ABSTRACT

Ketosis is currently regarded as a major metabolic disorder of dairy cows, reflective of the animal’s efforts to adapt to energy deficit while transitioning into lactation. Currently viewed as a pathology by some, ketosis is associatively implicated in milk production losses and peripartal health complications that increase the risk of early removal of cows from the herd, thus carrying economic losses for dairy farmers, and jeopardizing the sustainability of the dairy industry. Despite decades of intense research in the mitigation of ketosis and its sequelae, our ability to lessen its purported impacts remains limited. Moreover, the association of ketosis to reduced milk production and peripartal disease is often erratic and likely mired by concurrent potential confounders. In this review we discuss the potential reasons for these apparent paradoxes in the light of currently available evidence, with a focus on the limitations of observational research, and the necessary steps to unambiguously identify the impacts of ketosis on cow health and performance via controlled randomized experimentation. A nuanced perspective is proposed that considers the dissociation of ketosis—as a disease—from healthy hyperketonemia. Furthermore, in consideration of a growing body of evidence that highlights positives roles of ketones in the mitigation of metabolic dysfunction and chronic diseases, we consider the hypothetical functions of ketones as health-promoting metabolites and ponder on their potential usefulness to enhance dairy cow health and productivity.

Keywords: Ketosis, hyperketonemia, paradigm shift, dairy cow

INTRODUCTION

“There are no real paradoxes in science; the apparent paradoxes are merely nature’s polite way, sotto voce, of informing us that our understanding is incomplete or erroneous” (Gold, 2001). While these words were used by astrophysicist Thomas Gold when discussing the controversies around the origin of hydrocarbons in the earth’s mantle, they seem fitting in the context of the contemporary controversies surrounding peripartal ketosis of dairy cows. Indeed, although ketosis was first described over a century ago, its implications on health and productivity in dairy cows remain incompletely understood, and our views—as we shall argue herein—, muddled by contradicting pieces of seemingly reliable evidence. While in some cases ketosis is related to the onset of peripartal disease and reduced productive and reproductive performance, in other situations, the ketotic cow appears to sustain normal health and performance. It would appear then that a distinction between pathology and normal physiology needs to be made if we are to fully understand the roles of ketones and the implications of hyperketonemia in the peripartal dairy cow. Herein, we discuss the reasons behind our arguably limited understanding of ketosis and the putative difficulties in treating and preventing peripartal disease, while exploring the idea that the resolution of hyperketonemia itself may not be a meaningful single target moving forward. Mapping the road to achieving the resolution of the paradoxes around ketosis is the subject of this critical review.

Ketone Biology

**Ketone bodies.** The ketone bodies -Acetoacetate (AcAc), D-β-hydroxybutyrate (BHB), and Acetone- are energy-carrying substrates primarily synthetized in the hepatic and ruminal mitochondria that serve as metabolic fuels for cellular respiration in extra-hepatic tissues (Krebs, 1966). The (R)-enantiomer of BHB (i.e., D-BHB) is the natural configuration synthesized in cells. The (S)-enantiomer of βHB (i.e., L-BHB) is not readily oxidized and must be converted to the D form before it can be utilized (80–85% conversion rate; (Lincoln et al., 1987)). Although ketones can be synthesized from amino acids, fatty acids (FA) constitute by far the major source of ketones under most metabolic
situations (Figure 1) (Puchalska and Crawford, 2017). Ketones are water-soluble molecules made up of 2 R-groups attached to a carbonyl group (C = O). Of the 3 ketone bodies, AcAc and BHB are predominantly found in their acidic, deprotonated state, at physiological pH (pKa = 3.6 and 4.7, respectively). Because acetone does not have an ionizable hydrogen atom, it is a neutral molecule, with no acidity (i.e., no pKa).

**THE NATURALISTIC PERSPECTIVE OF KETOSIS**

Glucose is undoubtedly a chief metabolic substrate for mammalian life, and it can even be considered as essential and irreplaceable in cell types with specific glucose needs (e.g., the red blood cell). Fortunately, evolutionary history has imbued the action range of the mammalian metabolic machinery with remarkable plasticity for extracting energy from different substrates, arguably favoring survival in situations where availability of energy sources shifts. Ketones are one such substrate that appear to be a vital metabolic fuel across all domains of life (Puchalska and Crawford, 2017), and which can sustain cellular metabolism in several body tissues when glucose availability is reduced (Nelson et al., 2021). From this perspective, ketone production represents a versatile adaptation of metabolism to nutrient availability (glucose, amino acids, fatty acids), and a testament to the metabolic flexibility of mammalian physiology. Carnivores such as lions, bears, and minks, are leading examples of animals who naturally derive most of their energy supply from the breakdown of fat and protein (Tauson et al., 1994, Verbrugghe and Bakovic, 2013, Chazarin et al., 2019), both of which can be largely metabolized to ketones (Nelson et al., 2021). Similarly, ketones can represent an important substrate during mammalian early life (Nehlig, 2004), and furnish energy to several body organs in situations where glucose availability is reduced, or when it needs to be prioritized for use in situations such as lactation and bacterial infection (Bauman and Currie, 1980, Kvidera et al., 2017). Ketogenic states are in this manner a feature of normal adaptive metabolism across the mammalian spectrum.

**KETOGENESIS**

A detailed view of ketogenesis, including substrates, cofactors, end products and its regulation, is presented in Figure 1. Although ketogenesis yields the 3 different ketone bodies, typically only D-BHB and AcAc are readily available in meaningful quantities for metabolic use, which is mostly due to the loss of the highly volatile acetone via the airways. Indeed, the transformation of AcAc to acetone occurs via spontaneous decarboxylation and, as such, it represents a net loss of energy. Of the 2 remaining ketones, BHB represents by far the predominant form available for metabolic use. The interconversion of BHB and AcAc is dependent on the activity of β-hydroxybutyrate dehydrogenase (BDH), and is also tied to the local redox potential within the mitochondria (i.e., the NADH/NAD⁺ ratio). Because BHB and NAD⁺ production are concurrent events, BHB production favors NAD⁺ accumulation. Furthermore, the higher molecular stability of BHB compared with

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**Figure 1. Overview of ketone synthesis in the hepatic mitochondria.** The multi-step conversion of acetyl-CoA from fatty acids and amino acids to acetyl-CoA, acetone, and β-hydroxybutyrate, involves the use of the mitochondrial enzyme isoforms ACAT1, HMG-CoA synthase, HMG-CoA lyase, acetoacetate decarboxylase, and DBH1. The conversion of production of β-hydroxybutyrate from acetacetate uses NADH and yields NAD⁺. Hormonal regulation from glucagon and insulin is mediated by the transcription factor FOXA2. *Regulated, rate-determining step. ACAT1: acetoacetyl thiolase 1; DBH1: D-β-hydroxybutyrate dehydrogenase 1; HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA; CoASH: Coenzyme A; FOXA2: forkhead box A2.
AcAc, implies it is less prone to spontaneous reactions (AcAc is more reactive), making it a more dependable ketone for metabolic use. Overall, these factors favor ketone equilibration toward BHB accumulation, and explain its higher availability for transport and higher concentrations in circulation relative to AcAc. The ratio of BHB to AcAc is however not a fixed constant. Generally speaking, pathological states that modify the redox potential of the cells (e.g., ketoacidosis, severe hypoxia, liver disease, hepatic ischemia, etc.) will result in an altered redox potential of hepatic cells, manifested as abnormally high NADH concentrations and NADH/NAD+ ratios (Laffel, 1999). During conditions such as diabetic ketoacidosis (DKA) in humans, the ratio of BHB to AcAc may increase from 2.1 to as much as 10.1, due to highly reduced state of the hepatic mitochondria in the DKA patient (Laffel, 1999). It is also possible, however, to encounter increased BHB to AcAc ratios during non-pathological situations, such as during prolonged fasting (Laffel, 1999).

Limited data from sheep and cows suggests the ketone ratios can be several fold higher in liver and blood of ruminants, relative to non-ruminants such as humans, rats, and dogs, both during well-fed and fasted/ketoegenic conditions (Bergman, 1971). The reason for this apparent difference is not clear, and in this context it is worth considering that direct comparisons of cow metabolism to that of non-ruminants are complicated by differences in frequency of nutrient delivery/absorption, type of energy substrate availability, and tissue-specific differences in intermediary metabolism (e.g., differences in enzyme activity across metabolic pathways). The excellent reviews of Ballard and collaborators (1969) and Bauman (1976) are recommended on this topic (Ballard et al., 1969, Bauman, 1976). Furthermore, differences in the methodology used for ketone measurements (e.g., enzymatic assay vs. others) cannot be ruled out as contributing factors to this limited comparability, particularly considering the high variation in the data from reports across studies (Bergman, 1971). Finally, given the more unstable nature of the AcAc molecule relative to BHB, it is conceivable that variations in the timing of analytical assays across studies may result in variations in the accuracy of AcAc measurements (i.e., potential underestimation) and this explain some of the variation between ruminants and non-ruminants. Considering the above-mentioned reasons, the implications of such inter-species differences may be difficult to fully ascertain, and therefore, comparisons between ruminants and non-ruminants should be made carefully. Caveats aside, a higher ketone ratio is generally indicative of a cellular environment that supports BHB accumulation, such a high ratio of NADH and NAD+ (i.e., an elevated hepatic availability of NADH) that favors BHB synthesis from AcAc.

When summarizing the ketone ratio in normal versus ketotic cows, Bergman (1971) reported a decreased ratio of BHB to AcAc in the ketotic animals, an observation that stands in complete opposition to the reported increase in the ketone ratio during non-ruminant ketosis and human-specific ketoacidosis (Bergman, 1971, Laffel, 1999). As highlighted earlier, direct comparisons of ketosis in ruminants and non-ruminants must be carefully performed; furthermore, the method of induction of ketosis (e.g., starvation induced in humans vs. naturally occurring in a cow entering lactation) may result in different metabolic outcomes. In a study aimed at inducing ketosis via starvation in dairy cows, Baird and collaborators (1972) reported increased total ketones during the progression of starvation (6-d period), accompanied by a progressive reduction in the blood ratio of BHB to AcAc (Baird et al., 1972). This suggests that as total ketone production increased, progressively higher proportions of AcAc (i.e., accumulation) were favored. We speculate this may be due to either a higher peripheral extraction and utilization of BHB by extrahepatic tissues, or to local changes in the hepatocytes that limit the conversion of AcAc to BHB, such as a potential limitation in NAD+ availability (Williamson et al., 1967, Heitmann et al., 1987).

REGULATION OF KETOGENESIS

Although the rates of ketogenesis are largely dependent on the flow of oxidizable substrates reaching the liver (i.e., FA, and amino acids), the rate of ketone formation is regulated hormonally to match needs during specific physiological states. During states of energy insufficiency such as the early postpartum, insulin secretion and insulin action are reduced, whereas glucagon secretion and action are increased (De Boer et al., 1985). This arrangement of hormonal signals —where glucagon action predominates— promotes ketogenesis indirectly by stimulating lipolysis (via hormone sensitive lipase stimulation) and the inhibition of lipogenesis (i.e., acetyl CoA carboxylase inactivation; ACC), as well by favoring fatty acid β-oxidation (via limited ability of ACC to impede FA transport for mitochondrial oxidation; Laffel, 1999; Nelson et al., 2021). It should be noted, however, that the effects of glucagon on ruminant lipolysis appear to be equivocal (Brockman et al., 1978; She et al., 1999), as suggested by disparate effects when evaluated in vivo vs. in vitro (Basset, 1971; Bauman, 1976). Furthermore, the lipolytic effects of glucagon may depend on the presence of insulin (a more potent, opposing, signal), and appear to be weak at the onset of lactation in dairy
cows (She et al., 1999). On the other hand, glucagon could directly promote ketogenesis directly by inducing transcriptional activation of enzymes in the ketone synthesis pathway. The 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase reaction is the rate-limiting step in ketone synthesis (Figure 1), and it is tightly regulated by insulin (i.e., reduces transcription; Howell and Stoffel, 2009) and glucagon (i.e., promotes transcription; von Meyenn et al., 2013) in non-ruminants. The effects of these hormones on HMG-CoA synthase activity are in turn mediated by the forkhead box A2 (FOXA2) protein, a transcription factor with central roles in hepatic lipid metabolism (Wolfrum et al., 2004; Wolfrum and Stoffel, 2006; Bochkis et al., 2008). This protein is transcriptionally active in the fasted state of non-ruminants (with presumably similar activities in ruminants undergoing energy deficit) and induces the expression of enzymes involved in fatty acid oxidation, ketogenesis, and very-low density lipoprotein synthesis. Similar to the above-mentioned effects of glucagon on lipolysis in ruminants, the relatively weak effects of glucagon relative to insulin may also result in limited effects on ketogenesis, although evidence for this possibility is relative scarce (She et al., 1999).

**KETONE SYNTHESIS IN OTHER TISSUES**

In ruminants such as the dairy cow, the main ketogenic organs are the liver and the rumen (Bergman, 1971); although recent evidence highlights a modest contribution of hindgut fermentation to the BHB pool (Abeята et al., 2023). Although it is unclear what the relative contributions of this ketone-producing compartments are to systemic ketone availability, the importance of the rumen as a ketogenic organ is highlighted by the elevations in plasma BHB that occur in the few hours following a meal to raise ketones above basal concentrations (Seely et al., 2021). This observation also carries the implicit deduction that it is part of normal dairy cow physiology to sustain active ketogenesis and to experience fluctuating concentrations of circulating ketones on a daily basis. Even though either BHB or AcAc have been detected as products of metabolism in other organs such as skeletal muscle, lungs, and the mammary gland, these tissues are not regarded as ketogenic organs because the most likely origin of ketones appears to be their interconversion by BDH action (i.e., pseudo-ketogenesis). This idea is also supported by the observation that non-hepatic peripheral tissues cannot directly utilize acetyl CoA for ketone production because of the limited expression of the key mitochondrial enzyme HMG-CoA synthase, necessary for ketogenesis (Newman and Verdin, 2017; Qi et al., 2022). Although information on the mechanisms of ketones synthesis in ruminants is limited relative to that available in non-ruminants, it is generally understood that the events necessary for the synthesis of AcAc and BHB are virtually identical across mammals, which is supported by the verified output of both ketones from the rumen epithelium (Bergman, 1971, Beck et al., 1984). Volatile fatty acids (VFA) produced during fermentation are known to be metabolized in the cells of the rumen wall, particularly butyrate (Yamdaghi et al., 1968, Giesecke et al., 1979, Beck et al., 1984), of which 74–90% is oxidized by ruminal epithelial cells (Remond et al., 1995). While acetate is considered as a poor precursor for ketogenesis (Goosen, 1976), the conversion of butyrate to AcAc and BHB occurs readily in the rumen epithelium, amounting to an 83% conversion rate when calculated from the BHB enrichment of portal blood (Krehbiel et al., 1992). Although complete oxidation of butyrate to CO2 may also take place, the extent of oxidation and relative proportions of the final products appear to variable (Beck et al., 1984, Krehbiel et al., 1992, Remond et al., 1995). As for the ketone ratios, some in vitro studies using ruminal epithelial cells suggests that AcAc and BHB may be produced at a similar rate in the bovine rumen (i.e., 1 to 1 ratio), although others reported a higher ratio (1:4), closer to that found in blood (Beck et al., 1984). Finally, in terms of ketogenic capacity, a reduction in butyrate oxidation efficiency appear to occur as a function of increasing butyrate concentrations (Krehbiel et al., 1992). This suggests that the ketogenic capacity of the ruminal epithelial cells may be saturated at high fluxes of butyrate, whose availability can outpace the oxidative capacity of the epithelial cell (Remond et al., 1995).

**KETONE OXIDATION**

Upon mitochondrial synthesis, ketones exit the hepatic cells via the monocarboxylate transporter 1 (MCT1), a proton-coupled transporter located in the plasma membrane that belongs to the MCT or SLC16 solute carrier family (Halestrap, 2012). By virtue of their water-solubility, AcAc and BHB do not require lipoproteins for transport, and it is thus assumed that they dissolve in the aqueous phase of blood to reach peripheral tissues such as kidney, skeletal muscle, heart, brain, and the mammary gland in lactating animals (Bergman, 1971, Nelson et al., 2021). In these organs, ketones may constitute a vital energy source to sustain cellular metabolism while allowing glucose sparing for other physiological purposes (e.g., red blood cells; the activated immune system; the lactogenic mammary gland). The importance of ketones in this context is best represented in organs such as the brain. While fatty acids cannot traverse the blood-brain barrier, ketones can
passively diffuse though it (Pardridge, 1991). Available ketones can be utilized when glucose supply is limited—such as during prolonged fasting—and may account for up to 2/3 of the brain’s energy needs to replace glucose as the main fuel (Owen et al., 1967, Laffel, 1999). Other organs, such as the heart, may use fatty acids for oxidation, although they can also utilize ketones (Frayn et al., 2006). The liver, on the other hand, stands alone as the selfless organ that synthesizes ketone bodies, but which does not rely on them as a significant source of energy. The mechanisms responsible for this metabolic adaptation of the liver involve the limited expression of key enzymes involved in ketolysis, such as succinyl-CoA:3-oxoacid CoA transferase (SCOT; also known as β-ketoacyl transferase), across all studied animals (Fukao et al., 1997, Puchalska and Crawford, 2017). A specific deficiency of the BDH enzyme in ruminant liver mitochondria has been reported by some (Nielsen and Fleischer, 1969); however, it is difficult to reconcile this observation with the known fact that the ruminant liver produces BHB, the end product of mitochondrial BDH during ketogenesis. Overall, ketones may account for at least 30% of total respiratory CO2 production (Bergman, 1971) in lactating dairy cows, although

![Figure 2. Ketogenesis in the cells of the ruminal epithelium of ruminants. Pathways adapted from Remond and collaborators (1985). Remond et al., 1995.](image-url)
we speculate this number may be even higher in the modern dairy cow sustaining increased energy demands relative to those of the genetic populations available when these data were generated.

Ketones can enter target tissue cells via the monocarboxylate transporters MCT1 and MCT2, located in the plasma membrane (Pierre and Pellerin, 2005, Halestrap, 2012). Once inside of the cell, ketone lysis can occur (Figure 3). β-hydroxybutyrate is oxidized to acetocetate via the enzyme BDH, to yield AcAc and NADH (Lehninger et al., 1960). Acetoacetate is oxidized to acetocacetyl-CoA via the SCOT enzyme, which is transcriptionally absent in the liver but present in several ketone-oxidizing tissues (Fukao et al., 1997, Puchalska and Crawford, 2017). The formation of AcAc is favored because the free energy of AcAc-CoA hydrolysis is greater than that of succinyl-CoA, a necessary co-substrate for its synthesis. Finally, the thiolase (ACT1) reaction converts acetocacetyl-CoA to 2 molecules of acetyl CoA. Overall, although the ketone oxidation process essentially resembles ketogenesis in reverse, the HMG-CoA synthase and lyase steps are effectively bypassed and substituted with the succinyl CoA-dependent SCOT reaction, an event that is absent during ketogenesis. Acetyl-CoA, the end product of ketolysis, can enter the citric acid cycle, and following oxidative phosphorylation, will yield 20 and 22.5 ATP per each molecule of AcAc and BHB, respectively (Board et al., 2017). Because acetone does not convert back to acetyl-CoA (not thermodynamically favorable), this ketone is typically either excreted through urine or exhaled out of the body (Brockman, 1979). As noted by Puchalska and Crawford (2017), the conversion of ketones to an activated/oxidizable form (i.e., AcAc-CoA) does not require the use of ATP, unlike glucose and fatty acid oxidation events which require ATP-driven activation (i.e., hexokinase and Acyl-CoA synthetases, respectively). This means that ketone oxidative flux occurs due to mass action, whereby substrate abundance (i.e., AcAc) for ketolysis and a rapid acetyl-CoA consumption in the TCA cycle favor oxidation by SCOT.

Readers are directed to the detailed review of Puchalska and Crawford (2017), for a more in-depth description of the ketone oxidation process.

**Other physiological roles of ketones: feeding behavior**

This section will briefly summarize the better-characterized effects of ketones the regulation of appetite and feeding behavior, while subsequent sections will focus on the more recently characterized functions that may have ramifications on cow health. Because BHB is the predominant ketone in circulation, and given that AcAc will commonly be derived from it in most tissues, we will discuss the general effects of ketones, and provide distinctions between these 2 metabolites where applicable.

Although ketones are generally recognized as appetite suppression signals (Langhans et al., 1985), the mechanisms responsible for appetite regulation are complex and appear to remain incompletely defined (Paoli et al., 2015, Deemer et al., 2020). The complexity and confusion around their action is highlighted by the observation that ketosis can induce activation of both feeding and satiety centers in the hypothalamus, namely orexigenic and anorexigenic neurons (Paoli et al., 2015). These neurons act as signal integrators in the hypothalamic arcuate nucleus, and include the orexigenic neurons neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons, as well as the anorexigenic neurons pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) neurons (Paoli et al., 2015).

Regarding the ability of ketones to stimulate feeding behavior, several signals appear to potentially be at play. The orexigenic response may be attained during ketosis by mechanisms that increase the release of adiponectin, brain γ-aminobutyric acid, and that result in AMP-activated protein kinase phosphorylation (Paoli et al., 2015, Deemer et al., 2020). The power of these signals to induce feeding behavior is however questionable, particularly in light of the observation that the induction of ketosis typically results in either neutral or anorectic effects in several species, including cows (Langhans et al., 1985, Zarrin et al., 2013, Ruiz-González et al., 2022).

The specific mechanisms behind the putative hypophagic effects of ketones during ketosis seem to include the attenuation of ghrelin signaling (i.e., suppression of hunger signals) via stimulation of POMC/CART neurons, the decreased expression of NPY and AgRP in the hypothalamus (i.e., attenuation of feeding signals; Ralliff et al., 2009; Sumithran et al., 2011). The administration of exogenous ketones, ketogenic diets, and fasting, appears to induce appetite suppression via intestinal peptides such as cholecystokinin (CCK), peptide YY, and glucagon-like-peptide 1 (GLP-1) secretion (i.e., indirect regulatory mechanisms), although it is not completely clear whether these effects can be attributed to ketones specifically or rather to the ketogenic state. Some of the most definitive proof of the hypophagic effects of ketones comes from data in vagotomized and sham/vagotomized rats in which vagal signaling suppression effects of BHB were abrogated when vagal signaling to the hypothalamus was suppressed (Langhans et al., 1985). This critical study demon-
strated that the anorectic effect of BHB originates in the liver and is mediated by hepatic vagal afferents.

Considering the equivocal effects of ketones on appetite, we contemplate the possibility that the signaling actions of ketones may be dependent on the physiological state of the animal. For example, it is likely that ketone administration would have differential effects on feed intake depending upon whether or not the animal is in a positive or negative energy balance. Ultimately these potential differences will need to be evaluated in studies that compare the physiological response to ketones during distinct physiological states, such as the onset of lactation and post-peak lactation, where marked differences in hormonal signaling (e.g., insulin, somatotropin, catecholamines, etc) and energy demands are likely to be found.

While compelling information regarding the specific effects of ketones on the regulation of appetite in dairy cows are lacking, evidence from some randomized controlled trials (RCT) suggest ketones may be indeed involved in the regulation of feeding behaviors. For example, data from Smith and collaborators (2007) shows that the administration of the insulin sensitizing compound 2, 4-thiazolidinedione to peripartal dairy cows reduced lipolysis and circulating BHB concentrations, events that coincided with increased voluntary dry matter intake (Smith et al., 2007). Although other classical studies focused on the induction of ketosis using ketone precursors (e.g., (Drackley et al., 1991, Drackley et al., 1992) would be ideal points of reference for assessing effects of ketones on appetite regulation, animals in these experiments were also subjected to feed restriction—a necessity for achieving the ketosis phenotype with this model—, thereby making it unfeasible to make inferences regarding the effects of ketones in this situation. To circumvent some of the limitations of existing models for inducing and studying ketosis (this topic is specifically discussed in later sections), we have recently focused our attention on the use of dietary ketogenic precursors (i.e., calcium butyrate; CaBu) and the intravenous injection of ketones in the absence of feed restriction (Rico et al., 2021, Ruiz-González et al., 2022). Our preliminary data using increasing doses of CaBu-based in mid-lactation cows showed no effects of acute hyperketonemia on appetite when blood BHB rose to 3mM acutely (CaBu doses up to 4% of dietary DM), whereas appetite reduction was detected past this threshold, when blood BHB rose to 4.8mM at hour 2 from induction using CaBu at 6% of DM (Rico et al., 2021). Although a hypohagic effect of ketones appears likely, it is difficult to derive an unequivocal conclusion because the potential disruption of ruminal microorganisms and nutrient fermentation at the highest dose of butyrate cannot be ruled out as a

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**Figure 3.** Ketone lysis and oxidation in the mitochondria of extra-hepatic tissues. The step-by-step conversion of β-hydroxybutyrate to acetyl-CoA involves the use of the mitochondrial enzyme isoforms DBH1, SCOT, and ACAT1 to yield NADH and succinate. Acetyl-CoA can then be oxidized in the TCA cycle to yield NADH and FADH₂ for the production of ATP via oxidative phosphorylation. *Rate-determining step. DBH1: D-β-hydroxybutyrate dehydrogenase 1; SCOT: succinyl-CoA: 3-oxoacid-CoA transferase; ACAT1: acetoacetyl thiolase 1; Ox. Phos.: oxidized phosphorylation; CoASH: Coenzyme A.
potential factor reducing feed passage rates and physically limiting feed intake. A second preliminary study spanning 5 d of ketosis induction by intraruminal CaBu bolus administration (2X/day) resulted in episodic hyperketonemia that peaked above 2.0mM of blood BHB, and a transient reduction of DMI that converged back control levels by d 5 of ketosis induction (Ruiz-González et al., 2022). Finally, when assessing the effects of ketosis induction via direct intravenous BHB injection, Zarrin and collaborators (2013) reported not detecting any significant effects of continuous hyperketonemia (48 h, 1.74mM plasma BHB) on DMI (Zarrin et al., 2013). These results are borne out by our own recent evaluation using an intravenous Na-BHB injection, where we did not detect any effects of hyperketonemia (72 h induction, 1.4mM blood BHB; Barrientos Blanco et al., 2023). Collectively, the studies where ketone manipulation was achieved in absence of other confounding factors show variability in the hypophagic effects of ketosis. These differences could be attributed to the type of induction method (i.e., nutritional induction vs. intravenous induction), whereby the effects of ketones precursors may elicit or not a gastrointestinal response, as well as other physiological differences of animals related to stage of lactation, parity, etc. Differences in the method of induction are relevant to the study of ketosis in that they may produce disparate outcomes and lead to different interpretations of the effects of ketones. Indeed, the nutritional induction model may be useful in that it can integrate a wider range of events and signals related to digestion and absorption of ketogenic precursors; however, such methods may also elicit responses that may not necessarily be attributable to ketones. This point is exemplified by the observation that fasting-induced elevations in circulating ketones in humans can coincide with reductions in circulating leptin—an anorexigenic signal (Nasrallah and Horvath, 2014)— but when evaluated with a specific BHB intervention, it was demonstrated that reductions in leptin were independent of ketones (Kolaczynski et al., 1996). This underscores the idea that ketosis and hyperketonemia should not be understood as synonyms from a causative standpoint. Because intake regulation in ruminants is a complex process, a multitude of signaling factors are commonly involved (Allen et al., 2009). Considering that we cannot rule out any interactions between the nutritional induction methods and other dietary factors or consequences of nutrient digestion in dairy cows (e.g., propionate production and insulin release following a meal), the reading of the effects of ketones on appetite during induced ketosis may need to be considered carefully.

Ketosis as a disease: historical perspective.

Ketosis in cattle has been deemed a concern for the dairy industry for several decades. For a long time, it has been seen as primary disorder of peripartal cattle, not secondary to any other recognized disease, and similar to the ketosis of diabetes in humans (Shaw, 1943; 1956). While the first reports of cow acetoneemia date back to one hundred years ago (Sjollema and Van der Zande, 1923), what appears to be ketosis was reported as early as 1849 (Udall, 1943), with symptoms that characterized it as a nervous disease of the postpartum cow, earning it the name "mania puerperalis" (Shaw, 1956). The Sjollema and Van der Zande report (1923) initially described the disease as characterized by acetone odor, lack of appetite, dry feces, reduced milk production, and a state of excitation, where ketones, particularly circulating BHB were typically elevated. By studying starvation-induced ketosis authors from this time period came to the conclusion that the cow is rather resilient to nutrient deprivation ketosis, and that not much acetone is produced unless the animal is simultaneously impacted by certain disease conditions (i.e., cofactors; Carpenter, 1927, Robertson and Thin, 1953). In subsequent studies, Sjollema also described hypoglycemia as a characteristic that accompanied the state of ketonemia (Sjollema, 1927), and which was later differentiated into 2 different types of ketosis-related hypoglycemia, depending on whether it occurred during spontaneous ketosis or fasting ketosis (Kronfeld, 1971). Furthermore, subclinical ketosis was further classified as either Type I, or II, in feeding cows, which may be differentiated by the sites of ketogenesis—alimentary, hepatic, mammary for Types I, II, and III, respectively—and by blood patterns of BHB, AcAc, free FA (FFA), and acetate (Kronfeld, 1971). These definitions illustrate the variation in the manifestation of ketosis, as well as the potential complexity of the etiology of ketosis and its potential treatments. Crucially, 2 general types of ketosis had been conceived: 1) acetoneemia (i.e., clinical spontaneous ketosis; pathological), which described a metabolic disease of well-fed cows, and 2) ketosis, a general increase in ketones bodies in body fluids (i.e., physiological; Krebs, 1966, Kronfeld, 1971). As we will argue in this review, the progressive uniformization of ketosis as a disease with 2 degrees of manifestation (i.e., subclinical or clinical), as well as the heavy reliance of BHB as a marker for ketosis-associated diseases—often at the expense of other concurrent factors such as accelerated lipolysis, inflammation, etc.—might have led us through a narrow path for understanding and preventing peripartal disorders. This argument constitutes a basis for the evaluation of potential factors relevant to
Ketosis is often regarded by many as a major metabolic disorder of dairy cows that reflects their inability to adapt to energy deficit while transitioning from gestation to lactation (Herdt, 2000). Defined as blood BHB $\geq$1.2mmol/L (i.e., hyperketonemia), ketosis is commonly considered to be the most prevalent metabolic disorder in modern US dairy cows experiencing energy deficit (Emery et al., 1964, Simensen et al., 1990, Duffield et al., 1998, McArt and Oetzel, 2015). Cows identified as ketotic have been also shown to experience increased oxidative stress and a heightened pro-inflammatory status reflective of ongoing metabolic dysfunction (Sun et al., 2021). Furthermore, ketosis is often referred to as a disease in and of itself (Gordon et al., 2013, Liang et al., 2017, Benedet et al., 2019), while being regarded as a gateway to the development of other serious clinical diseases, such as fatty liver, displaced abomasum, metritis, mastitis, and hypocalcemia—all of which can compromise productive and reproductive performance of cattle (Dohoo and Martin, 1984, Drackley, 1999, Herdt, 2000, Walsh et al., 2007, Duffield et al., 2009, McArt et al., 2013). Ketosis and associated diseases can thus collectively lead to premature removal of cows from the herd and result in substantial economic losses to dairy farms (McArt et al., 2013, McArt and Oetzel, 2015). Indeed, the total cost of each ketosis case and related diseases has been estimated at $375 for primiparous, and $256 for multiparous cows in the US (McArt & Oetzel, 2015). Regardless of the cost per case, it has been argued that subclinical ketosis is overall more costly than clinical ketosis because of the much larger incidence of the former, a fact that supports the idea that constant monitoring for subclinical manifestations may benefit most dairies (Duffield et al., 2009). Considering that the incidence of subclinical ketosis typically ranges between 40 and 60% (Emery et al., 1964, Simensen et al., 1990, Duffield et al., 1998, McArt et al., 2013), the collective economic burden of ketosis on the dairy industry can be estimated to represent hundreds of millions of dollars annually in the United States alone. The global importance of ketosis mitigation is further highlighted by the recent systematic review by Cainzos and collaborators (2022), and although costs estimated across countries cannot be combined into a single figure, it is widely accepted that ketosis constitutes a disease of dairy cattle with a major impact on the profitability of dairy farms worldwide (Cainzos et al., 2022).
2003, Hammon et al., 2006, Schwartz et al., 2010, Ster et al., 2012, McFadden and Rico, 2019). Indeed, FFA are mechanistically linked to the accumulation of hepatic triglyceride (TAG) and lipotoxic lipid mediators (e.g., ceramides and diacylglycerols), some of which are implicated in the pathogenesis of fatty liver and insulin resistance of cows at higher risk for metabolic dysfunction and disease (e.g., overweight cows) (Roche et al., 2009, Rico et al., 2015, Rico et al., 2017, Samii et al., 2019). Similarly, saturated FA (SFA), can contribute to the development of an inflammatory state via activation of the NLRP3 inflammasome in humans and other non-ruminants (Ralston et al., 2017, Gianfrancesco et al., 2019), and may even compromise immune function in dairy cows (Lee et al., 2003, Contreras et al., 2010, Bradford and Swartz, 2020). In agreement with the above, recent evidence in dairy cows supports the negative role of FFA on immune function and insulin sensitivity, independently of energy balance (Vanacker et al., 2023).

A good biomarker is often described as an independent correlate to the outcome of interest, which means it needs to be a reliable predictor regardless of whether other co-factors are simultaneously present or not. Independent predictors should also exhibit high sensitivity (i.e., detect diseased animals) and high specificity (i.e., no false-positives) (FDA-NIH Biomarker Working Group, 2016). Unfortunately, peripartal concentrations of circulating FFA and ketones are not independent from each other, and their respective sensitivity and specificity are often low. These limitations in predictive accuracy are further illustrated by studies in which hyperketonemia was unrelated to milk production or infectious diseases (Bicalho et al., 2017), and others in which ketosis did not correlate with milk production or fertility, or even was positively associated to lactation performance (Rathbun et al., 2017, Pineiro et al., 2019, Rodriguez et al., 2022). The erratic nature of the relationship between ketosis and is best illustrated by Benedet and collaborators (2019), who summarized information from 8 different studies, with have reporting a negative relationship and the remaining half showing a positive relationship between these 2 factors (Benedet et al., 2019). Together, currently available data from observational and randomized controlled studies suggest that ketones are unreliable associative markers for peripartal disease and reduced cow performance. The reasons behind the limitations of BHB as a predictor can perhaps be best understood when considering this molecule may come from 2 origins (rumen and liver), as well as by the contemporary nature of a myriad of events (e.g., hyperlipidemia, inflammation, oxidative stress, etc.) that occur during peripartal hyperketonemia. Together, these factors muddle the nature of these relationships, and underscore the precarious predictive power of ketones as biomarkers (see Figure 4). Because of the lipotoxic effects of FFA, and the possibility of FFA-induced cellular dysfunction during the peripartum, the separation of the effects of ketones from those of other concurrent metabolic and physiological changes —e.g., excess lipolysis and FFA, oxidative stress, inflammation, cellular dysfunction— may simply be impossible to derive from observational data. With these considerations in mind, we posit that insofar as the effects of ketones on cow metabolism and physiology are confounded by co-factors, the value of BHB as an indicator of peripartal dysfunction will likely remain questionable. This argument is further illustrated in Figure 5.

**Treatment of ketosis.**

**Issues with Existing Treatment Approaches**

Considering the direct involvement of energy deficit and lipolysis on ketone production, it has been long thought that the efficient treatment of ketosis should aim to reduce excess negative energy balance (NEB) and lipolysis, while improve gluconeogenesis (Cote, 1971, Herdt and Emery, 1992). The current treatment of ketosis thus focuses on the acute resolution of hyperketonemia and NEB via the use of glucogenic precursors —such as propylene glycol (PG) and glycerol (Johnson, 1954, Maplesden, 1954, Emery et al., 1964, Piantoni and Allen, 2015)— which are metabolized to glucose in the liver and may thus reduce hepatic ketone synthesis (Sauer et al., 1973, Grummer et al., 1994, Chung et al., 2009). Indeed, PG (100%; 300 mL/d for 5 d) is often considered to be the most efficacious treatment for ketosis available (Gordon et al., 2013). This course of treatment can increase the odds of resolving SCK (McArt et al., 2012b, Capel et al., 2021), preventing SCK from advancing toward CK (McArt et al., 2011), and potentially improving milk yield relative to untreated cows (Nielsen and Ingvartsen, 2004). Despite such promising results, palliative or prophylactic treatments are costly (McArt et al., 2014), and often do not reduce disease incidence or normalize of milk yield (Studer et al., 1993, Miyoshi et al., 2001, Pickett et al., 2003), while their efficacy may differ for younger and older cows and by level of hyperketonemia and glycemia (Gordon et al., 2017b, Denis-Robichaud et al., 2022). Although renewed efforts have been made to identifying better treatments for ketosis via improving glucose production or availability (e.g., combination of PG with dextrose, recombinant insulin, vitamin B12 and phosphorus), these have had limited success to improve outcomes beyond those achieved with PG alone (Gordon et al., 2017a, Capel et al., 2021, Denis-Robichaud...
et al., 2022). These findings indicate that the correction of hyperketonemia with glucogenic precursors does not guarantee the restoration of health and productivity in dairy cows, and further supports the notion that other poorly understood metabolic or physiological factors—different from hyperketonemia alone—can contribute to metabolic dysfunction and negative peripartal outcomes. In fact, reduced serum vitamin D₃ status, increased levels of pro-inflammatory cytokines ([Tumor necrosis factor alfa (TNF-α) and 6 (1L-6)], as well as endotoxin (i.e., lipopolysaccharide; LPS) and lipopolysaccharide binding protein (LBP), have all been observed prepartum in cows that later develop ketosis and other peripartal disorders (Abuajamieh et al., 2016, Zhang et al., 2016, Wisnieski et al., 2020). These findings support the concept that factors predisposing cows to metabolic, physiological, and immune dysfunction may indeed precede the negative manifestations of production and health commonly credited to ketosis.

A shifting paradigm? Reconciling conflicting pieces of evidence. Our current understanding of the etiology of dysfunctional energy metabolism and its negative effects on milk production and health places ketones as central players. This paradigm has developed progressively over the past few decades as epidemiological studies have identified the contemporaneous occurrence of ketosis (i.e., hyperketonemia) and metabolic dysfunction during peripartum. Ketones are

Figure 4. Overview of concurrent metabolic events during peripartal ketogenesis, and their physiological and pathological consequences at the onset of lactation. Active lipolysis is promoted by reduced insulin action and increased growth hormone and glucagon signaling. Fatty acids reaching the liver may be contemporarily converted to ketones or incorporated into potentially lipotoxic lipids, such as ceramide and diacylglycerol. The pathogenic development of insulin resistance, inflammation, oxidative stress, and overall cellular dysfunction can contribute to compromised productivity, fertility, and health. These events are mechanistically linked to fatty acids but not to ketones. Esterification of fatty acids to triacylglycerols can actively occur and lead to fatty liver (not shown here).
indeed a necessary adaptation to energy deficit, and in this context, ketosis (understood as excessive ketones), can be seen as a manifestation of dairy cows failing to adequately adapt to the challenge of negative energy balance (NEB). Indeed, the contemporary discussions on ketosis address a crucial question: what is the actual role of ketones in the pathogenesis of peripartal disorders of dairy cows? The excellent reviews of Horst et al., 2023, and Mann and McArt, 2023, are recommended to readers interested in gaining an appreciation of the state of knowledge and controversies surrounding ketosis and its significance. Herein, we posit that our understanding of the factors leading to the development of peripartal dysfunction—including that of ketosis—is currently incomplete and insufficient to allow for the mitigation of peripartal metabolic disorders. This postulate is based on 4 salient observations: (1) the nature of the relation of circulating ketones with production and health is inconsistent; (2) unresolved inflammation and high blood levels of lipotoxic free fatty acids (FFA) typically ensue contemporaneously with—and can even precede—ketosis. This is relevant because excess FFA and unresolved inflammation can cause metabolic dysfunction, compromising production and health independently of ketones; (3) current treatments for alleviating ketosis have limited and variable effectiveness in improving production and health outcomes; (4) increased ketone availability can have positive effects on metabolic health via the attenuation of inflammation and the improvement of insulin sensitivity. These effects run contrary to the presumed negative roles of ketones, as derived from epidemiological research.

Based on the above, we suggest that the current paradigm regarding the role of ketones on peripartal dysfunction needs to be revised. A non-ketone-centric and more nuanced understanding of the factors leading cows to negative productive and health trajectories appears paramount, particularly in view of mounting evidence for positive effects of ketones on metabolism and health in non-ruminants and humans, which, to date, remain largely unaddressed in the dairy cow.
The classical view that ketones represent a detriment to health is not unique to livestock sciences; indeed, for several decades ketones have been considered noxious substances to animal metabolism, commonly associated with the onset of ketoacidosis in diabetic patients (Møller, 2020). Nevertheless, the myriad of metabolic and health-promoting effects recently discovered has revealed a more multidimensional nature of ketones, and has prompted a fundamental change in the way these molecules are perceived (Schugar and Crawford, 2012; Youm et al., 2015a; Goldberg et al., 2017a; Puchalska and Crawford, 2017). The negative perception surrounding ketones in dairy cow biology constitutes the current paradigm in animal sciences—which is largely based on epidemiological data—and appears to be strikingly similar to that historically held in human biomedicine. Unfortunately, we have not yet made the necessary progress for establishing whether a similar change in paradigm is conceivable in the dairy cow, particularly as our current efforts are mostly focused on the suppression of ketosis, which little focus on the evaluation of any potentially beneficial effects on physiology or health. Furthermore, because peripartal hyperketonemia occurs simultaneously with FFA elevations and inflammation, it has been difficult to independently and unequivocally assess the effects of ketones on metabolism, production, and health. The temporal concurrence of these events arguably limits our ability to clearly attribute poor health or performance outcomes to any single factor, including hyperketonemia (Figure 4). An important question arises, therefore, as to whether the metabolic perturbations leading to poor performance and health of peripartal cows occur because of—or despite—ketone accumulation. This question, however, cannot be answered explicitly at the moment, mostly because our focus has been historically heavily placed on the resolution of ketosis and not on the understanding of the effects of ketones themselves. Given the current discrepancies in outcomes provided by observational research, and illustrated in the previous sections, it is paramount to directly assess the effects of ketones individually and in conjunction with other factors, to attempt a fruitful reconciliation of the conflicting pieces of evidence. On the one hand, it is conceivable that ketones may contribute to metabolic dysfunction in particular scenarios, such as in situations that challenge and activate the immune system, including bacterial DNA and endotoxin exposure (e.g., leaky gut; Chirivi et al., 2023). In such moments where cows are experiencing reduced appetite and acute immune activation, ketones may exacerbate anorexia and may even counter the necessary inflammatory responses to respond to infection. On the other hand, however, it is also possible to envisage situations in which ketones may act as anti-inflammatory and insulin-sensitizing molecules in health cows with normal appetite and milk production, and even curtail the negative effects of excess FFA (i.e., lipotoxicity) and inflammation, to promote good health during the early postpartum. Such a nuanced understanding of ketosis is not only necessary to better prevent peripartal disease (i.e., the potential identification of better and earlier treatment targets), but also to ideally leverage the presumed positive effects that ketones, particularly, BHB, may exert on physiology and cow health. The definitive answer to these questions may only be attainable once a new body of controlled research that addresses these possibilities, is available.

At the heart of all discrepancies: what different types of evidence can tell us
We have thus far illustrated the nature of the paradoxical relationship between ketones and cow health and performance. In this section, we aim to contemplate the reasons behind the many discrepancies within the paradigm holding ketosis as a disease. Understanding how and when our interpretation of available information could limit our ability to solve the real issues surrounding peripartal health risks is crucial to determine novel focal areas and approaches for mitigation. Because much of our contemporary understanding of ketosis is based on the findings from observational studies (OR), it is critically important to understand the usefulness and potential limitations that this type of research may hold. Similarly, when trying to overcome some of the limitations here stated, it is important to understand why randomized controlled research can illuminate our understanding of ketosis and its consequences. For a detailed description of these types of research and their strengths and weaknesses, readers are directed to the classical epidemiological textbook of Dohoo and collaborators (2003), as well as to the recent work of Gershon and collaborators (2021), in which these 2 distinct types of research are contextualized to their use in healthcare decisions (Dohoo et al., 2003, Gershon et al., 2021). Furthermore, readers are directed to the excellent review by Horst et al. (2021), in which the authors thoroughly elaborate on the limitations of the historical overreliance on observational research to guide our understanding of peripartal disorders, namely hypocalcemia, hyperlipidemia and ketosis.

Observational studies are those in which individuals are observed for outcomes of interest, usually in the course of routine medical care (Gershon et al., 2021). Between-group differences in outcomes of interest (e.g., blood glucose, inflammatory markers, disease propensity, etc.) can be used to interpret the potential impact of the study factors (e.g., obese vs. lean) on the population being studied. Such insights can be powerful in
that they may be easily generalized, particularly when powered by data from large populations. Furthermore, these studies can be extremely useful in situations in which conducting a RCT is not feasible. Despite their advantages, OR is often limited by the difficulty to control for unmeasured confounding or other bias; this is because individuals are not randomized to study groups, they simply belong to a useful classification for study (e.g., hyperketonemia vs. normoketonemia). Lastly, OR is vulnerable to misclassification error or lack the necessary data to establish causal effects. While OR may not be generally about to answer the question of causation, some consider that estimation of causal effects is possible in situations where a dose-response can be observed that attenuates residual confounding, or when observed associations meet the Bradford Hill criteria (Guyatt et al., 2012, Fedak et al., 2015). The risk associated with a heavy reliance on OR to make inferences on causality is best elaborated by Young and Karr (2011), who indicate that observational claims fail to replicate at the alarming rate of 80% when tested via randomized experimentation. Furthermore, the authors suggested that “any claim coming from an observational study is most likely to be wrong – wrong in the sense that it will not replicate if tested rigorously” (Young and Karr, 2011). Based on the above considerations, a healthy skepticism on claims made from observational research may be warranted, particularly when these claims are used to attribute causality and to guide treatment efforts.

Randomized controlled experiments are generally considered powerful in that the allow us to make inferences about the causal effect of an intervention on outcomes because there is random distribution of measured and unmeasured confounding characteristics (Gershon et al., 2021). A major advantage of RCT is the randomization of individuals to intervention groups; this results in a random distribution of measured and unmeasured confounding factors that ultimately reduces bias. The major implication of randomization is that it grants the possibility of inferring causality, granted the study is sufficiently powered identify true effects, minimizing false negatives and false positives. Despite this critical advantage over OR, RCT may have limited generalizability if recruited individuals represent a select population, or if the study conditions do not fully reflect real life circumstances (Gershon et al., 2021).

Despite the differences in inferential outcomes mentioned above, the assessment of the usefulness of observational and controlled research does not need to be antagonistic. This is because these 2 kids of research can be complimentary, balancing for the limitations and strengths of each other. This also means that there is not necessarily a “better” type of research, but rather tradeoffs to consider when interpreting the results that arise from either of them. In the context of ketosis, observational research has made it possible to assess the relationship between ketosis and peripartal complications in dairy cows. As such, it can be said that this type of research has provided us with specific lines of inquiry to pursue via controlled experimentation. In so far as observational studies that identify ketosis as a correlate of disease and reduced performance are not interpreted as proof that ketosis—or more specifically, hyperketonemia—is causative of these outcomes, there may not be inferential concerns when looking at such studies. The questions that arise from observational research should then be tested in randomized controlled evaluations that can establish whether ketosis causes the above-mentioned issues, or whether it is itself a mere consequence of other root causes. If the latter were to be the case, then the focus on the resolution of ketosis may be unnecessary and / or unproductive unless said root causes are not also addressed. Figure 5 presents a short summary of the above-mentioned points regarding the use of OR and RCT, as well as the potential implications for the study of ketosis in dairy cows.

**Randomized controlled research in the dairy cow** Based on the limitations of observational research to answer the question of causality during ketosis, it appears necessary to use complimentary research approaches that may compensate for such limitations and provide more definite answers. As suggested in Figure 5, RCT stands as the de facto solution to this necessity, as it introduces the critical factor of random allocation of experimental interventions to study subjects (e.g., directly-infused ketones). This approach is of course not novel in and of itself, as it has been widely used in animal science research for many decades. The study of peripartal ketosis itself has seen the development of models aimed at recapitulating and studying the disease, such as that used by Drackley and collaborators in the 1990s (Drackley et al., 1991; Drackley et al., 1992). This methodology for the study of ketosis warrants recognition because of its success in recapitulating several metabolic features of ketosis, sus as hyperlipidemia, fatty liver, reduced milk production, and elevations in circulating concentrations of ketones. This model was developed on the basis of the relationship between dry matter intake and disease in the first week postpartum, and as such, it can be viewed as model of dietary energy restriction (ranging from 25 to 50% of feed restriction). In addition to energy deprivation, this induction model also necessitates the use of the ketogenic precursor 1,3-butaneediol (FRBD) model may carry some
inferential limitations when it comes to assessing the individual effects of ketones on health and productivity. First, by relying on the combination of feed restriction and ketogenic precursors, the previously-mentioned issue of confounding is introduced by design. This means that cows experience negative energy balance, rampant lipolysis, and fatty liver, all while simultaneously exhibiting elevations in circulating ketones. Attributing causation to any single factor—including ketones—is simply not possible under these conditions. Furthermore, feed restriction models likely impact feeding behavior in ways that may differ from those that take place during inflammation or disease. Regardless of this limitation in the context of ketosis, it is worth pointing out that the FRBD model appears to be very useful for understanding the response of dairy cows to energy deprivation and accelerated lipolysis during the first week of the postpartum, which remains a critical period to modern dairy cows. Second, despite the severity of feed restriction and the simultaneous use of a hepatic ketogenic precursor, the FRBD model falls short of achieving the levels of hyperketonemia characteristic of either SCK or CK (Drackley et al., 1991, Drackley et al., 1992). This second limitation is perhaps irrelevant however, if one considers that the current blood BHB thresholds for ketosis have limited predictive value for cow performance and disease, as highlighted in previous sections of this review.

A more specific evaluation of the effects of hyperketonemia on dairy cow metabolism and its consequences on outcomes of interest requires a targeted evaluation of ketones as a single intervention, that is, manipulate ketone concentrations specifically while minimizing other changes. Examples of this type of approach include the direct intravenous infusion of BHB salts, such as performed by Zarrin and collaborators, Swartz and collaborators, and more recently, by our group (Zarrin et al., 2014, Swartz et al., 2021, Barrientos et al., 2023, unpublished). Although the studies from the first 2 groups did not focus on production outcomes, their results showed the attenuation of inflammation responses of BHB-infused cows in response to intramammary bacterial infection, whether this was a mock infection (LPS injection, Zarrin et al., 2014) or a live-bacteria infection (S. uberis; Swartz et al., 2021). Their results further suggest that hyperketonemia may limit the ability of dairy cows to fend off some bacterial challenges, as indicted by increased bacterial load in milk. This effect, in turn, appears to agree with experiments that reported a more severe course of experimental mastitis (E. coli infection) during feed restriction-induced ketosis, relative to non-ketonicemic dairy cows (e.g., Kremer et al., 1993). Of note, however, direct comparability of across these studies should be done carefully, as it may be limited by the potential differences in immune responses that can arise from live bacteria vs. LPS administration. While these hyperketonemia-inducing approaches have the advantage of directly evaluating the effects of ketones on physiology and metabolism, they are not without potential drawbacks. A salient limitation of ketone manipulation (exogenous ketone supply) for the study of ketosis is that it negates the negative energy balance that characterizes dairy cows entering lactation, and who could ultimately succumb to peripartal disease. Although the control of ketones for the study of ketosis may help shed light on the specific effects of ketones on cow physiology, this approach may limit our ability to characterize their effects during states of energy deprivation. Furthermore, unintended effects of infusing salts of ketones may occur following the co-injection of the solutes that carry the ketones (e.g., Ca²⁺, Na⁺), such as the induction of blood hypertonicity, alkalinization of blood pH; together, these changes may lead to progressive reductions in the tone of immune cells, the effectiveness of immune responses, and may thus compromise metabolic health independently of ketones (Goff, 2008, Jobin et al., 2021).

A second group of methodologies for the study of hyperketonemia includes the nutritional induction of ketosis (NK) via dietary ketogenic precursors. In contrast to the FRBD model, in which ketosis is induced via hepatic ketogenesis, we have recently utilized a simple model of NK induction that relies on the ability of the ruminal epithelium to synthesize ketones (Rico et al., 2021, Ruiz-González et al., 2022, Barrientos et al., 2023), and uses calcium butyrate as a nutritional precursor during acute (12h) or episodic (2X boluses over 5 d) induction in rumen-fistulated and non-fistulated cows. Our preliminary studies using the NK model has so far been able to recapitulate hyperketonemia at an above the blood BHB concentrations that characterize SCK and CK, which is a desirable starting point for the study of nutritionally-induced ketosis (Rico et al., 2021, Ruiz-González et al., 2022). Of note, the model induced transient reductions in DMI and milk production, both of which returned to control levels by d 4 and 5 of induction, respectively (Ruiz-González et al., 2022). In terms of health, no acute signs of impairment were observed during hyperketonemia, including no differences pain scores, rectal temperatures or respiration, or changes in blood pH and respiration gases. Assuming these findings can be replicated, the lack of effects on blood pH and gases represents an advantage of the NK model relative to direct intravenous infusions since any good model of ketosis should minimize secondary effects not related to the specific manipulation of ketones alone. Nevertheless, with our limited data, we cannot rule out the potential drawbacks of the NK model. For example,
butyrate, or ketones themselves, may impact gut physiology and appetite in ways that may differ from those taking place in cows with natural hyperketonemia. As mentioned in the previous sections, the reported effects of ketogenic diets in humans may induce appetite suppression via bottom-upregulatory mechanisms, such as intestinal CCK, peptide YY, and GLP-1 stimulation. Similarly, we can envision that the NK model could induce compositional changes in the gut microbiome (i.e., ruminal or lower gastrointestinal tract), which may lead to alterations not observed during naturally occurring hyperketonemia. We cannot rule out that these mechanisms may operate in the dairy cow under the NK model, particularly in view of the apparent lack of effects on appetite reported with intravenous infusion models. Definitive answers to these possibilities will need to be explored in future investigations.

The above-mentioned potential limitations of the methodologies used to induce ketosis should be considered when interpreting and assessing the quality of available evidence. These potential issues further highlight the importance of keeping a healthy skepticism regarding claims we might make on the basis of limited data, regardless of whether they suggest positive or negative effects of ketones in dairy cows. A larger body of work, perhaps using different induction methodologies, is needed, so that we may draw more definite conclusions based on the convergence of results arising from several lines of evidence.

**Building a case for ketones**

*The putative positive effects of ketones on metabolism and health* As described in the previous sections, ketones have a crucial role as energy substrates to support peripheral tissues in the postpartum, a moment in which glucose utilization is prioritized for use in the mammary gland, and therefore, less readily available to sustain basal energy metabolism (Drackley et al., 2001; Laffel, 1999; White & Venkatesh, 2011). Furthermore, there is a growing body of evidence in the literature suggesting that ketones can also exert other positive effects by acting as anti-inflammatory, neuroprotective, and insulin sensitizing mediators, to treat and prevent chronic diseases in humans and other non-ruminant animal models (Park et al., 2011; Youm et al., 2015b; Goldberg et al., 2017b; Yamanashi et al., 2017; Møller, 2020). Such evidence has prompted a renewed interest in the use of ketone esters and their dietary precursors (e.g., ketogenic diets; 1,3, butanediol) in clinical science and medicine for the treatment of obesity, fatty liver, and even of neurodegenerative diseases (Schugar and Crawford, 2012; Puchalska and Crawford, 2017; Møller, 2020). While it is still unknown whether ketones can exert similar effects in ruminants, existing data indicates that ketotic dairy cows can display normal milk production and health—a finding supported by our data (Rico et al., 2021, Ruiz-González et al., 2022)—, which runs contrary to the current paradigm holding ketosis as a disease state that compromises healthy metabolism. We therefore postulate that ketones may play yet unidentified positive functions on cow metabolism and physiology that go beyond their mere role as energy substrates, and which warrant investigation. Furthermore, we contemplate the possibility that increased ketogenesis and ketone availability may serve a therapeutic approach to mitigate conditions such as peripartal oxidative stress and uncontrolled inflammation.

Given the dearth of available information regarding the effects of ketones and ketosis in dairy cows (i.e., causative support), the potentially beneficial effects of these molecules on cow metabolism may only be approximated by extrapolation from research in non-ruminants, such as mice and humans. The major pleiotropic effects of increased ketone availability include the attenuation of inflammation and oxidative stress, and the enhancement of endothelial and mitochondrial function (Newman and Verdin, 2014, Youm et al., 2015, Yurista et al., 2021).

The anti-inflammatory action of BHB is exerted via attenuation of signaling in the NOD-like receptor protein 3 (NLRP3) inflammasome pathway, a multiprotein complex that triggers the activation of inflammatory caspases, including caspase-1 (Youm et al., 2015, Bae et al., 2016). Specific BHB uptake and metabolism in immune cells (e.g., macrophages and neutrophils) (Newsholme et al., 1986) appears to mediate a protective role during inflammatory conditions such as gout, attenuating pain and inflammation by reducing Toll-like receptor 4-mediated interleukin1β (IL1β) release and NLRP3 inflammasome assembly (Youm et al., 2015, Goldberg et al., 2017).

The protective roles of BHB against oxidative stress are mediated by its action on stress response pathways. The mechanisms include the inhibition of class-I histone deacetylases (HDACS), and are related activation of forkhead box O3 (FOXO3a) and metallothionein 2 (Mt2) promoter genes, increased expression of super oxide dismutase, and ultimately to reduced mitochondrial stress (Shimazu et al., 2013, Newman and Verdin, 2014, Uchihashi et al., 2017). Furthermore, by acting as an alternative energy substrate, BHB can improve redox state and mitochondrial membrane potential, thus helping sustain normal mitochondrial function (Horton et al., 2019).
Building a case against ketones.

The putative negative effects of ketones on metabolism and health. In view of the negative associations between ketosis and dairy cow health and productivity, understanding the mechanisms mediating such potential effects becomes paramount. Unfortunately, as mentioned in previous sections, the study of ketosis in dairy cows has been historically limited in its ability to demonstrate causal relationships between hyperketonemia and metabolic dysfunction, and to date the root causes of peripartal dysfunction remain unclear (Horst et al., 2021). This is the case for observational studies that characterize the metabolism and physiology of cows that are already experiencing ketosis, as well as that from controlled studies in which confounding factors (e.g., hyperlipidemia, fatty liver) do not permit the evaluation of the effects of ketones. This section will therefore attempt to summarize the available literature in which causal effects of hyperketonemia may be derived, to then build a framework for the understanding of the potentially negative roles of ketones in the pathophysiology of peripartal diseases.

Given the mechanistic vast body of evidence suggesting anti-inflammatory roles for ketones, it might seem counterintuitive to suggest that antiinflammation could be detrimental. Nevertheless, we conceive the possibility that the attenuation of inflammatory responses could limit the ability of the animal to mount a strong response against acute immune challenges (i.e., bacterial infection). Support for this possibility comes from Swartz and collaborators (2021), who reported that an intravenous infusion of BHB salts at the SCK level might have limited the immune response to a mammary *S. uberis* insult, as indicated by a delayed febrile response and higher bacterial counts in milk, responses interpreted as a BHB-induced tolerance to bacterial challenge (Swartz et al., 2021). Similarly, the study of mammary immune response to LPS challenge by Zarrin and collaborators (2014) found a reduced influx of leucocytes from blood into milk (i.e., lower milk SCC) relative to the NaCl control, along with a more pronounced mRNA of the cytokines interleukin 8 (IL-8; pro-inflammatory) and interleukin 10 (IL-10; anti-inflammatory; Zarrin et al., 2014). These results seem to align with in vitro models that have reported reduced neutrophil bacterial killing abilities, lymphocyte function, and phagocytosis in bovine milk macrophages (Khucinski et al., 1988, Franklin et al., 1991, Grinberg et al., 2008). However, it should be noticed that the negative effects of ketones on immune function were variable and dose dependent and that culture conditions maybe not necessarily reflect physiological conditions for critical metabolites such as glucose. Furthermore, some of these findings are contradicted by other studies in which immune function of mononuclear leukocytes (i.e., IgM response) was unchanged or even enhanced by ketone supplementation (Nonnecke et al., 1992), and other in vivo studies where there was no observable relationship between ketones and glucose to immune function (e.g., serum and milk IgG, and total peripheral leukocytes; Ropstad et al., 1989). Nevertheless, it should be noted that these 2 studies are limited by the fact that they only address very specific aspects of immune responses, and that Ropstad et al. (1989) based their claims on observational data, which, as we discuss in this review, cannot possibly be used to draw inferences on causation.

Given the variation stated above, a clear definitive answer remains elusive, particularly as we consider the limited number of in vivo RCT available to date. Contemplating the evidence for potentially negative effects of ketones on immune activity, we suggest this relationship be considered in a careful and nuanced manner. Clear distinctions may need to be made when discussing the effects of ketones in situations such as chronic inflammation (potentially positive effect) or during the acute response to an infectious challenge (potentially negative effect).

The future of ketosis and ketone research. Is ketosis a disease, a cause of disease, or neither? Could ketosis be a misnomer distracting us from a wider understanding of the origins and the course of metabolic dysfunction in the peripartum? More than a simple issue of semantics or any rhetorical intent, we consider these are crucial questions worth asking, particularly after more than a century of having identified ketosis as a negative condition afflicting dairy cows. In this context, we put forward the idea that our collective focus on ketosis and its resolution may be limited in reach and that it could inadvertently dilute the efficacy of our efforts to find other novel solutions for the prevention and treatment of peripartal disease and its consequences. The multifactorial nature of the changes that occur during this high-risk period needs to be matched by a multifactorial view of interacting causes, where resolving ketosis—an arguably natural response to energy deficit—may not need to be at the center of our focus. We also ponder on the question of whether the convenience of ketone measurements at the farm may have contributed to our emphasis on hyperketonemia (consider that lipotopic FFA are not part of cow-side tests), which ultimately may turn out to be a downstream consequence of other root causes (e.g., infection, uncontrolled inflammation, energy deficit, etc.). Were this the case, ketones could also be viewed as mere passengers, or even as firefighters overwhelmed while trying to put down too big of a fire. Given the limits of
observational research, we propose that the possibility that ketones are—or not—bad actors be sufficiently evaluated via controlled research, particularly considering the growing body of evidence indicating that ketones can play important roles in regulating animal metabolism to promote good health. A nuanced view of ketosis that allows room for a wider understanding of causes and consequences might in turn enhance our efficacy in preventing and treating periparturient disorders, and take us in new directions conducive to sustaining and enhancing dairy cow health, welfare, and performance.

REFERENCES


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