Yeast Microbial Protein Supplementation in Transition Dairy Cows: Effects on Metabolic Status and Follicular Microenvironment

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It's not about the final product, it's about the journey, the experience, the thrill of putting something together saying, 'yes! I did this!'

- Seán McLoughlin

ABSTRACT

Metabolic stress in transitioning dairy cows is characterized by low glucose and high β-hydroxybutyrate (BHBA) and non-esterified fatty acids (NEFAs). Such a metabolic milieu appears to have negative effects on the ovarian follicle and overall fertility. We hypothesized that supplementing transition cows with rumen protected yeast-derived microbial protein (YMP) alleviates metabolic stress and enhances ovarian follicular function. For this, we supplemented the diet of transition dairy cows with (n=6; supplemented) or without (n=13; control) YMP. Supplemented cows received 50g of YMP daily for 3 weeks before calving and 200g daily for 4 weeks after calving. Blood samples were collected at weeks -3, 0, +1 and +7 relative to calving. Liver biopsies were taken at weeks -3, 0 and +7 relative to calving. Follicular fluid from the dominant follicle of a synchronized wave of each cow was collected at 7 weeks post-partum. Glucose concentrations decreased and BHBA levels increased from week -3 to +1 relative to calving in control cows ($P < 0.05$), but not in YMP-supplemented cows. In the liver, the mRNA abundance of genes associated with hepatic gluconeogenesis were higher (*G6PC*; $P < 0.05$ and *PCK1*; $P < 0.10$) in supplemented cows compared to control cows. At 7 weeks post-partum, supplemented cows had lower levels of NEFAs in the follicular fluid of the dominant follicle compared to control cows ($P < 0.05$). Also, follicular fluid of supplemented cows had lower proportions of palmitic acid (C16:0) and oleic acid (C18:1) ($P < 0.05$). Lastly, the dominant follicle of the supplemented cows had higher levels of 17β-estradiol ($P < 0.05$) and higher estradiol:progesterone ratio ($P < 0.01$) compared to control cows. Overall, the supplementation with YMP improved the consistency of the metabolic status of transition dairy cows by enhancing gluconeogenesis and stabilizing lipid mobilization. Importantly, this alleviation of metabolic stress was associated with improved follicular microenvironment at 7 weeks post-partum, the period immediately before the expected breeding of these cows.

RÉSUMÉ

Le stress métabolique chez les vaches laitières en transition se distingue par une faible glycémie et des niveaux sanguins élevés de β-hydroxybutyrate (BHBA) et d'acides gras non estérifiés (NEFA). Cet état métabolique semble avoir des effets négatifs sur le follicule ovarien et la fertilité globale. Nous avons émis l'hypothèse qu'un supplément avec la protéine microbienne dérivée de la levure (YMP) puisse alléger le stress métabolique et améliorer la fonction folliculaire ovarienne des vaches laitière en transition. Pour cela, nous avons complété le régime alimentaire des vaches laitières en transition avec (n = 6 supplémentées) ou sans (n = 13 témoins) YMP. Les vaches supplémentées ont reçu 50 g d'YMP par jour pendant 3 semaines avant le vêlage et 200 g par jour pendant 4 semaines après le vêlage. Des échantillons de sang ont été prélevés aux semaines -3, 0, +1 et +7 relativement au vêlage. Les biopsies hépatiques ont été effectuées aux semaines -3, 0 et +7 par rapport au vêlage. De plus, nous avons synchronisé les cycles d'oestrus des vaches et recueilli le liquide folliculaire du follicule dominant 7 semaines après le vêlage. Les glycémies ont diminué et les taux de BHBA ont augmenté de la semaine -3 à +1 par rapport au vêlage chez les vaches témoins ($P < 0,05$), mais pas chez les vaches supplémentées avec YMP. Dans le tissu hépatique, l'abondance de l'ARNm des gènes associés à la gluconéogenèse hépatique était plus élevée chez les vaches supplémentées (G6PC, $P < 0,05$ et PCK1, $P < 0,10$) comparativement aux vaches témoins. Sept semaines après la parturition, les vaches supplémentées présentaient des concentrations de NEFA plus basses dans le liquide folliculaire du follicule dominant que les vaches témoins ($P < 0,05$). De plus, le liquide folliculaire des vaches supplémentées avait des proportions plus basses d'acide palmitique (C16: 0) et d'acide oléique (C18: 1) ($P < 0,05$). Enfin, le follicule dominant des vaches supplémentées présentait des taux plus élevés de 17β-œstradiol ($P < 0,05$) et un ratio d'estradiol : progestérone plus élevé ($P < 0,01$) que chez les vaches témoins. En résumé, le supplément alimentaire d'YMP a amélioré la cohérence de l'état métabolique des vaches laitières en transition en améliorant la gluconéogenèse et en stabilisant la mobilisation des lipides. Ce soulagement du stress métabolique était surtout associé avec un meilleur microenvironnement folliculaire à 7 semaines post-partum, soit la période précédant immédiatement l'insémination prévue de ces vaches.

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LIST OF ABBREVIATIONS

AA: Amino Acid

AI: Artificial Insemination

ATP: Adenosine Triphosphate

CAC: Citric Acid Cycle

CL: Corpus Luteum

CP: Crude Protein

CYP11A1: Cholesterol Side-Chain Cleavage Enzyme (P450scc)

CYP19A1: Aromatase

DA: Displaced Abomasum

DIM: Days in Milk

DMI: Dry Matter Intake

E2: 17β-Estradiol

FBP: Fructose-1-6-Biphosphotase

FSH: Follicle Stimulating Hormone

FSHR: Follicle Stimulating Hormone Receptor

G6PC: Glucose-6-Phosphatase

GnRH: Gonadotropin Releasing Hormone

IFNτ: Interferon-tau

IGF-1: Insulin-like Growth Factor-1

IL-6: Interleukin 6

LH: Luteinizing Hormone

LHR: Luteinizing Hormone Receptor

NEFA: Non-Esterified Fatty Acid

NEB: Negative Energy Balance

NPB: Negative Protein Balance

OAA: Oxaloacetate

P4: Progesterone

PC: Pyruvate Carboxylase

PCK1: Phosphoenolpyruvate Carboxykinase-1

PCK2: Phosphoenolpyruvate Carboxykinase-2

$PGF_{2\alpha}$: Prostaglandin

RDP: Rumen Degradable Protein

RUP: Rumen Undegradable Protein

SCK: Sub-Clinical Ketosis

TAG: Triglyceride

VFA: Volatile Fatty Acid

YMP: Yeast Microbial Protein

I. INTRODUCTION

Canada is home to one of the most prestigious dairy industries in the world. Contributing $19.9 billion to Canada's GDP and providing over 220,000 jobs, the Canadian Dairy Industry is a staple of the Canadian Economy [1]. In terms of milk yield, Canadian dairy cattle performance has increased over 130% in the past 40 years [1]. This attractive feature is one of the reasons why Canadian Dairy Genetics are valued as some of the highest quality on the international market. In 2015, the export of genetic material in the form of cattle, embryos, and semen was valued at $140 million [1]. Yet, despite all the interest in our dairy cattle, fewer than 42% of dairy cows in Canada achieve their third lactation [2]. This fact is astonishing, when one considers that farmers will only begin to achieve a profit, accounting for the costs of rearing and/or replacement, mid-way through a cow's second lactation [3]. For animal removal, reproductive problems, at 15.81%, is cited as the most common reason for the involuntary culling of dairy cattle [2]. In order for a cow to continue producing milk, subsequent pregnancies must be achieved within a certain period of time to maintain profitability, usually within 465 days [4]. The metabolism of the modern Canadian dairy cow, in many cases, is unable to balance high milk yields with a reproductive performance capable of achieving and sustaining a pregnancy. As a result, the high milk production in Canadian dairy cattle is challenging their fertility. To mediate the effects of the energy deficit, a specific period in a dairy cow's lactation must be carefully managed. This period, known as the transition period, is classically defined as 3 weeks ante-partum to 3 weeks post-partum and includes the dramatic metabolic shift a cow experiences when she goes from a dry, non-lactating animal to a high performance, lactating animal [5]. The transition period is associated with the highest rate of morbidities and mortalities in dairy cattle [6]. This thesis will examine the transition period, the various management techniques through nutrition, and ultimately, the effect of a poor or proper transition period on the reproductive capabilities of Canadian dairy cattle.

II. REVIEW OF THE LITERATURE

1. The Transition Cow

The transition cow is classically defined as a dairy cow during the period of 3 weeks ante-partum to 3 weeks post-partum. During this period, a dairy farm will experience 75% of all health problems and 36% of its mortalities [2, 6]. The risk of illness is augmented due to the substantial energy that is put into milk production, energy that would normally be utilized to healing and illness abatement. The risk of illness starts at the beginning of the transition period, in the last month of the cow's pregnancy. At the end of the third trimester, the calf and placenta grow exponentially in size, which puts physical constraints on the cow's dry matter intake (DMI), resulting in reduced energy balance (Figure 1) [7]. Additional energy is also required to sustain the final growth of the calf. Because of these constraints, a cow can enter negative energy balance (NEB) before parturition. A cow in NEB will thus begin to mobilize her body reserves in order to meet energy requirements. The main tissue that is mobilized is adipose tissue and high rates of lipolysis result in an increase in circulatory levels of non-esterified fatty acids (NEFAs), which can lead to triglyceride (TAG) accumulation on the liver. NEFAs are used extensively in milk production during early lactation, accounting for up to 40% of milk fat production [8]. However, when NEFAs are not used in lactogenesis, they will be taken up by the liver and undergo beta-oxidation to produce energy carries, such as ATP [8]. In the liver, NEFA oxidation has three fates: 1) complete oxidation, resulting in carbon dioxide and ATP, 2) incomplete oxidation, resulting in the production of ketone bodies, or 3) re-esterification, resulting in the production and accumulation of TAG on the liver [9].

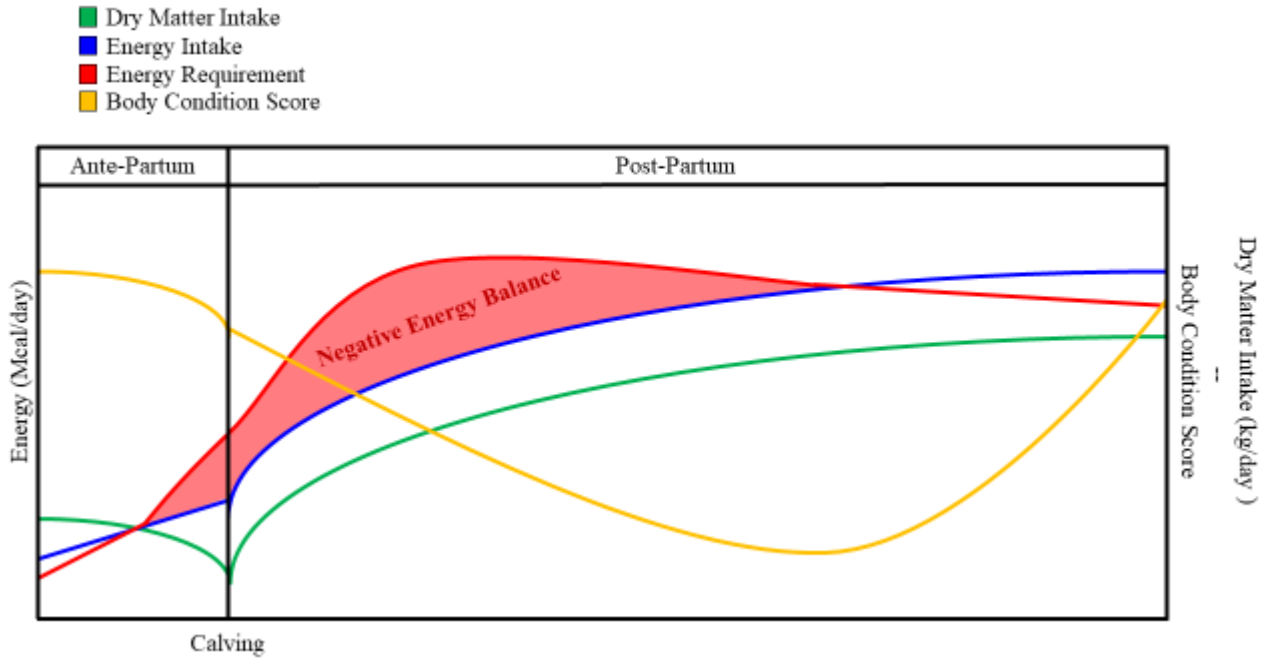

Figure 1. Dry Matter Intake, Energy Intake, Energy Requirement and Body Condition Score throughout the Transition Period (Adapted from Chung et al., 2008 and Hoffman, 2000) [10, 11].

Despite the energy requirement of fresh cows and the initiation of lipolysis, the liver has a limited ability to process the NEFAs created by lipolysis [12]. When NEFAs are metabolized in the liver for use as energy, TAGs are produced, however, when the liver's limit to process the NEFAs is reached, TAGs will begin to accumulate on the surface of the liver, rather than continue to be processed for energy [12]. The accumulation of TAGs is a normal phenomenon, yet, if a NEB promotes severe fat mobilization, the accumulation of TAGs on the liver can reduce hepatic functions such as gluconeogenesis and NEFA oxidation, further diminishing the liver's ability to produce energy [13].

This reduction in performance leads to incomplete oxidation of the NEFAs being metabolized. The incomplete oxidation of NEFAs produces ketone bodies such as β-hydroxybutyrate (BHBA) [9]. The reduced ability to metabolize and the accumulation of ketone bodies have the potential to lead to further metabolic disorders, thereby increasing susceptibility to disease and reducing reproductive efficiency [9]. In order to reduce the effect of metabolic disorders on the health and reproductive success of dairy cows, the transition period must be managed efficiently to ensure the NEB does not reach severe levels. Various

farms employ different management strategies to ensure the health of the transition cows, however, a key component is nutrition management.

2. Nutrition

2.1. Negative Nutrient Balance

2.1.1. Energy

The NEB a cow experiences during the transition period is unavoidable. At the beginning of the transition period, there is a noticeable drop in DMI in dairy cows (Figure 1). Transition dairy cows require approximately 13.7 Mcal/day for maintenance and gestation requirements, yet as calving approaches, the decrease in DMI can result in energy intakes of less than 11.1 Mcal/day [14]. The decrease in DMI, despite higher requirements, is not only due to hormonal stress, colostrogenesis, and the development of mammary tissue, but also because of the exponential growth of the calf and associated reproductive tissues in the final stage of gestation [7]. Simply put, dairy cows cannot physically consume the energy they require at the end of pregnancy.

Following parturition, dairy cattle lose the physical barrier to their DMI, but their energy output demands increase exponentially with the onset of milk production. The National Research Council (NRC) describes the amount of energy a fresh cow requires for maintenance and milk production as 27.9 Mcal/day [14]. Once again, the cow is physically unable to consume enough DMI to meet these demands. However, this offset in requirements may last much longer than the reduced DMI at the end of pregnancy, with a range from 24 days in milk (DIM) to 66 DIM [15]. Lactogenesis demands an increase in glucose synthesis and directs nutrients to the milk rather than for use by the cow. In Figure 1, while a gradual increase in DMI is observed, the energy demands drastically increase in a short period of time. It is this difference in energy consumed versus energy required that defines the NEB. The NEB will be present until the energy in the DMI matches the energy output in production [15]. It should be clearly noted, however, that the NEB experienced by each individual cow can vary drastically even within a herd and therefore management of this time period an important area of research.

As mentioned previously, the NEB experienced by dairy cows is a natural phenomenon, but the issues in management arise when the cow experiences a severe NEB. During this state, TAGs are mobilized from adipose reserves and circulatory levels of NEFAs increase. For this paper, a severe NEB is achieved by a cow when circulatory levels of BHBA reach levels

higher than 1200 μmol/L. This is the threshold level for sub-clinical ketosis (SCK), which will be described in greater detail below [16]. Dairy cows are particularly at risk for experiencing SCK during the early lactation due to a phenomenon known as insulin resistance. To support the demand in milk production, glucose is diverted to the mammary gland so that lactose can be produced [17]. For this to occur, peripheral insulin resistance is initiated in other tissues [17]. These tissues will then have to receive the majority of their energy from the NEFAs and TAGs from lipolysis, and if fat mobilization reaches the point where the liver is unable to process NEFAs efficiently, the increasing level of ketone bodies in circulatory will also become an alternative energy source for peripheral tissues [17].

2.1.2. Protein

Another component of the transition period is the negative protein balance (NPB) [18]. NPB is inferred under the NEB status, however, it should also be given direct consideration, as protein supplies amino acids (AAs) which are used in energy production and to sustain milk yield [19]. A study by Komaragiri and Erdman found that transition dairy cows have the potential to mobilize, not only 54 kg of body fat reserves during the transition period, but also 21 kg of body protein, which peaked at 2 weeks post-partum, the same time that the NEB can be at its most severe [20]. Therefore, cows are not only mobilizing their fat reserves to meet energy requirements, but are also mobilizing their protein reserves to meet AA requirements [18]. This is unsurprising when one considers that Methionine and Lysine are the rate limiting AAs for milk production and with insufficient DMI post-partum, protein mobilization is required to meet these AA requirements [14].

Cows receive a majority of their AAs from the microbial protein of rumen microorganisms [19]. Microbe colonization is critical to the function and performance of the rumen. Rather than directly digesting dry matter herself, 70-80% of a cow's DMI is digested by the bacteria, protozoa, and some fungi that, in turn, ferment the DMI into products the cow can efficiently digest [19]. The three major products of microbial fermentation are: 1) gases (such as carbon dioxide and methane), 2) volatile fatty acids (VFAs), and 3) ammonia [19]. While gases are eructated from the rumen as waste and VFAs are directly absorbed through the rumen wall to be used as a major energy source for the cow, ammonia is first transformed into microbial protein to sustain the microbial population [19]. However, this ammonia is indirectly consumed by the cow as microbial protein of the microbes that leave the rumen to be digested in the small intestine [19]. Microbial protein is responsible for 60% of the protein digested by the cow and supplies an ideal AA profile for the protein requirements of lactating dairy cows,

providing, among other essential AAs, a source of Methionine and Lysine [19, 21]. Therefore, as stated by Clark et al., the sources of protein and AAs in the dairy cow's diet must be carefully considered, and the sources must either encourage the growth and proliferation of the rumen microbial population or compliment the AA profile of those proteins [21, 22].

The crude protein (CP) in the diet of dairy cattle can be separated into two categories: rumen undegradable protein (RUP), which is not degraded in the rumen and moves onto the cow's digestive tract unchanged, and rumen degradable protein (RDP), which is used as a food source for the microbial population [14]. RUP and RDP are both required in varying amounts. In the post-partum diet, the NRC recommends that 7.5-9.5% of the diet be RUP, in order to increase AA absorption in the cow's small intestine, and that 10.5% of the diet be RDP in order to stimulate microbial growth and microbial product synthesis (i.e. VFAs and microbial protein) [14].

While protein is required for milk production, high levels of CP in the diet increase the levels of ammonia in the blood and urine [23]. Ammonia has a negative effect on the DMI and milk yield of dairy cattle, among other negative effects which will be discussed below [20]. Therefore, it is critical to balance the CP, RDP and RUP of a specific feed source with the diet of post-partum transition cows. In order to do so, the producer must care for the microbial population first and foremost by feeding the required amounts and balancing the RDP and RUP, all while maintain optimum rumen health to stimulate the production of microbial protein [24].

The key factor in feeding proteins is to improve the supply of AAs, both essential and non-essential to the cow during the NEB/NPB. While the AA profile of microbial protein is ideal for the requirements of milk production, microbial protein alone cannot meet the demands of milk production and dietary CP must be managed in addition to microbial protein [21]. Furthermore, the decrease in DMI and resulting NEB that occurs post-partum, reduces the ability of the microbial population in the rumen to produce products such as VFAs as well as produce adequate amounts of microbial protein [14, 25]. During the transition period the microbial protein that is produced is absorbed in the small intestine as AAs, which are then used in milk production and gluconeogenesis [21]. Propionate, a VFA, is the major precursor of gluconeogenesis when cows are not experiencing a NEB. However, during the early post-partum period, AAs contribute to 20% of glucose production through gluconeogenesis [26,

27]. This role of AAs helps to support the cow through her NEB by improving the energy status. Still, AAs also serve other important roles. Limited energy and AA availability reduces the expression of genes related to hepatic function, reduces the ability of the liver to detoxify free radicals, and increases TAG storage in the liver during NEB, thereby increasing the risk of metabolic disorders [12]. Supplementation with specific AAs has been shown to improve the immune response in transition dairy cows by increasing Il-10 secretions [28]. Il-10 downregulates the expression of pro-inflammatory cytokines, and in mice, has been reported to suppress antigen immune responses [28]. This result is of particular interest to studies concerning the transition period, since post-partum inflammation that is not resolved within the first 3-4 days post-partum, increases the risk of metabolic disorders, and consequently, negatively affect the performance of the animal [12].

In order to improve the energy and immune status of transition dairy cows, protein supplements that best match the profile of rumen microbial proteins should be considered.

2.2 Liver Metabolism

The onset of calving brings about drastic metabolic changes that occur over a short period of time. It is the liver which processes energy sources, whether they are internal or external sources, and metabolizes them into an appropriate energy units that can be used by the cow. The issue in dairy cattle, is that the external energy sources, which provide more appropriate and less stressful energy units, cannot match the energy output of the high milk production. Therefore, a majority of the energy used during early lactation will come from internal sources [12].

In the later stages of lactation, dairy cows enter a period of positive energy balance, during which, they take in more energy than is required by maintenance and production. The excess energy is stored as TAGs in the lipid droplets of white adipose tissue but can also be stored on the cardiac and skeletal muscles as well as on the liver [29, 30].

Lipolysis occurs when TAG stored in fat deposits undergo hydrolysis to NEFAs that are released into circulation. Three hydrolases are capable of performing this activity: hormone-sensitive lipase (HSL), monoglyceride lipase (MGL) and adipose triglyceride lipase (ATGL) ([29, 30]. NEFAs then travel through circulation to be metabolized in the liver, heart and muscle [30]. In cardiac and skeletal muscle, NEFAs are used for cellular energy production through beta-oxidation followed by the citric acid cycle (CAC) and oxidative phosphorylation [30]. In the liver, NEFAs are taken up into hepatocytes and translocated into

mitochondria where beta-oxidation occurs to produce acetyl-CoA [30]. Alternatively, NEFAs are retained in the cytoplasm and are re-esterified to TAGs to be secreted as a VLDL as source of energy for other tissues such as heart and muscle [31, 32]. The beta-oxidation product Acetyl-CoA then enters the CAC followed by oxidative phosphorylation to produce ATP [32]. Utilization of NEFA for ATP production is limited to energy needs of hepatocytes [31]. In the cases of highly conditioned cows or cows with drastic decreases in body condition there are higher levels of fat mobilization post-partum and hepatocytes can become overwhelmed by the amount of NEFAs arriving to be metabolized [31]. When this occurs, some NEFAs are partially oxidized and ketones (BHBA) are produced or NEFAs will be esterified back into TAGs which accumulate on the liver as storage until they can be processed [31]. The storage of TAGs on the liver further impedes the efficiency of metabolism such that the cow can develop fatty liver disease [31]. Likewise, the overproduction of BHBA will initiate ketosis. Both of these diseases will be discuss in a further section below.

The other main metabolic pathway that is drastically altered in transition dairy cows is gluconeogenesis [33-36]. As mentioned previously, the rumen environment makes it difficult for energy units to pass through unaltered. The microbial populations in the rumen produce VFAs, which the cow absorbs through the rumen wall or in the small intestine. The VFA propionate is the main source of carbons for glucose production through gluconeogenesis [35, 36]. Gluconeogenesis also uses AAs and lactate to produce glucose for organs like the brain and for lactose synthesis in milk production [34]. Propionate enters the gluconeogenic cycle by metabolism through succinate which is then transformed into oxaloacetate (OAA) in the CAC (Figure 2) [35]. Many AAs will also undergo transformation to OAA in order to enter the gluconeogenic cycle (Figure 2) [35]. Aschenbach et al. reviewed the process extensively and found that the cytosolic enzyme phosphoenolpyruvate carboxkinase-1 (PCK1) is responsible for the entry of AAs and propionate into the gluconeogenic cycle, while the mitochondrial enzyme phosphoenolpyruvate carboxykinase-2 (PCK2) is responsible for the entry of lactate, both of which require OAA as the precursor in order to produce phosphoenolpyruvate, which removes the carbons of the energy sources from the CAC and moves them towards the production of glucose [34]. Gluconeogenesis shares many enzymes with glycolysis, however, the rate limiting enzymes for gluconeogenesis and glycolysis are unique to one of the two pathways and allow the process to move in the direction of glucose production or glucose breakdown [37]. The rate limiting enzymes for gluconeogenesis are

PC, PCK, fructose-1-6-biphosphatase (FBP), and glucose-6-phosphatase (G6PC) [37]. Responsible for the final steps of gluconeogenesis FBP catalyzes the conversion of fructose-1,6-biphosphate to fructose-6-phosphate and G6PC catalyzes the conversion of glucose-6-phosphate to the final glucose unit [37]. When studying the hepatic gene expression involved during the fat mobilization post-partum, Weber et al. found that *PC*, *PCK1*, *PCK2*, and *G6PC* all increased in the post-partum period compared to the ante-partum period, due to the increased demand for glucose, with expression of *G6PC* continuing to increase until the end of the study at 7 weeks post-partum [9].

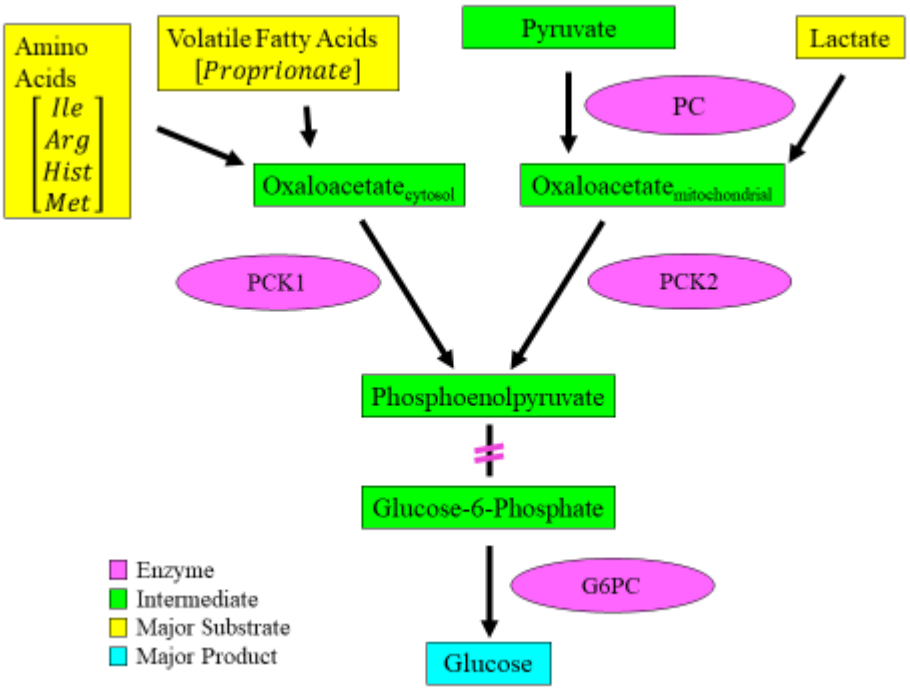

Figure 2. The Entry of Major Energy Precursors into Gluconeogenesis (Adapted from Lehninger et al., 2013 and White, 2015) [35, 37].

Because they are ultimately crucial in the production of glucose, *PC*, *PCK* and *G6PC* have been the classic hepatic gluconeogenic genes of interest when examining the effect of various nutrients on hepatic gluconeogenesis. Many studies comparing feed additives have found increases in the mRNA levels of these three genes, which coincided with positive health traits such as lower circulatory levels of NEFA, BHBA and improved circulatory levels of glucose [38-41]. Therefore, no study examining a nutritive supplement to transition dairy cows' diets and examining the hepatic tissue should overlook these genes critical to gluconeogenesis.

2.3 Metabolic Disorders in the Transition Cow

When the NEB experienced post-partum is not managed through adequate nutrition, modern dairy cows become at risk for various metabolic disorders. Cows that experience metabolic disorders have lower milk production, lower immune responses, and lower reproductive potential compared to cows that do not experience metabolic disorders during the transition period [6, 42, 43]. Interestingly, a study by Vanholder et al. reported that both the clinical (showing phenotypic symptoms) and sub-clinical (no visible symptoms) metabolic disorders can have similar effects on production and reproduction [44].

The displaced abomasum (DA) is a severe metabolic disease that requires surgical intervention [45]. DAs are most likely to occur in the first two months of early lactation [45]. At this point, a large amount of tissue has left the body cavity of the cow in a rather short period of time [46]. The risk of DA is augmented since fresh cow diets tend to have less forage and more energy density in them, to attempt to meet the energy requirements of early lactation [45]. The lack of forage coupled with the large amount of space suddenly available inside the abdominal cavity can allow the abomasum to shift in position and expand with gases that are unable to escape. A decrease in DMI coupled with a swelling on the right or left side of the body are indicative of a DA, so diagnosis can be done easily, followed by a quick intervention [47]. In the modern dairy system, it is rare that DAs are fatal. DAs occur in approximately 3% of cows in the first and second lactation, and the risk of DA increases to 5% in subsequent lactations [48]. Also, as a result of complications following surgery and increased risk for other diseases, 12-17% of cows treated for DAs die or are culled within 30 days of surgery [47]. In 2017, DAs were listed as the cause for 0.4% of the voluntarily culled cows in Canada [1].

On the other end of the spectrum, fatty liver is very difficult to diagnose and only be done by liver biopsy [49]. Clinical fatty liver (>10% liver TAG on wet weight basis) rarely occurs due to post-partum fat mobilization alone. Instead, clinical fatty liver, or fat cow syndrome, is believed to develop in the ante-partum phase in grossly overweight cows [49]. These cows, with excess fat reserves, will decrease their DMI significantly earlier than cows with a moderate or ideal ante-partum Body Condition Score (BCS) [14]. Thus, fat mobilization will begin before parturition and the TAGs begin to accumulate on the liver [14, 49]. Lowered milk production, ketosis, and severe BCS loss can result from fatty liver, but if fatty liver is allowed to progress or is present in a severe form, liver failure, kidney failure or even cardiac arrest can occur, causing the death of the cow [49]. Instances like these are rare, and instead

what is most prevalent is moderate fatty liver (5-10% liver TAG on wet weight basis) which is experienced in up to 50% of transition dairy cows [49]. These cows will also experience severe NEB, ketosis, lowered immune response, and decreased reproductive potential [49].

And finally, in the middle of this spectrum are the relatively easily diagnosed, but not always recorded diseases. Ketosis or hyperketonemia is a metabolic disease which occurs between 3 and 6 weeks post-partum at the peak of the NEB and is diagnosed through the measurement of circulatory BHBA levels [50]. SCK is diagnosed at a concentration of >1200 μmol/L and clinical ketosis is diagnosed at >3000 μmol/L [16]. Clinical ketosis is a rare occurrence in the modern dairy farm, at most it will be reported in 5-10% of the herd throughout the post-partum period. SCK, on the other hand, has been shown to be prevalent in up to 55% of the herd [50]. While ketosis is associated with many negative metabolic issues such as hypoglycemia, insulin resistance, decreased milk production, decreased fertility, increased mortality, and overall an increased risk of experiencing other metabolic diseases, research is still being conducted to determine what negative effects ketosis has directly on the body [6].

One place where it may have a clear direct and negative effect is in the immune response of transition cows [44, 51]. Immune cells use glucose as an energy source and are unable to use ketone bodies as energy sources, unlike other cells in the body. Therefore, when considering that glucose levels decrease post-partum and ketone levels increase, it is not surprising that ketones are capable of reducing neutrophil function and lymphocyte blastogenesis [51]. An *in vitro* study performed by Vanholder et al., in which cultures of granulosa cells were dosed with BHBA, found that cell numbers increased with the dosages up to SCK levels [52]. However, steroid production (i.e. progesterone (P4) and 17β-estradiol (E2)) decreased significantly, thereby indicating the negative effect of higher levels of BHBA on the reproductive function of the cows [52]. A more in-depth examination of the effects of ketosis and NEB on reproductive function will be discussed further on.

All this being said, it should be noted that each case of ketosis, when including all other factors that ketosis is associated to, only costs producers approximately 50-100$ CAD [53]. When this may not seem severe on a per cow basis, as mentioned earlier, the incidence of SCK can be at over 55% of the herd and there is also the possibility of a repeated illness [50]. Therefore, while the initial cost may be manageable, the overall cost of widespread ketosis in the herd can be substantial and even detrimental to the producer. Future studies, specifically

continuous nutrition trials, are required in order to continuously improve nutritional programs aimed at reducing the incidences of ketosis and other metabolic diseases.

3. Fertility

3.1. The Bovine Estrous Cycle

In order to achieve pregnancy, the hormonal changes of the estrous cycle are essential for all mammalian species. To understand the issues involved when hormonal control does not work, we must understand what hormonal changes are required during a healthy estrous cycle. The estrous cycle is controlled by the hypothalamic-pituitary-ovarian axis [54]. Gonadotropin-releasing-hormone (GnRH) is secreted by the hypothalamus which stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Both hormones are required for the estrous cycle, however, they are required at different amounts during different times. FSH and LH are released under the influence of a pulsatile release of GnRH, with a rapid pulse favoring the release of LH (1 pulse/hour) and a slower pulse favoring the release of FSH (1 pulse/ 3 hours) [54]. FSH is required during the growth and maturation of follicle, while the LH surge is the classic indicator for the final event of a follicle's life: ovulation [55].

The development of follicles begins when the animal is in utero, so that it is born possessing up to 150,000 follicles, most of which being primordial [56]. Primordial follicles are those that are classified as a follicle with a single layer of flat/ellipsoid granulosa cells and no oocyte development [57]. After this stage is reached, FSH receptors begin to appear on the granulosa cells [58, 59]. The primary follicle then develops into a secondary follicle. Secondary follicles are follicles classified as having a second layer of cuboidal granulosa cells, with the layer being incomplete or complete [57, 59]. Finally, as the name suggests tertiary follicles are follicles that have more than two layers of granulosa cells [57]. Tertiary follicles are also known as pre-antral follicles since, after this stage, the follicles will form a fluid filled antrum that is differentially regulated from the replicating granulosa cells [59]. Approximately 16-24 antral follicles will reach the size of >5 mm [60]. One of these follicles will eventually reach the size of 8 mm [61]. During this time, inhibin B is produced by the follicles, indirectly altering the expression of FSH receptors on the growing antral follicles, until a peak in production is achieved when a single follicle reaches 10-12 mm in size [62]. This follicle is known as the dominant follicle and the follicles that did achieve 5 mm but not 8 mm are called subordinate follicles [61]. The dominant follicle now grows at a faster rate, while the subordinate follicles grow more slowly and eventually regress [61]. The dominant

follicle is also characterized by a decrease in FSHR expression, and an increase in CYP11A1, CYP19A1 (aromatase), and LHR expressions [63]. These and other proteins allow for the accumulation of E2 and P4 in the follicular fluid of the antral follicle [63]. This accumulation is necessary to stimulate the hypothalamic-pituitary-ovarian feedback loop that increases the release of GnRH to stimulate the LH surge that would then initiate ovulation. Yet, in order for ovulation to be achieved the corpus luteum (CL) from the previous cycle must be in regression and no longer producing sufficient amounts of P4 to prevent ovulation [64].

The CL is made of granulosa and theca cells from a follicle that has ovulated [65]. Following ovulation, granulosa cells begin the transformation into large luteal cells with prostaglandin ($PGF_{2\alpha}$) receptors and begin producing P4 [66]. In order for a CL to be sustained, compatible signaling between the ovary, uterus, and embryo must occur. In the most basic sense, the embryo must develop from the spherical phase to the tubular phase and then achieve the filamentous phase [67]. At this stage, the filamentous embryo is capable of producing interferon-tau (IFNτ), which occurs between day 10 and 25 of pregnancy [67]. IFNτ inhibits the expression of E2 receptors and oxytocin receptors on the uterine epithelium and also inhibits the release of $PGF_{2\alpha}$ from the uterine epithelia [67]. $PGF_{2\alpha}$ is luteolytic and if produced will bind to receptors on the CL and initiate the regression of the CL [67]. With the CL in regression, P4 production drops precipitously and the negative feedback loops preventing GnRH production are removed as well [67].

However, throughout the lifespan of the CL, one or two follicular waves can be observed, where a dominant follicle is selected as described previously [68]. Under the influence of the existing CL, the dominant follicle, which can reach the pre-ovulatory size of 12-20 mm in diameter, cannot complete the ovulatory process [63]. Nonetheless, once the uterus has produced $PGF_{2\alpha}$ and the CL begins regression the hormone profile shifts again and a surge of FSH and E2 are observed with the induction of a new follicular wave, which has the potential of achieving ovulation. E2 will switch from having a negative feedback inhibitory effect on GnRH to a positive one, will stimulate LH receptor synthesis, and will inevitably induce the LH surge [69]. When a dominant follicle is selected and reaches pre-ovulatory size, circulatory levels of E2 will reach a peak and then decrease [70]. Estrus or heat can be detected in the animal at this point in time, with classic estrus signs being a display of mucus, bawling, increased activity, and willingness to be mounted. Displays of estrus last from 18-20 hours and during this time the LH surge peaks at approximately 60ng/ml [71]. The LH surge initiates a complex downstream effect on the ovary by completing several steps including:

follicular rupture, induction of oocyte meiotic maturation, and, most classically, the lutenization of granulosa and theca cells to form the CL [72].

It is only when all of these various mechanisms work in tandem that a proper estrous cycle can be experienced. Without all of these complex pathways achieving their goal, neither ovulation nor pregnancy can be achieved. Therefore, ensuring that each step of estrous is completed as required is in the best interest of the farmer when it comes to the profitability of their herd. This statement is all the more true when attempting to achieve pregnancy after the cow has calved. The next sections will cover reproduction resumption following calving and the problems it is associated with.

3.2. The Bovine Estrous Cycle after Calving

The event of parturition causes major changes in the reproductive system of the cow. Unlike the onset of milk production, which occurs alongside calving, the resumption of ovarian cyclicity takes several days [73]. It is also ovarian cyclicity that is responsible for the healing and cleaning process of uterine involution [74]. In total, it can take upwards of 73 days after calving, depending on the health status of the cow, for the cow to be capable of pregnancy once again [74].

Olsen et al. separated the resumption of ovarian cyclicity into three periods: 1) Puerperal 2) Intermediate phase, 3) Post-ovulatory [75].

The three periods are separated rather logically. The puerperal period is defined as the time period from parturition to point where the CL undergoes regression and the pituitary gland becomes sensitive to GnRH stimulus and can produce FSH again [75]. Likewise, the intermediate period is defined as the time period from when the pituitary gland becomes sensitive to GnRH stimulus to the time of the first ovulation [75]. Finally, the post-ovulatory period is defined as the time period between the first ovulation to the point where uterine involution is achieved and can be the longest period [75].

Following parturition, and only once the CL is no longer functional, increasing levels of FSH can be observed [75]. These increasing levels of FSH initiate the first follicular wave post-partum, though it should be noted that E2 levels remain lower than normally experienced in an estrous cycle [76]. The first ovulation post-partum does not follow a classic estrous cycle profile, as there is no CL for P4 production and uterine involution has yet to be completed. However, despite these factors, the ovary functions as described in the previous section.

Much like a normal estrous cycle (Figure 3), the first ovulation post-partum is dependent on the size of the follicles, the receptivity of the hypothalamus and pituitary glands to hormone levels, and finally the LH surge [77]. Follicular waves can also be experienced during this time, however, most cows will have their first ovulation after calving by 21 days post-partum, with 30-80% of cows ovulating with the first wave post-partum [73, 78]. Also different from a normal estrous cycle is that this cycle tends to be shorter, with an earlier release of $PGF_{2\alpha}$ and a lack of estrous behavior due to low E2 levels [73]. Once the intermediate period has been completed, another shorter estrous cycle is observed with the first post-partum CL only lasting 10 days, rather than 18-20 days [73]. Likewise, the second ovulation post calving occurs sooner, at approximately 11 days into the first estrous cycle, rather than day 20-22 [73]. Once this second ovulation occurs, the ovary is considered to have resumed cyclicity and estrus should be able to be observed by day 35-60 [79, 80]. However, while the ovary may be prepared for the next pregnancy at this point, the uterus and the cow may require additional time.

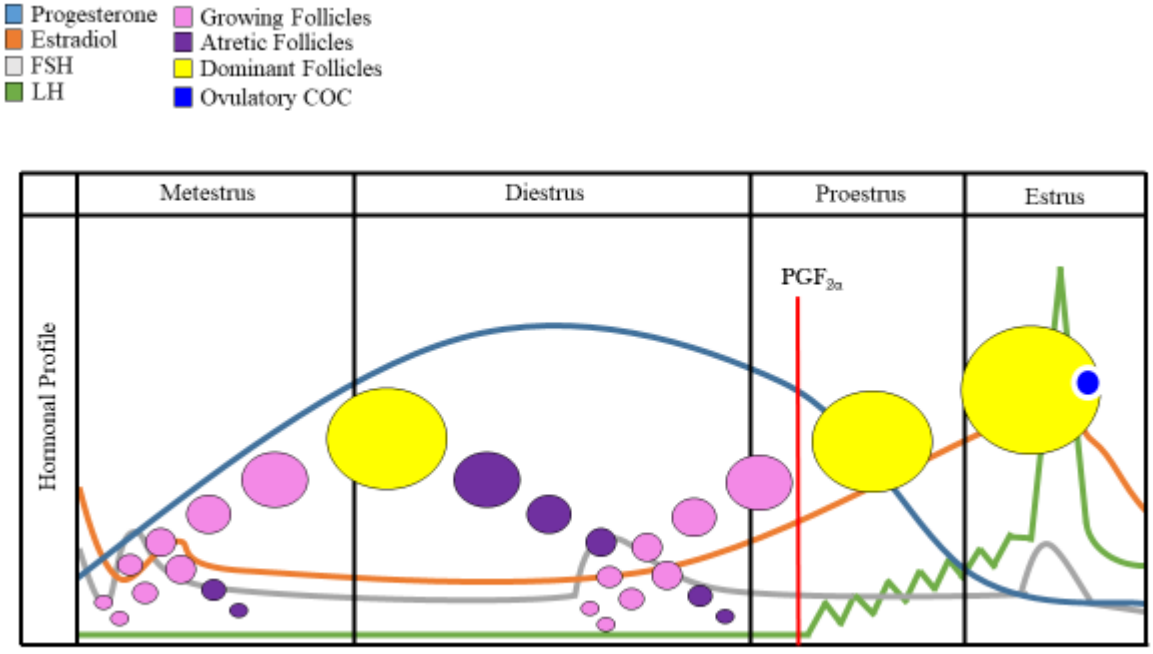

Figure 3. The Bovine Estrous Cycle (Adapted from Eilts, 2012) [71].

Uterine involution is defined as the return of a gravid uterus, including the cervix and the previously gravid horn, to the size of a non-gravid uterus [74, 81]. The first days post-partum are defined by a drastic decrease in the size of the uterus and an further expulsion of the fluids and tissues remaining inside the uterus [81]. Uterine involution also includes necrosis

and removal of caruncular tissues and repair of endometrial tissue [82]. Following the first ovulation, Morrow et al. noticed that by day 25 post-partum, the shape of the uterus was almost exactly that of a non-pregnant and cycling uterus, with complete involution being achieved by day 30 post-partum [83]. However, in Leslie's review, it is clearly stated that the ideal involution is a rare exception to the rule [76]. The "ideal" involution period is rarely achieved because parturition is a drastic event that exposes the normally sanitary environment of the uterus to the impossible to sanitize outer environment [84]. It is the norm, rather than the exception, that the reproductive pathway of the cow is exposed to bacterial contamination during the process of calving [84]. Controlling and healing any bacterial contamination increases the amount of time required for complete involution of the uterus and the surrounding tissues [84]. Instead of expecting total involution and ovarian cyclicity at approximately 30 days post-partum, farmers instead expect their animals to achieve involution and ovarian cyclicity by 45 days post-partum [83, 85]. Therefore, most farmers will breed their cows for the first time between 45 and 60 days post-partum [85].

Once again, pregnancy can only be achieved at this time point if all events have conspired accordingly. Unfortunately, post-partum diseases during the second half of the transition period can drastically change the reproductive potential of an animal [6]. And, we must recall that a cow is more likely to experience a disease during the second half of the transition period than any other time during her lactation [6]. The following sections will discuss the follicular microenvironment and the effect that metabolic diseases experienced during the transition period have on the concurrent and subsequent reproductive activity of the animal.

3.3. The Follicular Environment

The follicular environment has a direct effect on the development and competence of the oocyte within the follicle. The antrum is a cavity that contains the follicular fluid, which has been extracted via osmosis from theca cells [86]. The follicular fluid also collects the products of granulosa and theca cell activity, particularly steroid hormones [87]. The follicular fluid maintains the microenvironment of the follicle to provide ideal conditions to the developing oocyte [88]. Follicular fluid helps to maintain the meiotic arrest of the pre-ovulatory oocyte, prevents proteolysis in the follicle, and improves a myriad of factors related to the post-ovulatory act of fertilization [89]. Therefore, follicular fluid provides a unique insight into the health and developmental potential of the pre-ovulatory oocyte.

The follicular fluid contains many hormones as well as metabolites that come from both outside and inside the follicle. It is to be expected that varying sizes of follicles contain varying profiles of these hormones and metabolites [87]. Whether the follicle is dominant or atretic will also alter the profiles [70]. While Henderson et al. did not consider atretic follicles, they did perform an in depth characterization of the levels of hormones that are found in different sizes of bovine follicles [87]. For gonadotropin concentrations in the bovine follicular fluid, Henderson et al. found that levels of FSH and LH decreased in follicular fluid with relation to size, with the lowest amounts being found in large follicles (65ng/ml of FSH and 3.3 ng/ml of LH) [87]. On the other hand, prolactin levels increased with the size of the follicle, with the highest levels being measured at 30 ng/ml [87]. Additionally, levels of the steroids androstenedione and testosterone decreased dramatically with the size of the follicle [87]. While P4 levels increased slightly over time (from 35 ng/ml to 39 ng/ml), E2 had the most dramatic increases over time, increasing from 5 ng/ml in small follicles to 69 ng/ml in large follicles [87]. From this data we see a shift from androgen production to E2 production due to increased aromatase activity in granulosa cells as the follicles increase in size. However, little can be said about the profile of the dominant follicle versus atretic follicles from this data.

A study performed by Noseir, examined the differences in the hormone profiles between cows that had two follicular waves during their estrous cycle and those that had three waves [70]. Unlike Henderson et al., Noseir compared pre-ovulatory follicles to growing and atretic follicles [70, 87]. Unfortunately, Noseir only measured the hormone profile in the serum, which gives us an incomplete assessment [70]. However, much like Henderson et al., Noseir did observe an increase in E2 levels in the serum and found that cows with atretic follicles had E2 concentrations ranging from 4.14 – 5.70 pg/ml, while cows that had a pre-ovulatory follicle had E2 levels from 5.46 – 6.57 pg/ml [70, 87]. Additionally, in a project completed by Gasperin et al., who did measure E2 levels in follicular fluid, it was reported that the largest follicle before deviation had significantly higher levels of E2 compared to second largest follicle (190.1±54.9 vs 80.3±48.4 ng/ml) [90]. Likewise, after deviation, Gasperin et al. reported that the dominant follicle had significantly higher levels of E2 compared to the subordinate follicle (249.4±39.7 vs 3.98±3.1 ng/ml) [90]. Thus, E2 levels can be considered a marker of the health and function of the dominant follicle [88, 90].

Another key component of the follicular fluid is the fatty acid profile. Fatty acids are involved in many aspects of follicular function, including, but not limited to a precursor for

hormone synthesis, an energy source, and a building block for cell membranes [91]. Renaville et al. found that palmitic (C16:0; 15.96% of fatty acid profile), stearic (C18:0; 26.31% of fatty acid profile), oleic (C18:1n9; 12.68% of fatty acid profile), and linoleic (C18:2n6; 30.57% of fatty acid profile) were the most prevalent fatty acids in the follicular fluid [91]. In 2010, Renaville et al. performed a study in which they separated follicles based on E2 activity and follicle size (>4 mm collected at 3 days after a new follicular wave was induced, and >6 mm collected at 6 days after a new follicular wave was induced) [91]. Here, Renaville et al. found that the fatty acid profile estrogen active follicles collected at day 6 had lower percentages of palmitic, oleic, and linoleic acids, and had higher proportions of stearic acid compared to estrogen inactive follicles collected on either day 3 or day 6 (no estrogen active follicles were found on day 3) [91].

While much research has been done to determine the differences between dominant and subordinate follicles and estrogen active and estrogen inactive follicles, another popular method when it comes to identifying the health and function of follicles is comparing the differences between heifers and multiparous cows [70, 87, 90-92]. Heifers have a pregnancy rate at first AI of approximately 65%, while multiparous cows have a pregnancy rate at first AI of approximately 33% when under good management [93]. This can be attributed to the fact that when heifers are being bred for the first time, they are not experiencing a lactation simultaneously. The metabolic activity of lactation increases the metabolism of hormones and other key metabolites used for energy by the liver and ultimately decreases the reproductive potential of the cow [92]. Ideally, by achieving a follicular environment that is as close to a heifer's as possible, the multiparous cow will be more likely to conceive.

While Sartori et al. did not measure hormone levels in the follicular fluid, they did find that serum levels of E2 and P4 were lower in lactating cows compared to heifers (E2: 7.9 ± 0.8 vs. 11.3 ± 0.6 pg/mL; P4: 5.6 ± 0.5 vs. 7.3 ± 0.4 ng/mL) [94]. Similarly, Wolfenson et al. reported that E2, P4, and pre-ovulatory LH were all found to have higher levels in the serum of heifers compared to lactating cows [95].

In regards to differences between the fatty acid profile of cows and heifers, Bender et al. found that in the follicular fluid of the dominant follicle, palmitic, stearic, oleic, and linoleic acid levels were all significantly higher in cows compared to heifers [96]. However, the composition is what is of interest at this time. Most notably of the composition findings by

Bender et al., was that lactating cows had a higher proportion of palmitic acid (49.699 ± 6.836 % vs. 30.471 ± 5.092 %) in their dominant follicles compared to heifers [96].

Because of the drastic differences between the composition of the follicular fluid of the dominant follicles of lactating cows and heifers, the effect of lactation and the stress of the transition period cannot be understated. Heifers provide insight into the ideal follicular environment and should be considered when examining the effects of nutrition supplements on the profile of the follicular fluid in lactating dairy cows. However, as we will see in the next section, lactation and the NEB are not the only stressors that have an effect on the reproductive potential of dairy cows, but also many metabolic disorders which occur due to the NEB will impact the dairy cow's fertility.

3.4. The Impact of Metabolic Disorders on Reproductive Potential

The difficulty in assessing the problems of ovarian and uterine resumption post-partum is that many factors can affect the potential fertility of dairy cattle. These factors include, but are not limited to parity, season, NEB, BCS changes, milk yield, and instance of disease [73]. In Crowe's review, he determined that the events that effect the time to first ovulation were first, acute BCS loss post-partum (10.9-18.7%), followed by clinical ketosis (11.3 %), and then a smaller percentages included the occurrence of clinical diseases, abnormal vaginal discharge and dystocia [73]. All of these can be related back to the experience of NEB and metabolic disorders. Severe NEB's initiate severe BCS loss, which in turn supports the development of metabolic disorders such as ketosis and fatty liver [49].

While a study performed by Opsomer et al. is dated, the numbers it presents are staggering when it comes to post-partum ovarian resumption [97]. Studying over 400 modern Holstein dairy cows, Opsomer et al. reported that only 53.5% of high yielding cows presented normal cyclicity post-partum, while 20.5% had delayed 1st ovulation and 20% showed a prolonged luteal phase [97]. With average milk production having increased exponentially since this data was published in 1998, it is safe to assume that today, normal cyclicity post-partum can be observed in fewer than 53.5% of dairy cows.

It has also been hypothesized that folliculogenesis during this time period is affected by energy status of the cow [98, 99]. At the very least, increased liver metabolism due to the demands of milk production, decreases steroid half-life [100]. Thus, with increased feed intake post-partum, E2 and P4 levels decrease [100]. Improper hormone profiles during this time can lead to anovulation, multiple ovulations, decreased luteal function, decreased E2

production in dominant follicles, and reduced $PGF_{2\alpha}$ production in the uterus [100]. Also, reviewers have suggested that post-partum illnesses may decrease follicle growth and E2 production in the dominant follicle [73]. Reviews also point out the failure of LH secretion due to metabolic stress that reduces the GnRH pulse, thereby favoring FSH instead of LH secretion [73, 100]. While FSH is required for follicle growth, LH is also required for the dominant follicle survival, ovulation, and subsequent CL formation. Without the required levels of E2 produced by the dominant follicles, GnRH cannot initiate the LH surge that begins the cascade of activity to achieve ovulation.

Another expression of issues in resumption of cyclicity is the development of cysts on the ovary [101]. Simply put, cysts are dominant follicles that have failed to ovulate and continue to grow despite the lack of a P4 negative feedback and high levels of E2 that are produced by the cyst [102]. Unfortunately, the cyst also prevents the secretion of FSH and no follicular waves emerge in the presence of a cyst [103]. Two options are available if a cyst occurs: a cyst can be removed through veterinary and/or exogenous hormone intervention, thereby allowing the ovary to 'reset' itself and a new follicular wave to begin, or producers can wait until the cyst becomes estrogen-inactive [104]. In the latter case, the cyst will remain, but a new follicular wave will begin once hormonal cues are re-established [104]. However, not only does this method take up valuable time for the producer, but a wave following a cyst has a greater chance experiencing anovulation and becoming cystic once again [101, 104]. While cysts are more likely to occur during the resumption of ovarian cyclicity, it should be noted that cysts can occur at any time point and that some cows are simply prone to cysts [101].

Along the line of hormone half-life in fast metabolizing cows, it is believed that NEBs encourage the shift of energy/nutrients from reproductive maintenance to basic maintenance and milk production [98-100]. Without energy to support folliculogenesis, fewer follicles are observed progressing in the follicular waves, dominant follicles tend to be smaller in size, time to 1st ovulation is extended, and ovulation can be inhibited altogether [105, 106]. Low circulatory levels of glucose also decrease the GnRH pulse generation, thereby decreasing the amount of LH secretion for folliculogenesis and ovulation [107]. Also, with low insulin, there is a reduction in the synthesis of insulin-like growth factor-1 (IGF-1) in the liver, which also has an impact on the reproductive potential of an animal, since IGF-1 is required for follicular growth, steroidogenesis and is a critical component of the LH surge cascade [107]. Observing the transition cow's blood profile, we see that these blood metabolites decrease significantly

during early post-partum when the first follicular wave and subsequent ovulation is experienced post-partum [101, 108].

Finally, it must be made clear the follicle and oocyte development begins 3-4 months before its potential ovulation [98]. Therefore, the follicle of potential at the time of first breeding (approximately 45-60 DIM) would have been exposed to the effects of the NEB experienced post calving. Thus, not only could a NEB have negative effects on the cow herself, but also on the follicle and oocyte that ovulates around this period (i.e. the potential calf). It is plausible that such negative effects may even impact the future production and health of the calf borne out of that oocyte.

One of the metabolic disorders that cows are most likely to experience post-partum is ketosis. As mentioned previously, SCK is experienced when circulating blood levels of BHBA reach levels greater than 1200 μmol/L and clinical ketosis is experienced with circulatory blood levels higher than 3000 μmol/L [16]. Many studies have examined the effects of BHBA on pregnancy rate post-partum, however Walsh et al. categorized ketosis at the sub-clinical level [109]. Their lengthy investigation brought to light striking results in regards to the effect of BHBA levels and NEB on pregnancy [109]. Cows that had BHBA levels higher than 1000 μmol/L in the first and second week post-partum were 50% less likely to become pregnancy after the first insemination (at approximately 80 days post-partum) [109]. However, it should be made clear that this study, while it did make use of AI and estrus detection, did not use synchronization protocols [109]. Therefore, the results presented by Walsh et al. are the result of no hormonal interference [109]. Walsh et al. were able to confidently conclude that duration of the negative effects of SCK experienced in early lactation was much longer that originally hypothesized [109].

One of the theories behind the length of the effects of SCK is that it not only effects of the metabolic capabilities of the cow, but also the immunity of the animal, and increases the susceptibility of the cow to non-metabolic diseases [16]. Several *in vitro* and *in vivo* studies have shown that SCK levels of BHBA and severe NEB levels of NEFA affect the function of immune cells [110-112]. *In vivo*, Wathes et al. reported that increased circulatory levels of BHBA and NEFA was correlated to lowered numbers of leukocytes [111]. *In vitro*, Grinberg et al. exposed bovine neutrophils to levels of BHBA that match SCK levels and found that in increasing the amount of BHBA, reduced levels of phagocytosis and overall killing ability [112]. Still, both *in vitro* and *in vivo* experiments have only been completed in the short term,

so their long term effects have yet to be thoroughly examined. However, the short term decrease in immune capabilities does support the increased disease susceptibility experienced by these animals, which does have the potential for long term effects.

Another important long term effect to consider, is the effect on oocyte competence. The oocyte may seem secluded from any changes experienced during the transition period, however, important nutrients are diverted away from the reproductive system to milk production and basic maintenance at this time [17, 113]. The ovary experiences these changes because the follicular fluid that surrounds the oocyte is created through osmotic pressure from blood capillaries in the theca cells [86]. Therefore both the oocyte and the follicle experience any changes in the circulatory metabolite profiles, which can be observed in changes in the follicular fluid profile and lipid metabolism [43, 92]. Following the onset of lactation, glucose is diverted to the mammary gland for lactose production and Leroy et al. have observed decreased levels of intrafollicular glucose at the time of parturition [114]. Leroy et al. went on to examine the effects of low glucose levels on *in vitro* oocyte maturation and found that lower glucose levels led to reduced cleavage rates and decreased amounts of blastocyst development [115]. Therefore, it can be assumed that lower glucose levels during the time of calving can affect future embryo viability and subsequent pregnancy rates. Leroy et al. have also shown that the NEFA concentrations and profiles in the follicular fluid are comparable to those found in serum [116]. In this study, Leroy et al. also reported that increased levels of saturated NEFAs *in vitro* reduced oocyte competence and embryo viability [116]. Additionally, our group has previously compared the follicular environment of multiparous cows and heifers [92]. While NEFAs were not examined in this study, multiparous cows, which had significantly lower levels of glucose and higher levels of BHBA in their follicular fluid compared to heifers, also had reduced IGF-1 signaling abilities in the granulosa cells of the dominant follicle compared to heifers [92]. IGF-1 is a major component of ovarian function and altered IGF-1 signaling in granulosa cells suggests an impact on the development of the oocyte in the dominant follicle of dairy cows [92].

However, although severe NEBs are characterized by significant decreases in the circulatory levels of glucose coupled with significant increases in the circulatory levels of NEFAs and BHBAs, so far, the combined effect has yet to be studied. It would be of interest to observe if there is additional oocyte competency issues when these three main factors are taken into account simultaneously.

Although it must be stressed that the changes in metabolic and circulatory profiles following parturition are a natural phenomenon, the issue in modern dairy cows is the in extent to which these changes are experienced. These changes cannot be avoided, yet, they can be mediated. Much research has been dedicated to the development and application of various protocols to limit the extent of the NEB. However, some techniques must be examined critically. For example, although results have been inconsistent, increased CP levels in post-partum diets (higher than 15%) have helped to sustain milk production, but have also increased the levels of blood urea in circulation, which has led to decreased reproductive potential rather than improving it [14]. The next section will examine what has been done so far to address the requirements of modern transition dairy cows and will assess where further research is also necessary.

4. What's been done?

Many different studies have been performed to attempt to improve the management conditions that are related to the NEB experienced post-partum and the infertility experienced at the time of expected breeding [117, 118]. Some studies have examined management decisions such as dry period length and found that cows that were on shorter dry periods (28-34 days) had lower NEFA levels post-partum, higher overall BCS, and less weight loss over the post-partum period [119]. Cows on shorter dry off periods were also earlier in achieving ovarian resumption [120]. However, it should be noted that these animals experienced a reduction in 5 kg/d of milk yield and, that despite ovarian resumption and a less severe NEB experienced, there was no difference in days open, number of services per conception, nor conception rate between the long and short dry periods [120]. Contrary to these results, DeGaris et al. performed a study where ante-partum cows were given pre-partum diets at three weeks ante-partum compared to two weeks ante-partum [121, 122]. It should be known that pre-partum diets mimic the post-partum diet in components and energy more closely than the dry ration [121]. Interestingly, DeGaris et al. found improved pregnancy rates in cows that had eaten the pre-partum diet for longer when pregnancies were confirmed 12 weeks post-partum [122]. This brings us to the main focus in fertility improvement in dairy cattle. Since the NEB is at the center of issues pertaining to fertility after calving, the role of nutrition in the management of NEB has been studied rigorously [42, 43, 118, 123].

In studying the pre-partum diet, various diets have been compared. Most significantly have been the comparison of *ad libitum* diets to restricted calorie diets. In ante-partum *ad libitum* diets, significantly greater weight loss and lipid mobilization post-partum are observed

compared to restricted diets [124]. On the other hand, ante-partum restricted calorie diets have greater DMI, improved energy balance status, and lower serum levels of NEFAs and BHBAs post-partum compared to *ad libitum* diets [124]. Interestingly, in Dann et al.'s study, milk production did not differ between ante-partum *ad libitum* or restricted calorie diets, a result that makes the technique of restricted calorie diets of interest to modern dairy farms [124].

Another important dietary alteration to consider, is that non-fiber carbohydrates theoretically stimulate the development of ruminal papillae [14]. This is ideal for the post-partum cow in order to increase the surface area for VFA absorption [14]. Following parturition, carbohydrates should increase in starch availability to meet the energetic demands, however, physically effective NDF must also be included in the post-partum diet [125]. Increasing the levels of non-fiber carbohydrates has also been shown to improve fertility by reducing the time required for ovarian resumption and the time until first estrus is observed [126]. However, further study would be required to confidently say that this diet improves fertility, because as we saw with the Gümen et al. study, ovarian resumption does not always equate overall fertility and the ability to achieve and maintain pregnancy [120].

While changes in the pre-partum diet still warrant further research, much less is known about the best way to manage the post-partum diet. The issue in feeding cows is that much of the diet that is fed to cows in their ration is transformed into microbial products that we have less control over with the current knowledge of rumen microbiota. Many studies have tried to directly increase specific components such as insulin [127]. Insulin is involved in the growth hormone-IGF-1 axis that is critical to follicular development and the resumption of ovulation [43]. Increasing the amount of starch in the diet does increase circulatory levels of insulin and has been found to improve the number of cycling cows by day 50 post-partum [43]. However, it should also be noted that high starch diets reduce the palatability and DMI of the ration [43]. Thus, carbohydrate content of post-partum diets must be considered with caution.

Another nutritional strategy used in the dairy industry is ionophores such as Monensin. While these are already used universally in the North American dairy industry, they warrant mention for their effect on the rumen microbiome. Monensin and other ionophores alters the rumen microbial environment to favor bacteria that are involved in propionate production [128]. While significant effects on the reproductive potential of animals that have been given

ionophores have yet to be reported, treated animals do have an overall reduction in the severity of the NEB experienced and a reduced risk of experiencing ketosis [129].

When it comes to improving the energy levels in the post-partum diet, increasing the fat levels in the diet is a practical solution. However, while fat does increase the energy density of the diet, it is quite difficult to feed. Not only can too much fat in the diet decrease the palatability and DMI, but due to the biohydrogenation of 70-85% of all unsaturated fats in the diet it is difficult to know the exact effects [106, 130]. Still, studies have shown promising results, with several observing an increase in pregnancy per artificial insemination (AI) in dairy cattle given a fat supplementation [106]. In a study by Silvestre et al., which supplemented fatty acids in the pre- and post-partum diets, in the form of sunflower and fish oil, higher rates of pregnancy and increased milk production were observed [131]. Additionally, *in vitro* embryo studies, involving fatty acid supplementation, have been promising, in that embryos cultured in fatty acids such as trans-octadenoic acid had improved quality and viability [106, 132]. Still, the outcomes of the implementation of fat in the pre- and post-partum diets are inconsistent at best [130]. This is one of the reasons why more recent studies have moved on to focus on rumen protected nutrients.

Rumen protected nutrients are, as the name suggests, protected from microbial digestion in the rumen. This can be done in a number of ways, including exposure to heat and having the size of the feed particles physically small enough to bypass the rumen environment and move on directly to the digestive system of the cow [21, 133]. Much attention has been given to rumen protected essential nutrients such as choline. Lima et al. found that supplementation with rumen protected choline throughout the transition period, specifically 25 days ante-partum to 80 days post-partum, improved liver metabolism, such that there was a decrease in TAG accumulation on the liver, a decrease in BCS loss, and a decrease in the number of incidences of ketosis, coupled with an increase in the amount of fat corrected milk [134]. These results seem astounding and too good to be true, but naturally, despite the decrease in the severity of the NEB experienced by these cows, Lima et al. found no effects on the reproductive potential of the animals [134]. Lima et al. observed no differences in the resumption of ovarian cyclicity, nor pregnancy per AI, nor maintenance of pregnancy [134].

Not only have essential nutrients like choline been tested for their effectiveness when protected from the rumen environment, but AAs like methionine and lysine have also been studied. Methionine and lysine are of particular interest for the lactating dairy cow because

they are the limiting AAs in the mammary gland [14, 135]. Unfortunately, results regarding methionine supplementation have been inconsistent. Recently, however, studies have presented promising results, such as Toledo et al. who recently reported that methionine decreased the incidence of pregnancy loss in high producing, multiparous dairy cows, thereby reigniting interest in AAs as rumen-protected supplements [136].

The interest in feeding specific AAs stems from the fact that feeding protein in the post-partum diet can be difficult to manage. Increases in CP in the diet can lead to increased ammonia and urea in the blood, which have negative impacts on the fertility of the animal [14]. Another option that has recently gained traction is the addition of yeast microbial proteins (YMPs) to the ration of lactating dairy cows [21]. YMPs are physically small enough to bypass the rumen environment by entering the liquid phase of the rumen thereby passing quickly through the environment and avoiding a majority of the transformation by rumen microbes. Additionally, YMPs have an AA profile that closely matches the AA profile of the rumen microbial proteins that are digested by the cow [21]. The final section of this review will discuss what has been done with regards to the supplementation of yeast and yeast microbial products to dairy cattle and the possible implications for management practices and fertility.

5. Yeast

Yeast has been used as a supplement in dairy cattle nutrition for over 25 years [137]. While the results of studies concerning the effectiveness of yeast products have been inconsistent, there is a general consensus that yeast does improve lactation performance [41]. The effectiveness of live yeast as a feed supplement has been related to the fact that the aerobic yeast use up oxygen in the rumen, thereby improving the rumen environment for the anaerobic bacteria that are responsible for VFA production [138]. Yet, most studies have been performed on cows during their lactation and only a few studies have considered the transition period. One of these studies, performed by Zaworski et al., examined the effect of various dosages of a yeast fermentation product on performance of transition cows [139]. Supplementation was given from 4 weeks ante-partum to 4 weeks post-partum [139]. They observed a tendency for supplemented cows to have higher glucose levels in circulation, coupled with decreased milk somatic cell count (SCC) and increased milk production [139]. Increased milk production was also related to an increase in protein, lactose and total solid production [139]. However, there were no differences in BHBA levels, NEFA levels, or BCS between supplemented and control groups [139]. Zaworski et al. also observed no differences

in circulatory levels of insulin [139]. Another study performed during the transition period, examined the effect of glycerol and yeast and a combination of both on performance, energy status, and genes associated with hepatic gluconeogenesis [41]. Ye et al. found that yeast supplementation did not alter DMI or MY, but significantly increased milk fat and protein percentages compared to control animals [41]. In combination with glycerol, yeast supplemented cows had higher plasma glucose levels, and lower BHBA and NEFA levels compared to control cows [41]. These results were coupled with an increased in hepatic *PCK1* mRNA levels and higher enzyme activities of hepatic PC [41]. However, the promising results were only found in the combination treatment (Glycerol and Yeast) and in the glycerol treatment alone [41]. Therefore, it can be assumed that the latter results are due to the effect of the glycerol component.

Still, none of these studies examined the effect of yeast on the reproductive parameters of transition cows. Also, the results of studies that have examined the effect of yeast supplementation on the fertility of dairy cows post-partum, have been even more inconsistent than the results in studies that have examined lactation performance. A study performed on Jersey cows found that yeast supplementation did not reduce the days to first breeding, nor did it reduce the number of services per pregnancy [140]. Lehloenya et al. also found no differences in the number of days to first and second ovulation between yeast supplemented cows and control cows [141]. A massive study by Bruno et al., which included 717 cows and examined the supplementation of yeast to the diet of heat stressed Holstein dairy cows post-partum found no differences in the resumption of ovarian cyclicity, conception rates, nor pregnancy losses in yeast supplemented cows versus control cows [142]. Of note, however, is that Bruno et al. also included a measurement for lameness [142]. Bruno et al. reported that yeast supplementation did not alter the percentage of lame cows, but reduced the severity of the lameness experienced [142]. A study performed by Sprecher et al., found that as the severity of the lameness increased, there was an increase in the days from parturition to first insemination, which resulted in an increase in the number of days from parturition to conception [143]. While this could be due to farmers being hesitant to breed cows with severe lameness, it nevertheless increases the risk of the cow being removed from the herd. Therefore, yeast supplementation could theoretically improve reproductive potential through this indirect pathway [142]. Another indirect way that yeast supplementation could improve fertility can be theorized when examining the results of a study performed by Yuan et al. [144]. They observed that yeast supplementation improved various measures of immunity in

transition dairy cows, such as decreasing levels of uterine *IL-6* mRNA [144]. By improving the immune response of the dairy cows, the improvement of the dairy cow's health can increase the reproductive potential of the animal [144].

Despite all of these negative and indirect results when it comes to assessing the effect of yeast supplementation on the fertility of dairy cows, one researcher has reported positive effects on the reproductive potential of transition dairy cows. In the first study, Al Ibrahim et al. fed a live yeast culture to transition dairy cows that were grouped by low and high BCS from days 14 ante-partum to 70 post-partum [145]. Alongside classic nutrition study parameters such as milk yield, and DMI, Al Ibrahim et al. performed daily ultrasounds from day 10 post-partum until first ovulation to observe the size and development of the first ovulatory follicle [145]. While Al Ibrahim et al. did not see any differences in DMI or milk yield between control and supplemented animals, they did observe an increase in circulatory levels of insulin, found that the E2 peak tended to be higher, and reported that the first ovulatory follicle tended to be larger in cows supplemented with the yeast culture [145]. The report of increased insulin in yeast supplemented cows contradicts the results from previous studies. While these results are the first, that we are currently aware of, to describe an improved reproductive potential associated with yeast supplementation, it should be noted that Al Ibrahim et al. did not find differences between control and supplemented groups with regards to the number of days to first ovulation, nor the number of follicular waves to first ovulation [145].

In a second study, Al Ibrahim et al., examined the supplementation of live yeast to a pasture based diet in post-partum dairy cows (day 0 to day 70 post-partum) [146]. In this study, Al Ibrahim et al. found that yeast supplementation tended to reduce circulatory levels of BHBA and the severity of NEB experienced, while significantly reducing the circulatory levels of NEFAs [146]. Blood glucose and insulin were also significantly increased in yeast supplemented cows [146]. Once again, they monitored the ovarian follicular activity with daily ultrasounds, beginning at 10 days post-partum until the first ovulation was observed [146]. Results from this study promoted Al Ibrahim et al. to suggest that their yeast supplementation improved the energy status of the cows, which other studies have not managed to do, and thus stimulated ovarian activity at an earlier time in the supplemented group compared to the control group [146, 147].

While the inconsistent results of studies performed using yeast supplementation does provide some positive results, no studies, to the knowledge of the authors, have examined the effect

of YMPs on the reproductive potential of transition dairy cows. As mentioned in the previous section, YMPs are physically small enough to bypass the rumen and provide an ideal AA profile that can be delivered directly to the cow's small intestine for direct absorption by the cow [21]. YMPs have the potential to stimulate hepatic gluconeogenesis and improve the energy tatus of transition cows [41, 139, 146]. From the studies examined here, we can assume that there may be benefits to the reproductive potential of cows supplemented with YMPs. Here we will investigate the effect of YMPs on the hepatic and ovarian functions of transition dairy cows, with regards to gluconeogenesis and hormone production, respectively.

III. HYPOTHESES & OBJECTIVES

Hypothesis:

The effect of the NEB on the fertility of transition dairy cows has been well documented. There is evidence that in reducing the severity of the metabolic stress through nutritional supplementation, it is possible to influence fertility, although the outcomes from those studies have been inconsistent. Based on this, we hypothesized that supplementation with rumen-protected YMP during the transition period improves the metabolic status, the ovarian microenvironment, and the function of the dominant follicle in lactating dairy cows

Objectives:

1. Compare the profile of circulatory metabolites related to metabolic stress between control and YMP supplemented transition cows
2. Compare the expression of hepatic gluconeogenic genes (*G6PC*, *PCK1*, *PCK2*, and *PC*) between control and YMP supplemented transition cows
3. Compare the microenvironment of the dominant follicle and reproductive performance between control and YMP supplemented transition cows

IV. ARTICLE

This manuscript is being prepared for submission to "Reproduction" for publication.

Yeast Microbial Protein Supplementation in Transition Dairy Cows: Effects on Metabolic Status and the Follicular Microenvironment

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Short title: The effects of YMP supplementation on the bovine dominant follicle

Keywords: Yeast Microbial Protein, bovine, Negative Energy Balance, liver biopsy, gluconeogenesis, dominant follicle

Abstract

Metabolic stress in transitioning dairy cows is characterized by low glucose and high β-hydroxybutyrate (BHBA) and non-esterified fatty acids (NEFAs). Such a metabolic milieu appears to have negative effects on the ovarian follicle and overall fertility. We hypothesized that supplementing transition cows with rumen protected yeast-derived microbial protein (YMP) alleviates metabolic stress and enhances ovarian follicular function. For this, we supplemented the diet of transition dairy cows with (n=6; supplemented) or without (n=13; control) YMP. Supplemented cows received 50g of YMP daily for 3 weeks before calving and 200g daily for 4 weeks after calving. Blood samples were collected at weeks -3, 0, +1 and +7 relative to calving. Liver biopsies were taken at weeks -3, 0 and +7 relative to calving. Follicular fluid from the dominant follicle of a synchronized wave in each cow was collected at 7 weeks post-partum. Glucose concentrations decreased and BHBA levels increased from week -3 to +1 relative to calving in control cows ($P < 0.05$), but not in YMP-supplemented cows. In the liver, the mRNA abundance of genes associated with hepatic gluconeogenesis were higher (*G6PC*; $P < 0.05$ and *PCK1*; $P < 0.10$) in supplemented cows compared to control cows. At 7 weeks post-partum, supplemented cows had lower levels of NEFAs in the follicular fluid of the dominant follicle compared to control cows ($P < 0.05$). Also, follicular fluid of supplemented cows had lower proportions of palmitic acid and oleic acid ($P < 0.05$) and higher proportion of linolenic acid ($P < 0.05$). Lastly, the dominant follicle of the supplemented cows had higher levels of 17β-estradiol ($P < 0.05$) and higher estradiol:progesterone ratio ($P < 0.01$) compared to control cows. Overall, the supplementation with YMP improved the metabolic status of transition dairy cows by enhancing gluconeogenesis and stabilizing lipid mobilization. Importantly, this alleviation of metabolic stress was associated with improved follicular microenvironment at 7 weeks post-partum, the period immediately before the expected breeding of these cows.

1. Introduction

The transition period in dairy cows is defined as 3 weeks before to 3 weeks after calving [5]. During this period, dairy cows can undergo the severe metabolic stress of a negative energy balance (NEB), due to their physical inability to consume enough feed to match the energy demands with the onset of milk production [7]. The modern Holstein dairy cow can easily reach over 40kg of milk production per day at the peak of her lactation, approximately 2 months post-partum [148]. In order to meet the energy demand, the mobilization of body reserves must occur, resulting in losses of up to 41.6 kg of body weight with an estimated loss of 31 kg of fat and 4.5 kg of muscle protein [149]. Cows that undergo severe metabolic stress are susceptible to diseases such as DAs, metritis, ketosis, mastitis, low milk production, and post-partum anovulation [6]. All of these diseases are costly to producers and have the potential for long lasting consequences.

The reproductive processes can be heavily impacted by severe metabolic stress. Modern dairy cows must reproduce approximately once every year to remain a productive asset in the herd. With a 283 day gestation length, cows must be bred starting by seven to eight weeks post-partum. This time of breeding falls around the time of peak lactation when a cow experiences the most metabolic stress. The ovarian follicles, harboring the oocyte for the potential calf, are developing under those stressful conditions [150]. It is therefore with the increase in milk production the fertility at the time of first breeding has declined severely over the past few decades [151]. In fact, reproductive issues are the number one reason for involuntary culling, accounting for 15.81% of involuntary culling in Eastern Canada [2].

Some studies have found that the increased rate of metabolism during peak lactation decreases the half-life of reproductive hormones, 17β-estradiol and progesterone, in circulation thereby affecting fertility [100]. Others have shown that cows experiencing a severe NEB have fewer follicles in follicular waves, smaller dominant follicles and frequent anovulation [105, 106]. Lower levels of glucose in the lactating cows have been linked to decreased GnRH pulse generation and decreased LH during the pre-ovulatory surge [107]. In an elegant study, Jorritsma et al. used fasting heifers as a model for the NEB experienced in lactating cows and found that ovarian function, specifically the E2:P4 ratio was significantly lower in fasted than control heifers [152]. This study clearly indicated that NEB negatively impacts follicular health.

In order to attempt to reduce the severity of the NEB and to improve fertility in transition dairy cows, several techniques have been implemented. Techniques such as restricted calorie ante-partum diets have been reported to improve post-partum DMI and lower serum levels of NEFAs and BHBAs post-partum, thereby improving the post-partum energy status [124]. Another study, Al Ibrahim et al., supplemented live yeast to transition dairy cows and reported that supplemented cows had larger follicles and higher pre-ovulatory levels of estradiol in the serum compared to control cows [145]. Recently, the technique of protecting supplementation from the rumen environment has become of interest. By protecting a supplement from the rumen environment, a cow is then capable of directly receiving the supplement in the small intestine for absorption [21]. One study, by Toledo et al., supplemented rumen protected methionine, an essential and limiting AA in milk production, to multiparous, lactating dairy cows and observed that, in comparison to control cows, supplemented cows had fewer incidences of pregnancy loss [136]. These results for rumen protected supplementation are promising, however, to our knowledge, no one has yet to examine the impact of yeast microbial protein (YMP) supplementation on the metabolic health and fertility of transition dairy cows. YMP has an AA profile similar to that of rumen microbial protein [21]. What protects YMP from the rumen environment, is that its small physical size allows it to by-pass digestion and transformation by rumen microbes so that the cow can directly receive the AAs through absorption in the small intestine [21]. In theory, the cow will then have the ability to use these AAs as precursors for energy production, specifically in use as gluconeogenic precursors. Thus, we hypothesized that supplementing transition dairy cows with YMP decreases the severity of the metabolic stress experienced by enhancing gluconeogenesis. We also hypothesized that supplementation improves the health and function of the dominant follicle at the time of expected breeding. The objectives of this study were to determine the effects of YMP supplementation on BCS, circulatory metabolites, genes associated with hepatic gluconeogenesis, and steroidogenic function of the dominant follicle at the time of expected breeding.

2. Materials & Methods

All animal procedures were approved by the Animal Care Committee of the Faculty of Agricultural and Environmental Sciences of McGill University.

2.1 Animals

We used nineteen multiparous Holstein cows from two commercial, tie-stall dairy farms. Cows were enrolled from 3 weeks ante-partum to 8 weeks post-partum. All cows had free access to water and were fed a dry cow diet of total mixed ration (TMR) twice daily (0600 and 1500h) and post-partum cows were fed a lactating diet of TMR ad libitum. Cows were assigned to either control (n=13) or supplemented (n=6) group. All Supplemented cows were supplemented daily with 125g of FOSTO (Bélisle Solution Nutrition Inc., St-Mathias sur Richelieu, CA) pellets (0.42% of DMI) containing 50g of YMP during the ante-partum period and were supplemented daily with 500g of FOSTO pellets (1.25% of DMI) containing 200g of YMP during the post-partum period. YMP was fed to Supplemented cows as a top dress at 0530h, 30 minutes before the morning feeding. Control cows were not fed YMP. Body condition scoring was performed by two independent and trained evaluators using a 5-point scale [153].

2.2 Blood Collection

Blood samples were collected from each cow at four time-points relative to calving: 3 weeks ante-partum, the week of calving, 1 week post-partum, and 7 weeks post-partum. Blood sampling was done via venipuncture of the coccygeal vein 30 minutes before morning feeding (0530h). We used 10-ml serum Monoject blood collection tubes or a 10-ml plasma Monoeject blood collection tubes (Monoject Blood Collection Tube, Mansfield, USA) and a 20G vacutainer needles (BD Vacutainer PrecisionGlide Multiple Sample Needle, Plymouth, UK) for blood sampling. Plasma samples were immediately centrifuged at 1,500 rpm for 15 minutes at room temperature and samples were flash frozen in liquid nitrogen. Another set of blood samples were allowed to clot at room temperature for approximately 30 minutes to separate serum and centrifuged at 1,500 rpm for 15 minutes at room temperature before being flash frozen. All plasma and serum samples were stored at -80°C until further analyses.

2.3 Liver Biopsies

Liver biopsies were collected from control and supplemented cows at 3 weeks ante-partum, the week of calving, and 7 weeks post-partum. All liver biopsies were performed between 0500h and 0800h. Biopsies were collected using percutaneous needle (Tru-Cut biopsy needle,

14G Needle, CareFusion, Vernon Hills, USA) at the 10th intercostal space. Prior to liver biopsy, a 7 inch area was clipped, washed with 70% isopropanol, and disinfected with iodine and a local anesthetic (2 ml Lidocaine HCL 2%, Bimeda, Cambridge, CA) was applied. A skin incision was made to ease needle insertion. Liver tissue collection (approximately 10-12mg) was repeated three times to ensure adequate amount of sample for RNA isolation and qPCR analysis. Samples were immediately flash frozen in liquid nitrogen and stored at -80°C until downstream analyses. Following the liver biopsy, each cow was treated with Blu-Kote antiseptic spray (Blu-Kote, Dr. Naylor, Parry Sound, CA).

2.4 Follicular Aspiration

At approximately 6 weeks post-partum a synchronization protocol was initiated to perform an ultrasound-guided follicular aspiration of the dominant follicle as described previously [92].

2.5 RNA Collection and qPCR

RNA was isolated from hepatic tissue using TRizol® (Thermo Fisher Scientific, CA). The integrity and yield of RNA were determined by spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, CA).

We then used Advanced iScript cDNA synthesis kit (Bio-Rad, Mississauga, CA) to synthesize cDNA from 250 ng RNA, according to the manufacturer's instructions. All primers were purchased from Integrated DNA Technologies (Skokie, USA) and qPCR assays were all completed on CFX384 (Bio-Rad, Mississauga, CA) and performed according to MIQE guidelines [154]. mRNA transcript abundance was analyzed by standard curve methods, with efficiencies between 90-110%, and taking the starting quantities (SQ) as given by the CFX manager TM Software (Bio-Rad, Mississauga, CA). Relative transcript abundance was calculated by dividing the SQ values of each sample by the mean SQ of three reference genes (B2M, L19, and RPLP0) (Table 1).

2.6 Metabolite & Hormone Analyses

Blood samples from 3 weeks ante-partum, the week of calving, and 1 week post-partum were analyzed at the Animal Health Laboratory (University of Guelph) for BHBA, glucose, albumin, globulin, cholesterol, glutamate dehydrogenase (GLDH), and aspartate aminotransferase (AST), using a Roche Cobas 6000 c501 automated chemistry analyzer (Roche, Mississauga, Canada). Analytical procedures were performed according to the manufacturer's guidelines.

Blood and follicular fluid samples from week 7 post-partum were analyzed for glucose and BHBA levels using the Freestyle Precision Neo (Abbott, Abbott Park, US). Glucose levels were read using Precision: Blood Glucose Test Strips (Abbott, Abbott Park, US) and BHBA levels were read using Precision β-Ketone: Blood β-Ketone Test Strips (Abbott, Abbott Park, US). This protocol was validated in lab, prior to use in this experiment.

Gas chromatography (Varian 3900 with 8400 Auto-sampler with flame ionization detector at 260°C, Varian Analytical Instruments, Walnut Creek, CA) was used to determine follicular fluid fatty acid profiles as described previously [155].

Follicular fluid NEFA levels was completed using a non-esterified fatty acid colorimetric kit (Catachem, Oxford, USA). The amount of all solvents and samples used was reduced by two-thirds, to modify the protocol for use in a 96-well plate, rather than cuvette. This modified protocol was validated in lab, prior to use in this experiment.

E2 concentration was determined using a multispecies E2 ELISA kit (Caymen Chemical, Ann Arbor, USA). Samples of follicular fluid were diluted between 1:500 and 1:5000. All plates were read on FilterMax™ F5 Multi-Mode Microplate Reader (Molecular Devices, San Jose, USA). The intra- and inter-assay coefficients of variation were 3.00% and 14.99% respectively.

P4 concentration as determined using a multispecies P4 ELISA kit (Caymen Chemical, Ann Arbor, USA). Samples of follicular fluid were diluted between 1:200 and 1:1000. All plates were read on FilterMax™ F5 Multi-Mode Microplate Reader (Molecular Devices, San Jose, USA). The intra- and inter-assay coefficients of variation were 5.54% and 10.44%, respectively.

2.7 Pregnancy per AI

Following the competition of the study, the number of inseminations per pregnancy was reordered for all cows involved in the trial. Cows that were sold or otherwise removed from the herd for non-reproductive reasons were not included in this record.

2.8 Statistical Analysis

Data were analyzed as repeated measures using an analysis of variance (ANOVA) with PROC MIXED of SAS 9.4 (SAS Institute, 2012). Normality of the residuals was confirmed by Shapiro-Wilk test and log transformed if found not-normal. All log transformed data was presented as raw data LSM ± SE. All data has been expressed as LSM ± SE. Variance

components of each reported trait can be found on Table S1. Significant differences were declared at $P < 0.05$ and tendencies were declared at $P < 0.10$.

The first model was used in the analyses of repeated measures:

$$Y_{ijkm} = \mu + TRT_i + FARM_j + COW_{k(ij)} + TIME_m + TRT*TIME_{im} + e_{ijkm}$$

Y_{ijkm} = the parameter being measured

μ = overall mean

TRT_i = the fixed effect of the i-th treatment (i = 1 or 2)

$FARM_j$ = the fixed effect of the j-th farm (j=1 or 2),

$COW_{k(ij)}$ = the random effect of the k-th cow within the i-th treatment and the j-th farm [$COW_{k(ij)} \sim N(0, \sigma^2 cow$].

$TIME_m$ = the fixed effect of the m-th time point (k= -3, 0, or 7 OR -3, 0, or 1)

$TRT*TIME_{im}$ = the fixed effect of interaction of i-th treatment and m-th time point

e_{ijkm} = the residual error [$e_{ijkm} \sim N(0, \sigma^2 e)$].

The second model was used in the analyses of single time point measures:

$$Y_{ijk} = \mu + TRT_i + FARM_j + e_{ijk}$$

Y_{ijk} = the parameter being measured

μ = overall mean

TRT_i = the fixed effect of the i-th treatment (i = 1 or 2)

$FARM_j$ = the fixed effect of the j-th farm (j=1 or 2),

e_{ijk} = the residual error [$e_{ijk} \sim N(0, \sigma^2 e)$].

3. Results

BCS profile from 3 weeks before to 7 weeks after calving

First, we analyzed the change in BCS through 3 weeks before to 7 weeks after calving in both control and YMP supplemented groups. Even though YMP supplementation had no effect on the overall BCS profile of control and supplemented cows, within control cows the BCS at 7 weeks post-partum was lower than that at 3 weeks before and the week of calving ($P < 0.01$; Figure 1). However, supplemented cows did not exhibit such a robust decline in BCS from 3 weeks before to 7 weeks after calving ($P > 0.05$; Figure 1).

Circulating concentrations of metabolic indicators

Given that YMP supplemented cows appeared to evade significant loss of BCS during early lactation, we examined the circulating profiles of metabolic indicators including glucose, BHBA, albumin-globulin ratio, AST, total cholesterol, and GLDH. While there was no group nor time effects, there was a significant group x time interaction on serum concentrations of glucose and BHBA (Figure 2). Control cows had lower levels of glucose ($P < 0.01$; Figure 2A) and higher levels of BHBA ($P < 0.05$; Figure 2B) at 1 week post-partum compared to 3 weeks ante-partum. However, YMP supplemented cows did not have such changes in glucose and BHBA levels ($P > 0.10$; Figure 2). In fact, there was a numerically increasing trend in glucose concentration and a decreasing trend in BHBA levels from the week of to 1 week after calving (Figure 2). Supplemented cows also had significantly higher Albumin:Globulin Ratio and AST levels ($P < 0.05$; Figure 3A and B), a tendency to have higher levels of Cholesterol and GLDH ($P < 0.10$; Figure 3C and D).

Hepatic Gluconeogenesis

As the YMP supplemented cows did not have postpartum decline in glucose levels we went to investigate the molecular basis of the maintenance of glucose levels in these cows. Since gluconeogenesis is the major pathway for maintaining adequate glucose concentrations in ruminants, we examined the gene expression of four genes involved in the gluconeogenic pathway in hepatic cells [156]. In log transformed data, *G6PC* was found to have significantly higher relative mRNA abundance in supplemented cows compared to control cows ($P < 0.001$; Figure 4A). The relative mRNA abundance of *G6PC* also had a tendency to be higher in supplemented cows at 7 weeks post-partum ($P < 0.1$; Figure 4A). Additionally, supplemented cows had a tendency to have higher relative mRNA abundance of *PCK1* compared to control cows ($P < 0.10$; Figure 4B). On the other hand, the relative transcript

abundance of *PC* was not different between control and supplemented cows (P > 0.1; Figure 4C). As expected, the relative mRNA abundance of *PCK2* did not differ between control and supplemented cows (P > 0.05; Figure 4D).

Ovarian functions and pregnancy rate

As our data indicated that YMP supplemented cows had favorable metabolic milieu and healthy liver function, we shifted our focus to the ovary and reproductive performance. First, we checked the circulating levels of glucose and BHBA at week 7 after calving, the period immediately prior to the period of expected breeding in typical dairy herds. There were no differences in circulating concentrations of glucose nor BHBA between control and supplemented cows (P > 0.1; Figure 5A and B).

We then examined the development and the microenvironment of the dominant follicle of the synchronized follicular wave in control and YMP supplemented cows. There were no differences (P > 0.1) between control and supplemented cows in the size of the dominant follicle (12.4 ± 0.9 vs 14.7 ± 1.7, respectively) nor the total number of follicles on the day of follicular aspiration (4.4 ± 1.0 vs 4.4 ± 1.7, respectively). Similar to blood samples, there were no differences between control and supplemented cows in glucose nor BHBA levels in the follicular fluid of their dominant follicle (P > 0.1; Figure 6A and B). On the other hand, there were lower levels of NEFA in the follicular fluid of supplemented compared to control cows (P < 0.05; Figure 6C). Further, the follicular fluid of the dominant follicle in YMP supplemented cows had lower proportions of C16:0 and C18:1 (P < 0.05; Figure 6D) and significantly higher proportions of C18:2 (P < 0.05; Figure 6D). The proportions of C18:0 and C18:3 in the follicular fluid of both control and supplemented cows did not differ (P > 0.1; Figure 6D).

As markers of ovarian functions, we analyzed the follicular fluid concentrations of 17β-estradiol and progesterone. The follicular fluid of the dominant follicle in supplemented cows had higher levels of 17β-estradiol compared to control cows (P < 0.01; Figure 7A). While there was no difference in follicular fluid concentrations of progesterone between the two groups of cows (P > 0.1; Figure 7B), the estradiol:progesterone ratio was higher in the follicular fluid of supplemented cows (P < 0.01; Figure 7C).

Finally, we examined the effect of YMP on the reproductive performance of the supplemented cows. Even though there was no statistical difference (P > 0.1), the pregnancy

rate per artificial insemination in supplemented cows was 22% higher than in control cows (45% vs. 23%, respectively; Figure 8).

4. Discussion

The transition period in dairy cows has been well documented as a period of metabolic stress due to the energy demands of early lactation. The NEB experienced during this time period is due to the fact that feed intake alone cannot meet the energy demands. Instead, dairy cows mobilize their body reserves for maintenance and lactation. The mobilization of body reserves is a physiological process, however, if the rates of mobilization are too severe, the resulting altered hormones and metabolites increase disease susceptibility and decrease production and reproductive performance. The objective of this study was to determine the effects of YMP supplementation on metabolic status through the transition period and ovarian health at the time of first breeding post-partum. YMP is regarded as a rumen protected protein that is diluted in the liquid phase of the rumen [21]. The liquid phase passes quickly through the rumen, thus YMP avoids the majority of transformation by rumen microbes [21]. Also, YMP has an AA profile similar to that of microbial crude protein and has been shown to improve milk fat percentage in lactating dairy (Table S2) [21]. To our knowledge, no one has yet to report of the effects of YMP supplementation on the reproductive performance of transition cows.

Following parturition, BCS loss continues up to 30 days post-partum [157]. The fact that BCS was lower at 7 weeks post-partum in control cows compared to supplementated cows indicates that the control animals had a prolonged period of NEB beyond 30 days post-partum or had a more severe NEB during early lactation. Taken together, our observations with regards to BCS, glucose levels and BHBA levels, indicate that YMP supplementation evaded such a carry-over effect of the NEB in supplemented cows, potentially reducing the negative impact on ovarian functions.

At the same time, supplemented cows had higher levels of AST and GLDH along with lower Albumin:Globulin ratio compared to control cows. While higher albumen:globulin ratio in control cows is indicative of chronic inflammation [158]. Conversely, higher levels of AST and GLDH in supplemented cows are indicative of hepatocyte injury and fatty liver, respectively [159, 160]. Nonetheless, the levels of these metabolites in both groups of cows were within the normal range for health fresh cows [161]. However, this does raise concerns regarding any on farm changes to the levels of YMP provided in the diet, in this case, these results should be considered carefully before increasing the amount of YMP supplementation. Taken together, our results indicate that both control and supplemented cows underwent metabolic stress as expected and that YMP supplementation does not completely eliminate

the metabolic stress associated with parturition and early lactation. Interestingly, supplemented cows had a tendency to have higher levels of cholesterol, which is a precursor for steroidogenesis in the ovary [162].

Lack of a significant decline in glucose concentration after parturition was associated with higher expression of *PCK1* and *G6PC*, in supplemented cows. The enzyme PCK1 is responsible for the entry of AAs and propionate into the gluconeogenic pathway and the enzyme G6PC catalyzes the final step of conversion of glucose-6-phosphate to glucose. There was no difference in the expression of *PC* nor *PCK2* between the two groups of cows. The enzyme PC catalyzes pyruvate to oxaloacetate and PCK2 is a mitochondrial enzyme that converts oxaloacetate to phosphoenolpyruvate. As most AAs yield CAC intermediates, which enter gluconeogenesis through oxaloacetate PCK1 and G6PC are more important enzymes for gluconeogenesis from AA metabolites. Analysis of enzymatic activity of these gluconeogenic would provide additional insight into liver metabolism, however this was beyond the scope of this project. Still, these results are consistent with a study investigating the effects of the supplementation of glycerol enriched yeast culture on metabolic status of transition cows [41]. This study also observed an increased expression of *PCK1*, in association with higher glucose and lower BHBA in supplemented cows [41]. Taken together, these results suggest that AAs from YMP supplementation could be directly contributing to gluconeogenesis, leading to improved metabolic status in transition cows.

Though the glucose and BHBA levels were similar in both groups of cows at 7 weeks post-partum, the positive effects on metabolic status of supplemented cows during early post-partum were associated with improved ovarian health. As metabolic indicators in circulation are represented in the follicular fluid of dominant follicles in dairy cattle, we sought to examine the microenvironment of the dominant follicle at 7 weeks post-partum, the period leading up to the breeding period in dairy cows [92]. While there was no difference in the size of the dominant follicle of a synchronized wave, the hormonal and metabolite profiles in the follicular microenvironment was indicative of better ovarian function in supplemented cows. Fatty acids are required for a multitude of critical functions in the follicle, including energy for cellular survival and steroid hormone synthesis. Supplemented cows had lower proportions of palmitic acid and oleic acid, coupled with a higher proportion of linoleic acid. Supplementation of cultured human and bovine granulosa cells with palmitic acid has been shown to increase apoptosis [163, 164]. Other studies have reported negative effects on bovine oocytes when exposed to higher levels of palmitic acid and linoleic acid [116, 132].

However, there is evidence that higher levels of oleic acid reduced the negative impact of palmitic acid on bovine oocytes [165]. These in vitro studies show that the effects of individual NEFA on granulosa cells and oocytes are complex and thus warrant studies investigating the combined effects of the NEFA supplemented in proportions similar to the ones observed in our study.

Despite similar glucose and BHBA levels, there were lower levels of NEFAs in the follicular fluid of supplemented cows. It was shown that estrogen active follicles have significantly lower amounts of NEFA levels in the follicular fluid compared to estrogen inactive follicles [166]. Additionally, in a study using fasting heifers to represent NEB in lactating cows, it was shown that the dominant follicles of fasted heifers had lower estrogen:progesterone ratios compared to control heifers [152]. In our study, the dominant follicles of supplemented cows had dramatically higher levels of estradiol and estrogen:progesterone ratio compared to control cows. Data from all these studies clearly indicate that alleviation of metabolic stress positively impacts the follicular microenvironment. When we examined the reproductive performance of the cows used in this study, the supplemented cows appeared to have higher pregnancy rates per artificial insemination than control cows, although the differences were not statistically significant. We suggest that additional studies with larger numbers of cows are needed to confirm the positive effects of YMP supplementation on fertility of lactating cows.

In conclusion, we found that YMP supplementation during the transition period improved the metabolic status of lactating dairy cows. This was associated with increased expression of gluconeogenic genes in the liver of supplemented cows. As a result of the improved metabolic status, the dominant follicles of supplemented cows at 7 weeks post-partum were more estrogenically active than those of control cows. Thus, using a multidisciplinary approach, here we provide the evidence that YMP supplementation is an ideal nutritional intervention to alleviate metabolic stress and improve ovarian follicular health in lactating dairy cows.

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6. Author Contributions

A.S-Y: contributed to ideas, designed experiments, took part in the field work, collected, assembled, analyzed, and interpreted data, prepared figures and wrote manuscript; Y.S: contributed to ideas, designed experiments, took part in the field work, collected and analyzed data; V.H: contributed to ideas, designed experiments, took part in the field work, and collected data; N.D., R.C.B. & R.M.: took part in field work and collected data; M.T. & E.M. took part in field work; V.B., A.M. & B.B.: contributed to ideas; R.D.: conceived the study, designed experiments, analyzed data, and edited manuscript.

7. Figure Legends

Figure 1. Supplementation with YMP during the transition period results in consistent BCS during early lactation. LSM (±SE) BCS of control (n=13) and supplemented (n=6) cows from 3 weeks before to 7 weeks after calving. The supplemented cows received rumen protected YMP from 3 weeks before to 4 weeks after calving. Double asterisk denotes P < 0.01 from week -3 and week 0 within control group. Gp = Group, Tm = Time, GT = Group x Time.

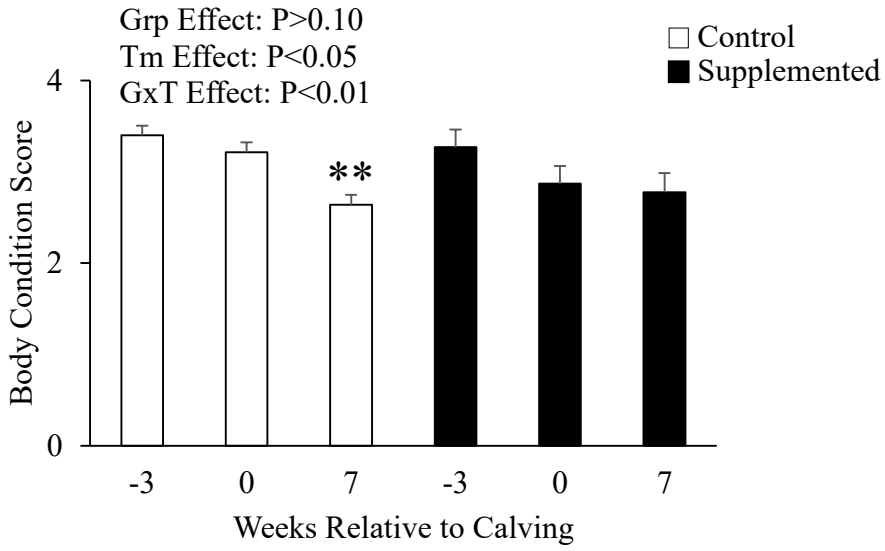


Figure 2. Supplementation with YMP during the transition period improves circulatory levels of Glucose (A) and BHBA (B). LSM (±SE) circulating concentrations of glucose (A) and BHBA (B) in control (n=13) and supplemented (n=6) cows from 3 weeks before to 1 week after calving. The supplemented cows received rumen protected YMP from 3 weeks before to 4 weeks after calving. Single asterisk denotes $P < 0.05$ from week -3. Double asterisk denotes $P < 0.01$ from week -3. Gp = Group, Tm = Time, GT = Group x Time.

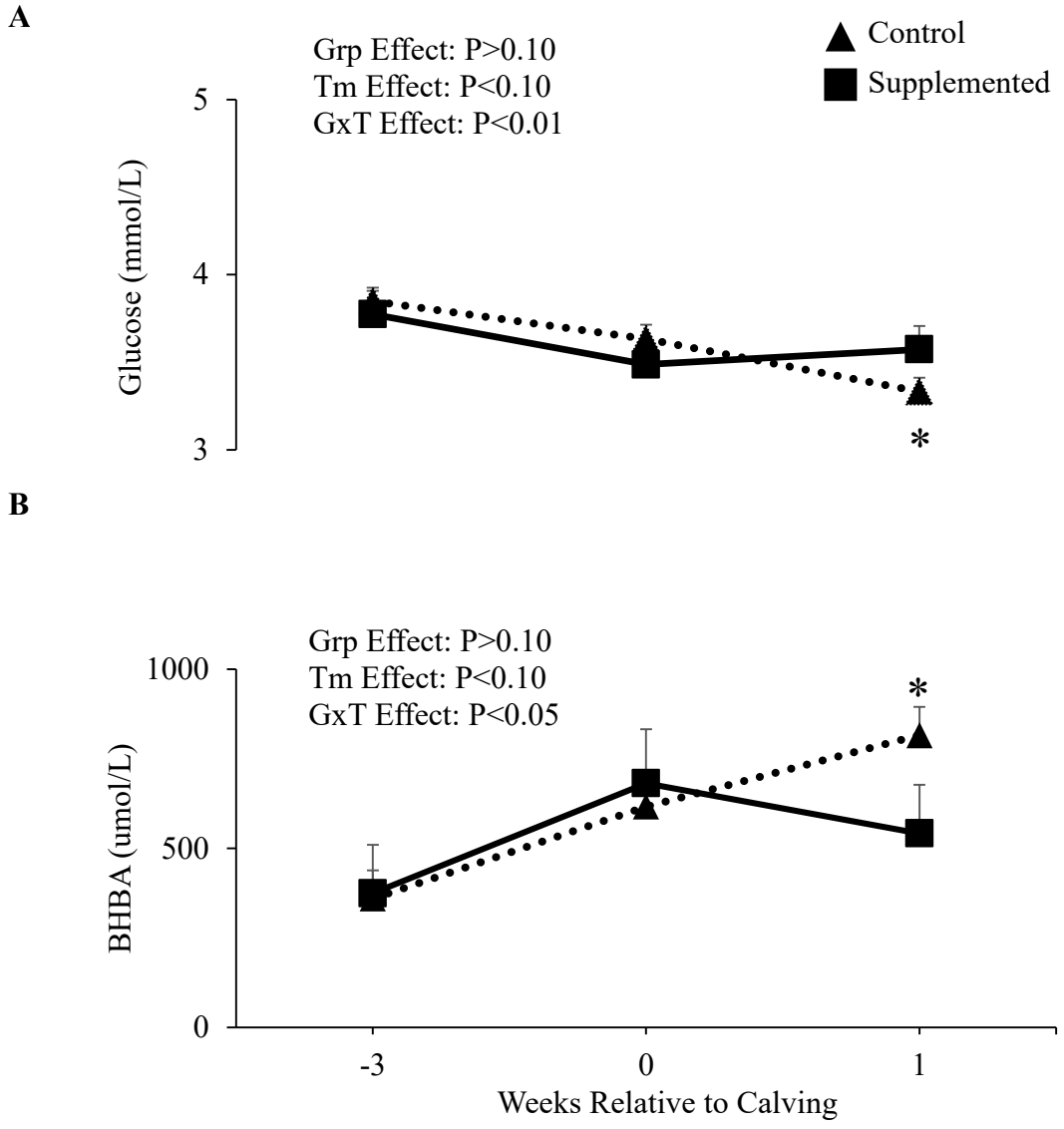


Figure 3. Supplementation with YMP during the transition period alters indicators of metabolic adjustments in the liver. LSM (±SE) ratio of Albumin:Globulin (A) and LSM (±SE) concentrations of AST (B), Cholesterol (C), and GLDH (D) in the circulation of control (n=13) and supplemented (n=6) cows from 3 weeks before to 1 week after calving. The supplemented cows received rumen protected YMP from 3 weeks before to 4 weeks after calving. Gp = Group, Tm = Time, GT = Group x Time.

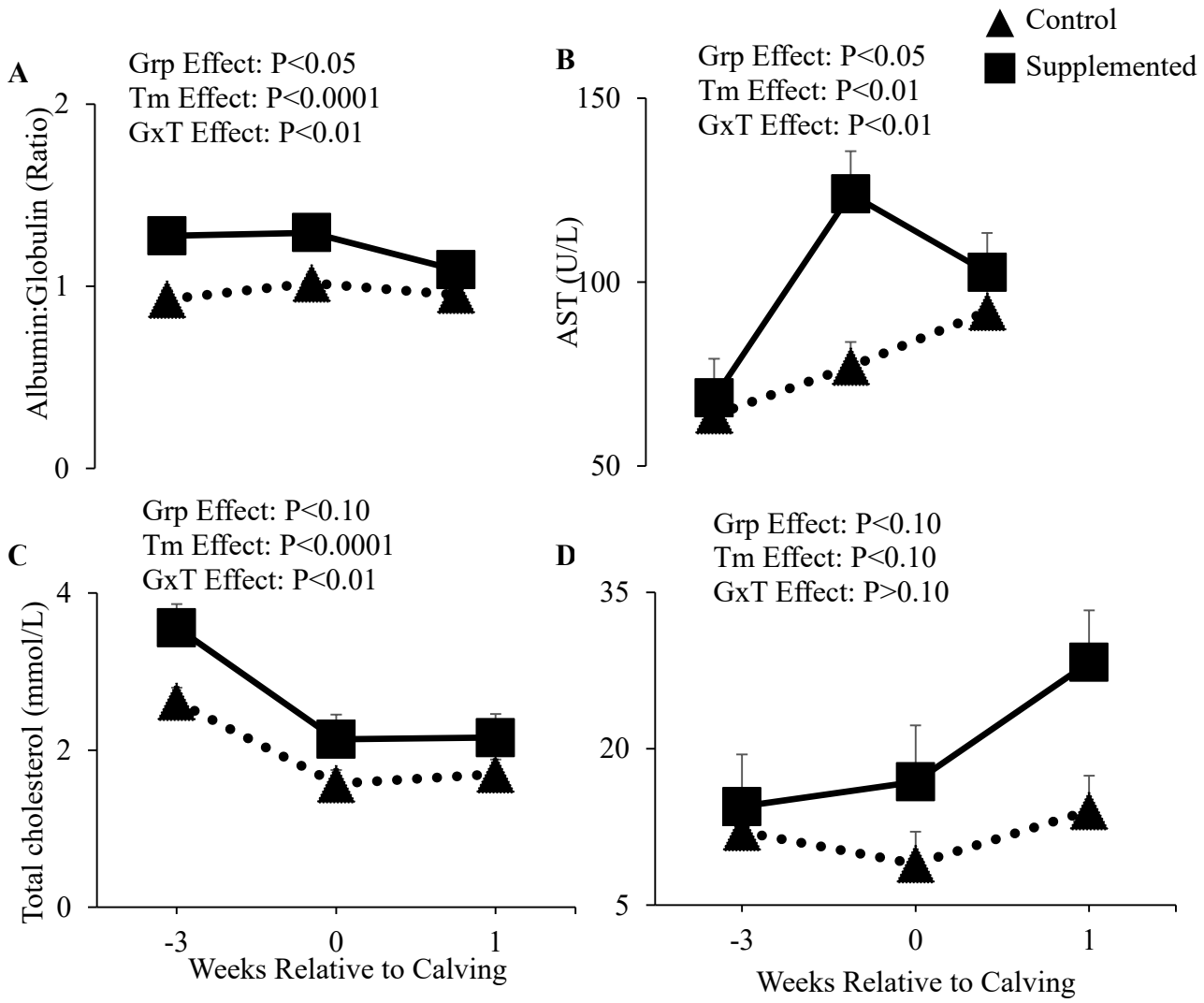


Figure 4. Supplementation with YMP during the transition period alters the mRNA abundance of genes involved in hepatic gluconeogenesis. LSM (±SE) relative mRNA abundance of genes associated with hepatic gluconeogenesis (*G6PC*, *PC*, *PCK1*, *PCK2*) in control (n=13) and supplemented (n=5) cows. *G6PC* data required log transformation and the LSM (±SE) of the raw data is presented here. The supplemented cows received rumen protected YMP from 3 weeks before to 4 weeks after calving. Hepatic tissue was collected by biopsy. Gp = Group, Tm = Time, GT = Group x Time.

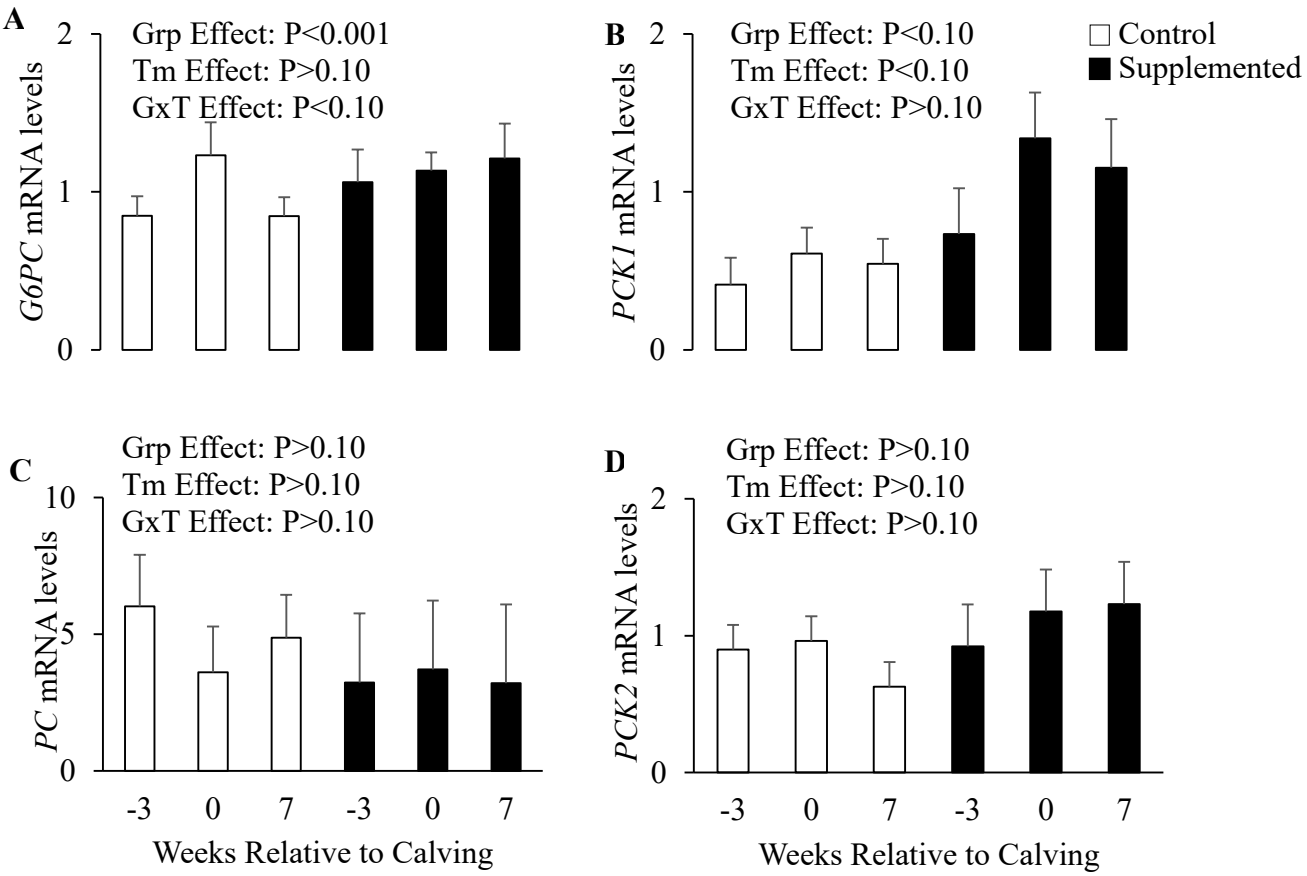


Figure 5. Supplementation with YMP during the transition period does not alter the levels of Glucose (A) and BHBA (C) in circulation at 7 weeks post-partum. LSM (±SE) circulating concentrations of glucose (A) and BHBA (B) in control (n=13) and supplemented (n=5) cows. BHBA data required log transformation and the LSM (±SE) of the raw data is presented here. The supplemented cows received rumen protected YMP from 3 weeks before to 4 weeks after calving.

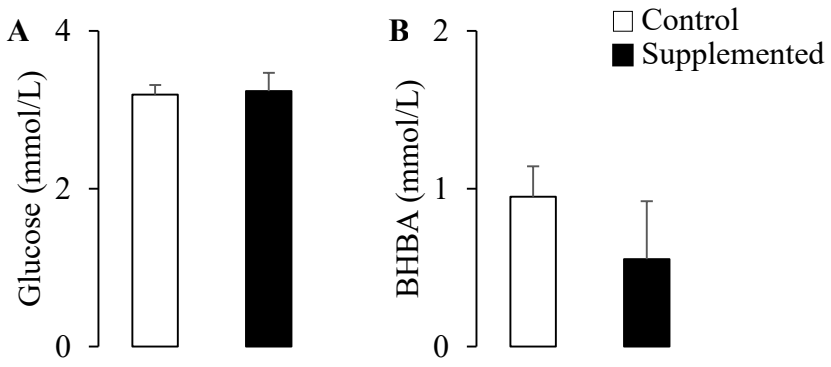


Figure 6. Supplementation with YMP during the transition period reduces NEFA levels and alters the NEFA profile of follicular fluid at 7 weeks post-partum. LSM (±SE) concentrations of Glucose (A), BHBA (B), NEFA (C) and proportions of NEFAs (D) in the follicular fluid of the dominant follicle in control (n=13) and supplemented (n=5) cows at 7 weeks post-partum. BHBA data required log transformation and the LSM (±SE) of the raw data is presented here. The supplemented cows received rumen protected YMP from 3 weeks before to 4 weeks after calving. Follicular fluid was collected by ultrasound guided follicular aspiration of the dominant follicle 6 days after synchronization of the follicular wave. Palmitic Acid = C16:0, Stearic Acid = C18:0, Oleic Acid = C18:1, Linoleic Acid = C18:2, Linolenic Acid = C18:3. Single asterisk denotes $P < 0.05$.

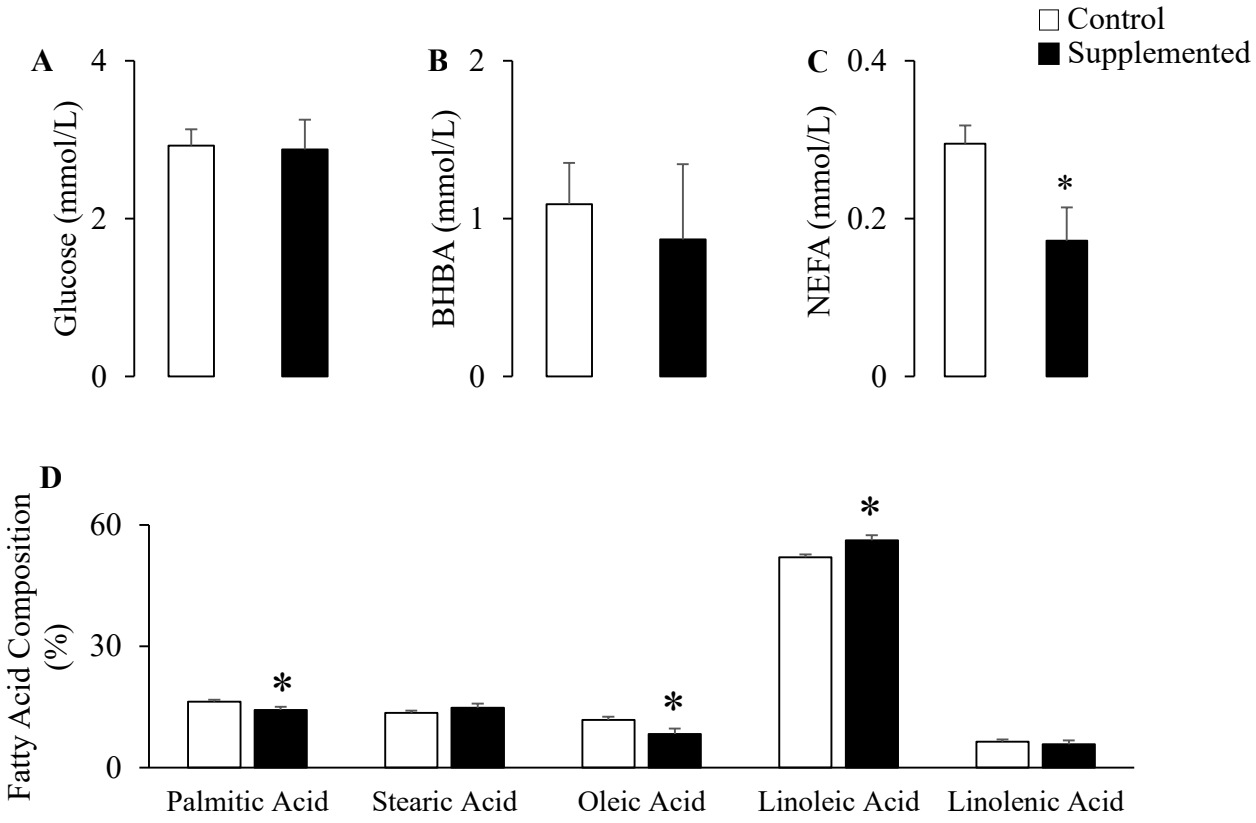


Figure 7. Supplementation with YMP during the transition period improves hormone function in the Dominant Follicle at 7 weeks post-partum. LSM (±SE) concentrations of 17β-estradiol (A) and progesterone (B) and LSM (±SE) ratios of estradiol:progesterone (C) of the dominant follicle in control (n=10) and supplemented (n=5) cows. Progesterone data required log transformation and the LSM (±SE) of the raw data is presented here. The supplemented cows received rumen protected YMP from 3 weeks before to 4 weeks after calving. Follicular fluid was collected by ultrasound guided follicular aspiration of the dominant follicle 6 days after synchronization of the follicular wave. Double asterisk denotes $P < 0.01$. Triple asterisk denotes $P < 0.001$.

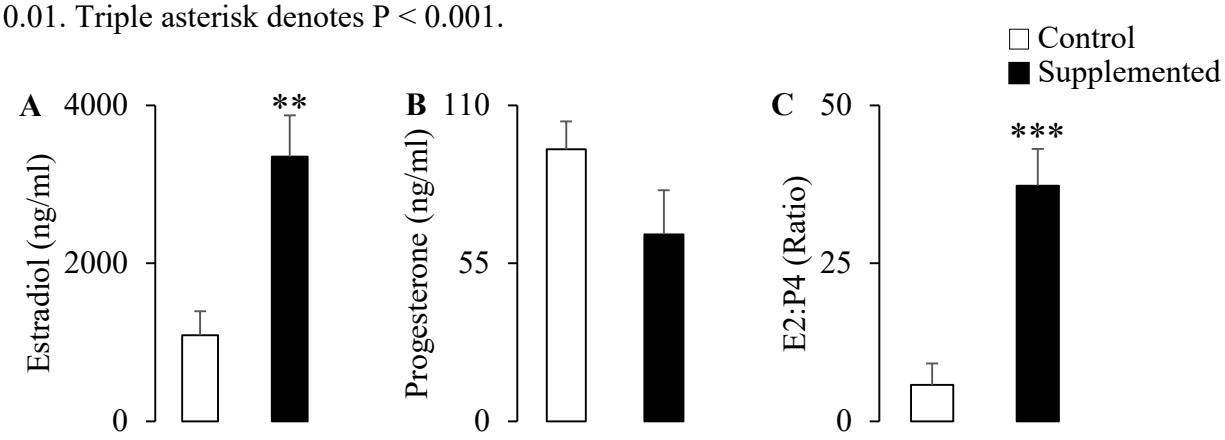
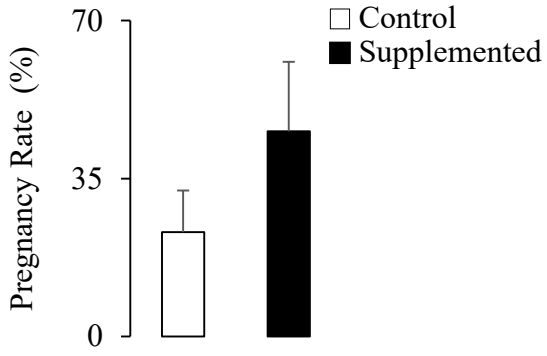


Figure 8. Supplementation with YMP during the transition period does not alter the number of pregnancies per insemination in the following lactation. LSM (±SE) percentage of pregnancies per artificial insemination in control (n=9) and supplemented (n=5) cows. The supplemented cows received rumen protected YMP from 3 weeks before to 4 weeks after calving.



8. Tables

Table 1: List of primer sets used in real-time PCR analyses

Gene Name	Primer Sequence (5' to 3')	
G6PC	F	ACTCCTCTGGGTAGCTGTGAT
	R	TCGGTATCCAAAACCCACCAG
PCK1	F	AGGGTCGCACCATGTATGTC
	R	AGTGGAGGCACTTGACGAAC
PCK2	F	CACCACCAACCCCAATGCTA
	R	GGGCACAAAAGCGAGAGTTG
PC	F	ACTGCAGCAAGTTCGGTT
	R	CACCATGACCTTCTTGATGGG
B2M	F	GCGTCCTCCAAAGATTCAAG
	R	CAGGTCTGACTGCTCCGATT
L19	F	GCCAACTCCCGTCAGCAGA
	R	TGGCTGTACCCTTCCGCTT
RPLP0	F	GGCGACCTGGAAGTCCAACT
	R	CCATCAGCACCACAGCCTTC

V. CONCLUSION

In this study, we examined the differences in physical traits, circulatory metabolites, hepatic gluconeogenesis and the follicular microenvironment between control cows and cows supplemented with YMP. We reported that supplemented animals did not experience the decreases in circulatory levels of glucose, nor did they experience the increases in circulatory levels of BHBA, that control animals did experience. Supplemented animals also had increased relative mRNA abundance of the genes related to hepatic gluconeogenesis (*G6PC*; *PCK-1*), underpinning the lack of the expected reduction in glucose levels in supplemented cows. We also reported that the improved levels of metabolites during the transition period had a dramatic effect on the NEFA and hormonal profile of the dominant follicle at 7 weeks post-partum. Low NEFA levels and high E2:P4 ratios in the follicular fluid of the dominant follicle support our hypothesis that supplementation with YMP during the transition period improves the health and function of the follicular microenvironment. While it would be of key interest that future studies examine both the hormone and NEFA profile of the follicular fluid and the alterations in gene expression of granulosa cells and oocytes that are developing in this microenvironment, our study demonstrates that YMP supplementation has a positive impact on the health and fertility of lactating Holstein dairy cows.

VI. APPENDIX

Supplementary Tables

Table S1: Variance Components of Each Reported Trait.

Trait	σ^2cow	σ^2SP(POW)	σ^2e
BCS	0	0.8732	0.1513
Glucose – Circulation	0	0.2027	0.07833
BHBA – Circulation	1387.66	0.2921	78865
Albumin	0	0.6824	9.3522
Globulin	17.2233	0.1419	13.6032
Albumin:Globulin Ratio	0	0.8791	0.03438
AST	61.6979	-0.1303	515.47
Cholesterol	0.2726	-0.04143	0.1234
GLDH	29.3062	-0.3677	93.9969
G6PC	0.1833	0.001002	0.1821
PCK1	0.1835	0.4957	0.1470
PC	13.3180	0.4940	18.6262
PCK2	0	0.9237	0.2870
Glucose at 7 weeks – Circulation			0.1903
BHBA at 7 weeks – Circulation			0.3744
Size of DF			10.2799
Follicle Number			10.5306
Glucose – FF			0.4979
BHBA – FF			0.8529
NEFA			0.006270
Palmitic Acid			2.1699
Stearic Acid			3.4252
Oleic Acid			6.1922
Linoleic Acid			5.4183
Linolenic Acid			2.9414
E2			819868
P4			1.8289
E2:P4 Ratio			113.01
Pregnancy/Insemination			760.60

Table S2: Amino acid (AA) composition of yeast-derived microbial protein (YMP).

AA	YMP
Essential AA	% of Total Essential AA
Arg	9.1
His	6.6
Ile	10.4
Leu	16.6
Lys	16.6
Met	3.3
Phe	10.1
Thr	11.7
Trp	2.4
Val	13.0
Non-Essential	% of Total Non-Essential AA
Ala	14.9
Asp	16.9
Cys	3.5
Glu	27.4
Gly	8.8
Pro	10.4
Ser	11.9
Tyr	6.2

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