). In this herd, when a 1st lactation cow was considered ketotic by the milk test, the cow was 5.1x as likely to be ketotic according to the blood test compared to a 1st lactation cow that was not ketotic according to the milk test. Cows in 2nd and 3rd and greater lactation were 10.9 and 10 times (respectively) more likely to be ketotic according to the blood test when indicated as ketotic by the milk test compared to cows that were not ketotic according to the milk test. This is interpreted similarly for the urine test (Table 2.10).

The expected survival curves for the time-to-ketosis (as defined by the blood test) adjusted for lactation group and using the milk or urine test as a predictor in the extended Cox regression model are shown in Figures 2.8 and 2.9. The median survival to ketosis defined by the
blood test in the model, adjusted for the milk test result and lactation was 8 d. The median survival to ketosis in the model, adjusted for the urine test result and lactation was infinite. This surprising result might be due to the sizeable amount of missing urine observations, which inadvertently removed many ketotic blood observations through complete case analysis. If the 75% survival point is compared instead, the 75% survival from the milk test and lactation adjusted model is shorter than that from the urine test and lactation adjusted model; which are 75% survival points of 4 and 7 d, respectively.

When comparing the hazard ratios of the time-varying covariate Cox regression model to the related sensitivities and specificities of the milk and urine tests, the larger hazard ratios for the urine test as a predictor for ketosis supports the better diagnostic test performance of the urine test compared to the milk test. Furthermore, it should be noted that a major shortfall for application of the urine test is the inability to consistently collect urine samples for testing. This highlights the challenges with the use of the urine test for on-farm testing programs.

As a study limitation, it must be noted that this was a field trial on an experimental station and thus generalizations are not unconditional, and future research in a commercial setting is needed to expand on the findings in this study. Treating cows with propylene glycol (PG) on the day of blood-detected ketosis (and 4 d following that) may have affected the milk and urine test performance compared to the blood in the 3 d following ketosis detection, possibly causing the differences presented in the results. The cows should not have been given a PG treatment in order to monitor the true path of the ketones in a ketotic cow. However withholding treatment from sick cows is an ethical issue, so refusing treatment may not be feasible. Sampling for 3 consecutive days following blood-defined ketosis was adequate, but would have benefitted from
a longer duration of sampling in order to track both the elevation and the elimination of ketones in the blood, milk, and urine.

Another limitation to this study was the sample size, the sample size was not estimated for this study, as it was used as the screening process for a separate randomized controlled trial (that had its own sample size estimation). In future research, a larger study population would decrease uncertainty in estimated test, and provide more power to detect any differences between the diagnostic tests.

CONCLUSION

The daily measurement of ketones in the blood, milk, and urine in early lactation dairy cows through the Precision Xtra® blood BHB meter, Keto-Test™ milk BHB strips, and KetoStix® urine AcAc strips highlighted the temporal changes within and between each cow-side diagnostic test. The milk and urine ketone tests diagnosed ketosis approximately 2 d later based on the herd’s median time-to-ketosis. The data suggests differences in the concentrations of ketones in milk and urine relative to fluctuations in blood BHB concentrations. Future research should examine this hypothesis on a larger scale, without treating ketotic cows (if ethically possible) and over the full duration of a case of ketosis.
**Table 2.1** Blood, milk, and urine sampling schedule (d0 = detection of blood-defined ketosis)

<table>
<thead>
<tr>
<th>Days from first diagnosis of ketosis</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample day (X)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2 Keto-Test™ BHB Colour Chart (µmol/L)
Table 2.3 Ketostix® Acetoacetate Colour Chart (mg/dL)

<table>
<thead>
<tr>
<th>Classification</th>
<th>mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>5</td>
</tr>
<tr>
<td>Trace</td>
<td>15</td>
</tr>
<tr>
<td>Small</td>
<td>40</td>
</tr>
<tr>
<td>Moderate</td>
<td>80</td>
</tr>
<tr>
<td>Large</td>
<td>160</td>
</tr>
</tbody>
</table>

![Acetoacetate Colour Chart](chart.png)
Table 2.4 Performance of 2 cow-side diagnostic tests\textsuperscript{1} for detection of ketosis in Holstein cows in early lactation, defined as a blood BHB concentration ≥ 1.2 mmol/L

<table>
<thead>
<tr>
<th>Test and threshold</th>
<th>n\textsuperscript{2} n ket\textsuperscript{3}</th>
<th>Sensitivity Estimate</th>
<th>95% CI</th>
<th>Specificity Estimate</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk BHB 100 µmol/L</td>
<td>1617 395</td>
<td>0.608</td>
<td>0.558-0.656</td>
<td>0.914</td>
<td>0.897-0.929</td>
</tr>
<tr>
<td>Urine AcAc 5 mg/dL</td>
<td>1484 339</td>
<td>0.941</td>
<td>0.91-0.964</td>
<td>0.707</td>
<td>0.679-0.733</td>
</tr>
<tr>
<td>15 mg/dL</td>
<td></td>
<td>0.773</td>
<td>0.725-0.816</td>
<td>0.935</td>
<td>0.92-0.949</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Keto-Test\textsuperscript{TM}, Elanco, Greenfield, IN; KetoStix\textsuperscript{®}, Bayer Corporation, Elkhart, IN.

\textsuperscript{2}Number of observations paired with a blood BHB measurement for each cow-side test

\textsuperscript{3}Number of observations with blood BHB concentration ≥1.2 mmol/L
Table 2.5 Performance of 2 cow-side diagnostic tests\(^1\) for detection of ketosis in fresh Holstein cows diagnosed with ketosis, defined as a blood BHB concentration \(\geq 1.2\) mmol/L (d=0 is the day of diagnosis)

<table>
<thead>
<tr>
<th>Test &amp; days post-detection</th>
<th>(n^2)</th>
<th>(n_{k}^3)</th>
<th>(n_{t}^4)</th>
<th>Estimate</th>
<th>95% CI</th>
<th>Estimate</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk BHB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>101</td>
<td>101</td>
<td>42</td>
<td>0.416</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>1</td>
<td>102</td>
<td>52</td>
<td>38</td>
<td>0.558</td>
<td>0.41-0.70</td>
<td>0.820</td>
<td>0.69-0.91</td>
</tr>
<tr>
<td>2</td>
<td>99</td>
<td>37</td>
<td>22</td>
<td>0.459</td>
<td>0.30-0.63</td>
<td>0.919</td>
<td>0.82-0.97</td>
</tr>
<tr>
<td>3</td>
<td>95</td>
<td>34</td>
<td>22</td>
<td>0.471</td>
<td>0.30-0.65</td>
<td>0.902</td>
<td>0.80-0.96</td>
</tr>
<tr>
<td>Urine AcAc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>88</td>
<td>88</td>
<td>62</td>
<td>0.705</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>1</td>
<td>85</td>
<td>40</td>
<td>38</td>
<td>0.725</td>
<td>0.56-0.85</td>
<td>0.800</td>
<td>0.65-0.90</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>34</td>
<td>29</td>
<td>0.618</td>
<td>0.44-0.78</td>
<td>0.867</td>
<td>0.75-0.94</td>
</tr>
<tr>
<td>3</td>
<td>88</td>
<td>31</td>
<td>32</td>
<td>0.742</td>
<td>0.55-0.88</td>
<td>0.839</td>
<td>0.72-0.92</td>
</tr>
</tbody>
</table>

\(^1\)Keto-Test\(^{TM}\), Elanco, Greenfield, IN; KetoStix\(^{®}\), Bayer Corporation, Elkhart, IN.
\(^2\)Number of observations paired with a blood BHB measurement for each cow-side test
\(^3\)Number of observations with blood BHB concentration \(\geq 1.2\) mmol/L
\(^4\)Number of observations with milk BHB concentration \(\geq 100\) µmol/L or with urine AcAc concentration \(\geq 15\) mg/dL
Table 2.6 Performance of 2 cow-side diagnostic tests\(^1\) for detection of ketosis in fresh Holstein cows diagnosed with ketosis after 3 DIM, defined as a blood BHB concentration $\geq 1.2$ mmol/L ($d=0$ is the day of diagnosis)

<table>
<thead>
<tr>
<th>Test &amp; days post-detection</th>
<th>n(^2)</th>
<th>n ket(^3)</th>
<th>n test(^4)</th>
<th>Sensitivity</th>
<th>95% CI</th>
<th>Specificity</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk BHB</td>
<td></td>
<td></td>
<td></td>
<td>Estimate</td>
<td>95% CI</td>
<td>Estimate</td>
<td>95% CI</td>
</tr>
<tr>
<td>0</td>
<td>63</td>
<td>63</td>
<td>23</td>
<td>0.365</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>1</td>
<td>64</td>
<td>26</td>
<td>20</td>
<td>0.50</td>
<td>0.30-0.70</td>
<td>0.816</td>
<td>0.66-0.92</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>15</td>
<td>7</td>
<td>0.267</td>
<td>0.08-0.55</td>
<td>0.936</td>
<td>0.82-0.99</td>
</tr>
<tr>
<td>3</td>
<td>59</td>
<td>17</td>
<td>10</td>
<td>0.353</td>
<td>0.14-0.62</td>
<td>0.905</td>
<td>0.77-0.97</td>
</tr>
<tr>
<td>Urine AcAc</td>
<td></td>
<td></td>
<td></td>
<td>Estimate</td>
<td>95% CI</td>
<td>Estimate</td>
<td>95% CI</td>
</tr>
<tr>
<td>0</td>
<td>59</td>
<td>59</td>
<td>39</td>
<td>0.661</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>1</td>
<td>57</td>
<td>24</td>
<td>23</td>
<td>0.708</td>
<td>0.49-0.87</td>
<td>0.818</td>
<td>0.64-0.93</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>14</td>
<td>9</td>
<td>0.357</td>
<td>0.13-0.65</td>
<td>0.913</td>
<td>0.79-0.98</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>16</td>
<td>15</td>
<td>0.688</td>
<td>0.41-0.89</td>
<td>0.897</td>
<td>0.76-0.97</td>
</tr>
</tbody>
</table>

\(^{1}\)Keto-Test\™, Elanco, Greenfield, IN; KetoStix®, Bayer Corporation, Elkhart, IN.

\(^{2}\)Number of observations paired with a blood BHB measurement for each cow-side test

\(^{3}\)Number of observations with blood BHB concentration $\geq 1.2$ mmol/L

\(^{4}\)Number of observations with milk BHB concentration $\geq 100$ µmol/L or with urine AcAc concentration $\geq 15$ mg/dL
Table 2.7 Median time-to-ketosis for 3 cow-side diagnostic tests\(^1\) for detection of ketosis in Holstein cows in early lactation

<table>
<thead>
<tr>
<th>Diagnostic test</th>
<th>n</th>
<th>Events</th>
<th>Median survival time (d)</th>
<th>95% CI (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood BHB</td>
<td>148</td>
<td>110</td>
<td>5</td>
<td>5 - 7</td>
</tr>
<tr>
<td>Milk BHB</td>
<td>148</td>
<td>101</td>
<td>7</td>
<td>6 - 11</td>
</tr>
<tr>
<td>Urine AcAc</td>
<td>148</td>
<td>96</td>
<td>7</td>
<td>6 - 13</td>
</tr>
</tbody>
</table>

\(^1\) Precision Xtra\(^\circ\), Abbott Laboratories, Abbott Park, IL: Ketosis cut point of BHB ≥ 1.2 mmol/L; Keto-Test\(^\text{TM}\), Elanco, Greenfield, IN: Ketosis test cut point of BHB ≥ 100 µmol/L; KetoStix\(^\circ\), Bayer Corporation, Elkhart, IN: Ketosis test cut point of AcAc ≥ 15 mg/dL.
Table 2.8 Median time-to-ketosis for 3 cow-side diagnostic tests\(^1\) for detection of ketosis, stratified by lactation, in Holstein cows in early lactation

<table>
<thead>
<tr>
<th>Test and lactation</th>
<th>P-value(^2)</th>
<th>n</th>
<th>Events</th>
<th>Median survival time (d)</th>
<th>95% CI (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood BHB</td>
<td>&lt;0.001</td>
<td>74</td>
<td>43</td>
<td>7.5</td>
<td>5 - (\infty)</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>36</td>
<td>33</td>
<td>5</td>
<td>4 - 6</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>33</td>
<td>34</td>
<td>3.5</td>
<td>3 - 7</td>
</tr>
<tr>
<td>Milk BHB</td>
<td>0.252</td>
<td>74</td>
<td>46</td>
<td>10</td>
<td>6 - 18</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>36</td>
<td>26</td>
<td>9</td>
<td>5 - 27</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>38</td>
<td>29</td>
<td>4</td>
<td>4 - 11</td>
</tr>
<tr>
<td>Urine AcAc</td>
<td>0.037</td>
<td>74</td>
<td>39</td>
<td>8</td>
<td>5 - (\infty)</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>36</td>
<td>26</td>
<td>9</td>
<td>5 - 18</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>38</td>
<td>31</td>
<td>6</td>
<td>4 - 10</td>
</tr>
</tbody>
</table>
| \(^1\)Precision Xtra®, Abbott Laboratories, Abbott Park, IL: Ketosis cut point of BHB \(\geq\) 1.2 mmol/L; Keto-Test™, Elanco, Greenfield, IN: Ketosis test cut point of BHB \(\geq\) 100 \(\mu\)mol/L; KetoStix®, Bayer Corporation, Elkhart, IN: Ketosis test cut point of AcAc \(\geq\) 15 mg/dL. | \(^2\)P-value of the log-rank test
Table 2.9 Hazard ratios for the effect of lactation on a ketotic outcome using 3 cow-side diagnostic tests

<table>
<thead>
<tr>
<th>Test and lactation</th>
<th>Hazard ratio</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood BHB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (referent)</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>2.29</td>
<td>0.237</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3+</td>
<td>2.46</td>
<td>0.233</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Milk BHB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (referent)</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>1.04</td>
<td>0.248</td>
<td>0.869</td>
</tr>
<tr>
<td>3+</td>
<td>1.50</td>
<td>0.240</td>
<td>0.091</td>
</tr>
<tr>
<td>Urine AcAc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (referent)</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>1.29</td>
<td>0.254</td>
<td>0.315</td>
</tr>
<tr>
<td>3+</td>
<td>1.79</td>
<td>0.241</td>
<td>0.016</td>
</tr>
</tbody>
</table>

Precision Xtra®, Abbott Laboratories, Abbott Park, IL: Ketosis cut point of BHB ≥ 1.2 mmol/L; Keto-Test™, Elanco, Greenfield, IN: Ketosis test cut point of BHB ≥ 100 µmol/L; KetoStix®, Bayer Corporation, Elkhart, IN: Ketosis test cut point of AcAc ≥ 15 mg/dL.
**Table 2.10** Hazard ratios from the time-varying covariate Cox regression examining the time to ketosis as indicated by blood BHB measurements\(^1\), using lactation and the milk or urine tests\(^2\) as predictors

<table>
<thead>
<tr>
<th>Test comparison and lactation</th>
<th>Hazard ratio</th>
<th>Coefficient</th>
<th>HR indicated by milk/urine</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood w/ Milk</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk BHB</td>
<td>5.13</td>
<td>1.635</td>
<td>5.13</td>
<td>0.207</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>2.12</td>
<td>0.751</td>
<td>10.87</td>
<td>0.242</td>
<td>0.002</td>
</tr>
<tr>
<td>3+</td>
<td>1.94</td>
<td>0.664</td>
<td>9.96</td>
<td>0.242</td>
<td>0.006</td>
</tr>
<tr>
<td><strong>Blood w/ Urine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine AcAc</td>
<td>14.87</td>
<td>2.699</td>
<td>14.87</td>
<td>0.236</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>2.74</td>
<td>1.007</td>
<td>40.69</td>
<td>0.250</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3+</td>
<td>1.16</td>
<td>0.151</td>
<td>17.29</td>
<td>0.24</td>
<td>0.537</td>
</tr>
</tbody>
</table>

*Reference population is cows in lactation 1 with a negative milk/urine test.

1 Precision Xtra®, Abbott Laboratories, Abbott Park, IL: Ketosis cut point of BHB ≥ 1.2 mmol/L

2 Keto-Test™, Elanco, Greenfield, IN: Ketosis test cut point of BHB ≥ 100 µmol/L; KetoStix®, Bayer Corporation, Elkhart, IN: Ketosis test cut point of AcAc ≥ 15 mg/dL.
Figure 2.1 Relative frequency distribution of blood β-hydroxybutyrate (BHB) concentrations in 1729 blood samples from 148 fresh Holstein cows.
Figure 2.2 Kaplan-Meier estimated survival curve for ketosis according to the blood test\(^1\) in fresh Holstein cows. A plus sign (+) indicates when cows were censored/removed from the data set without showing a ketotic test.

\(^1\) Precision Xtra®, Abbott Laboratories, Abbott Park, IL: Ketosis cut point of BHB $\geq$ 1.2 mmol/L
Figure 2.3 Kaplan-Meier estimated survival curve for ketosis according to the milk test\(^1\) in fresh Holstein cows. A plus sign (+) indicates when cows were censored/removed from the data set without showing a ketotic test.

\(^1\)Keto-Test™, Elanco, Greenfield, IN: Ketosis test cut point of BHB $\geq 100$ $\mu$mol/L
Figure 2.4 Kaplan-Meier estimated survival curve for ketosis according to the urine test\(^1\) in fresh Holstein cows. A plus sign (+) indicates when cows were censored/removed from the data set without showing a ketotic test.

\(^1\) KetoStix®, Bayer Corporation, Elkhart, IN: Ketosis test cut point of AcAc ≥ 15 mg/dL
**Figure 2.5:** Lactation-stratified Kaplan-Meier estimated survival curve for ketosis according to the blood test\(^1\) in fresh Holstein cows. A plus sign (+) indicates when cows were censored/removed from the data set without showing a ketotic test. (Strata 1 = 1\(^{st}\) lactation 1; strata 2 = 2\(^{nd}\) lactation; strata 3 = 3\(^{rd}\) lactations)

\(^1\) Precision Xtra®, Abbott Laboratories, Abbott Park, IL: Ketosis cut point of BHB \(\geq 1.2\) mmol/L
Figure 2.6 Lactation-stratified Kaplan-Meier estimated survival curve for ketosis according to the milk test$^1$ in fresh Holstein cows. A plus sign (+) indicates when cows were censored/removed from the data set without showing a ketotic test. (Strata 1 = 1st lactation; strata 2 = 2nd lactation; strata 3 = 3rd + lactations)

$^1$Keto-Test™, Elanco, Greenfield, IN: Ketosis test cut point of BHB $\geq 100 \mu$mol/L
Figure 2.7 Lactation-stratified Kaplan-Meier estimated survival curve for ketosis according to the urine test\(^1\) in fresh Holstein cows. A plus sign (+) indicates when cows were censored/removed from the data set without showing a ketotic test. (Strata 1 = 1\(^{\text{st}}\) lactation; strata 2 = 2\(^{\text{nd}}\) lactation; strata 3 = 3\(^{\text{rd}}\) + lactations)

\(^1\) KetoStix®, Bayer Corporation, Elkhart, IN: Ketosis test cut point of AcAc $\geq$ 15 mg/dL
Figure 2.8 Expected survival curve from the extended Cox regression model for ketosis according to the blood test\(^1\) with the milk test\(^2\) and lactation as predictors. A plus sign (+) indicates when cows were censored/removed from the data set without showing a ketotic test.

\(^{1}\) Precision Xtra®, Abbott Laboratories, Abbott Park, IL: Ketosis cut point of BHB $\geq 1.2$ mmol/L
\(^{2}\) Keto-Test™, Elanco, Greenfield, IN: Ketosis test cut point of BHB $\geq 100$ µmol/L
**Figure 2.9** Expected survival curve from the extended Cox regression model for ketosis according to the blood test\(^1\) with the urine test\(^2\) and lactation as predictors. A plus sign (+) indicates when cows were censored/removed from the data set without showing a ketotic test.

\(^1\) Precision Xtra®, Abbott Laboratories, Abbott Park, IL: Ketosis cut point of BHB ≥ 1.2 mmol/L

\(^2\) KetoStix®, Bayer Corporation, Elkhart, IN: Ketosis test cut point of AcAc ≥ 15 mg/dL.
REFERENCES


CHAPTER 3: REDUCING MILKING FREQUENCY AS AN ADJUNCT TREATMENT FOR KETOSIS IN LACTATING DAIRY COWS

INTRODUCTION

Transition cows experience a decrease in dry matter intake (DMI), reduced immune function, a period of insulin resistance, and a drastic increase in energy demand once lactation begins (Baird, 1982; Herdt, 2000). Due to these changes, transition dairy cows are typically unable to fulfill their nutrient intake requirements to meet their energy demands, causing fresh cows to experience a period of negative energy balance (NEB) (Herdt, 2000; Duffield, 2000). Some elevation of ketones is normal during periods of NEB, but poor adaptation to NEB can result in an excessive build-up of ketone bodies, leading to the development of hyperketonemia (Herdt, 2000). Ketosis is defined by a concentration of circulating ketone bodies $\geq 1.2$ mmol/L, that is associated with an increased risk of undesirable outcomes such as early culling, left displaced abomasum (LDA), metritis, increased severity of mastitis, or a decrease in reproductive performance (Suriyasathapom et al., 2000; LeBlanc et al., 2005; Walsh et al., 2007; Duffield et al., 2009; LeBlanc, 2010).

The goal of ketosis treatment is to provide glucose, stimulate gluconeogenesis, and decrease lipolysis (Herdt & Emery, 1992; Gordon et al., 2013). The current recommendation for treating ketosis is to provide a 300 mL oral drench of propylene glycol for 5 d, which resolves approximately 50% of ketotic cases by the fifth day of treatment (McArt et al., 2011; Gordon et al., 2013). There has been little advancement beyond the findings by McArt et al. (2011) and Gordon et al. (2017a;b) for ketosis treatment. Virtually all research on ketosis treatments have focused on supplementing cows with different forms of carbohydrates (which can be converted into glucose) or with products that may stimulate gluconeogenesis.
Ketosis is caused by poor adaptation to an imbalance of energy input and output. However, providing affected cows with a product that can be converted to glucose has only moderate success. A temporary reduction in energy demand could reduce metabolic challenge and more effectively resolve ketosis.

Reducing milking frequency (MF) in dairy cattle has been heavily researched, dating back to as early as the 1960’s. While there is no research that has examined reduced milking as a treatment for ketosis, many researchers have shown effects of reducing MF on improved metabolism and metabolic health. Once-daily milked (ODM) cows have lower plasma non-esterified fatty acids (NEFA) and BHB concentrations, and higher plasma glucose compared to twice-daily milked (TDM) cows in early lactation (Loiselle et al., 2009; Schlamberger et al., 2010; Kay et al., 2013; Phyn et al., 2014). Some researchers have also observed plasma BHB and NEFA concentrations to remain lower for ODM cows than TDM cows 1 to 2 wk after the ODM cows had been returned to a TDM schedule (Loiselle et al., 2009; Schlamberger et al., 2010; Phyn et al., 2014).

Negative energy balance develops due to a cow’s inability to meet its metabolic needs through feed intake alone, so the effect reducing MF may have on DMI is important. Once-daily milking is normally practiced in pasture-based dairying systems thus there is limited information on ODM’s effect on DMI. Of the few studies that have examined ODM in an indoor housing system, Loiselle et al. (2009) report no difference in DMI between ODM and TDM in early lactation, and McNamara et al. (2008) found ODM cows consumed 1.4 kg/d less TMR than TDM cows during the first 4 wk of lactation.

Regardless of the duration of ODM, an immediate decrease in milk yield is the most predictable outcome in almost all studies (Davis et al., 1999; Stelwagen et al., 2013). Reported
short-term milk yield losses during ODM in early lactation range from 13 to 40% while MF is reduced (Rémond et al., 1999; Rémond & Pomiès, 2007; Loiselle et al., 2009; Schlamberger et al., 2010; O’Driscoll et al., 2012; Kay et al., 2013; Stelwagen et al., 2013; Phyn et al., 2014). There is also a risk for long-term milk yield loss from periods of short-term reduction in MF. However, the duration of ODM that minimizes the negative carryover effect on milk production requires further examination, as milk yield and energy-corrected milk (ECM) yield losses range from 0 to 16% in short-term, early lactation studies.

The objective of this study was to measure the effect of reducing milking frequency from 2 to 1 milking per day for 2 wk, in conjunction with a treatment of 5 d of 300 mL oral propylene glycol, in ketotic dairy cows. The main outcomes of interest were the resolution of ketosis and milk yield. We hypothesized that reducing milking frequency as a treatment for ketosis would decrease the time to recovery of ketosis and decrease the incidence of relapse of ketosis, which would then mitigate the effects on subsequent milk yield.

MATERIALS AND METHODS

Study Population

Early lactation Holstein cows at the University of Guelph, Livestock Research and Innovation Centre – Dairy Facility were used for this randomized controlled trial. Fresh primiparous and multiparous cows were enrolled from November 2016 to September 2017. To be eligible for enrolment, fresh cows needed to be free from milk fever, displaced abomasum, or any other serious health issue within their first 3 days in milk (DIM). Cows that received Caesarean sections were also excluded.
The *a priori* sample size estimation was calculated based on improved ketosis cure-rates. For the treatment to be practically considered by the industry, an estimated success rate of 75% was used for the treatment, versus the current standard of approximately 50% success within 4 to 5 d of treatment (McArt et al., 2011; Gordon et al., 2013). Detection of this treatment effect, with power of 80%, and 95% confidence interval yielded a minimal sample size number of 58 animals with ketosis in each treatment group. Allowing for a small number of losses to follow-up, it was determined that 60 cows with ketosis were needed for each treatment group. The research methods and study protocol were approved by the University of Guelph Animal Care Committee (AUP#3617) and were followed by the researchers and the dairy facility staff.

**Data Collection and Study Design**

In the days prior to calving, cows were moved from a free stall pen to individual box stall pens bedded with straw, to allow for closer monitoring by the facility staff. Post-calving, fresh cows remained in their box stalls, and were milked via pipeline in the box stall. All fresh cows sampled during the study were moved to the free-stall milking robot pen once deemed sufficiently healthy to leave the box stall pens (typically around 4 to 6 DIM). Cows in the box stall pens were fed once daily between 0800 and 1000 h, and daily feed intakes were measured manually. The robot pen had a 2-row free stall design with a DeLaval VMST™ robot located at one end. The stalls had mattress-padded beds topped with 5 to 8 cm of chopped straw, cleaned daily, and re-bedded once/wk. All cows in the robot pen were milked by one DeLaval VMST™ robot equipped with Herd Navigator™. Cows in the robot pen consumed their total mixed ration (TMR) from Insentec (Insentec, Marknesse, the Netherlands) feed bins and received 1.5 kg (as-fed) of grain at each milking, to a maximum of 3 kg/d (as-fed) daily. TMR was fed once daily
between 0900 and 1100 h. Settings in the robot were adjusted for the cows enrolled on ODM to allow for them to receive their full 3 kg of grain at their single milking. Diets are described in Tables 3.1, 3.2, and 3.3.

Post-calving, cows were screened for ketosis daily from 3 to 16 DIM using a Precision Xtra® meter (Abbott Laboratories, Abbott Park, IL). Enrolment on the trial would occur on the first day that blood BHB concentration was ≥ 1.2 mmol/L (Oetzel, 2004; Duffield et al., 2009). All cows (screened and treated) were sampled in the morning between 0700 and 1000 h, before the morning feeding, however the feed bins were never without feed. Blood was collected from the coccygeal vein using a 20 gauge needle attached to a 1 mL syringe; approximately 0.1 – 0.5 mL of blood was collected in this process. Immediately after blood was collected, it was tested using a BHB strip and a Precision Xtra® meter, which has been validated for use in cattle (Iwersen et al., 2009; Voyvoda & Erdogan, 2010).

Upon detection of ketosis, a health event was added into the farm’s Dairy Comp 305 system (D305; Valley Ag Software, Tulare, CA, USA), including the detected blood BHBA measurement. This ensured that cows appeared on the daily treatment list for the farm employees. Cows were then randomly allocated to a daily MF: the one milking per day (ODM) treatment group or the 2 milkings per day (TDM) treatment group. To ensure that treatment allocation was random, a coin flip was utilized to determine a random 10-treatment set, assigning 5 slots to each treatment. When one treatment had been flipped 5 times, the remaining slots were assigned to the other MF. This set of 10 was used to allocate cows to their treatment group, and was repeated until the trial was finished. First lactation cows had a separate treatment allocation chart from multiparous cows to ensure that these animals were equally represented in each treatment group. Regardless of their MF assignment, all ketotic cows were treated with 300 mL
of oral propylene glycol once per day for 5 d beginning in the afternoon on the day of their first ketotic test.

Specific milking protocols were input into the DeLaval VMS™ robot’s settings for the once and twice per day milking groups. This was to ensure that the milking protocol was followed accurately for all animals enrolled on the treatment trial. Milking protocols were changed immediately after a cow’s first ketotic blood test. Animals enrolled in the ODM group were given a 23 h milking interval, meaning that these cows could not re-enter the robot to be milked for 23 h after a milking. The robot’s overdue milking alert was programmed to alert at the 23 h mark as well. Similarly, the cows enrolled in the TDM group were given an 11 h milking interval; with the robot alerting cows to be overdue at the 11 h mark. The DeLaval VMS™ milking robot’s milking priority queue labels cows on a basis of colour, with yellow indicating that a cow is eligible to be milked, and red indicating that a cow is overdue to be milked. By labelling trial cows as overdue for milking as soon as they were eligible to be milked, it made it easier for staff to identify which cows needed to be milked sooner than others. The milking status of cows was regularly checked and cows were fetched if overdue for the robot throughout the day between 0500 and 1900 h. This ensured that cows were milked as closely as possible to the 24 h or the 12 h mark. In both groups, cows that had been incompletely milked were required to wait until their next designated milking.

Cows that were still in the special needs box stall pens at the time of their ketosis diagnosis had a sign attached to their stall indicating their specific MF. Cows located in the special needs pens enrolled in the ODM group were only milked in the morning.

Once a cow was enrolled in the treatment trial, they were tested for ketosis for the first 3 d following their diagnosis, and then every 3rd day, to a total of 21 d (see Table 3.4). The MF
treatment ran for 14 d, and time-constraint settings on the robot were removed on the morning of the 15th day of the trial. The additional sampling wk (d 15 – 21) was used to observe any possible relapses of ketosis from either group. The research team performed all cow sampling. Propylene glycol (PG) treatment was administered by barn staff who had been trained in PG.

To ensure that the health and welfare of the cows on trial remained a priority, cows that did not recover from ketosis, or recovered and relapsed, were given additional 5 d of PG treatments. However, to allow time for assessment of the MF treatments on the health of the cows, all cows on trial could not be re-treated with additional PG until at least the 9th day of trial. Following a PG re-treatment cycle (if needed), a 3rd PG treatment cycle could not occur until 1 d after the previous treatment finished (6 d after the 2nd treatment began). Subjects could be given a maximum of 4 PG treatments within the 21 d period, with the final re-treatment beginning on d 21 of trial if needed. As another welfare measure, cows that developed more severe illnesses (DA, milk fever, or pneumonia) during the trial, became seriously injured, or had blood BHB concentrations ≥ 5.0 mmol/L or greater (that were not decreasing after 2 or 3 d), were removed from the trial to ensure that the cow could be more extensively treated.

Cows on the trial remained in the robot pen until at least 21 d after enrolment. Milk production was monitored for the first 15 wk of lactation following ketosis detection in all trial cows. From wk 4 to 15, cows could be milked in the sick/fresh pen box stall system, the robot milker, or in the rotary parlour. Daily milk yield values were collected from the milking equipment software system. Weekly milk recording data (CanWest DHI) were available for cows on this trial. These data were used for analyses of milk fat percentage, milk protein percentage, linear score (LS), and energy corrected milk yield for the 15-wk period. Energy-
corrected milk (ECM) was calculated by standardizing milk production to 3.5% fat and 3.2% protein using the formula:

$$ECM \text{ kg} = (0.3246 \times \text{kg milk}) + (12.86 \times \text{kg fat}) + (7.04 \times \text{kg protein})$$ (Bernard, 1997).

Feed intake data were collected for each cow for 21 d after enrolment. The Insentec feed bins in the robot pen recorded the time, duration, and amount of feed consumed each time a cow’s transponder (located on a band around their neck) triggered the feed bin gate to open (validated by Chapinal et al., 2007). Daily intakes in the box stall pens were measured once daily before feeding. Feed samples from both areas were collected once per wk to determine the feed dry matter percent. The robot measured the amount of grain dispensed to each cow during milkings, but could not measure actual consumption, based on this, we calculated dry matter intakes with the assumption that all pellets dispensed were consumed.

The facility’s Dairy Comp 305 software was used to collect information on health events within the first 60 DIM, 305M (for cows that remained in the herd past 200 DIM), and reproductive events. Staff recorded disease events in dairy comp based off of vet diagnosis and/or based on disease definitions from Kelton, Lissemore, & Martin, 1998.

**Statistical Analysis**

All data were recorded in Microsoft Excel (Microsoft Corp., Redmond WA). Data for diseases, reproductive performance, and 305M were extracted from DC305 into Microsoft Excel for all of the enrolled cows. The mean milk yields for the period of 15 wk following trial enrolment were collected as daily measurements from DeLaval DelPro™ and were compiled into weekly averages. Data for milk fat percentage, milk protein percentage, and LS were recorded from DHI. The energy corrected milk (ECM) yield was calculated using the milk data
collected and compiled for the weekly milk yield and the weekly data received from DHI on the
fat and protein composition of each cow’s milk. Feed intake data were collected and analysed for
the first 3 wk upon trial enrolment for each cow. Cows were excluded from all analyses if they
were enrolled onto the trial for 4 d or less before being removed. Cows were excluded from the
feed intake analyses if they were missing intake data for the first 5 d of trial or more. Cows that
had feed intake data before d 4 but ended the trial early were still included in the dataset (until
their were removal from trial). All statistical analyses were performed in SAS (Version 9.4, SAS

Descriptive statistics were generated with the MEANS and FREQ procedures in SAS and
SUM function in Microsoft Excel. Univariable analysis of the association of treatment with
ketosis resolution, disease events, and reproductive events was conducted using Fisher’s Exact
and Chi-squared statistics before building statistical models.

A binary logistic regression model (GLIMMIX procedure in SAS) was used to evaluate
the resolution of ketosis outcome for treatment. Cows were either classified as ketotic or non-
ketotic based on whether blood BHB ≥ 1.2 mmol/L. Day of trial (DOT) = 0 was excluded from
this statistical analysis as all cows had a blood BHB ≥ 1.2 mmol/L on this date. Linear regression
models (MIXED procedure in SAS) were used to evaluate all continuous outcomes (BHB
concentration, milk production, milk components, and LS) for the effects of treatment.

In the models analyzing the resolution of ketosis and BHB concentration outcomes, in
addition to the treatment and DOT variables as linear and quadratics, the potential explanatory
variable parity (1st or 2nd and greater) was offered to the model, as well as all two-way and three-
way interactions with treatment. In the models analyzing milk production and milk components,
and LS outcomes, the potential explanatory variable parity (1st, 2nd , and 3rd and greater) was
added to the model in addition to the treatment and wk variables. In the model examining the 305M outcome on treatment; parity and previous lactation 305M were offered to the model as independent, potential explanatory variables. The 305M model was evaluated with, and without the previous lactation’s 305M variable, 1st lactation animals were not included in the model with previous lactation 305M.

Independent variables and their respective interaction terms were manually removed by backward stepwise elimination if biologically unimportant or statistically insignificant (p > 0.05).

Repeated measures were accounted for with a heterogeneous Toeplitz covariance structure for the resolution of ketosis, BHB concentration, milk fat percentage and LS outcomes, which was selected based on providing the lowest Akaike’s Information Criterion for the final model. Repeated measures were accounted for with an unstructured covariance structure for milk yield, ECM, and milk protein percentage, also selected based on providing the lowest Akaike’s Information Criterion for the final model. All final models included the variables treatment and parity nested within cow as repeated measures.

The ANOVA assumptions were then assessed by conducting analyses of the residuals through the UNIVARIATE procedure in SAS. Examination of the kurtosis, skewness, extreme observations, residual plots, and tests for normality (Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling) determined whether the ANOVA assumptions were met. Based on the residual analyses, outcome variables were transformed and outliers were examined, if necessary, outliers were removed if they appeared to be a reporting error.

Treatment means, standard error, and P-values are reported from these analyses. Medians are presented instead of means for the effect of treatment on BHB concentration due to the log
transformation performed on the model. Differences in data are considered significant at $P \leq 0.05$, and considered a trend at $P \leq 0.10$.

**RESULTS**

A total of 104 cows tested positive for ketosis during the screening period and were deemed fit to enter the trial, and 94 cows completed the full 3-wk trial period. There were 55 animals enrolled in the ODM treatment group, of which 21 were primiparous cows, and 34 were multiparous cows; 5 cows from this group did not complete the trial, 4 of which left the trial within the first wk. There were 49 animals enrolled in the TDM treatment group, 18 of which were primiparous cows, and 31 were multiparous cows; 5 cows from this group did not complete the trial, 2 of which left the trial within the first wk (see Figure 3.1). All 6 cows that left the trial within the first wk were excluded from the data analysis. The mean DIM at enrolment was 5.3 for the ODM cows (5.3 for both primiparous and multiparous) and 4.9 for the TDM cows (4.3 for primiparous, 5.2 for multiparous). The mean blood BHB concentration at enrolment was 1.6 for the ODM cows (1.5 for primiparous, 1.6 for multiparous) and 1.6 for the TDM cows (1.6 for primiparous and multiparous).

Health events occurring within the first 60 DIM are described in Table 3.5. Forty-five percent (21/47) of the ODM cows and 30% (13/43) of the TDM cows were pregnant after the first insemination.

*Resolution of Ketosis*

The final model evaluating the effect of the treatment on the resolution of ketosis required the “day of trial variable” to be included as a quadratic. Interaction of DOT by treatment
by parity, as well was \((\text{DOT})^2\) by treatment by parity were detected \((P=0.0026, P=0.0029\) respectively). Therefore the treatment effect varied by both parity and DOT, results were stratified by parity.

ODM primiparous cows were less likely to have blood BHB \(\geq 1.2\) mmol/L from d 2 to d 18 of trial, and tended to be less likely to have blood BHB \(\geq 1.2\) mmol/L on d 21 than the TDM primiparous cows (Table 3.6). ODM multiparous cows were less likely to have blood BHB \(\geq 1.2\) mmol/L for the entire duration of the trial (from d 1 to d 21, Table 3.6). In both lactation groups, the difference between the two treatment groups persisted beyond the treatment period (after d 14). The curves shown in Figure 3.2 show the effect of the MF treatment during the 2-wk treatment period and 1-wk follow-up period on ketosis status.

As explained in the methods, cows were eligible to receive additional 5-d PG treatments if they were ketotic on the 9th day of trial, or later, and cows could be given a maximum of 3 re-treatments (solely based on time). Overall, TDM received more additional 5-d PG treatments than ODM cows. Sixty-four percent (30/47) of the TDM cows required one additional treatment, 50\% (22/44) required a 2nd, and 37\% (16/43) required a 3rd additional treatment of PG. While 39\% (20/51) of the ODM cows required one additional treatment, and 14\% required a 2nd additional treatment, none of the ODM cows required a 3rd additional treatment. The distribution of the timing of the 1st and 2nd additional treatments are shown in Figures 3.3 and 3.4, all 3rd re-treatments began on the 21st day of trial.

**Blood BHB Concentration**

A log transformation was required for the final model, and the “day of trial” variable needed to be included as a quadratic. There was an effect of treatment on blood BHB
concentration ($P=0.002$). There was an interaction of DOT by treatment by parity and the quadratic for DOT by treatment by parity. Therefore the treatment effect varied by both parity and DOT.

Following trial enrolment, blood BHB concentrations were lower in ODM primiparous cows compared to TDM primiparous cows from d 1 to 18 of trial (Table 3.7). Blood BHB concentrations were also lower in ODM multiparous cows compared to TDM multiparous cows through the full trial period. Primiparous animals on ODM maintained significantly lower BHB concentrations than TDM for 4 d after the ODM treatment ended, and ODM multiparous cows maintained lower BHB concentrations than TDM for the week following ODM treatment. Figure 3.5 shows the median BHB concentrations for each treatment group over the 14 d treatment and 7 d close observation period.

*Milk Production and Components*

Over the course of the trial, there were DHI-collected samples from all 104 cows enrolled onto the trial, and 93 of these cows remained in the lactating herd for the entire 15-wk monitoring period post-calving. Six of the 104 trial cows were completely excluded from the milk production and milk component analyses, as they were a part of the trial for 4 d or less before they were removed.

There was an interaction of treatment with wk of trial (following enrolment) for milk yield ($P=0.001$). Cows in the ODM treatment group produced less milk than cows in the TDM treatment group for all 15-wk of lactation following trial enrolment (Table 3.8). The difference in milk yield between treatment groups progressively decreased over the 15-wk period, beginning with ODM cows producing 9.1 kg/d less milk than TDM cows at wk 1, and ending with ODM
cows producing about 5 kg/d less milk than TDM cows by wk 15. Figure 3.6 shows the mean milk yields between treatment groups over the 15-wk period.

There were no interactions with treatment in the models for fat percentage, protein percentage, or LS. Milk fat percentage for ODM cows tended to be 2.9% greater than TDM cows \( (P=0.086) \) for the 15-wk period (Table 3.9). Protein percentage in ODM cows was 3.6% greater than TDM cows \( (P=0.003) \) during the 15-wk observation period (Table 3.9). Parity had no effect on milk fat percentage or protein percentage \( (P=0.85 \& P=0.53 \text{ respectively}) \). ODM cows tended to have LS 0.51 points greater than TDM cows \( (P=0.062, \text{ Table 3.9}) \). LS in both treatment groups were still well below the threshold for subclinical mastitis (LS = 4 or SCC= 200,000, Duffield et al. 2009).

There was an interaction of treatment with wk of trial (following enrolment) for energy-corrected milk yield \( (P<0.001) \) in the model. Cows in the ODM treatment group produced less ECM than cows in the TDM treatment group for all 15-wk of lactation following trial enrolment (Table 3.10). The largest difference in ECM yield between groups occurred in wk 1, with ODM cows producing 11.3kg/d less ECM than TDM cows \( (P<0.0001) \). Similar to the trend from milk yield, the difference in ECM decreased over the 15-wk period, with the smallest difference in ECM yield occurring at wk 15, where ODM cows produced 2.4 kg/d less ECM than TDM cows. Figure 3.7 shows the weekly ECM yields (kg/d) over the 15-wk following trial enrolment.

The 305M data comprised of 85 data points collected from all cows that remained in the herd past 200 DIM, 34 of which came from primiparous cows. The 305M outcome was modelled with and without the previous 305 milk data as an explanatory variable. ODM cows tended to produce 537 kg (or 5%) less milk over a lactation than TDM cows \( (10 254 \pm 203.8 \text{ kg and } 10 791 \pm 206.1 \text{ kg for ODM and TDM cows respectively, } P=0.07) \) when the previous 305M were
excluded from the model. When the previous 305M were included in the model, ODM cows produced 360 kg less milk than TDM cows, but the difference was not significant (10 910 ± 245.6 kg and 11 220 ± 250.1 kg for ODM and TDM cows respectively, \( P=0.31 \)).

Dry Matter Intake

Of the 104 cows enrolled on the trial 92 cows were included in the feed intake analyses. Six cows were excluded because they were enrolled onto the trial for 4 d or less, 6 more cows were excluded because they were missing feed data for at least the first 5 d of trial. In the evaluation of the DMI, it was noticed that 8 cows in the ODM group were restricted from consuming the full 3 kg (as-fed) of pellets at the robot and were limited to 1.5 kg of pellets instead for the first 7 to 14 d of trial. This occurred due to a minor programming error when these cows were moved over to the robot. The DMI and milk production data were analyzed with and without these cows in the dataset; we found that their removal did not affect the models, so they were kept in the analyses.

Dry matter intake did not differ \( (P = 0.16) \) between ODM (17.8 ± 0.37 kg/d) and TDM (17.6 ± 0.39 kg/d) cows during the 21 d trial, but there was an interaction for treatment with DOT \( (P=0.008, \text{Figure 3.8}) \). Dry matter intake did not differ from d 0 to 18 of trial, but there was a tendency for ODM cows to consume approximately 1 to 1.2 kg more feed (dry matter) than TDM cows on d 19, 20, and 21 (Table 3.11).

DISCUSSION

Our approach in implementing ODM was to improve the metabolic status of ketotic dairy cows in early lactation in an intensive farming environment. This is in contrast to most other
studies examining ODM where ODM has been tested as a preventative measure for metabolic stress, or been focused on the effects from milking once daily as a labour-saving measure in an extensive farming system where less emphasis is placed on milk production per cow (Clark et al., 2006; Stelwagen et al., 2013).

In this study, the hypothesized effects of reducing MF as an adjunct treatment for ketosis on ketosis resolution were confirmed. However, reducing MF as an adjunct treatment for ketosis did not maintain milk yield. The findings of this study support the hypothesis that reducing MF from 2 to 1 time per day, in conjunction with a 5 d treatment of PG *per os* (as a treatment for ketosis), more effectively decreases blood BHB concentrations below 1.2 mmol/L compared to treating cows with PG alone.

The effect of ODM on reducing ketotic tests was observed almost immediately. By the 6\textsuperscript{th} DOT, ODM primiparous cows were < 0.001 times as likely to have blood BHB ≥ 1.2 mmol/L compared to TDM primiparous cows. ODM multiparous cows were 0.247 times as likely to have blood BHB ≥ 1.2 mmol/L compared to TDM multiparous cows by the 6\textsuperscript{th} DOT. Most of the cows that decreased blood BHB below 1.2 mmol/L had done-so by the 6\textsuperscript{th} DOT, indicating 2 wk of treatment is not necessary to gain the metabolic benefits of ODM and could possibly be implemented for the same duration as PG treatment. The PG retreatment data supports the efficacy of the ODM treatment on resolving ketosis (or reducing the amount of ketotic tests), and shows the inadequacy of extended PG treatments on resolving ketosis. However, this study lacked a negative control group. Consequently, it was not possible to compare the resolution of ketosis in cows without administration of PG.

Cows milked once daily during the treatment period had markedly lower BHB concentrations, and were more likely to have blood BHB < 1.2 mmol/L than cows milked twice
daily, both during the treatment period, and for the wk following treatment. Research conducted by Rémon et al. (1999), McNamara et al. (2008), Loiselle et al. (2009), Schlamberger et al. (2010), and Kay et al. (2013) observed that healthy cows milked 1x/d for a 1 to 4 wk period in early lactation, had lower blood BHB concentrations than their TDM counterparts. Loiselle et al. (2009) observed blood BHB concentrations in ODM cows to persist at a lesser level than TDM cows for an additional wk post treatment. These findings are analogous to studies where researchers have evaluated the metabolic effects of incompletely milking cows within the first 5 d of lactation (Carbonneau et al., 2012; Morin et al., 2018). These researchers have similarly reported that their method for decreasing energy demand by partially milking cows reduced the incidence of hyperketonemia and reduced blood BHB concentrations compared to conventionally milking cows (Carbonneau et al., 2012; Morin et al., 2018).

There was no analysis of blood NEFA or glucose in the current study, but short-term early lactation studies by Rémon et al. (1999), McNamara et al. (2008), Loiselle et al. (2009), Schlamberger et al. (2010), Kay et al. (2013), and Phyn et al. (2014) observed higher blood glucose and lower blood NEFA concentrations in healthy ODM cows compared to TDM cows. Carbonneau et al. (2012) observed the same effect when cows were incompletely milked for the first 5 d of lactation. This provides further support to the hypothesis that reducing MF can improve energy balance.

Milking cows 1x/d for a period of 2 wk upon ketosis detection in early lactation reduced milk yield during ODM, and this effect persisted after cows were returned to TDM. Daily ECM yield was reduced by 24.6% for ODM cows compared to TDM cows during the 2 wk treatment period. The sudden decrease in milk yield during ODM is consistent with previous studies that have compared once and twice daily milking, regardless of duration of ODM. Our observed
decrease in milk yield of 24% falls in the middle of the 13 to 40% range that has been reported in the literature for short-term, early lactation studies (Stelwagen & Knight, 1997; Rémond et al., 1999; Davis et al., 1999; Patton et al., 2006; Rémond & Pomiès, 2007; Loiselle et al., 2009; Schlamberger et al., 2010; O’Driscoll et al., 2012; Kay et al., 2013; Stelwagen et al., 2013; Phyn et al., 2014).

The weekly milk yields along with the results from the 305M analyses indicate that a 2-wk period of ODM in early lactation has a negative carry-over effect on milk production lasting at least 13 wk. Cows in the ODM group produced 17.8% less milk, and 14.4% less ECM than cows in the TDM treatment group over the 13 wk after treatment. Cows in the ODM group gradually increased their milk yield by 5.7 kg/d and ECM yield by 6.2 kg/d by wk 15 (13th wk post-treatment), but did not achieve the same yields as TDM cows by the end of the 13 wk. The steady increase in milk production over the 13 wk suggests that the ODM cows had not yet reached peak yield. TDM cows increased their daily milk yield by 1.5 kg/d, but decreased their ECM by 2.7 kg/d by the end of the 13-wk period. The tendency for an only 5% decrease in the 305M found in our study supports that ODM cows continued to increase their milk yield after the 13-wk of observation post-treatment. The 305M data that included previous 305-d milk yield as a variable reported a smaller difference in 305M between groups, but was not significant. This was likely due to inadequate power due to the smaller sample size for the 305M analysis. It is plausible that these ODM cows would have eventually “caught up” to the TDM cows later in lactation. However, this prolonged duration of monitoring was not possible in this trial.

The published short term, early lactation ODM trials all have very different study designs, from treatment durations and observation periods, to management systems (pasture, pasture and supplement, or TMR), and different breeds. These differences help to explain the
variability in milk production losses between trials, and make it difficult to compare our cumulative milk yield losses. However, the results from the literature and from our trial all encourage a shorter duration of ODM for reduced risk of prolonged milk yield reductions.

ODM cows had higher milk protein percentage than TDM cows over the full 15 wk of observation in our trial; this outcome is consistent with many other short-term ODM trials (Clark et al., 2006; Loiselle et al., 2009; Schlamberger et al., 2010; Kay et al., 2013; Phyn et al., 2014). ODM cows tended to have higher fat percentage than TDM cows over the 15 wk of observation in our trial; Rémond et al. (1999), Rémond & Pomiès (2007), and Loiselle et al. (2009) report similar results. The reasoning for greater fat content in milk of ODM is not clear, but is hypothesized to simply be due to a concentration effect associated with lower milk volume (Stelwagen et al., 2013).

Prior to our research, there had not been any early lactation trials that had implemented two wk of ODM. Studies of longer duration (3-4 wk) report long-term milk yield losses of 6 to 16% and long-term ECM yield losses of 5 to 8% (Patton et al., 2006; McNamara et al., 2008; Schlamberger et al., 2010; Kay et al., 2013; Phyn et al., 2014). Of the few studies that tested ODM over 1 wk, Rémond & Pomiès, (2007) found no differences in long term milk yield, while Loiselle et al. (2009) observed an 8% decrease in milk production in ODM cows, but when milk yield was adjusted for components, the ECM yields were similar for ODM and TDM cows. Additionally, researchers that examined 5 d of incomplete milking protocols found no difference in long term milk yield and ECM yield between milking groups (Carbonneau et al., 2012; Krug et al., 2018). Due to this information, along with the negative effect ketosis has on milk production, we were unsure of the magnitude of the impact (if any) 2 wk of ODM would have on long term milk production. The goal of this trial was to implement a treatment duration that was
sufficient to ensure that it was possible to measure an effect, if it existed, on ketosis and blood BHB concentration, and thus ketosis persistence, and as a result, we opted for a 2-wk treatment period instead of one. Given the rapid effect of ODM on both reducing ketones and milk yield in this study, it would be valuable for subsequent research to study a shorter duration of ODM treatment, restricting it to perhaps 5 or 7 d.

Our study observed a tendency for greater LS in ODM cows compared to TDM cows over the 15-wk period (both during treatment and post-treatment). Stelwagen et al. (2013) reported somatic cell counts/LS are consistently elevated during ODM compared to TDM, but other researchers who have examined the possible long-term impact of short-term ODM on SCC all report no differences between ODM and TDM cows after treatment (Rémond et al., 1999; Rémond & Pomiès 2007; Loiselle et al., 2009; Schlamberger et al., 2010; Kay et al., 2013; Phyn et al., 2014). However, the LS increase observed was not concerning, as the LS in both treatments were well below the mastitis threshold of LS ≥ 4 (or SCC ≥ 200,000). High-producing cows milked once daily are more likely to leak outside of the parlor (Gleeson et al., 2007; Tucker et al., 2007). Preventing incompletely milked cows from returning to the robot until the next designated milking may have put some of the ODM cows at a greater risk for milk leakage and/or increased udder distension. Udder tension has been shown to cause a mild inflammatory response and an increase in neutrophils in the milk (Stelwagen and Lacy-Hulbert, 1996; Stelwagen et al., 2011). Additionally, milk leakage creates a greater risk for bacterial challenge in the teat canals, but these factors don’t explain why ODM cows maintained higher LS well past treatment.

There was no difference between the DMI of ODM and TDM cows during the experimental period and there was no difference between the DMI of ODM and TDM cows in
the first 4 d after trial, but there was a tendency for ODM cows to consume 1 to 1.2 kg more feed (DM) in the last 3 d of observation (d 19 to 21). While a portion of the ODM cows were restricted from 1.5 kg of pellets for the first 7 to 14 d of trial, these cows appeared to have compensated for this loss through TMR intake instead. Loiselle et al. (2009) and Rémond et al. (2004) also report no difference in DMI between ODM and TDM cows in early lactation during their respective experimental periods, and Patton et al. (2006) didn’t detect a difference in DMI between ODM and TrDM cows in early lactation either.

The tendency for increased DMI in ODM cows in the last 3 d of observation may have occurred, because, unbeknownst to us, during week 3 of trial, the robot allowed previously ODM cows possible access to 3 kg of pellets at each milking, with no maximum limit to pellet allowance. TDM cows were not given this access, and instead were only allowed 1.5 kg of pellets at each milking in week 3, and most were prevented from consuming more than 3 kg of pellets per day. This may have been associated with the tendency for ODM cows to consume more grain in the last 3 d of observation, but it is also possible that ODM cows consumed less PMR during this period. We know that ketosis (or elevated levels of BHB) can cause a decrease in appetite (Andersson, 1988; Duffield, 2000). A larger proportion of cows in the TDM group had ketosis in the last wk of the trial compared to the ODM group. The higher levels of BHB in the TDM group may have slightly suppressed cow appetite in the TDM group, which may also explain why we observed this tendency.

Cows experience a drastic increase in energy demand in early lactation, which cannot be filled through feed consumption alone (Duffield et al., 2009; LeBlanc, 2010). As all of the cows in our trial were ketotic when they began treatment, we know that they hadn’t successfully adapted to NEB, and were failing to meet their metabolic needs. Cows in the TDM group were
consuming an average of 17.6 kg of dry matter feed/d, yet it is evident through their blood BHB concentrations that they were still poorly adapting to NEB. The likely reason we did not observe a decrease in DMI due to ODM is because cows in the ODM group still required all of the feed they were consuming to meet their metabolic demands. Reducing MF from two milkings per day to one milking per day, was associated with the decrease in metabolic demand for milk production, which likely supported a more positive energy balance.

One limitation to this study was the sample size. While our power calculations recommended a population of 120 cows for the trial, 104 cows were enrolled due to both time and researcher constraints. However, despite not meeting the estimated sample size, the efficacy of ODM was more effective than predicted in terms of percent reduction on ketosis, and thus the actual sample size was adequate for assessing the primary outcome. This trial did not have sufficient power to detect differences in health events or reproductive performance. A substantially larger study size in the future would allow for more definitive conclusions on the impact of ODM on some of the secondary outcomes, such as milk components, 305M, DMI, disease outcomes, and reproductive performance.

CONCLUSION

In this experiment, milking frequency was reduced from 2 milkings per day to 1 milking per day for a 2-wk period as an adjunct treatment for ketosis. The results indicate that this treatment approach was more successful in reducing ketotic tests and decreasing BHB concentrations than from treating with PG alone. However, ODM was associated with a reduction in milk yield that carried past the treatment period. Future research should investigate a shortened milk reduction treatment period to determine whether the shorter period can provide
positive effects of ketosis resolution while reducing (or eliminating) the long-term negative reduction on milk production.
Table 3.1 Diets fed to cows enrolled in a randomized controlled trial reducing milking frequency as an adjunct treatment for ketosis at the LRIC Dairy Research Facility

<table>
<thead>
<tr>
<th>Item</th>
<th>Robot Pen TMR</th>
<th>Fresh cow TMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients, % of diet DM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Straw, long chop</td>
<td>2.06</td>
<td>3.63</td>
</tr>
<tr>
<td>Haylage</td>
<td>37.54</td>
<td>32.02</td>
</tr>
<tr>
<td>Corn Silage</td>
<td>37.82</td>
<td>31.4</td>
</tr>
<tr>
<td>High moisture corn</td>
<td>10.28</td>
<td>19.37</td>
</tr>
<tr>
<td>Dairy supplement mix*</td>
<td>2.27</td>
<td>2.3</td>
</tr>
<tr>
<td>Soy plus*</td>
<td>6.28</td>
<td>5.75</td>
</tr>
<tr>
<td>Soybean meal*</td>
<td>2.47</td>
<td>3.64</td>
</tr>
<tr>
<td>Canola*</td>
<td>0.84</td>
<td>1.33</td>
</tr>
<tr>
<td>Fish meal (herring)*</td>
<td>0.44</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Formulated Composition (DM%)               |               |               |
| Forage                                    | 77.44         | 67.05         |
| ME (%Rqd)                                 | 104.49        | 104.73        |
| NFC                                       | 38.87         | 41.65         |
| Starch                                    | 21.98         | 25.84         |
| Sugar                                     | 1.91          | 2.1           |
| ADF                                       | 22.1          | 20.33         |
| NDF                                       | 33.64         | 31.44         |
| MP Supply (g)                             | 2157.71       | 2641.52       |
| CP                                        | 16.28         | 16.1          |
| SP (%CP)                                  | 48.15         | 46.68         |
| RDP                                       | 10.25         | 9.79          |
| Ca                                        | 0.8           | 0.8           |
| P                                         | 0.4           | 0.4           |
| Mg                                        | 0.35          | 0.35          |
| K                                         | 1.26          | 1.25          |
| NEL (Mcal/kg DM)                          | 1.62          | 1.65          |

*Included in the dairy supplement mix, see Table 3.2 for the supplement break-down
Table 3.2 Formulated Ingredient and nutrient composition of dairy supplements fed to cows enrolled in a randomized controlled trial reducing milking frequency as an adjunct treatment for ketosis at the LRIC Dairy Research Facility

<table>
<thead>
<tr>
<th>Item</th>
<th>Dairy Supplement</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Robot Pen</td>
<td>Fresh cow pens</td>
<td></td>
</tr>
<tr>
<td>Ingredients, % of mix DM</td>
<td>51.06</td>
<td>42.3</td>
<td></td>
</tr>
<tr>
<td>Soy plus</td>
<td>20.06</td>
<td>26.8</td>
<td></td>
</tr>
<tr>
<td>Soybean meal</td>
<td>6.85</td>
<td>9.81</td>
<td></td>
</tr>
<tr>
<td>Canola</td>
<td>4.67</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>Sodium Sesquicarbonate</td>
<td>4.13</td>
<td>3.37</td>
<td></td>
</tr>
<tr>
<td>Fish meal (herring)</td>
<td>3.61</td>
<td>4.13</td>
<td></td>
</tr>
<tr>
<td>Monocalcium Phosphate</td>
<td>3.02</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Diamond V Yeast XP</td>
<td>2.03</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Magnesium Oxide</td>
<td>1.24</td>
<td>1.31</td>
<td></td>
</tr>
<tr>
<td>Tallow</td>
<td>1.1</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Vitamin-mineral micro-premix</td>
<td>0.9</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>Calcium Carbonate</td>
<td>0.53</td>
<td>2.03</td>
<td></td>
</tr>
<tr>
<td>Metasmart (Methionine)</td>
<td>0.37</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>Sulphur (99.5%) granular</td>
<td>0.35</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Rumensin/Coban</td>
<td>0.05</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Selplex 2000 (Selenium Yeast)</td>
<td>0.03</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>DCAD+ (Potassium Carbonate)</td>
<td>-</td>
<td>0.76</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3 Nutrient composition of the robot pellets fed to cows housed in the robotic milker pen and enrolled in a randomized controlled trial reducing milking frequency as an adjunct treatment for ketosis at the LRIC Dairy Research Facility

<table>
<thead>
<tr>
<th>Item</th>
<th>Formulated Composition (%DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM%</td>
<td>88.98</td>
</tr>
<tr>
<td>NEL (Mcal/Kg DM)</td>
<td>1.48</td>
</tr>
<tr>
<td>CP</td>
<td>18.16</td>
</tr>
<tr>
<td>SP</td>
<td>5.08</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.93</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.24</td>
</tr>
<tr>
<td>Crude fat</td>
<td>2.02</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>9.71</td>
</tr>
<tr>
<td>ADF</td>
<td>12.73</td>
</tr>
<tr>
<td>NGF</td>
<td>24.44</td>
</tr>
<tr>
<td>Ca</td>
<td>0.58</td>
</tr>
<tr>
<td>P</td>
<td>0.46</td>
</tr>
<tr>
<td>Ash</td>
<td>5.53</td>
</tr>
<tr>
<td>Na</td>
<td>0.22</td>
</tr>
<tr>
<td>Cl</td>
<td>0.35</td>
</tr>
<tr>
<td>K</td>
<td>1.04</td>
</tr>
<tr>
<td>Mg</td>
<td>0.38</td>
</tr>
<tr>
<td>S</td>
<td>0.24</td>
</tr>
</tbody>
</table>
Table 3.4 Treatment trial blood sampling schedule to measure beta-hydroxybutyrate. D 0 = detection of ketosis and start of randomized treatment (once daily milking, or twice daily milking).

| Days from first diagnosis of ketosis | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|-------------------------------------|---|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Sample day (X)                      | X | X | X | X | X | X | X | X | X | X | X  | X  | X  | X  | X  | X  | X  | X  | X  | X  | X  | X  |
**Table 3.5** Disease events occurring within the first 3 to 60 DIM for ketotic cows milked once (ODM) and twice (TDM) daily in early lactation

<table>
<thead>
<tr>
<th>Clinical Disease</th>
<th>Number of cows</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ODM (n=51)</td>
</tr>
<tr>
<td>Retained placenta</td>
<td>4</td>
</tr>
<tr>
<td>Milk fever</td>
<td>0</td>
</tr>
<tr>
<td>Mastitis</td>
<td>7</td>
</tr>
<tr>
<td>Displaced abomasum</td>
<td>1</td>
</tr>
<tr>
<td>Metritis</td>
<td>2</td>
</tr>
<tr>
<td>Lameness</td>
<td>4</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>1</td>
</tr>
<tr>
<td>Early cull (sold or died)</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 3.6 Proportion of cows with ketosis in a randomized controlled trial where cows were milked twice daily (TDM, control), or once daily (ODM) for two wk, and twice daily thereafter, following the detection of ketosis; as determined by the binary logistic regression model. Day of trial represents the number of days post-ketosis detection.

<table>
<thead>
<tr>
<th>Parity</th>
<th>Day of trial</th>
<th>ODM</th>
<th>TDM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.50</td>
<td>0.53</td>
<td>0.78</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>0.067</td>
<td>0.48</td>
<td>0.0037</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>0.0067</td>
<td>0.43</td>
<td>0.0015</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>2.7x10^-5</td>
<td>0.32</td>
<td>0.0012</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>1.10x10^-6</td>
<td>0.26</td>
<td>0.0011</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>4.7x10^-7</td>
<td>0.22</td>
<td>0.001</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>2.1x10^-6</td>
<td>0.22</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1</td>
<td>18</td>
<td>9.6x10^-5</td>
<td>0.25</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1</td>
<td>21</td>
<td>0.044</td>
<td>0.30</td>
<td>0.054</td>
</tr>
<tr>
<td>2+</td>
<td>1</td>
<td>0.39</td>
<td>0.58</td>
<td>0.048</td>
</tr>
<tr>
<td>2+</td>
<td>2</td>
<td>0.36</td>
<td>0.58</td>
<td>0.009</td>
</tr>
<tr>
<td>2+</td>
<td>3</td>
<td>0.33</td>
<td>0.59</td>
<td>0.0013</td>
</tr>
<tr>
<td>2+</td>
<td>6</td>
<td>0.27</td>
<td>0.60</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2+</td>
<td>9</td>
<td>0.24</td>
<td>0.62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2+</td>
<td>12</td>
<td>0.22</td>
<td>0.63</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2+</td>
<td>15</td>
<td>0.21</td>
<td>0.64</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2+</td>
<td>18</td>
<td>0.22</td>
<td>0.65</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2+</td>
<td>21</td>
<td>0.24</td>
<td>0.66</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Day of trial = 0 was excluded from the model as all animals were ketotic on d=0
There were 3-way interactions for treatment by parity by day of trial ($P=0.0026$) & treatment by parity by day of trial ($P=0.0029$)
Table 3.7 Blood BHB concentrations over the 21-d trial period for Holstein cows in a randomized controlled trial where cows were milked twice daily (TDM, control), or once daily (ODM) for two wk, and twice daily thereafter, following the detection of ketosis.

<table>
<thead>
<tr>
<th>Parity</th>
<th>Day of Trial</th>
<th>Median BHB Concentration (mmol/L)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>ODM 0.92  TDM 1.22</td>
<td>0.013</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>ODM 0.84  TDM 1.16</td>
<td>0.006</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>ODM 0.78  TDM 1.10</td>
<td>0.004</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>ODM 0.64  TDM 0.96</td>
<td>0.0051</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>ODM 0.57  TDM 0.87</td>
<td>0.009</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>ODM 0.54  TDM 0.82</td>
<td>0.014</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>ODM 0.55  TDM 0.81</td>
<td>0.021</td>
</tr>
<tr>
<td>1</td>
<td>18</td>
<td>ODM 0.59  TDM 0.83</td>
<td>0.045</td>
</tr>
<tr>
<td>1</td>
<td>21</td>
<td>ODM 0.69  TDM 0.89</td>
<td>0.169</td>
</tr>
<tr>
<td>2+</td>
<td>1</td>
<td>ODM 1.01  TDM 1.40</td>
<td>0.0045</td>
</tr>
<tr>
<td>2+</td>
<td>2</td>
<td>ODM 0.97  TDM 1.43</td>
<td>0.001</td>
</tr>
<tr>
<td>2+</td>
<td>3</td>
<td>ODM 0.94  TDM 1.45</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2+</td>
<td>6</td>
<td>ODM 0.87  TDM 1.52</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2+</td>
<td>9</td>
<td>ODM 0.82  TDM 1.58</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2+</td>
<td>12</td>
<td>ODM 0.79  TDM 1.62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2+</td>
<td>15</td>
<td>ODM 0.78  TDM 1.65</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2+</td>
<td>18</td>
<td>ODM 0.80  TDM 1.67</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2+</td>
<td>21</td>
<td>ODM 0.83  TDM 1.67</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

There were interactions for day of trial by treatment ($P=0.017$), day of trial by parity ($P<0.001$), day of trial^2 by treatment ($P=0.04$), & day of trial^2 by parity ($P=0.003$)
Table 3.8 Milk yield (LSM ± SE) for Holstein cows in a randomized controlled trial where cows were milked twice daily (TDM, control), or once daily (ODM) for two wk, and twice daily thereafter, over a period of fifteen wk following the detection of ketosis/trial enrolment.

<table>
<thead>
<tr>
<th>Week</th>
<th>ODM (n=51)</th>
<th>TDM (n=47)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27.9 ± 0.78</td>
<td>37.0 ± 0.82</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>28.3 ± 0.75</td>
<td>37.1 ± 0.78</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3</td>
<td>28.7 ± 0.71</td>
<td>37.2 ± 0.74</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>29.1 ± 0.68</td>
<td>37.4 ± 0.71</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5</td>
<td>29.5 ± 0.65</td>
<td>37.5 ± 0.68</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6</td>
<td>29.9 ± 0.63</td>
<td>37.6 ± 0.66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>7</td>
<td>30.3 ± 0.61</td>
<td>37.7 ± 0.64</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>8</td>
<td>30.8 ± 0.60</td>
<td>37.8 ± 0.63</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>9</td>
<td>31.2 ± 0.60</td>
<td>37.9 ± 0.62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10</td>
<td>31.6 ± 0.60</td>
<td>38.0 ± 0.62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>11</td>
<td>32.0 ± 0.60</td>
<td>38.1 ± 0.63</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>12</td>
<td>32.4 ± 0.62</td>
<td>38.2 ± 0.64</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>13</td>
<td>32.8 ± 0.63</td>
<td>38.3 ± 0.66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>14</td>
<td>33.2 ± 0.66</td>
<td>38.4 ± 0.68</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>15</td>
<td>33.6 ± 0.68</td>
<td>38.6 ± 0.71</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

1 Sample sizes at wk=1
2 At wk 15, n=43 for ODM and n=42 for TDM
There was an interaction of week by treatment (P=0.001)
Table 3.9 Milk fat percent, protein percent, and linear score (LSM ± SE) for Holstein cows in a randomized controlled trial where cows were milked twice daily (TDM, control), or once daily (ODM) for two wk, and twice daily thereafter over a period of fifteen wk following the detection of ketosis/trial enrolment.

| Item         | ODM (n=51)
|--------------|-----------
| Fat (%)      | 4.30 ± 0.052 |
| Protein (%)  | 3.15 ± 0.025 |
| Linear Score | 3.21 ± 0.19 |
|              | TDM (n=47)
| Fat (%)      | 4.18 ± 0.054 |
| Protein (%)  | 3.04 ± 0.026 |
| Linear Score | 2.70 ± 0.19 |
| P-value      | 0.086 0.003 0.062 |

1 Sample sizes at wk=1
2 At wk 15, n=43 for ODM and n=42 for TDM
Table 3.10 Energy-corrected milk yield (ECM) (LSM ± SE) for Holstein cows in a randomized controlled trial where cows were milked twice daily (TDM, control), or once daily (ODM) for two wk, and twice daily thereafter, over a period of fifteen wk following the detection of ketosis/trial enrolment.

<table>
<thead>
<tr>
<th>Week</th>
<th>ECM yield (kg/d)</th>
<th>ODM (n=51)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>TDM (n=47)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>33.5 ± 0.99</td>
<td>44.8 ± 1.02</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>33.9 ± 0.94</td>
<td>44.6 ± 0.96</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>34.4 ± 0.89</td>
<td>44.4 ± 0.91</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>34.8 ± 0.84</td>
<td>44.2 ± 0.87</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>35.3 ± 0.81</td>
<td>44.0 ± 0.83</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>35.7 ± 0.77</td>
<td>43.8 ± 0.80</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>36.2 ± 0.75</td>
<td>43.6 ± 0.77</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>36.6 ± 0.73</td>
<td>43.4 ± 0.75</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>37.0 ± 0.71</td>
<td>43.2 ± 0.74</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>37.5 ± 0.71</td>
<td>43.0 ± 0.74</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>37.9 ± 0.72</td>
<td>42.9 ± 0.74</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>38.4 ± 0.73</td>
<td>42.7 ± 0.76</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>38.8 ± 0.75</td>
<td>42.5 ± 0.78</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>39.3 ± 0.78</td>
<td>42.3 ± 0.81</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>39.7 ± 0.81</td>
<td>42.1 ± 0.84</td>
<td>0.043</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Sample sizes at wk=1

*At wk 15, n=43 for ODM and n=42 for TDM

There was an interaction of week by treatment ($P<0.001$)
Table 3.11 Daily dry matter intake (DMI) (LSM ± SE) over the 21-d trial period for Holstein cows in a randomized controlled trial where cows were milked twice daily (TDM, control), or once daily (ODM) for two wk, and twice daily thereafter, following the detection of ketosis.

<table>
<thead>
<tr>
<th>Day of trial</th>
<th>ODM (n=48)</th>
<th>TDM (n=44)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.52 ± 0.45</td>
<td>15.42 ± 0.47</td>
<td>0.16</td>
</tr>
<tr>
<td>1</td>
<td>14.82 ± 0.43</td>
<td>15.63 ± 0.45</td>
<td>0.20</td>
</tr>
<tr>
<td>2</td>
<td>15.13 ± 0.42</td>
<td>15.83 ± 0.44</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>15.44 ± 0.41</td>
<td>16.03 ± 0.42</td>
<td>0.31</td>
</tr>
<tr>
<td>4</td>
<td>15.75 ± 0.39</td>
<td>16.24 ± 0.41</td>
<td>0.39</td>
</tr>
<tr>
<td>5</td>
<td>16.06 ± 0.39</td>
<td>16.44 ± 0.40</td>
<td>0.49</td>
</tr>
<tr>
<td>6</td>
<td>16.37 ± 0.38</td>
<td>16.65 ± 0.40</td>
<td>0.60</td>
</tr>
<tr>
<td>7</td>
<td>16.67 ± 0.37</td>
<td>16.85 ± 0.39</td>
<td>0.74</td>
</tr>
<tr>
<td>8</td>
<td>16.98 ± 0.37</td>
<td>17.06 ± 0.39</td>
<td>0.89</td>
</tr>
<tr>
<td>9</td>
<td>17.29 ± 0.37</td>
<td>17.26 ± 0.39</td>
<td>0.96</td>
</tr>
<tr>
<td>10</td>
<td>17.60 ± 0.37</td>
<td>17.47 ± 0.39</td>
<td>0.80</td>
</tr>
<tr>
<td>11</td>
<td>17.91 ± 0.37</td>
<td>17.67 ± 0.39</td>
<td>0.66</td>
</tr>
<tr>
<td>12</td>
<td>18.22 ± 0.38</td>
<td>17.87 ± 0.39</td>
<td>0.53</td>
</tr>
<tr>
<td>13</td>
<td>18.52 ± 0.38</td>
<td>18.08 ± 0.40</td>
<td>0.42</td>
</tr>
<tr>
<td>14</td>
<td>18.83 ± 0.39</td>
<td>18.28 ± 0.41</td>
<td>0.33</td>
</tr>
<tr>
<td>15</td>
<td>19.14 ± 0.40</td>
<td>18.49 ± 0.42</td>
<td>0.26</td>
</tr>
<tr>
<td>16</td>
<td>19.45 ± 0.41</td>
<td>18.69 ± 0.43</td>
<td>0.20</td>
</tr>
<tr>
<td>17</td>
<td>19.76 ± 0.42</td>
<td>18.90 ± 0.44</td>
<td>0.16</td>
</tr>
<tr>
<td>18</td>
<td>20.07 ± 0.44</td>
<td>19.10 ± 0.46</td>
<td>0.13</td>
</tr>
<tr>
<td>19</td>
<td>20.37 ± 0.45</td>
<td>19.30 ± 0.48</td>
<td>0.10</td>
</tr>
<tr>
<td>20</td>
<td>20.68 ± 0.47</td>
<td>19.51 ± 0.49</td>
<td>0.086</td>
</tr>
<tr>
<td>21</td>
<td>20.99 ± 0.48</td>
<td>19.71 ± 0.51</td>
<td>0.072</td>
</tr>
</tbody>
</table>

There was an interaction of day of trial by treatment ($P=0.008$)
Figure 3.1 Flowchart showing total enrolment, reasons for exclusion from trial, and reasons for removal during trial for Holstein cows in a randomized controlled trial where cows were milked twice daily (TDM, control), or once daily (ODM) for two wk, and twice daily thereafter.

Cows Screened for Ketosis
Starting at 3 DIM: 148

Non-Ketotic Cows
Never tested ≥ 1.2 mmol/L: 44

Ketotic Cows
Tested ≥ 1.2 mmol/L: 104

Excluded from the Trial
Researcher or recording error: 3
Temperament: 1
Other: 2

Enrolled onto the Trial
Once-daily milking: 55
Twice-daily milking: 49

Removed during the first week of trial
Once-daily milked: 4
Milk fever: 1
Displaced abomasum: 3
Twice-daily milked: 2
Milk fever: 1
Excessively high ketones: 1

Removed during the second week of trial
Once-daily milked: 1
Moved to the wrong pen: 1
Twice-daily milked: 3
Culled due to injury: 1
Displaced abomasum: 1
Temperament: 1

1 All occurred within the same calendar wk (feed issue)
2 Ketones ≥ 5.0 mmol/L for 3 or more consecutive days
Figure 3.2 Proportion of primiparous (lact 1) and multiparous (lact 2+) Holstein cows diagnosed with ketosis in a randomized controlled trial where cows were milked twice daily (TDM, control), or once daily (ODM) for two wk, and twice daily thereafter, following the detection of ketosis.

There were 3-way interactions for treatment by parity by day of trial ($P=0.0026$) & treatment by parity by day of trial$^2$ ($P=0.0029$)
Figure 3.3 Distribution of the number of 2\textsuperscript{nd} propylene glycol (PG) treatments (1\textsuperscript{st} additional treatment) for Holstein cows in a randomized controlled trial where ketotic cows were milked twice daily (TDM, control), or once daily (ODM) for two wk, and twice daily thereafter during the 21 d trial period.
**Figure 3.4** Distribution of the number of 3\textsuperscript{rd} propylene glycol (PG) treatments (2\textsuperscript{nd} additional treatment) for Holstein cows in a randomized controlled trial where ketotic cows were milked twice daily (TDM, control), or once daily (ODM) for two wk, and twice daily thereafter during the 21 d trial period.
**Figure 3.5** Blood BHB concentrations for primiparous (Lact 1) and multiparous (Lact 2+) Holstein cows (diagnosed with ketosis on d=0) in a randomized controlled trial where cows were milked twice daily (TDM, control), or once daily (ODM) for two wk, and twice daily thereafter, following the detection of ketosis.

BHB at d=0 are the treatment group means, not the medians calculated by the model. There were interactions for day of trial by treatment ($P=0.017$), day of trial by parity ($P<0.001$), day of trial$^2$ by treatment ($P=0.04$), & day of trial$^2$ by parity ($P=0.003$)
**Figure 3.6** Milk yield (kg/cow per d) from Holstein cows in a randomized controlled trial where cows were milked twice daily (TDM, control), or once daily (ODM) for two wk, and twice daily thereafter, following the detection of ketosis.

There was an interaction of week by treatment ($P=0.001$)
Figure 3.7 Energy-corrected milk yield (kg/cow per d) from Holstein cows in a randomized controlled trial where cows were milked twice daily (TDM, control), or once daily (ODM) for two wk, and twice daily thereafter, following the detection of ketosis.

There was an interaction of week by treatment ($P<0.001$)
Figure 3.8 Dry matter intake (kg/d) of Holstein cows in a randomized controlled trial where cows were milked twice daily (TDM, control), or once daily (ODM) for two wk, and twice daily thereafter, following the detection of ketosis.

There was an interaction of day of trial by treatment ($P=0.008$)
REFERENCES


CHAPTER 4: GENERAL DISCUSSION

Important Findings

Ketosis can have lasting negative impacts of the health, productivity, and welfare of dairy cows, resulting in substantial financial losses per case, and affects approximately 40% of lactations affected by ketosis in North America (LeBlanc, 2010; McArt, et al., 2012; McArt, et al., 2015). These health and production factors pose large monetary losses to dairy producers if ketosis is not properly managed. This means that early and accurate detection followed by rapid and effective treatment, are crucial factors in successfully managing and resolving cases of ketosis and reducing losses on dairy farms.

The objectives of this thesis were to compare the test performance of the blood, milk, and urine ketone tests over time, and identify any changes in ketones over time in ketotic cows in early lactation (Chapter 2), and to evaluate the effect of reducing milking frequency from two to one milking per day for two weeks (in conjunction with 5 d of oral PG) in ketotic dairy cows on ketosis resolution and milk production (Chapter 3).

The prospective cohort study evaluating the blood, milk, and urine ketone tests, indicated that there may be differences in the changes in concentrations of ketones in the milk and urine compared to those in the blood over time, specifically in relation to fluctuations in blood ketones in a newly ketotic cow (Chapter 2). More specifically, the data in Chapter 2 showed a possible delay in the increase of ketones in the milk and urine compared to the blood when a cow became ketotic, and it also showed that the ketones may decrease in the milk and urine before they decrease in the blood. While the semi-quantitative milk (Keto-Test™) and urine (KetoStix®) ketone tests have definite limitations when it comes to diagnostic accuracy compared to the quantitative laboratory gold-standard test or the Precision Xtra® ketone meter, these findings
may explain some of the variation/discrepancies in the diagnostic accuracy of the milk and urine tests reported in the literature beyond that of misclassification bias. Most researchers that have compared the diagnostic accuracy of the Keto-Test™ or KetoStix® tests to the laboratory gold-standard ketone test and typically have only sampled each subject once within the desired screening period (e.g. within the first 15 DIM). Due to this, one cannot be certain when ketotic cows became ketotic, which may affect how the milk and urine tests performed in each study (Geishauser et al., 2000; Carrier et al., 2004; Iwersen et al., 2009).

The production of ketones in the blood has been well-studied, but the physiologic pathways of ketones in the blood, milk, and urine over time still is not fully understood (Baird, 1982; Herdt, 2000; Tatone et. al., 2016). The change in the concentration of ketones in the blood, milk, and urine over time had not been compared beyond Samiei et al. (2010) prior to the current study, and had not ever been compared on a daily-basis. The findings in Chapter 2 highlight possible temporal changes within the blood, milk, and urine tests, and the differences between each test.

Our randomized controlled trial evaluating ODM as an adjunct treatment for ketosis in Chapter 3 indicated that 2 wk of ODM (with 5 d oral PG) was more effective at resolving ketosis (reducing blood BHB below 1.2 mmol/L) than treating cows with PG alone (Chapter 3). ODM cows were less likely to have blood BHB ≥ 1.2 mmol/L, and had blood BHB < 1.2 mmol/L more quickly compared to cows in the TDM treatment group. ODM cows had blood BHB concentrations 1.5 to 2.2 times lower than TDM cows by the end of the 2 wk of treatment. Additionally, the ODM treatment maintained blood BHB concentrations < 1.2 mmol/L in most cows after they returned to a TDM schedule. On the 21st day of trial (or one wk following the end of ODM), 4.4% of primiparous and 24% of multiparous cows in the ODM treatment group
had blood BHB $\geq 1.2$ mmol/L, while 30% of primiparous and 66% of multiparous cows in the TDM group had blood BHB $\geq 1.2$ mmol/L. These positive metabolic effects are in line with other studies (Loiselle et al., 2009; Schlamberger et al., 2010; Kay et al., 2013; Phyn et al., 2014) that have examined ODM in early lactation.

The 2-wk period of ODM was also associated with a reduction in milk yield, and ECM yield, which carried past the treatment period and remained for all 15 wk of observation. Due to this outcome, this treatment is not practical for use in its current 2-wk length, as the financial losses due to milk loss from ODM outweigh the possible losses due to unresolved ketosis. But, the ODM treatment worked quickly, and most cows that had blood BHB < 1.2 mmol/L had achieved that by the 6th day of trial, which indicates that a full 2 wk is not necessary for this treatment. This study did not examine milk production over full lactations, therefore we could not assess whether milk production remained lower in ODM cows beyond the 13 wk post-treatment, or if ODM cows managed to perform similarly, or better than TDM cows later in lactation.

The tendency for cows from the ODM group to have higher linear scores than TDM cows both during and after treatment raises slight concern, as no other studies have reported an increase in LS after treatment (Rémont et al., 1999; Rémont & Pomiès 2007; Loiselle et al., 2009; Schlamberger et al., 2010; Kay et al., 2013; Phyn et al., 2014).

**Limitations and Future Studies**

There are several questions raised by this work that could be addressed in future studies. Since the Chapter 2 study was connected to the randomized controlled trial in Chapter 3, the sampling schedule changed following ketosis diagnosis to better-fit the treatment trial. This
resulting in only 3 consecutive days of observations following a ketotic blood test, then tests every 3rd day. Additionally, cows were treated with PG on the day of blood-ketosis detection, and some cows were also given an ODM schedule. Treating ketotic cows on the 1st day of detection may have been associated with the blood BHB concentrations for a large portion of the ketotic cows to decrease below 1.2 mmol/L within 1 to 2 d, and may have had an effect on the milk and urine test performance and/or prevented us from seeing the true nature of the milk and urine ketones compared to the blood. Additionally, the sample size was estimated for the treatment trial, not for this prospective study, and there may not have been adequate power for all of the evaluations performed.

Future research should monitor daily blood, milk, and urine ketone concentrations in a much larger population of ketotic cows, and it should follow them from 2 to 3 d after calving to ketosis detection and over the full duration of a case of ketosis (or up to 30 DIM). Future research should avoid treating cows, or compare test performance between treated and untreated cows during these observations (if ethically possible). If the study population were large enough, it would be beneficial to compare the trends in ketone concentration in both treated and untreated cows over an extended period of time. Ideally, future research will be able to better-observe the differences (or lack thereof) in the concentrations of ketones in the blood, milk, and urine, leading up-to, and during a case of ketosis.

In Chapter 3, preventing incompletely milked cows from returning to the robot to be completely milked may have been associated with a number of limitations in our study. Inadvertently incompletely milking some of the cows in the robot may have been a factor causing larger LS in ODM cows, by putting ODM cows at a greater risk for milk leakage and/or increased udder distension. The incomplete milking of some cows may have also been associated
with larger reductions in milk yield and udder discomfort due to increased udder pressure. Many cows that were milked in the robot were introduced to a robotic milking system for the first time (or any milking system for heifers) and cows received one training session with farm employees to ensure they were comfortable in the robot and completely milked on their first visit. This new environment can be stressful, and some animals may have had trouble letting their milk down in the robot (especially heifers) and ended up incompletely milked. We had additional issues when the robots broke down overnight (which happened ~1x/month) as some cows ended up unmilked for almost 30 hr. Future studies using robotic milkers should adjust robotic milking settings to ensure cows that are incompletely milked cows are re-milked within a 1 to 2 hr window of the last milking to ensure the milking intervals are not affected.

The behavioural effects of reducing milking frequency have not been examined much in the literature, and were not examined in this study, but would be beneficial in determining whether reducing milking frequency is a suitable option for ketosis treatment in future research.

Future research should examine a shorter period of ODM (still in conjunction with PG), of perhaps 5 to 7 d to determine if the shorter treatment period still successfully reduces blood BHB concentrations below 1.2 mmol/L (and maintains them), and examine whether milk yield is negatively affected by ODM after a much shorter treatment period. Five days would be ideal, as it is the same number of days as the PG treatment, which would make it simpler to implement as a treatment protocol on farms. This future research should also use a larger sample size. A larger sample size (if powered properly) would allow for the evaluation of health, reproductive, and behavioural (such as bawling/vocalizations in and near the milking parlour, gait score, lying behaviour, and lying time) outcomes, which are generally lacking in the literature. It would also give greater power to the outcomes for ketosis resolution and milk production. Lastly, future
studies should implement this treatment in commercial herds with milking parlours to investigate the feasibility of this treatment beyond research.

**Summary and Conclusion**

In summary, measuring blood BHB concentrations with the use of a handheld meter is still the most diagnostically accurate method for the cow-side diagnosis of ketosis in dairy cattle. Blood BHB concentrations should be measured at least twice within the first two weeks of lactation to ensure most (if not all) ketotic cows are found and promptly treated. If blood BHB measurement isn’t an ideal (or practical) option for producers, measuring ketones in the milk or urine using ketone test strips are moderately accurate alternative options for ketosis screening. In order to ensure most (or all) ketotic cows are detected, milk and/or urine ketone tests should be done more than the two times (within the first two weeks of lactation) recommended for the blood test to account for the reduced diagnostic accuracy.

Daily observation of ketones in the blood, milk, and urine found delays in the time-to-ketosis diagnoses in the milk and urine tests compared to the blood test. Additionally, our observations indicated that ketone concentrations in the milk and urine might react differently over time in relation to fluctuations in blood BHB concentrations. This data may help provide a better understanding on the production and elimination of ketones in the blood, milk, and urine, and might help veterinarians and producers plan better screening programs for at-risk cattle based on the test being used.

We also found that reducing milking frequency from 2 to 1 milking per day for 2 weeks (in conjunction with 5 d PG) in ketotic cows, more effectively reduces blood BHB concentrations below 1.2 mmol/L than by treating cows with PG alone. The ODM treatment also
had no effect on DMI and it reduced milk and ECM yield both during and after the treatment period. While ODM is not practical for a period of 2 weeks in early lactation, our research shows promise that a much shorter period of ODM could be successful in resolving ketosis without negatively affecting long term milk production.

When we focused on the biology of the development of ketosis, we learned that the most successful way to treat ketosis was by controlling both energy input and output, instead of controlling energy input alone. These findings may assist veterinarians and producers in developing more effective treatment protocols for ketotic cows by balancing both energy input and output.
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