Impacts of Reducing the Dry Period to 40 days and Eliminating the Far-Off Diet on Milk Production, Rumen and Blood Parameters, Liver Gene Expression and Rumen Microbiome Profile of Holstein Dairy Cows

by

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Abstract

Effects of a short 40-d dry period with only a close-up diet (SHORT) and a conventional 60-d dry period with a 39-d far-off and a 21-d close-up diet (CONV) on milk production, feed intake, blood and rumen parameters, liver gene expression and rumen microbiota profile were compared in 11 second-parity and 15 third and later parity cows. Milk production was recorded daily during the first 16 wks of lactation. Differential liver gene expression was assessed by affymetrix microarray analysis and DNA extracted from rumen samples was subjected to Illumina sequencing for exploring the microbiome profile. The SHORT treatment reduced milk yield and DMI after calving in third and later parity cows, but not in second-parity cows when compared to the CONV treatment. Cows on the SHORT treatment had higher concentrations of NEFA in blood plasma and tended to have higher liver TAG immediately after calving. These effects tended to be greater in third- and later parity cows compared to second-parity cows. Expression patterns of genes involved in β-oxidation at the first week of lactation compared to those at three weeks before calving showed lower hepatic β-oxidation capacity in cows on the SHORT treatment compared to those on the CONV treatment. During this period, the expression of DGAT, a key gene in the triglyceride synthesis, increased in SHORT-treatment cows while it remained unchanged in CONV-treatment cows. The expression patterns of genes involved in gluconeogenesis showed a higher capacity at first week after calving in cows on the SHORT compared to those on the CONV treatment. Our study also showed that the SHORT treatment increased the relative abundance of Firmicutes and reduced the relative abundance of Bacteroidetes compared to the CONV treatment and reduced the shifting of rumen microbiota from before to after calving. Results also demonstrated that the rumen microbiota was more stable in the SHORT treatment during the transition period. Based on these results, a 40-d dry period management with only a close-up diet might be beneficial for second parity cows. However, this treatment may be detrimental for older cows as excessive energy intake and fat deposition during the dry period in these animals result in lower milk production and higher mobilization of NEFA and accumulation of fat in the liver.

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DEDICATION

I dedicate this thesis to my parents.

You are the reason I’ve made it this far!

Thank you.

FOREWORD

This thesis was prepared following a manuscript format. The thesis is comprised of four manuscripts each corresponding to a chapter. Manuscript I and II have been published by the Canadian Journal of Animal Science. Manuscript III and IV will be submitted to the Journal of Dairy Science and PLOS ONE, respectively. All manuscripts are formatted in accordance with the guidelines of the Canadian Journal of Animal Science.

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A:P	Acetic acid to propionic acid ratio
ABCA1	ATP-binding cassette transporter A1
ACADVL	Acyl-CoA dehydrogenase, very long chain
ACOX2	Acyl-CoA oxidase 2
ACTB	Beta actin
ADF	Acid detergent fiber
ADIPOR2	Adiponectin receptor 2
ALDOA	Aldolase A, fructose-bisphosphate
ANGPTL4	Aangiopoietin-like 4
APOA	Apolipoprotein A
APOC2	Apolipoprotein C-II
ASCL1	Acyl-CoA synthetase, long chain 1
AUC	Area under the curve
BHBA	Beta hydroxybutyric acid
BRP44L	Mitochondrial pyruvate carrier 1
BW	Body weight
CBW	Calf birth weight
CD36	CD36 molecule
CONV	A 60-d dry period during which a 39-d far-off and a 21-d close-up was fed
CPT	Carnitine palmitoyltransferase
CRAT	Carnitine acetyltransferase
CREB3L3	cAMP responsive element binding protein 3-like 3

CROT	Carnitine octanoyltransferase
CYC1	Cytochrom c-1
CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1
DGAT	Diacylglycerol O-acyltransferase
DM	Dry matter
DMI	Dry matter intake
EB	Energy balance
ELOVL5	Very long chain fatty acid 5
F6P	Fructose-6-phosphate
FADS2	Fatty acid desaturase 2
FBP	Fructose-1,6-bisphosphatase
FDPS	Farnesyl diphosphate synthase
G6PC	Glucose-6-phosphatase
GHR	Growth hormone receptor
GK	Glucokinase
GNMT	Glycine N-methyltransferase
GYS2	Glycogen synthase 2
HDL	High-density lipoprotein
IGF1	Insulin-like growth factor 1
IGFBP	IGF-binding protein
LCFA	Long chain fatty acids
LDHA	Lactate dehydrogenase A
LOS	Lactose and other solids

LPIN1	Lipin1
LPL	Lipoprotein lipase
ME1	Malic enzyme 1
MLYCD	Malonyl-CoA decarboxylase
MP	Metabolizable protein
NDF	Neutral detergent fiber
NEFA	Non-esterified fatty acid
NEL	Net energy for lactation
OUT	Operational taxonomy unit
PAR 2	Parity 2
PAR 3+	Parity 3 and older
PC	Pyruvate carboxylase
PCK	Phosphoenolpyruvate carboxykinase
PCR	Polymerase chain reaction
PDHA1	Pyruvate dehydrogenase
PERMANOVA	Permutational multivariate analysis of variance
PFKL	Phosphofructokinase
PLIN2	Perilipin 2
PLS-DA	Partial least square discriminant analysis
PPARGC1A	peroxisome proliferative-activated receptor-γ coactivator 1 α
PPARα	Peroxisome proliferative-activated receptor-α
PPARγ	Peroxisome proliferative-activated receptor-γ
PPP1R3C	Protein phosphatase 1, regulatory subunit 3C

SCC	Somatic cell count
SCD	Stearoyl-CoA desaturase
SHORT	A 40-d dry period during which only a close-up diet was fed
SLC22A5	Solute carrier family 22 (organic cation/carnitine transporter), member 5
SLC2A2	Solute carrier family 2
SREBP1	Sterol regulatory element-binding protein 1
TAG	Triacylglyceride
THRSP	Thyroid hormone responsive
UGP2	UDP-glucose pyrophosphorylase 2
VFA	Volatile fatty acids
VLDL	Very low-density lipoprotein
WK	Week

INTRODUCTION

Dry period management plays an important role in dairy cows health and performance especially in early lactation (Collier et al., 2012). Disorders such as ketosis, fatty liver, retained placenta and milk fever can be caused by poor management during the dry period (Grummer, 1995; Drackley, 1999). Traditionally a 60-d dry period has been applied since World War II (Knight, 1998) but recent studies are suggesting that the length of dry period can be shorter (Santschi et al. 2011). There is no unanimous agreement on this length; however, it has been shown that the required length of dry period, depending on the management, can be shortened to a range between 30 to 40 days (Bachman, 2002; Gulay et al. 2003; Annen et al. 2004; Santschi et al. 2011). Animal nutrient and energy requirements during this period are another aspect that has been a focus of different studies. Some studies suggest restricting the feed and energy intake of dairy cows during the dry period (Drackley, 1999; Dann et al., 2005; Janovick et al., 2011) while others suggest increasing the energy content of diet to metabolically prepare animals for the lactation diet (Grummer, 1995; Rastani et al., 2005; Santschi et al. 2011).

Although remarkable advances have improved our understanding of physiology and metabolism of dairy cows during the transition period, there are still unknown phenomena that affect responses of animal to different dry period managements. There are very limited studies regarding the rumen microbiota profile during the dry period and how it changes during the transition period in cows with different dry period managements. Liver metabolism during the transition period is another area that requires further research as it plays an important role in energy hemostasis in early lactation. Metabolism of mobilized fat from adipose tissues to the liver around calving requires changes in the expression of genes to adapt to the new metabolic needs in the body. It is important to discover the network of genes that work together to improve response of animal. To the best knowledge of authors there has not been a study that compares all aspects of production, physiology, gut microbiology, and liver gene expression of dairy cows with a short 40-d dry period with a single close-up diet and/or a conventional 60-d dry period with separate far-off and close-up diets , during the transition period. Therefore, this study was conducted to explore these comparisons..

CHAPTER ONE

LITERTURE REVIEW

1.1 Why is a dry period needed?

It is well-recognized in the literature that a dry period is required for high milk production in the subsequent lactation of dairy cows. There are four hypotheses to explain why a dry period is required, as summarized by Collier et al. (2012). Woodward and Dawson made the first hypothesis in 1926 that dairy cows need a non-lactating period to increase the body energy reserves for the subsequent lactation. However, this was rejected by other researchers (Swanson, 1965; Lotan and Alder, 1976) who reported lower milk production of cows even with enhanced body weight with a 0- to 30-d dry period compared to a 60-d dry period. The second hypothesis focused on hormones and suggested that lower milk production in cows with continuous milking is due to the constant exposure of galactopoietic hormones resulting in delayed involution of mammary gland. This was also disproved later by studies that showed lower milk production in continuous milked quarters compared to those with a planned dry period even though they were under the same hormonal influence (Smith et al. 1967). The third theory to explain the lower milk production in the continuous milked quarters was the reduction in mammary epithelial cells as a result of eliminating the dry period. However, this hypothesis was disapproved by Swanson et al. (1967) who showed that a 6-wk difference in dry period length did not lead to significant differences in dry fat-free tissue weight, total DNA, or abundance of alveolar cells in the mammary gland. Authors of this paper suggested that lower milk production in continuously milked quarters during the subsequent lactation is probably the result of a reduced function of mammary parenchyma rather than subnormal structure. Swanson et al. (1967) hypothesized that the main reason behind reduced milk yields in continuous milked quarters is the lower secretory activity per unit of mammary tissues. This also was proven by Capuco et al. (1997) who reported that replacing senescent mammary epithelial cells is required for optimum milk production and a dry period is necessary for increasing the total mammary parenchymal tissue. This hypothesis is the basis of current dry period management recommendations.

1.2 The optimum length of the dry period

As described, a dry period is essential for high milk production in the subsequent lactation. Traditionally, a 60-d dry period has been applied since World War II (Knight, 1998). Due to genetic progress in dairy cows during the last decades and also due to the demonstrated health and economic benefits (Santschi et al., 2011) of a shorter dry period, the optimum dry period length has been reevaluated. A far-off diet is usually fed for about 40 d and contains low energy and protein mostly, whereas a close-up diet is fed for about 21 d and contains high energy and protein to prepare the rumen microbiota for a high grain and high energy diet after calving. However, as these changes in diets in occur in a short period of time, there may not be enough time for rumen microbiota to adapt (Dirksen, et al., 1985) This may lead to lower feed intake during early postpartum (Jolicoeur et al. 2014), and consequently to metabolic disorders, such as ketosis (Santschi et al., 2011). However, in contrast to a traditional 60-d dry period, a shorter dry period provides the opportunity of using only one diet during the dry period and eliminating switching diets from far-off to close-up. Also, it has been suggested that a shorter dry period may reduce the incidence of mastitis due to lower milk production at the time of drying-off (Bernier-Dodier et al. 2011). Drying-off when milk production is high places substantial pressure on the udder and teat canals, resulting in open canals with leaking milk that consequently cause intramammary infection (Newman et al. 2010). In addition, cows with a short dry period have more milk production in their previous lactation compared to a conventional 60-d dry period, as studies have shown that shortening the dry period from 60 to 35-d increases net income by $245.18 per cow when all economic parameters such as quota price, milk price, breading cost, replacement cost and housing are considered (Santschi et al. 2011). As stated above, it has been suggested that a short dry period may reduce mastitis, because with short dry period management, the amount of produced milk at the time of drying off is reduced, leading to a decrease in the concentrations of fat, casein, lactose and citrate which enhances the natural defense system in these animals (Collier et al. 2012). The response to a shorter dry period is inconsistent. Factors, such as if the cows are prepared for a short dry period, diet, length of dry period and parity of the cows may impact cow response during the dry period. Sorensen and Enevoldsen (1991) reported lower milk production as a result of a short dry period and others reported better health and performance of animal compared to the conventional 60-d dry period (Bachman, 2002; Gulay et al. 2003; Annen et al. 2004). This inconsistency in response to a short dry period demonstrates the need for additional research to identify the optimum dry period length and its relevance to the animal condition in terms of age, production and health. In current study we examined the impact of a 40-d dry period and compared it to the conventional 60-d dry period.

1.3 Diet management during the dry period

A conventional dry period is divided by two phases known as far-off and close-up (Watters et al. 2008). The length of far-off period is around 39-d during which dairy cows receive a relatively low protein and low energy diet to facilitate drying off and to prevent excessive energy and protein intake. The far-off period is followed by close-up period which occurs during the last 21-d of gestation. The energy and protein content of diet during the close-up period is higher than that of the far-off diet to prepare how? the rumen papillae and rumen microbiome for high grain diet that will be fed after calving. The increase in nutrient density of the diet is also required for the increased mammary parenchymal growth that occurs in last few weeks of gestation (Vandehaar et al. 1999). The recommended energy and protein content of diet (DM basis) during the far-off and close-up periods are 1.25 Mcal/Kg NE_L, 12% CP, and 1.62 Mcal/Kg NE_L and 12-13% CP, respectively (NRC, 2001). Two-year old heifers require more energy and protein than multiparous cows since their feed intake as a percentage of body weight is lower. Guidelines for close-up heifer diets are 15% CP and 1.59 Mcal/Kg NE_L (NRC, 2001).

1.4 Effect of a short dry period on:

1.4.1 Milk production

Due to the lack of knowledge about all factors during dry period that can affect milk production in subsequent lactation, the optimum number of dry days based on influential factors is not still clear. Hence, different studies with different experimental designs and conditions such as the actual composition of far-off, close-up and lactation diets have reported inconsistent results on milk production. van Knegsel et al. (2013) performed a meta analysis of 24 studies with short and omitted dry period managements, of which, 22 studies reported milk production. The meta analysis showed that across studies, cows with approximately 30-d dry periods or omitted dry periods produced 1.4 and 5.9 kg/d less milk compared to cows with a conventional 6-8 week dry period, respectively. They argue that most of these studies did not consider age of dairy cows as factor in dry period management and milk production. There are studies that have shown that a short dry period has a more negative impact on younger cows compared to the older animals (Annen et al. 2004; Pezeshki et al. 2007; Santschi et al. 2011). They suggested that primiparous cows might need dry period longer than 30 days for additional mammary growth. It has been shown that additional milk production in the previous lactation compensates for the lower milk production in cows with short dry period (Annen et al. 2004; Andersen et al. 2005; Rastani et al. 2005). Atashi, et al. (2013) studied 41,842 cows and reported that cows with 0-50 d dry period had lower initial milk yield during the first two months of lactation compared to cows with 51-60 d dry period. It has been suggested that a lower milk production during the first few weeks of lactation improves animal health by creating less metabolic pressure (Bachman and Schairer, 2003; van Knegsel et al. 2013).

1.4.2 Milk components

1.4.2.1 Fat

Santschi et al. (2011) and Anen et al. (2004) demonstrated that shortening the dry period from 60 to 30 or 35 days, has no effect on milk fat percentage in following lactation. Other researchers who did not consider the parity effect, also, reported that cows with a shorter than 60-d dry period had similar milk fat percentage compared to cows with conventional dry period (Gulay et al. 2003; Watters et al. 2008; Klusmeyer et al. 2009). van Knegsel et al. (2013) performed a meta-analysis and reported that dry period length has no effect on milk fat percentage in dairy cows. Studies have also shown that shortening the dry period from a range of 55-60 d to 28-35 d has no significant effect on milk fat yield in subsequent lactation (Gulay et al. 2003; Annen et al. 2004; Rastani et al. 2005; Pezeshki et al. 2007; Watters et al. 2008; Klusmeyer et al. 2009;

Santschi et al. 2011). In contrast, Rastani et al. (2003) reported higher milk fat percentages in cows with a short 28-d dry period compared to a conventional 60-d dry period probably due to higher blood concentrations of NEFA and their uptake by mammary gland. Unfortunately, the number of studies that reported the effect of a short dry period in different parities is limited.

1.4.2.2 Protein

Watters et al. (2008) reported that shortening the dry period from 55 to 34 d increased milk protein percentage in both primiparous and multiparous cows. Santschi et al. (2011) also reported a higher milk protein percentage during early lactation in primiparous but not in multiparous cows with 35-d dry period compared to those with 60-d dry period. A comprehensive meta-analysis showed that cows with dry period lengths between 28 to 35 d had higher milk protein percentages compared to cows with dry periods between 49 to 63 d (van Knegsel et al. 2013). A study of 65,971 lactations showed that cows with a dry period of 51-60 d produced more milk protein compared to cows with dry periods less than 35 d (Atashi et al. 2013). Not all studies agreed that a short dry period increases the milk protein. Pezeshki et al. (2007) reported lower milk protein yields when the dry period length was shortened from 56 to 35 d. In another study, the same authors demonstrated that cows with a 28 d dry period produced same amount of milk protein as cows with a 49 d dry period (Pezeshki et al. 2008).

1.4.2.3 Lactose

Lactose is the main component of the milk that regulates the osmolarity and determines the milk volume (Collier et al. 2012). Studies have shown that shortening the dry period from 65 d to 35 d reduces the lactose concentration in milk, but has no effect on lactose yield (Jolicoeur et al.

2014). Rastani et al. (2005) reported that cows with a 56 d dry period produced 0.21 kg/d more lactose compared to cows with a 28 d dry period. They also reported that cows with the short dry period produced 0.19 kg/d more lactose compared to cows with no planned dry period (Rastani et al. 2005). It has been reported that milk lactose percentage increases with time from wk 2 to wk 9 or to 14 postpartum in cows with 60 or 65 d dry period, respectively (Lu et al. 2013, Weber et al. 2013). In the other hand, Adrien et al. (2012) reported that lactose percentages in milk decrease in cows with a 127± 82 d dry period from wk 2 to 8. Lu et al. (2013) demonstrated that there was no difference in milk lactose percentage between cows with a 60 d dry period compared to continuously milked cows without a dry period.

1.4.2.4 Somatic Cell Count (SCC)

Mastitis is the most important cause for low milk production and quality and also one of the most important reasons for culling which results in large economic losses in dairy industry (Vissio et al. 2014). The somatic cell counts (SCC) is composed of sloughed epithelial cells and white blood cells, and can be used as an marker for diagnosing mastitis (Vissio et al. 2014). An uninfected quarter has less than 300,000 of SCC. The normal pattern of SCC during lactation cycle starts with high amount, followed by a decrease at peak and mid lactation and again an increase at the end of lactation (Peters, 2002). There are different factors that affect the SCC, for instance the prepartum diet, milk yield, parity and length of dry period. Milk yield can affect SCC, probably as a result of a dilution effect, as the milk yield in non-infected quarters increases, at the peak of lactation, the SCC decreases (Schepers et al. 1997). Providing a 150% of energy requirement during a 65 d dry period in multiparous cows has been shown to increase milk SCC in the subsequent lactation compared to cows that received 80 or 100% of their energy requirement during the dry period (Janovick and Drackley, 2010). In the same experiment, regardless of energy content of diet, multiparous cows had higher SCC compared to primiparous cows. Studies on shortening the dry period have shown that multiparous cows with a 40 to 42 d dry period had higher milk SCC compared to cows with a 56 to 60 d dry period, while there was no effect of dry period length on second lactation cows (Pezeshki et al. 2007, Santschi et al. 2011). These authors were not able to explain the reasons behind it. Other studies have reported no effect of the dry period length on milk SCC (Bernier-Dodier et al. 2011, Steeneveld et al. 2013, Jolicoeur et al. 2014). The SCC raises dramatically in the last 4 to 5 weeks of gestation in cows with extended lactation due to a shortened or omitted dry period (Remond et al. 1997a; Andersen et al. 2005; Annen et al. 2008). However, studies have shown that this higher SCC does not affect milk SCC after calving (Andersen et al. 2005; Annen et al. 2007 and 2008; Watters et al. 2008).

1.4.3 Blood metabolites

1.4.3.1 Glucose

Glucose demand increases dramatically by three times between 250 d of gestation and 4 d of lactation as a result of increased energy requirements of the gravid uterus and onset of milk production (Overton and Waldron, 2004). Glucose hemostasis in ruminants is controlled by hepatic gluconeogenesis, glucose oxidation in peripheral tissues and glucose absorption in the small intestine (Overton and Waldron, 2004). Propionate, lactate, glucogenic amino acids (mostly Ala and Gly), and glycerol are the main precursors for hepatic gluconeogenesis, from which 50-60%, 15-20%, 2.5-10% and 2-4% of glucose originates, respectively (Reynolds et al. 2003; Larsen and Kristensen, 2009) Studies have shown that the energy intake and also the length of dry period affect blood glucose concentration during the transition period (Dann et al. 2005). Rastani et al. (2005) reported that cows with a 28 d dry period had higher blood glucose concentrations compared to cows with a 56-d dry period. On the other hand, other studies have shown that shortening the dry period from 63 to 35 d had no effect on blood glucose levels during both the pre- and post-calving period (Jolicoeur et al. 2014). Pezeshki et al. (2007) also reported no difference in blood glucose level between cows with 56, 42 and 35 d dry periods. They also reported that primiparous and multiparous cows had similar blood glucose concentrations that responded similarly to the length of dry period.

1.4.3.2 NEFA

Mobilized fatty acids are required for animal to meet energy demands in early lactation. Feed intake decreases during the last three weeks of gestation by 39% and it drops dramatically by 89% within the last week before calving, and increases gradually during 3 to 4 wks after calving (Hayirli et al. 2002). Among the factors that may affect dry matter intake, day of gestation, parity, BCS, and concentrations of NDF and ether extract (EE) in diet play the main role in reducing the feed intake before calving (Hayirli et al. 2002). As DMI decreases linearly as BCS, RUP and NDF increase and it decreases 4 times as EE increases, and increases 4 times as RDP increases (Hayirli et al. 2002). In addition, cows eat more than heifers. Low feed intake along with hormonal changes, such as increased estrogen, and other lipolytic hormones like prolactin, somatotropin and placental lactogen (Grummer et al. 1990) during the last few days before parturition, increased required energy of gravid uterus, and uterine contractions short before calving, initiate mobilization of NEFA from adipose tissues (McNamara and Cronje, 2000). After calving, the mobilization of fatty acids from adipose tissues are transported to the liver and mammary glands increases dramatically to compensate for the lack of energy required for milk production (McNamara and Cronje, 2000). In addition, the basal rate of esterification in adipose tissues decreases dramatically right after parturition to the third wk of lactation, probably in response to negative energy balance during this time (Rukkwamsuk et al. 1999). Esterification rate in adipose tissues increases in response to glucose, insulin or both (Rukkwamsuk et al. 1999). However, cows with excessive fat deposits are less responsive to these signals, because high concentration of fatty acids in blood reduces the insulin sensitivity in adipose tissues and muscles by changing the membrane fluidity, rotation of membrane proteins and hormone receptors responses (Drackley, 1999) This results in higher mobilization of NEFA even at the presence of glucose or insulin (Rukkwamsuk et al. 1999). Both multiparous and primiparous cows reach again to the positive energy balance in about 5 ± 2 wk (Grummer et al. 2010).

The reports on blood NEFA concentrations during the transition period are inconsistent. Watters et al. (2008) reported that NEFA concentrations during prepartum tended to be lower in cows with a 60 d dry period compared to cows with a 35 d dry period. However, after calving cows with a short dry period showed lower blood NEFA concentrations compared to the cows with a 60 d dry period (Watters et al. 2008). In another study, cows with a 28 d dry period had lower NEFA concentrations at 1 wk before calving compared to cows with a 56 d dry period, however, there was no difference between these two treatments after calving (Rastani et al. 2005). Comparisons in NEFA concentrations between heifers and multiparous cows showed that the blood concentrations of NEFA are higher in multiparous cows compared to heifers (Vandehaar et al. 1999; Grummer et al. 2004). Other studies reported no difference in blood NEFA concentrations between multiparous and primiparous cows during the transition period, which was probably due to providing high-energy diet during the dry period (Ospina et al. 2010; Adrien et al. 2012). It has been shown that increases in body fat, especially in obese cows, around calving result in excessive mobilization of fatty acids from adipose tissues, probably due to lowering the sensitivity to insulin (Rukkwamsuk et al. 1999; McNamara and Cronje, 2000). Rukkwamsuk et al. (1999) suggested that dairy cows in PAR 3+, which deposited more fat, are more prone to mobilization of NEFA compared to cows in PAR 2, which deposited energy as lean tissue rather than fat.

1.4.3.3 Beta hydroxyl butyric acid (BHBA)

The BHBA in blood comes from β-oxidation of fatty acids in the liver and also from oxidation of butyrate in rumen epithelial cells. The small intestine is barely able to oxidize the short chain fatty acids (SCFAs). Ruminal SCFAs can be aerobically oxidized to CO2 or anaerobically to ketonic acids (e.g. D-3-hydoxybutirate, acetoacetate) and lactate in the rumen epithelial cell wall (Sejrsen et al. 2006). However, the rumen intraepithelial aerobic breakdown of SCFA is negligible (Gabel et al. 2002; Sejrsen et al. 2006). The process of production of ketone bodies in epithelial cells of the rumen is called alimentary ketogenesis, and unlike hepatic ketogenesis that is considered a metabolic disorder, has benefits for ruminants. These include improving uptake of short chain fatty acids by the rumen and also by providing energy for rumen epithelial cells (Gabel et al. 2002). The BHBA concentration in blood fluctuates during the day (Oetzel, 2004), and it is likely to be affected by sampling time relative to feeding (Eicher et al. 1999). Generally, overfed cows during the dry period experience higher concentrations of BHBA in blood after calving compared to cows that were fed according to their requirements as a result of higher hepatic oxidation of mobilized NEFA to the liver (Ji et al. 2012; Khan et al. 2014). It has been shown that cows with a 35 d dry period had higher feed intakes and lower mobilization of body reserves, which resulted in lower blood concentration of BHBA and NEFA, compared to cows with a 63 d dry period (Jolicoeur et al. 2014).

1.4.3.4 Insulin

Insulin controls blood glucose by influencing its uptake by muscles and adipose tissues. Its concentration along with NEFA in blood can be used as an indicator for energy balance (Mashek and Beede, 2001). Insulin and NEFA concentrations in blood have opposite trends. This can be explained by anti-lipolytic effects of insulin and its inhibition of releasing fatty acids from adipose tissues (Holtenius, 1993; Chilliard et al. 1999; Holtenius et al. 2003). It has been also reported that there is a negative correlation of −0.35 between blood insulin and NEFA concentrations (Holcomb et al. 2001). Insulin sensitivity in adipose tissues and muscles in late gestation, and in early lactation diminishes the stimulation of mobilization of fatty acids from theses body resources (Bell and Bauman, 1997). Also, high concentrations of NEFA in blood can change membrane fluidity, which may affect rotation of membrane proteins and consequently hormone receptors responses (Drackley, 1999) and results in more mobilization of NEFA from adipose tissues. The amount of energy intake during the dry period affects blood insulin levels more during pre-calving rather than post-calving time (Janovick et al. 2011, Adrien et al. 2012, Ji et al. 2012). Age is another factor that plays a role in the blood concentration of insulin in dairy cows. It has been shown that insulin concentrations are higher in primiparous cows during prepartum and at the day of parturition (Janovick et al. 2011) than in multiparous cows. Pezeshki et al. (2007) reported no change in blood insulin concentration as a result of shortening the dry period from 56 d to 42 d or 35 d.

1.4.3.5 Lactate

Sources of blood lactate in dairy cows can include 1) the diet, when silages or other fermented feed are digested, 2) rumen fermentation, when high concentrate diets are consumed and 3) endogenous production (Huntington et al. 1980). To the best of the author's knowledge, there is no report of the effect of dry period length on blood lactate concentrations in dairy cows. As mentioned above, lactate contributes 15-20% of the glucose pool produced by hepatic gluconeogenesis. Krehbiel et al. (1992) reported that lactate provides 44% of carbon required for glucose synthesis in steers. Most of the lactate that is utilized for gluconeogenesis originates from catabolism of glucose by peripheral tissues or fractional catabolism of propionate by visceral epithelial tissues (Drackley et al. 2001).

1.4.4. Rumen fermentation metabolites

1.4.4.1. VFA

Three factors determine the amount of VFA in rumen: the amount of fermentation, the passage rate and the absorption through the rumen epithelium (Dijkstra et al. 1993). The amount of ingested feed affects the amount of fermentation in the rumen (Russell et al. 1992). The passage rate is another factor that influences the clearance of VFA from the rumen and it can be affected by the amount of feed and water intake (Russell et al. 1992; Sniffen et al. 1992; NRC, 2001) or/and particle break down as a result of rumination (Demment and Vansoest, 1985), density and hydration rate of feed particles. As dry matter intake increases or particle size decreases, the ruminal passage rate of feed increases (Sniffen et al. 1992). An Increased passage rate in rumen can result in two scenarios: reduction in ruminal digestion and fermentation (Vansoest, 1984), which may reduce VFA production, or/and increase in clearance of VFAs from rumen (Resende

Junior et al. 2006). Absorption of VFA from the rumen wall can be affected by pH, concentration and type of VFA and also absorptive surface area (Bugaut, 1987; Bergman, 1990). Studies have shown that at pH 7, according to Henderson-Hasselbalch equation, almost 99 percent of each VFA ionized (dissociated). This form of VFA does not transmit passively across the rumen epithelium cell wall, leading to very small absorption of the dissociated form of VFA (Dijkstra et al. 1993). The half-maximum rate of absorption of VFA in the rumen is at pH of 6 in which almost 94 percent of every VFA is converted to the undissociated form (Dijkstra et al. 1993). The rumen surface area is another important factor that can affect ruminal absorption of VFA and is positively correlated to the metabolic size of the animal (Van Soest, 1994). The three main volatile fatty acids in the rumen are acetic (55-70%), propionic (15-30%) and butyric (5-15%) acids. The ratio between acetate and propionate (A: P) shows the fermentation pattern in the rumen as a high amount of acetate reflects availability of high-fiber and low fermentable carbohydrates in the rumen whereas high rumen propionate level shows availability of high-fermentable carbohydrates that lead to lower digestion of fiber and potential acidosis. In a healthy condition this ratio should be more than 2.2 (Andrieu and Wilde, 2008).

Dry period management can have long-term effects on animal physiology and performance that have been shown it is mostly as a result of improved energy status of animal after calving by improving feed intake and rumen fermentation (Mashek and Beede, 2001; Shoshani et al. 2014). Absorption of each VFA from the rumen is correlated with their concentrations in rumen fluid (Dijkstra et al. 1993). As concentration increases, the absorption of acetate and propionate decrease because their absorption capacity through epithelial cells can reach the maximum due to limiting availability of ions for transporting (Dijkstra et al. 1993).

1.4.5 Rumen pH fluctuation

Ruminal pH fluctuates within 0.5 to 1 pH unit during a 24 hr period (Dado and Allen, 1993; Nocek et al. 2002). The main factors that reduce ruminal pH are availability of high fermentable carbohydrate, low buffering, absorption and flow rate (Russell, 1998). Rumen pH is correlated positively to the acetate and ratio of acetate to propionate and negatively to the propionate and lactate concentrartions (Mohammed et al. 2012). The relationship between acetate and propionate is a reflection of the ratio of low or high fermentable carbohydrate in the diet. When a large portion of the diet is fiber, the number of fibrolytic bacteria increase, which result in higher acetate production in rumen whereas a large amount of high-fermentable carbohydrate in the diet stimulate the growth of lactate-utilizer and propionate-producer bacteria (Mohammed et al. 2012). The rumen pH in dairy cows can also be affected by parity, as salivation is higher in multiparous cows. This is related to longer ruminating times of these cows compared to younger animals (Beauchemin, 1991, Maekawa et al. 2002). Saliva and also intrinsic buffering of the feed provide buffering for the rumen and keep a stable pH condition (Giger-Reverdin et al. 2002).

1.4.6 Feed intake

Low feed consumption during late gestation and early lactation has a positive correlation with metabolic disorders such as fatty liver, milk fever and ketosis in early lactation (Grummer, 1995; Drackley, 1999). Feed intake is reduced around 1 wk before calving (Bertics et al. 1992; Drackley, 1999) probably as a result of oxidation of nutrients in liver that sends signals to the brain through the vagus nerve and control feed intake (Alen and Bradford, 2005). All attempts should be made to minimize the reduction of feed intake before calving since it has a positive correlation with higher feed intake after cavling (Grummer, 1995; Mashek and Grummer, 2003).

The National Research Council (NRC 2001) recommends a dry period with a far-off and a close-up period, which the first phase consists of a high fiber and low energy diet to facilitate drying off and prevent excessive energy intake. During the close-up period, cows receive a higher energy and protein diet, to offset reduced feed intake and higher nutrient requirements. It has been suggested that a short dry period provides better health conditions for animals by using one diet instead of separate far-off and close-up diets during the dry period to reduce adaptation stress and increase feed intake and consequently health and performance of the animal (Shoshani et al. 2014; Rastani et al. 2005). Another benefit of a short dry period is that it provides a required 5 to 6 weeks of feeding a high-energy diet for rumen papillae to reach their maximum length (Xu, 1999) and highest absorbance of volatile fatty acids (Grummer, 1995; Mashek and Beede, 2001). In addition, longer close-up period gives enough time to the rumen microbiota to adapt to high concentrate diet after calving (Goff and Horst, 1997).

1.4.7 Liver TAG

Dairy cow livers have a limited capacity to oxidize fatty acids and to export them as very low-density lipoproteins (VLDL) (van den Top et al. 1995). Fatty liver, e.g. accumulation of TAG, occurs when the amount of esterification and storage becomes higher than oxidation or export of fatty acids in the liver (Grummer, 1993). Liver TAG reflects the concentration of NEFA in blood (Vazquezanon et al. 1994; Bobe et al. 2004). Janovick et al. (2011) reported that overfed-energy cows during the dry period experience higher liver TAG within the first 10 d after calving (van den Top et al. 1995). It has been reported that cows that were force-fed to keep feed intake as same as wk 3 pre-calving, experienced a 75% increase in liver TAG at day 1 after calving compared to 3 wk before calving (Bertics et al. 1992). This suggests that there are some other factors such as hormonal changes around parturition that may be responsible for altering metabolism in the body and initiate mobilization of NEFA from adipose tissues rather than negative energy balance (Grummer et al. 1990, Gulay et al. 2003). Increased concentrations of estrogen and other lipolytic hormones like prolactin and somatotropin as calving approaches and also the low blood insulin short after calving lead to mobilization of NEFA and accumulation of TAG in the liver (Herdt, 1988; Janovick et al. 2011; Weber et al. 2013). The normal concentration of liver TAG in dairy cows is less than 1% (on wet weight basis), however, during the first month of lactation due to mobilization of fatty acids to liver, the percentage of TAG can increase to 5%, in mild cases, or 10%, in moderate cases, or more than 10% in severe cases of fatty liver (Bobe et al. 2004). This severe fatty liver may impair hepatic functions such as gluconeogenesis. During early lactation moderate and severe fatty liver is common in high producing dairy cows and 50% of all cows show some accumulation of TAG in the liver (Bobe et al. 2004).

1.4.8 Liver gene expression

Providing diets with different energy content during the dry period affects energy balance of dairy cows during the dry period and early lactation. Higher propionate as a result of a high-grain diet and its transportations to liver, along with hormonal changes around calving and negative energy balance in early lactation and consequently mobilization of non-esterified fatty acids (NEFA) from adipose tissue to liver may act as ligands and activate key genes involved in lipid and carbohydrate metabolism, which may result in higher or lower gluconeogenesis or/and fatty acid oxidation.

1.4.8.1 Genes involved in carbohydrate metabolism pathways

Gluconeogenesis is the main process during which the required glucose of dairy cows is produced by converting the propionate, lactate, glycerol and amino acids to glucose (Overton et al. 1999; Larsen and Kristensen, 2013). Four key genes involved in gluconeogenesis are fructose-1,6-bisphosphatase (FBP), phosphoenolpyruvate carboxykinase 1 (PCK1, cytosolic), phosphoenolpyruvate carboxykinase 2 (PCK2, mitochondrial), glucose-6-phosphatase, catalytic subunit (G6PC) (Figure 2.1). Upregulation of these genes results in increasing gluconeogenesis and more glucose output. The protein encoded by the pyruvate carboxylase (PC) gene is essential for synthesis of oxaloacetate, which is the main carbon supplier for both gluconeogenesis and tricarboxylic acid cycle (Jitrapakdee et al. 2006). The activities of the PCK1 and PCK2 genes regulate if the oxaloacetate might be used for which of these pathways. Upregulation of PC along with PCK1 and PCK2 increases the gluconeogenesis as higher expression of PCK1 increases the glucose production from amino acids while upregulation of PCK2 uses lactate for glucose production. On the other hand, upregulation of PC with no change in PCK isoforms results in the utilization of oxaloacetate for increasing the oxidative capacity of the tricarboxylic acid cycle (White et al. 2012). The expression of PCK gene has not been reported until wk 4 of lactation, whereas upregulation of PC has reported right after calving at the first day of lactation (Loor, 2010). Other genes involved in gluconeogenesis that are also involved in glycolysis consist of aldolase A, fructose-bisphosphate (ALDOA), that catalyzes the conversion of fructose-1,6-biphosphate to glyceraldehyde 3-phosphate and dihydroacetone phosphate, and pyruvate kinase (PKLR) that catalyzes the conversion between phosphoenolpyruvate (PEP) and pyruvate, and lactate dehydrogenase A (LDHA) gene that catalyzes the conversion between lactate and pyruvate. The expression of ALDOA has been reported in dairy cows to increase within the first 10 d of lactation (Loor et al. 2005).

1.4.8.2 Genes involved in lipid metabolism pathways

The bovine hepatic angiopoietin-like 4 (ANGPTL4) gene has an inhibitory effect on lipoprotein lipase (LPL) activity in adipose tissues that results in lower absorption of VLDL-TAG and, instead, in higher lipolysis (McCabe et al. 2012) and, eventually, transport to liver. Acyl-CoA synthetase, long chain 1 (ACSL1), carnitine palmitoyl-transferase 1A (CPT1A), carnitine palmitoyl-transferase 1B (CPT1B) and acyl-CoA dehydrogenase very long chain (ACADVL) are four key genes in hepatic fatty acid beta-oxidation (Figure 2.1). The protein encoded by the ACSL1 gene is responsible for the activation of mobilized fatty acids to liver (Voet and Voet, 1995). After activation, they are able to attach and be transferred to mitochondria by CPT1 (Price et al. 2003). The CPT1 gene has three family members, i.e. CPT1A, CPT1B, and CPT1C, that are known as liver, muscle and brain types. This naming is based on the place first time these genes have been found at, however, for example expression of CPT1A has been reported also in liver (Price et al. 2003). The next step in β-oxidation is the forming a trans-αβ double bond in long fatty acids by ACADVL (Khan et al. 2014). The protein encoded by ATP-binding cassette (ABC) is responsible for esterification of fatty acids that are more than the capacity of the mitochondria to oxidize (Violante et al. 2013). These esterified fatty acids then are transferred by carnitine octanoyltransferase (CROT), peroxisomal carnitine acetyltransferase (CRAT) to peroxisome for beta-oxidation (Violante et al. 2013). Proteins encoded by stearoyl-CoA desaturase (SCD) (Legrand et al. 1997), very long chain fatty acid 5 (ELOVL5) (Moon et al. 2009), fatty acid desaturase 2 (FADS2) (Jacobi et al. 2011), and diacylglycerol O-acyltransferase

(DGAT) are responsible for fatty acid biosynthesis and TAG in liver. Apolipoprotein A-I (APOA1) and ATP-binding cassette transporter A1 (ABCA1) are the two key genes in cholesterol homeostasis (Iio et al. 2012).

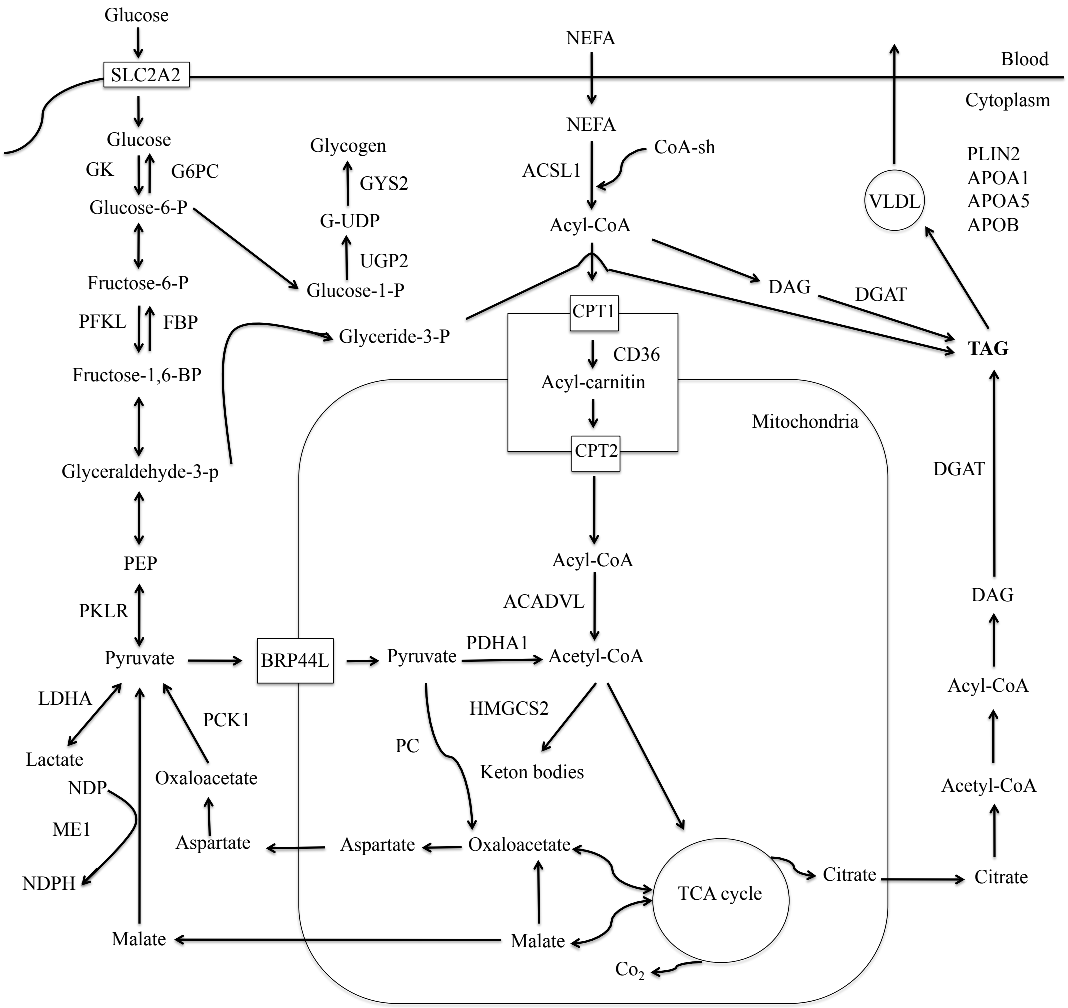

Figure 1.1. Glucose, NEFA and TAG metabolism pathways in hepatocytes

1.4.9 Rumen microbiome

The microbiome profile of rumen around calving is not well known and there is no report of effect of shortening the dry period on rumen microbiota. The transition period is considered as 3 wk before to 3 wk after calving when dairy cows face huge metabolic changes (Drackley, 2009). These include hormonal changes, reduction in feed intake, onset of milk production and negative energy balance. All have been suggested to shape rumen microbiota (Freestone and Lyte, 2010; Jami et al. 2014; Pitta et al. 2014). Jami et al. (2014) reported a high correlation between bacterial composition, especially abundances of Bacteroidetes and Firmicutes phyla, with milk production and fat-yield. These two phyla are the most abundant bacterial groups in the rumen, with Firmicutes having the highest abundance (Ozutsumi et al. 2005; Tajima et al. 2007; Brulc et al. 2009). A relatively high abundance of Firmicutes has been reported when high concentrate diets are fed, whereas higher abundance of Bacteriodetes has been observed when high fiber diets are fed in cows (Ozutsumi et al. 2005; Tajima et al. 2007; Brulc et al. 2009). In agreement, Pitta et al. (2014) reported a higher concentration of Bacteroidetes compared to Firmicutes in the rumen of cows with a diet consist of 80% forage and 20% concentrate. Changes in the ratio of Bacteroidetes to Firmicutes in gut, as shown by mice studies, probably have an impact on extracting energy from diet since Firmicutes are more efficient in harvesting the energy of diet compared to Bacteroidetes (Turnbaugh et al. 2006). Studies have shown that the availability of highly fermentable carbohydrates stimulates the bacterial growth favoring the growth of amylolytic and other starch utilizer bacteria and is associated with more bacterial richness (Fernando et al. 2010). Although the abundance of some specific bacteria such as amylolytic and fibrolytic may change by switching diets from a high-fiber to a high-fermentable carbohydrate, the number of bacteria species may remain similar (Fernando et al. 2010).

1.5 Different techniques in rumen microbial population study

1.5.1 Real-time PCR (polymerase chain reaction)

Real-time PCR has revolutionized microbiome detection and quantification by increasing its sensitivity and specificity through using bacteria-specific primers. In this method, amplification and detection occur at the same time, and allow the collection of data during the PCR process. In addition, the amplification products in the real-time PCR can be evaluated without gel electrophoresis, which means less required experimental time and higher throughput.

A singleplex or multiplex assay can be used to perform real-time PCR. These assays refer to amplification of one or multiple target genes in a single reaction tube and may be chosen based on researcher needs in terms of speed, cost and accuracy. The main advantage of using a multiplex assay is that it maximizes the throughput in each run and reduces the cost over time. Other benefits of using this assay are reducing 1) the false negatives of the control gene are amplified in the same reaction, 2) contamination by less sample handling process, 3) the required amount of initial template. A singleplex assay is faster to set up, easier to design and initially cheaper than multiplex. There are two types of fluorescent chemistry used in the real-time PCR reaction, DNA-binding dye (such as SYBR Green) and dye-labeled probes (such as TaqMan hydrolysis probes). The selection of fluorescent chemistry depends on whether a singleplex assay is chosen or a multiplex assay.

The entire process in a real-time PCR is divided into 4 phases, including the linear ground, early exponential phase, exponential phase, and plateau phase. During the linear ground phase, which is usually the first 10-15 cycles, the intensity of the fluorescent signal is still less than background. During the early exponential phase the fluorescence intensity increases and reaches a threshold that is higher than background. The cycle at which the intensity becomes higher than threshold called threshold cycle or Ct and shows the starting copy number of the target template. At the exponential phase, also known as the log-linear phase, the reaction reaches the optimal point of amplification as the DNA products double in each cycle. When the reagent materials are utilized and the output of PCR reaction reduces and curve reaches to the plateau phase at which there is no more fluorescence detection. There are two types of quantification by real-time PCR, absolute and relative. The absolute quantification method uses a standard curve made by serial dilutions of a template. This standard curve provides a linear relationship between the Ct value and concentration of PCR product. The main assumption in this method is that the amplification efficiency of the standard curve should be similar to the amplification efficiency of the samples. In addition, the highest and lowest concentrations of serial dilutions should be within a range of samples concentration. In contrast to the absolute quantification method, relative quantification uses a reference sample or external standard and measures the changes in expression of the samples relative to these references. In this method amplification efficiency plays an important role in calculating the experiment results.

1.5.2 Sequencing

The main restraint of the real-time PCR technique is that the number of bacteria that can be quantified in each reaction is limited to the designed probe or primers. However, sequencing techniques give much broader spectra of microbiota in a sample. The purpose of sequencing is to determine the DNA sequence to differentiate between different sequences and eventually reveal different genes or microorganisms. The automated Sanger sequencing is considered as the first-generation technology and all new techniques after that are known as next-generation sequencing (NGS). These technologies differ in template preparation, sequencing, imaging, genome alignment and assembly methods. The NGS techniques have been introduced about a decade ago to the market and since then have been highly improved in terms of cost per base, speed, throughput, and read length. Pyrosequencing, widely known as 454, was the first next-generation sequencing technology that was introduced to the market in 2005. Solexa/Illumina platform and Sequencing by Oligo Ligation Detection (SOLiD) were other technologies that were introduced to the market in 2006 and 2007, respectively. Three years later in 2010, Ion Torrent and PacBio technologies appeared in the market. The two common technologies used in recent researches are Pyrosequencing and Illumina that have their own advantages and disadvantages.

1.5.2.1 Pyrosequencing

As mentioned above, the pyrosequencing technology was the first method that appeared in the market by 454 Life Sciences. The running time is around 23 h, which is considered short. The read length in this method can be as long as 1 kb, which makes it easy to assemble and map to the reference genome. These features made pyrosequencing a good candidate for metagenomics projects. However, this method has lost its popularity during the last few years due to low throughput data (1 million read that is about 700 Mb), high reagent cost and high error rate. Roche, the provider company, is going to stop providing technical support for this technology by the middle of 2016, which is the main disadvantage of this platform.

1.5.2.2 Illumina

Illumina is the frontier in next-generation sequencing technology. The company has developed two sequencing systems, MiSeq and HiSeq. Illumina MiSeq technology allows a read length of 2×300 bp and is able to generate 25 million clusters (15 Gb) within 65 hrs. Illumina HiSeq read length is shorter, 2×100 bp, however, the number of clusters can be up to 3 billion with about 600 Gb outputs, which increases the sequencing coverage (the average number of reads that align to known reference bases) and degree of confidence at individual base positions. The latest system may take 11 days to complete a run. Illumina sequencing has provided the lowest price per base-pair and has enabled us to study the genomes of entire rumen microbial communities. This technology has a wide variety of applications such as metagenomics, DNA sequencing, quantitative and qualitative sequencing-based transcriptome analysis, single nucleotide polymorphism (SNP) discovery and structural variation analysis, cytogenetic analysis, DNA-protein interaction analysis (ChIP-Seq), sequencing-based methylation analysis, small RNA discovery and analysis, de novo, metatranscriptomics. One of the biggest challenges in this technology especially in metagenomics studies is the sequence complexity. The 16S metagenomics libraries are low diverse and must be mixed with more complex sequence libraries such as PhiX.

1.6.0 Different techniques in gene expression studies

An important section of our study focuses on liver gene expression in dairy cows with different dry period managements. There are different techniques for exploring gene expressions that are introduced briefly in following sections.

1.6.1 Real-time reverse-transcription polymerase chain reaction (RT-PCR)

The fluorescent chemistries and reaction phases used in real-time RT-PCR are similar to real-time PCR that was explained before (see section 5.1). The difference between the two real-time PCR methods used in the microbiome and gene expression studies is that in the later method mRNA must first be extracted, and then be reversely transcribed to complimentary DNA (cDNA). However, in microbiome studies, DNA can be extracted directly from the samples and be used as a template.

Quantification of gene expression by RT-PCR can be performed using one-step or two-step RT-PCR that refers to whether the reverse transcription and real-time PCR amplification occurs in the same or separate tubes. The two-step RT-PCR produces cDNA templates from original mRNA that can be used for further gene expression studies using real-time or microarray. In a two-step RT-PCR, oligo(dt) primers, random primers, a mixture of the two or gene-specific primes can be used while in one-step RT-PCR only gene-specific primers can be used for amplification. In general, RT-PCR technique is considered as a sensitive, reliable and rapid method, which is able to detect and quantify, even a single mRNA in a tube. RT-PCR is used to confirm the mRNA expression results of other studies such as microarray.

1.6.2 Microarray

This technology has provided the opportunity of quantifying expression of thousands of genes in only one experiment, and is able to give an overall picture of gene expression profile of a sample. Since the quality of the microarray results may be varied between different labs based on the type of platform and procedure used and also limited sensitivity and specificity of technology due to the relatively small dynamic range of fluorescent microarrays (10^2 - 10^3), the scanned results need to be validated by other methods such as real-time PCR. Different expression microarray profiling platforms, such as affymetrics and Illumina, are available in the market. Affymetrics technology uses a silica wafer surface for probe attachments while Illumina uses microscopic beads. Typically, microarray platforms consist of chips where an arrayed series of thousands probes are located on. These probes comprise oligonucleotides that are part of target genes. For instance, affymetrics arrays consist of up to 1.3 million different oligonucleotide probes. This platform is considered as one of the most used in the market. Using affymetrics GeneChip Bovine genome array allows studying of 23,000 genes in bovine tissue specimens. An advantage of using a microarray over real-time PCR is that the expression levels of many genes can be evaluated at the same time. Additionally, the required initial amount of RNA for microarray study can be very small. On the other hand, microarray technology has some limitations such as 1) it is mainly a scanning technique, 2) has a low sensitivity to low-abundance genes, 3) it is limited in measuring heterogeneous cells expression.

1.7 Concluding remarks

In dairy cows a dry period is required for replacing senescent mammary epithelial cells and increasing the total mammary parenchymal that result in optimum milk production in the subsequent lactation. The traditional 60-d dry period during which a 39-d far-off and a 21-d close-up diet is fed has been applied since World War II, however, due to genetic progress in dairy cows during the last decades and also the health and economic benefits of a shorter dry period, the optimum dry period length has been reevaluated. It has been suggested that a short dry period provides better health conditions for animals by using one diet instead of separate far-off and close-up diets during the dry period to reduce adaptation stress to new diet and increase feed intake and consequently health and performance of animal. Another benefit of a short dry period is that it provides a required 5 to 6 weeks of feeding a high-energy diet for rumen papillae to reach their maximum length and highest absorbance of volatile fatty acids. In addition, a longer close-up diet gives enough time to rumen microbiota to adapt to high concentrate diet after calving. Another benefit of providing a short dry period with a longer close-up diet for dairy cows is that it helps in hepatic adaptations for higher fat metabolism and glucose synthesis, which is necessary in early lactation. There are limited studies that show dairy cows in different parities have different responses to the dry period management. As a result, it is important to consider the effects of shortening the dry period on physiology, milk production and components, rumen fermentation and microbiome, and liver gene expression of dairy cows in different parities. There are limited studies that reported each or some of these aspects separately, however, because results vary between different studies, it is very important to consider all these factors in one study and systematically explore the effects of shortening the dry period. To the best of authors knowledge there is no such study that considers all these aspects.

1.8 Hypotheses

A 40-d dry period with only a close-up diet increases feed intake before and after calving, which consequently reduces the negative energy balance after calving and enhances milk production in dairy cows compared to a 60-d dry period with separate far-off and close-up diets.

Shortening the dry period to 40 d and using only a close-up diet throughout this period improves energy availability, decreases lipolysis in adipose tissues and ketogenesis, and also reduces accumulation of triacylglyceride (TAG) in the liver in dairy cows during early lactation compared to a 60-d dry period with a far-off and a close-up diet. We also hypothesize that these changes are affected by parity as growing parity 2 cows have different nutrient requirements compared with mature parity 3 and later cows.

A short 40-d dry period with a single close-up diet stimulate expression of genes involved in lipid and carbohydrate metabolism and accelerates liver adaptation to higher lipid metabolism and higher gluconeogenesis compared to a 60-d dry period with separate far-off and close-up diets.

Shortening the dry period to 40 d with only a close-up diet reduces the compositional and functional shifts in the rumen microbiome during the transition from prepartum to postpartum compared to a conventional 60-d dry period, during which a 39-d far-off and a 21-d close-up diets are fed.

1.9 Objectives

To determine whether these dry-period management options differ in their effects on milk production, feed intake and energy balance during the transition period, and early and mid-lactation.

To determine whether a 40-d dry period during which only a close-up diet is fed improves metabolic health and energy availability regarding to rumen fermentation, plasma concentrations of glucose, insulin, BHBA, NEFA, urea, and lactate and TAG in the liver compared with a 60-d dry period with separate far-off and close-up diets, and whether the interaction between parity and dry cow management affects these measures.

To determine whether a 40-d dry period increases expression of genes involved in hepatic β-oxidation and gluconeogenesis compared to a conventional 60-d dry period.

To compare the effects of a 40-d dry period management with a single close-up diet and a conventional 60-d dry period with separate far-off and close-up diets on rumen microbiome during pre- and post-calving period and their shifts during this time. Also, to characterize changes in the rumen microbiota profile during the transition period within each treatment. Additionally, to describe the rumen microbiome profile of cows in parity 2 compared with parity 3 and later. Moreover, to compare the functions of rumen microbiota in the two treatments and during the transition period.

CHAPTER TWO

MANUSCRIPT I

Effects of dry period management on milk production, dry matter intake, and energy balance of dairy cows

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2.1 ABSTRACT

Effects of dry-period management on milk production, feed intake, and energy balance were determined in 11 second-parity (PAR2) and 15 third or later parity (PAR3+) cows. Cows were paired based on expected calving date, and randomly assigned to treatments including a conventional 60-d dry period with a 39-d far-off and a 21-d close-up diet (CONV), and a short 40-d dry period with only a close-up diet (SHORT). Treatment did not affect DMI and energy balance across the 6 wk before calving and 4 wk after calving. At the first 16 wk of lactation, cows on the SHORT treatment had lower milk yield compared to cows on the CONV treatment that was mostly due to the lower production in PAR3+ cows. Also, PAR3+ cows on the SHORT treatment had lower milk protein yields and higher somatic cell counts than the other cows. In general, PAR2 cows on the SHORT treatment had similar DMI, fat corrected milk yields, and protein and fat yields and percentages compared to cows in both parity groups on the CONV treatment. This suggests that the SHORT treatment may be suitable for PAR2 cows, but not for older cows.

Key words: Dry period, dairy cows, Milk production, Feed intake, Energy balance

2.2 INTRODUCTION

Many studies suggest that a non-lactating/dry period before a subsequent lactation is needed to maximize milk production during that lactation (Keown and Everett, 1986; Funk et al. 1987; Makuza and McDaniel, 1996). Eliminating this period can result in 12 to 25% reduction in milk yield in the next lactation (Smith et al. 1967; Remond et al. 1992, 1997). Traditionally a 60-d dry period consisting of a far-off and a close-up period has been recommended (Watters et al., 2008). During the far-off period, cows receive a diet with high fiber and a low energy content to facilitate drying off and to prevent excessive energy intake (Grummer et al., 2004). During the close-up period, cows receive a higher energy and protein diet compared to the far-off period, to compensate for lower feed intake and higher nutrient requirements. Different recommendations in regard to the length and nutritional management during the dry period exist (Santschi et al. 2011). Results of using a short dry period on milk production during the subsequent lactation have been inconsistent, probably because factors such as the length of dry period (21 to 42 d), energy content of diets (1.52 to 1.59 Mcal NE_L/kg of DM) and parity (1 to 8) of dairy cows used in differ between studies (Kuhn et al. 2006; Pezeshki et al. 2007; Pezeshki et al. 2008; Santschi et al. 2011; Steeneveld et al. 2013). Several studies suggest a dry period of at least 40-d (Coppock et al. 1974; Sørensen and Enevoldsen, 1991), and not longer than 60-d (Hurley, 1989). Some experiments indicate that shortening the dry period from 60-d to 28-d does not affect milk production in the following lactation (Bachman, 2002; Gulay et al. 2003; Annen et al. 2004). Alternately, some experiments estimate a 10% decrease in milk production when the dry period was reduced from a range of 50 to 57 d to a range of 34 to 30 d (Sorensen and Enevoldsen, 1991). Several studies recommend a dry period with a 40-d duration (Schairer, 2001; Bachman, 2002; Gulay et al. 2003). Even if a short dry period does not increase the milk production or even slightly decrease the milk production in subsequent lactation, this may be compensated by longer milk production during the previous lactation (Watters et al. 2008; Klusmeyer et al. 2009; Santschi et al. 2011).

Grummer (1995) suggested that dairy cows might benefit from an increased dietary nutrient density during the dry period. In contrast, Drackley (1999) suggested that a restricted energy intake during the prepartum period increases feed intake after calving and reduces health risks in early lactation dairy cows. Since then, many studies have been conducted to investigate the effects of dietary energy density (Dann et al. 2005; Dann et al. 2006; Douglas et al. 2006; Janovick et al. 2011) and energy intake during a 60-d dry period on animal health, physiology and milk production (Douglas et al. 2006; Janovick and Drackley, 2010; Janovick et al. 2011). These studies have reported that overfeeding energy during the dry period increases the mobilization of body fat and the energy deficit, and lowers feed intake after calving. A short dry period provides an opportunity for using one diet instead of separate far-off and close-up diets during the dry period, as this reduces switching diets and, consequently, may reduce adaptation stress and increase feed intake (Rastani et al. 2005; Shoshani et al. 2014).

Rumen papillae need 5 to 6 weeks of feeding a high-energy diet to reach their maximum length (Xu and Allen, 1999). It is, therefore, suggested that a 21 d close-up period may not be enough for optimal rumen papillae development and that a longer close-up period enhances this development at the time of calving, and, as a result, also enhances absorbance of volatile fatty acids, rumen conditions, and feed intake (Grummer, 1995; Mashek and Beede, 2001). Moreover, because of similarities between the close-up diet and lactation diet, a longer close-up period may facilitate the adaptation of rumen microbiota to high grain diets after calving (Goff and Horst, 1997).

Reduction of feed intake starts shortly before calving (Bertics et al. 1992; Drackley, 1999) and continues until a few weeks after calving. It has been suggested that a high feed intake during the dry period is very critical, as it increases feed intake during early lactation, and reduces the risk of metabolic disorders like fatty liver, milk fever and ketosis after calving (Grummer, 1995; Drackley, 1999).

We hypothesize that a 40-d dry period and a close-up diet throughout the entire 40-d dry period increases feed intake before and after calving, which consequently reduces negative energy balance after calving and enhances milk production in dairy cows compared to a 60-d dry period with separate far-off and close-up diets. The objective of this research was to determine whether these dry-period management options differ in their effects on milk production, feed intake and energy balance during the transition period, and early and mid-lactation.

2.3 MATERIAL AND METHODS

Animals and Experiment Design

The study was conducted at the Glenlea Research Station, University of Manitoba. It was approved by the University of Manitoba Animal Care Committee, and followed the guidelines of the Canadian Council for Animal Care (CCAC, 1993). Twenty-six Holstein dairy cows (11 in their second parity (PAR 2) and 15 in their third and higher parity (PAR 3+)) were paired based on expected calving date and randomly assigned to two treatments within each pair. Treatments included a conventional 60-d dry period with separate 39-d far-off and 21-d close-up periods (CONV) and a short 40-d dry period during which only the close-up diet was fed (SHORT). In total, 5 and 6 PAR 2 cows received the SHORT and CONV treatments, respectively, and 8 and 7 PAR 3+ cows received the SHORT and CONV treatments, respectively. At the beginning of experiment, at 60 d and 40 d before calving, cows on the CONV and SHORT treatments had body weights of 737 ± 60 and 782 ± 69 kg (mean ± SD), respectively, and body condition scores between 2.75 and 4.00 with an average of 3.36 ± 0.11 and 3.40 ± 0.11, respectively, on a 1 to 5 scale (Edmonson et al. 1989). Cows were housed in tie stalls during the entire experiment. The diet fed during lactation did not differ between treatments. Cows were dried off within 4 d by decreasing feed delivery, reducing the dietary energy content and reducing milking frequency. Cefa-Dri (cephapirin benzathine; Boehringer Ingelheim Ltd., Canada) was used as intramammary infusion in dry cows. Nutrient and ingredient compositions of diets and feeds are presented in Tables 2.1 and 2.2. Actual dry period lengths for SHORT treatment were 34 ± 6 d (mean ± SD) with a minimum of 23 d and maximum of 44 d and for the CONV treatment they were 59 ± 4 d (mean ± SD) with a minimum of 53 d and max of 68 d. This variation in dry period lengths is because some cows calved either earlier or later than expected calving dates. Diets were fed as total mixed rations and were provided *ad libitum* twice a day at 0900 and 1500. Cows had unlimited access to water. During early lactation, a total of 5 health issues were observed. Two PAR 2 cows in each treatment, experienced left displaced abomasum and went under surgery. In addition, one cow in PAR 3+ on the SHORT treatment had a retained placenta. Two cows in PAR 2 in both treatments experienced clinical mastitis. Moreover, there was a cow with twins in PAR 2 on the SHORT treatment.

Table 2.1. Nutrient composition of far-off, close-up, and lactation diets (mean ± SD)

Item	Nutrient Composition[1]		
	Far-off	Close-up	Lactation
Crude protein, %	14.8±0.4	14.9±1.9	17.7±1.3
Neutral detergent fiber, %	50.2±1.1	38.1±7.4	31.2±5.6
Acid detergent fiber, %	32.2±1.2	23.2±5.1	19.3±3.3
NFC[2], %	26.2±1.6	36.2±6.1	39.4±3.9
Crude fat, %	1.7±0.3	3.3±1.0	4.2±0.7
Calcium, %	0.6±0.1	0.8±0.2	1.0±0.1
Phosphorous, %	0.3±0.0	0.4±0.1	0.4±0.0
Magnesium, %	0.2±0.0	0.4±0.1	0.4±0.1
Potassium, %	2.1±0.1	1.7±0.4	1.5±0.1
Ash, %	7.1±1.0	7.8±0.8	7.5±0.7
Net Energy for lactation, Mcal/kg	1.28	1.39	1.68

[1] Far-off diet ingredients (% of DM): timothy hay, 89.5; soybean-meal, 8.2; mineral, 2.2; forage to concentrate ratio, 90 to 10; Close-up diet ingredients (% of DM): timothy hay, 43.0; barley silage, 25.3; Transrite (from Masterfeeds), 31.6; forage to concentrate ratio, 68 to 32; Lactation diet ingredients (% of DM): mixed hay, 20.4; corn silage, 32.2; roasted soybeans, 4.4; Energy supplement, 39.5; protein supplement, 2.2; forage to ratio concentrate ratio, 53 to 47.

[2] Non fiber carbohydrate= 100 - (NDF% + CP % + Crude fat % + Ash %)

Table 2.2 Nutrient composition of the supplements, forages (dry matter basis; mean ± SD)

Item	Energy Sup.	Protein Sup.	Barley Silage	Alfalfa Silage	Corn Silage	Grass Hay	Soybeans	Soybean-Meal
Crude protein, %	20.3±1.27	33.4±1.72	9.9±1.48	15.3±1.75	7.6±0.51	11.8±2.42	38.7±6.12	51.1±0.18
Crude fat, %	4.5±0.61	9.2±0.89	2.0±0.90	2.1±0.86	2.7±0.36	1.8±0.12	17.0±7.38	1.2±0.21
Ash, %	7.2±1.18	14.3±0.68	7.6±3.78	9.6±1.96	4.9±0.46	7.4±0.98	5.3±0.92	6.6±0.47
Calcium, %	1.1±0.14	3.0±0.21	0.3±0.17	1.2±0.35	0.2±0.05	0.6±0.32	0.4±0.20	0.5±0.18
Phosphorous, %	0.7±0.08	1.1±0.09	0.4±0.06	0.2±0.05	0.2±0.04	0.2±0.03	0.6±0.10	0.8±0.06
Magnesium, %	0.5±0.12	0.3±0.03	0.2±0.06	0.4±0.08	0.2±0.02	0.2±0.05	0.3±0.06	0.4±0.03
Potassium, %	0.8±0.07	1.1±0.05	1.4±0.83	2.7±0.59	1.1±0.13	2.1±0.43	1.9±0.14	2.4±0.25
Sodium, %	0.5±0.05	2.1±0.43	0.1±0.08	0.1±0.03	0.02±0.01	0.04±0.03	0.03±0.01	0.1±0.05
Acid detergent fiber, %	7.3±0.95	10.9±0.66	27.0±1.46	39.6±3.18	20.8±1.87	35.9±1.12	14.6±4.33	7.8±0.28
Neutral detergent fiber, %	14.6±1.22	29.1±0.34	44.0±0.6	47.9±6.39	36.2±2.66	55.2±0.05	17.1±0.35	9.5±1.08
NFC[1]	53.3±1.20	13.9±1.17	36.5±1.94	25.1±4.03	48.6±1.90	23.8±2.77	21.8±1.98	31.5±0.59

1. Non fiber carbohydrate= 100 - (NDF% + CP % + Crude fat % + Ash %)

Feed Intake and Analysis

Feed intakes of individual cows were initially measured daily from 6 wk before calving to 4 wk after calving by subtracting weights of feed offered from individual weight backs/orts from weights of feed offered to individual cows on a DM basis and calculated on a percentage of body weight basis.

Weekly samples were taken from diets, feeds and orts from individual cows. These samples were analyzed for DM by drying at 60°C for 48 h in a forced air oven. All dried samples for every month were pooled based on weight and ground with a Wiley mill using a 1-mm screen (Thomas-Wiley, Philadelphia, PA) and kept at -20°C for future analyses. Analytical DM for pooled samples for each month was determined (method 934.01; AOAC, 1990). All feed samples were analyzed for CP using the CuSO4/ TiO2 mixed catalyst Kjeldahl procedure (method 988.05; AOAC, 1990), NDF (Van Soest et al., 1991) using α-amylase (Sigma No. A3306, Sigma Chemical Co., St. Louis, MO), and sodium sulfite and corrected for ash concentration adapted for Ankom 200 Fiber An- alyzer (Ankom Technology, Fairport, NY), ADF (method 973.18; AOAC, 1990), ether extract (method 920.39; AOAC, 1990), and ash (method 942.05; AOAC, 1990).

Milk Production

Milk production was recorded daily during the first 16 wks of lactation using Tru Test regulation meters (Westfalia Surge, Mississauga, ON). Cows were milked twice daily and samples were collected from four consecutive milkings each week, preserved with 2-bromo-2-nitropropane-1,3 diol and stored at 4°C. Subsequently, samples were analyzed for fat, crude protein, lactose and other solids (LOS), and somatic cell counts (SCC) at the laboratory of the Dairy Farmers of

Manitoba (Winni- peg, Manitoba, Canada) by near-infrared analysis using the Milk-O-Scan 303AB (Foss Electric, Hillerød, Den- mark). This infrared analysis was calibrated by the Babcock method for milk fat analysis (AOAC 989.04, 1990) and the Kjeldahl method for nitrogen/protein nitrogen analysis in milk (AOAC 991.22, 1990). Milk production was corrected based on 4 percent fat using $\{0.4 \times \text{milk, kg/d} + 15 \times \text{fat, kg/d}\}$ (NRC, 2001). Milk yields for 16 wks of lactation were calculated as area under the curve (AUC) by the trapezoidal method using GraphPad Prism 6 (GraphPad Software, Inc, 2014).

Body Weight and Energy Balance

Body weight of individual cows was measured weekly using a scale starting at 6 wk before to 4 wk after calving. The energy balance (EB) of individual cattle was calculated weekly from 6 wk before to 4 wk after calving by multiplying dry matter intake of individual cows by the average NE_L content (NRC, 2001) of the diet and subtracting the energy required for maintenance $(0.08 \times BW^{0.75})$, milk production $\{\text{milk yield} \times [(0.0929 \times \text{fat\%}) + (0.0563 \times \text{protein\%}) + (0.0395 \times \text{lactose\%})]\}$, and pregnancy $[(0.00318 \times D - 0.0352) \times (CBW/45)]/0.218$, respectively, in which D is the day of gestation between 190 and 279, and CBW is calf birth weight in kilograms (NRC, 2001). Daily values were averaged into weekly means for statistical analyses, and energy balance was calculated from 6 wk before to 4 wk after calving. Also, NRC 2001 software was used to estimate the balance of required NE_L and MP during the dry period in both treatments.

Statistical Analyses

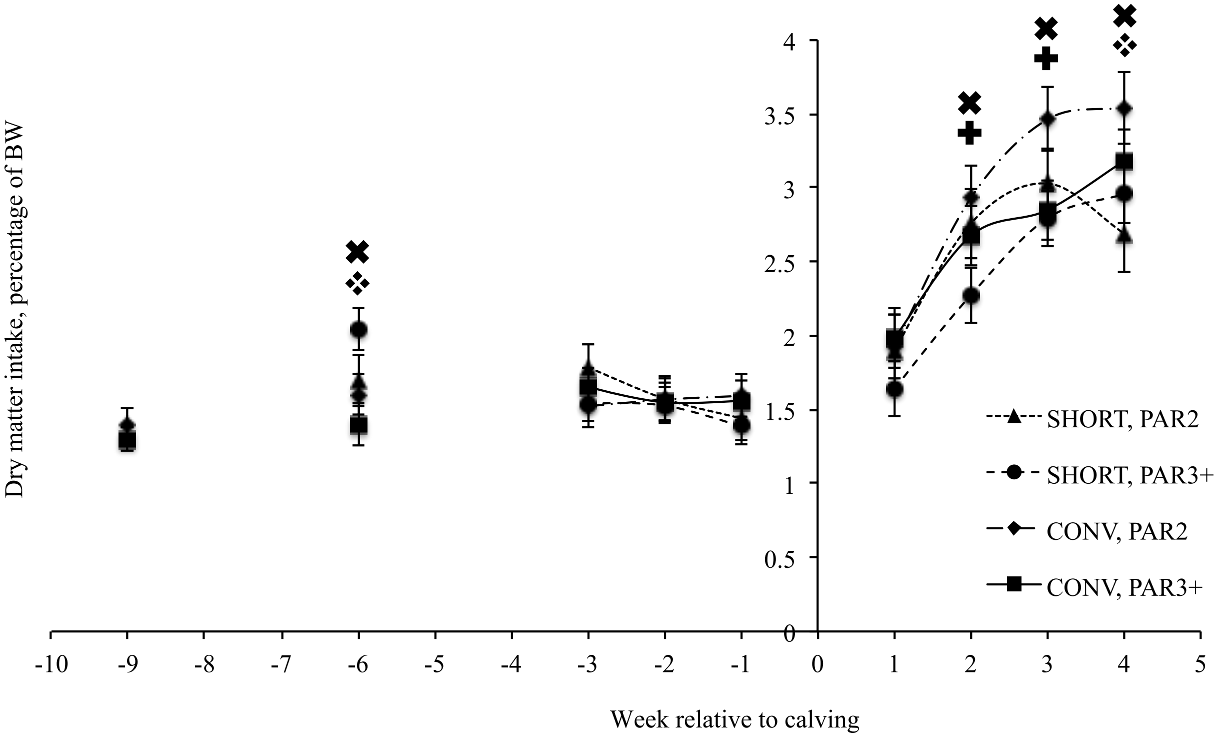
Data measured over time were subjected to ANOVA by using the REPEATED statement in the MIXED procedure of SAS (Littell et al., 1996), which was used to test the difference in variables across periods (pre- or post-partum) in a randomized block design (Littell et al. 1996). For each variable analyzed, cow nested within treatment and also block were considered as random effects. The statistical model used contained treatment, parity, week, interactions of treatment × parity, treatment × week, parity × week, and treatment × parity × week. When week intervals were not equally spaced, spatial exponential, spatial Gaussian, spatial linear, spatial linear log, spatial power, and spatial spherical covariance structures were compared, while when week intervals were equally spaced, variance components, compound symmetry, heterogeneous compound symmetry, unstructured, first-order autoregressive, heterogeneous first-order autoregressive, toeplitz, and first-order ante-dependence covariance structure were compared. The covariance structure with the smallest Akaike information criterion was used (Littell et al. 1996). When interactions of treatment × parity × week were significant, the SLICE option in LSMEANS statement was used to help explanation of changes within weeks. The SCC, fat and lactose and other solids (LOS) percentages were log-transformed to normalize the residuals. Effects were considered significant at $P \leq 0.05$. Trends were discussed at $P \leq 0.10$. Differences among the AUC for the four combinations of treatment and parity were tested for significance non-parametrically using the trapezoidal method using GraphPad Prism 6 (GraphPad Software, Inc, 2014).

2.4 RESULTS

Feed Intake, Body Weight and Energy Balance

The measured DMI expressed as amount per day and as a percentage of BW per day were not significantly different by treatment, parity nor their interaction across the 6 wk before calving and across the 4 wk after calving (Table 2.3). Since interaction of treatment, parity and week was significant for DMI ($P<0.01$), weekly comparisons between treatment, parity and their interactions were made to help interpretation of results (Figure 2.1). Feed intake as a percentage of BW was affected by treatment ($P<0.01$), and interaction of treatment and parity ($P<0.01$) at 6 wk before calving as cows on the SHORT treatment had higher DMI compared to cows on the CONV treatment. In addition, during this time, PAR3+ cows on the SHORT treatment had higher DMI compared to PAR2 cows on the same treatment and cows on the CONV treatment. There was no difference between treatments, parities and their interactions within wk -3, -2, -1, and +1, relative to calving. During the second and third week of lactation DMI as a percentage of body weight was affected by parity as PAR3+ cows had lower DMI compared to PAR2 cows that was due to lower feed intake in PAR3+ cows on the SHORT treatment ($P<0.1$). During the fourth week of lactation cows on the SHORT treatment had lower feed intakes as percentage of BW compared to cows on the CONV treatment ($P=0.02$; Figure 2.1). There was a trend in interaction of treatment and parity at wk +2, +3, and +4, as PAR3+ cows on the SHORT treatment had lowest DMI as a percentage of BW compared to others ($P<0.1$). The results of DMI as kg/d were similar to DMI as a percentage of BW as the effects of treatment, parity and their interactions were not significant across 6 wk before calving and also across 4 wk after calving. The average DMI as kg/d across weeks before calving in PAR2 and PAR3+ cows on the SHORT treatment were 12.5, 13.5, and in those on the CONV treatment were 12.1, and 12.5, respectively. These amounts across weeks after calving in PAR2 and PAR3+ cows on the SHORT treatment were 18.4, 17.1, and in those on the CONV treatment were 20.4, and 19.6, respectively. In addition, similar to the results of DMI as a percentage of body weight, at 6 wk before calving, cows on the SHORT treatment had higher DMI as kg/d compared to cows on the CONV treatment (13.0 vs. 12.3 kg/d; $P<0.01$). Also, at this time, PAR3+ cows on the SHORT treatment had the highest DMI (kg/d) compared to PAR2 cows on the same treatment and PAR2 and PAR3+ cows on the CONV treatment (16.8 vs. 12.4, 11.7, and 10.8, respectively; $P<0.01$). After calving, the opposite trend was observed as cows on the SHORT treatment had lower DMI (kg/d) compared to cows on the CONV treatment at wk 4 after calving (19.7 vs. 23.5; $P=0.02$).

Figure 2.1. Effect of dry period length and diet on dry matter intake (percentage of body weight). Symbols are: significant effects of treatment (❖), parity (✚), and their interactions (✖).



The average BW of cows on the SHORT and CONV treatments during prepartum were 811±64, 798±69, and of those during the postpartum were 709±50 and 707±62, respectively (mean ± SD). Body weight was affected by parity before calving, as cows in PAR 2 had lower BW compared to cows in PAR 3+ (*P*=0.02). There were no difference in BW between treatments, parities and their interactions across the 4 wk after calving (Table 2.3). Comparisons within each week revealed no difference between treatments, parities and their interactions in energy balance from 3 wks before calving to 4 wks after calving (Figure 2.2). However, energy balance was affected by treatment and interaction between treatment and parity at 6 wk before calving, since cows on the SHORT treatment had higher energy balance compared to cows on the CONV treatment at this time. Also, during this time PAR 3+ cows on the SHORT treatment tended to have higher energy balance compared to PAR 2 cows on the same treatment (P=0.09), and PAR 2 cows on the SHORT treatment tended to have higher energy balance compared to PAR 3+ cows on the CONV treatment (P=0.06; Figure 2.2).

Table 2.3. Effects of dry period management and parity on dry mater intake (DMI), body weight (BW) and energy balance (EB) during the 6 wks before calving and 4 wks of after calving

	Treatment[1]				SED[2]	*P*-value		
	SHORT		CONV					
Items	PAR 2	PAR 3+	PAR 2	PAR 3+		Trt	Parity	Trt × Parity
Before calving								
DMI (kg/d)*	12.5	13.5	12.1	12.5	1.10	0.30	0.37	0.76
DMI (% of BW)*	1.6	1.6	1.6	1.5	0.16	0.47	0.88	0.89
BW (kg)	782	830	765	827	31.50	0.64	0.02	0.77
EB wk −6 (Mcal NEl/d)	4.4	9.2	0.9	−0.5	2.18	<0.01	0.34	<0.01
EB wk −3 to −1 (Mcal NEl/d)	1.9	1.8	1.4	2.1	1.69	0.96	0.84	0.83
After calving								
DMI (kg/d)*	18.4	17.1	20.4	19.6	2.07	0.11	0.48	0.86
DMI (% of BW)*	2.6	2.4	3.0	2.7	0.28	0.11	0.24	0.78
BW (kg)	696	716	686	724	28.4	0.96	0.17	0.66
EB (Mcal NEl/d)	−14.0	−10.3	−9.7	−14.0	2.63	0.92	0.90	0.13

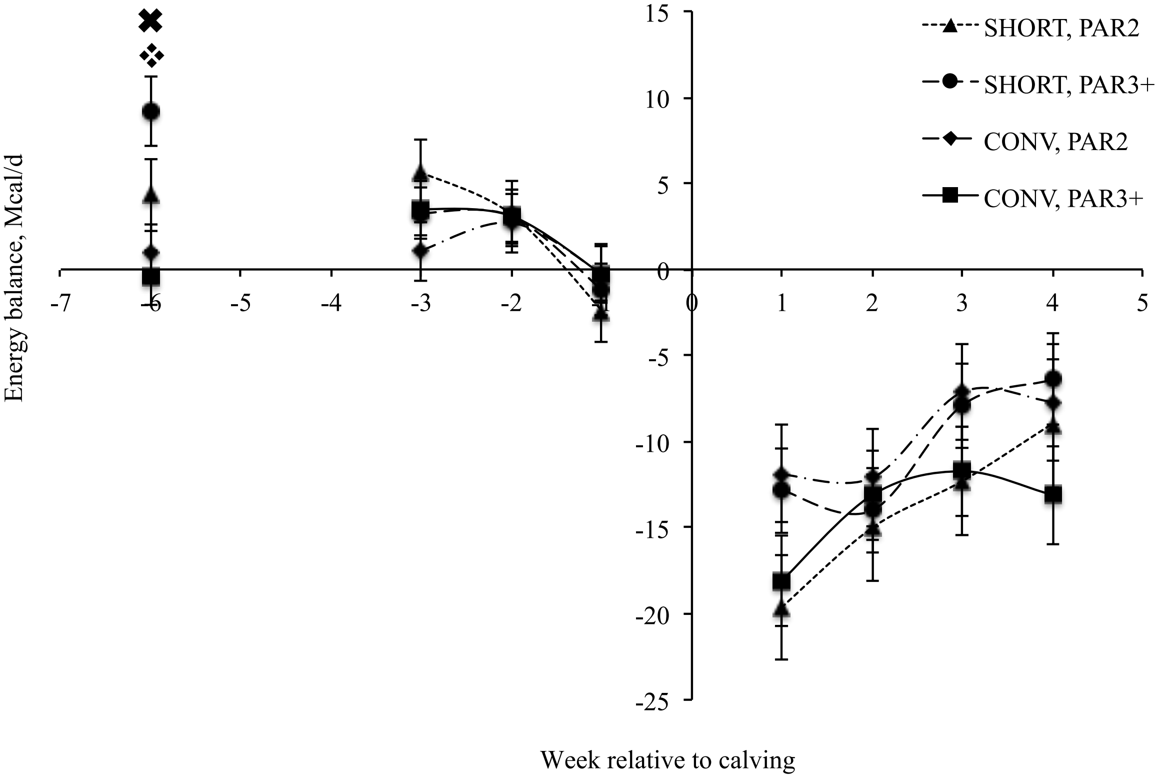
[1] Treatment consisted of a short 40-d dry period with a close-up diet throughout entire dry period (SHORT) and a conventional 60-d dry period with separate far-off and close-up diets (CONV). The PAR 2 shows cows in parity 2 and PAR 3+ shows cows in parity 3 and higher during the dry period

[2] SED: Standard error of difference.

* The interactions of Treatment×Parity×Time were significant ($P < 0.05$).

The estimated NE_L and MP balance by NRC 2001 software at 6 wks before calving showed that cows in PAR 2 and PAR 3+ on the SHORT treatment and cows in PAR 2 and PAR 3+ on the CONV treatment consumed 130, 134, 103 and 98 percent of required NE_L respectively, and 150, 142, 105, and 108 percent of their required MP, respectively. At 3 weeks before calving cows in PAR 2 and PAR 3+ on the SHORT treatment and cows in PAR 2 and PAR 3+ on the CONV treatment obtained 137, 122, 108, and 122 % of their NE_L requirement, respectively, and 150, 151, 130, and 143 % of their required MP, respectively. At 2 weeks before calving cows in PAR 2 and PAR 3+ on the SHORT treatment and cows in PAR 2 and PAR 3+ on the CONV treatment obtained 117, 121, 126, and 120 % of required NE_L, respectively, and 135, 150, 148 and 137 % of their required MP, respectively. Looking at the NE_L and MP requirements at week 1 before calving revealed that cows in PAR 2 and PAR 3+ on the SHORT treatment and cows in PAR 2 and PAR 3+ on the CONV treatment obtained 100, 95, 85, and 100 % of required NE_L respectively, and 125, 120, 120 and 115 % of their required MP, respectively.

Figure 2.2. Effect of dry period length and diet on energy balance. Symbols are: significant effects of treatment (❖), and interaction of treatment and parity (✖).



Milk Production

Treatments affected fat corrected milk yield across the first 16 wks of lactation, as cows on the SHORT treatment had lower milk production compared to cows on the CONV treatment (Table 2.4; *P*=0.03). In addition, parity tended to affect fat corrected milk yields as PAR 3+ cows tended to have lower fat corrected milk yields compared to PAR 2 cows (*P*<0.10). These effects were mainly due to the low milk yield of PAR 3+ cows on the SHORT treatment (Table 4, Figure 2.3). The AUC during the first 16 wks of lactation showed that PAR 3+ cows on the SHORT treatment, with 4330 kg, had the lowest fat corrected milk yields compared to other cows (*P*<0.01). The AUCs for cows in PAR 2 and PAR 3+ on the CONV treatment and cows in PAR 2 on the SHORT treatment were 4719, 4738 and 4789 kg, respectively. The day of peak lactation for the PAR 2 and PAR 3+ cows on the SHORT treatment and PAR 2 and PAR 3+ cows on the CONV treatment were 12, 35, 34, and 24 d, respectively, with peak yields of 53.2, 44.6, 49.4 and 51.0 kg, respectively.

Table 2.4. Effect of dry period management and parity on milk yield (FCM)[1], yields and percentages of fat, crude protein (CP), and lactose and other solids (LOS) and somatic cell count (SCC) during the first 16 wks of lactation

		Treatment[2]				SED[3]	*P*-value		
		SHORT		CONV					
Items		PAR 2	PAR 3+	PAR 2	PAR 3+		Trt	Parity	Trt × Parity
Milk yield (kg/d)		42.2	38.4	43.9	42.1	1.65	0.03	0.10	0.54
AUC[3]		4789[a]	4330[b]	4719[a]	4738[a]				
Yield (kg/d)									
	Fat	1.61	1.50	1.69	1.66	0.12	0.13	0.43	0.66
	CP	1.45	1.29	1.41	1.47	0.05	0.02	0.14	< 0.01
	LOS	2.53	2.33	2.53	2.55	0.12	0.18	0.31	0.20
Percentages									
	Fat	3.72	3.77	3.90	3.74	0.17	0.20	0.21	0.51
	CP	3.30	3.30	3.35	3.31	0.10	0.61	0.75	0.81
	LOS	5.67	5.65	5.80	5.72	0.05	<0.01	0.61	0.50
SCC (cells/mL)		579,121	883,120	205,194	118,873	312,514	< 0.01	0.39	0.09

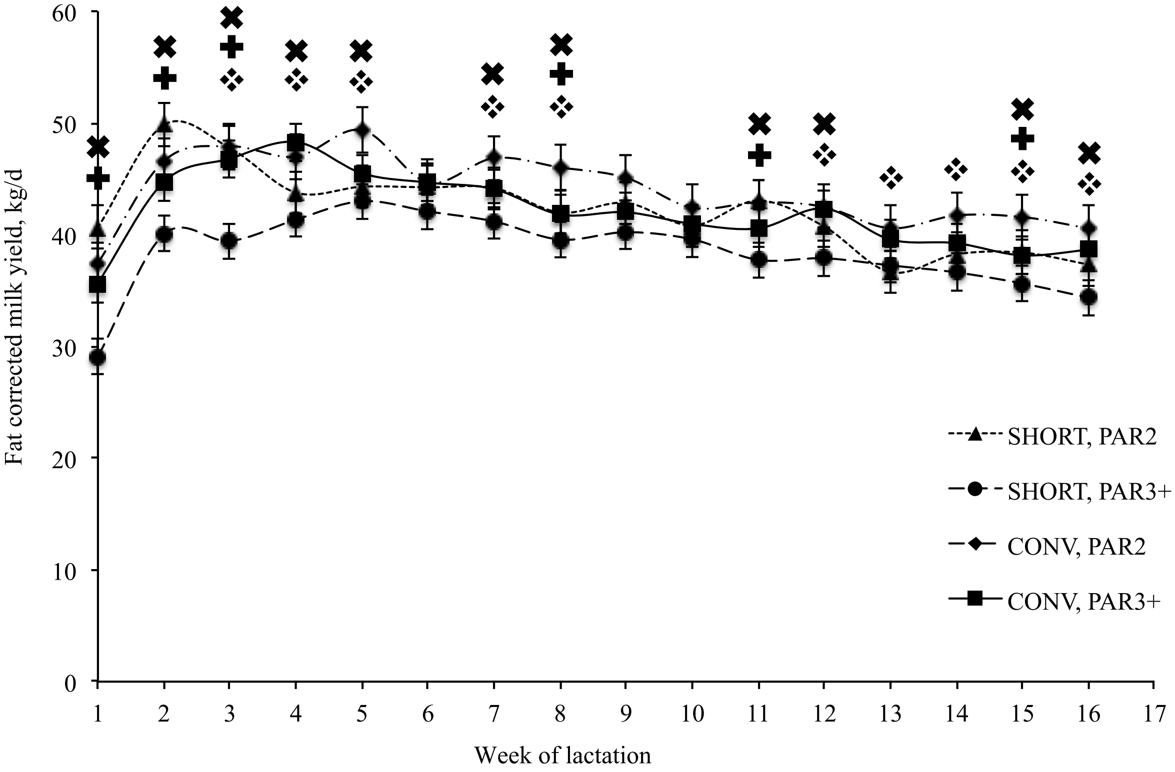
[1] Fat corrected milk according to 4 percent fat.

[2] Treatment consisted of a short 40-d dry period with a close-up diet throughout entire dry period (SHORT) and a conventional 60-d dry period with separate far-off and close-up diets (CONV). PAR 2 shows cows in parity 2 and PAR 3+ shows cows in parity 3 and higher during the dry period.

[3] SED: Standard error of difference

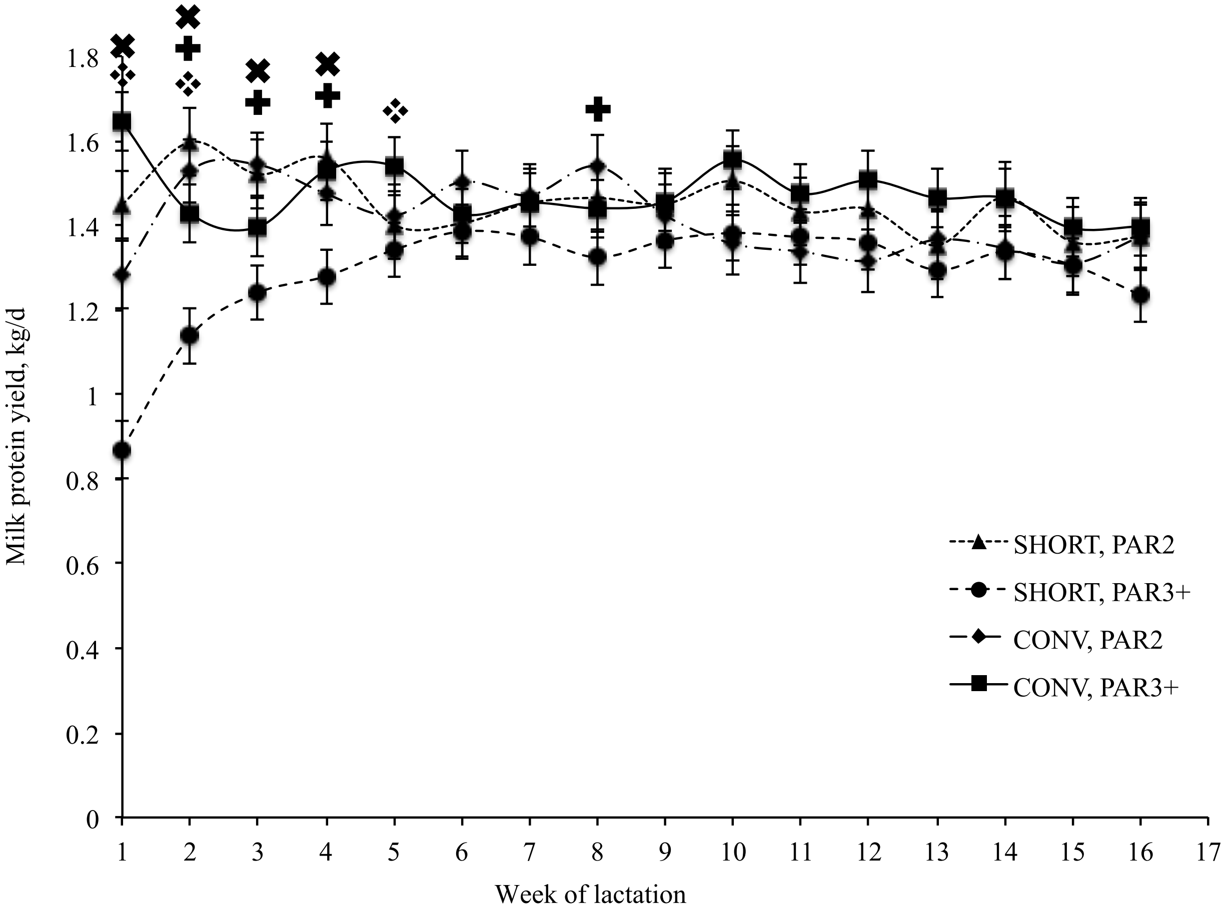
[4] Area under the curve calculated for fat corrected milk. Groups that do not share letters are not significantly different.

Figure 2.3. Effect of dry period length and diet on fat corrected milk yield across the first 16 wks of lactation. Symbols are: significant effects of treatment (❖), parity (✚), and their interactions (✖).



Milk fat yield and percentage were not affected by treatment, parity and their interaction across the first 16 wks of lactation (Table 2.4). Milk protein percentage was also not affected by treatment, parity and their interactions across the first 16 wks of lactation (Table 2.4). Milk protein yield was affected by treatment and the interaction of treatment and parity across the first 16 wk of lactation (Table 2.4). During this time protein yield was lower in cows on the SHORT treatment (*P*=0.02), which was mostly due to low milk protein yield in PAR 3+ cows compared to other cows (Table 2.4; *P*<0.01). The interaction of time, parity and week for milk protein yield was significant. Weekly comparisons of protein yield revealed that milk protein yield was affected by treatment at wk +1, +2, and +5 as cows on the CONV treatment had higher milk protein yield compared to cows on the SHORT treatment (*P*<0.1). Also, parity affected the milk protein yield at wk +2, +3, +4, and +8, as PAR3+ cows had lower protein yield compared to PAR2 cows. In addition, interaction of treatment and parity was significant within the first 4 wk of lactation, which was due to lower milk protein yield in PAR3+ cows on the SHORT treatment (Figure 2.4; P≤0.01).

Figure 2.4. Effect of dry period length and diet on milk protein yield across the first 16 wks of lactation. Symbols are: significant effects of treatment (❖), parity (✚), and their interactions (✖).



Across the first 16 wks of lactation, cows on the SHORT treatment had lower milk LOS percentages compared to those on the CONV treatment (*P*<0.01; Table 2.4). The LOS yield was not affected by treatment, parity and their interaction across the first 16 wks of lactation (Table 2.4).

The average milk SCC across the first 16 wks of lactation was affected by treatment, as cows on the SHORT treatment had higher SCC compared to cows on the CONV treatment (*P*<0.01). Also during this time, interaction between treatment and parity tended to affect SCC, as PAR 3+

cows on the SHORT treatment had higher SCC compared to PAR 3+ and PAR 2 cows on the CONV treatment (*P*=0.09; Table 2.4).

DISCUSSION

Feed Intake and Energy Balance

In contrast to our hypothesis, the SHORT treatment did not increase DMI before calving and after calving. This absence of a treatment effect on DMI in our study agrees with Gulay et al. (2003) and Rastani et al. (2005) who did not observe an effect of the dry period length on prepartum DMI. In our study, treatments differed not only in the length of the dry period, but also in the diet fed between days – 40 and – 21 relative to calving. Whereas cows on the SHORT treatment received the close-up diet, the cows on the CONV treatment received the lower energy far-off diet during this period. As a result, the energy intakes and, likely, the fat deposition of the cows on the SHORT treatment were higher than those of cows on the CONV treatment during this period. Increases in fat deposition during the close-up period have been associated with reductions in feed intake during early lactation (Douglas, 2006; Janovick and Drackley, 2010). This may be due to a higher mobilization of NEFA from adipose tissue leading to increased oxidation in the liver and consequently sending signals to brain to control feed intake (Allen and Bradford, 2005). Hence, a higher fat deposition in cows on the SHORT treatment during the dry period, may have prevented any increases in feed intake that this treatment otherwise could have caused. Dairy cows reach their mature weight later than the third parity (NRC, 2001), and therefore, parities differ in their growth and maintenance requirements. Cows in the second parity are still growing, and excessive energy intakes in these cows may, therefore, result in less fat deposition compared to older cows (Demment and Van Soest, 1984; Owens, 1993;

McNamara, 2000; NRC, 2001). This means that the excessive energy intake during – 40 and – 21 d before calving could have been less detrimental with regards to feed intake in PAR 2 cows compared to PAR 3+ cows.

Using models for the estimation of energy and protein requirements showed that at 6 weeks before calving cows on the SHORT treatment received almost 30 percent more energy compared to cows on the CONV treatment. During the last 3 weeks of gestation the difference in energy intake decreased between animals in different treatments since cows on the CONV treatment also started receiving the close-up diet. The lower energy balance in PAR 2 cows on the CONV treatment is probably due to lower feed intake in this group compared to others. During the last week of gestation cows in PAR 2 on the SHORT treatment and cows in PAR 3+ on the CONV treatment received close to 100 percent of their energy requirement. However, during this time, PAR 3+ cows on the SHORT treatment and PAR 2 cows on the CONV treatment experienced a negative energy balance. Unfortunately, information regarding to BW and DMI is not available for the period between – 40 and – 60 day relative to calving for cows on the SHORT treatment. As a result we were not able to estimate and compare the amounts of available energy and protein by animals between these two treatments during this period.

The higher energy balance of cows on the SHORT treatment at 6 wk before calving is due to the higher energy content of the diet fed to these cows at that time. In contrast with our hypothesis, treatment did not affect the energy balance during the first 4 wks of lactation. The absence of a treatment effect on the energy balance during the first 4 wks of lactation disagrees with the study by Jolicoeur et al. (2014) in which cows with 35-d dry period without dietary switches had lower energy deficit after calving due to higher feed intake compared to cows with 65-d dry period with a far-off and close–up diet. Although the energy content of the close-up and the far-off diets in the study by Jolicoeur et al. (2014) and our study were similar, the energy content of the lactating diet was lower in the earlier study. The higher energy content of lactation diet in our study may have been one of the reasons that DMI during the first 4 wks of lactation was lower in our study than in the earlier study. This may also explain why in our study cows on the SHORT treatment did not have the highest DMI, as they did in the study of Jolicoeur et al. (2014).

It has been suggested that cows with lower energy balance during the far-off period have a lower energy deficit during the first 10 days after calving (Dann et al. 2006). In contrast to our study, Pezeshki et al. (2007) reported that cows with a 35-d dry period that received close-up diet during the entire dry period, had a lower energy deficit after calving compared to cows with a 56-d dry period that received 21 d far-off and 35 d close-up diets. This effect was assumed to be as a result of a higher feed intake. However, in their study, the energy content of the close-up and the far-off diets were similar. Hence, in the study of Pezeshki et al. (2007) differences in energy intake and fat deposition between treatments might have been smaller compared to our study. Gulay et al. (2003) also reported that cows with a 30-d dry period had higher energy balance in the first 4 wks of lactation compared to cows with a 60-d dry period, possibly due to lower milk production in cows with 30-d dry period during this time.

Milk Production

Our study showed that dry period management affects milk production, as the production of fat corrected milk across the first 16 wks of lactation was lower in cows on the SHORT treatment compares to those on the CONV treatment. The AUC of milk production shows that PAR 3+ cows on the SHORT treatment produced about 400 kg less than others during the first 16 wks of lactation. Hence, the treatment effect on milk yield was mainly due to the low milk yield of PAR

3+ cows on the SHORT treatment. This agrees Mashek and Beede (2001) who concluded that excessive energy intake during the dry period reduces milk production.

There are only a limited number of studies that considered parity as a factor in the evaluation of dry period management (Dias and Allaire, 1982; Annen et al. 2004; Pezeshki et al. 2007). Feeding the high-energy close-up diet for the entire dry period may not have negative effects on PAR 2 cows as it has been observed in PAR 3+. Our results are in contrast to previous studies that reported that shortening the dry period from 60 to around 28 to 35 d has no effect on average daily milk production (Gulay et al. 2003; Rastani et al. 2005; Santschi et al. 2011). Kuhn et al. (2006) reported that shortening the dry period from 60 to 20 d or less reduces the milk production. Annen et al. (2004) and Pezeshki et al. (2007) reported that shortening the dry period decreased daily milk yield in primiparous cows but did not change milk yield in multiparous cows. It has been suggested that the dry period length in the second lactation must be between 44 to 76 d but this duration can decline as an animal ages (Dias and Allaire, 1982). Collier et al. (2012) reported that the first parity cows require a 60-d dry period, while the older cows subjected to a 30-d dry period had no negative impacts. Our data suggests a short dry period during which only our close-up diet is fed may be appropriate for second parity cows, but not for older cows. It is important to note that there was a variation in dry period length between 23 to 44 d in the SHORT treatment and 53 to 68 d in the CONV treatment that probably had an impact on the response of animals.

Milk Fat

Several other studies reported that shortening the dry period had no effect on milk fat yield (Gulay et al. 2003; Annen et al. 2004; Rastani et al. 2005; Pezeshki et al. 2007; Watters et al.

2008; Klusmeyer et al. 2009; Santschi et al. 2011). In agreement, we observed no difference in milk fat yield between treatments, parity and their interaction. These results are consistent with others who reported that regardless of parity, shortening dry period to 35 d (Santschi et al. 2011) or 30 d (Annen et al. 2004) did not alter fat percentage in dairy cows. Other studies that did not consider parity as a factor also reported that shortening the dry period below 60-d had no effect on fat percentage in the subsequent lactation (Gulay et al. 2003; Watters et al. 2008; Klusmeyer et al. 2009). In contrast, Rastani et al. (2003) demonstrated that cows with short dry periods tended to have higher fat percentages in following lactation.

Milk Protein

The lower milk yield by PAR 3+ cows on the SHORT treatment compared to others resulted in lower milk protein yield in this group in the first 16 wks of lactation. These results are in agreement with Pezeshki et al. (2007) who reported that shortening dry period from 56 to 35 d decreased milk protein yield. In contrast, Pezeshki et al. (2008) reported no effect of shortening dry period from 49 to 28 d on milk protein yield. Regardless of parity, Atashi et al. (2013) reported lower milk protein yields for cows with dry periods between 36 and 50 d compared to cows with dry periods between 51 to 60 d. Dann et al. (2006) demonstrated that as lactation progresses, the carryover effects of dry period feeding management decrease. This may explain the absence of difference in milk protein yield after the 6 wks of lactation observed in our study.

Milk protein percentage was similar between treatments and parities across 16 wks of lactation, which is in agreement with previous studies that reported that the length of dry period, does not affect milk protein content (Smith et al. 1967; Gulay et al. 2003; Pezeshki et al. 2007, 2008). Watters et al. (2008) reported a higher milk protein percentage in both primiparous and multiparous cows in response to shortening dry period from 55 to 34 d. Likewise, Rastani et al. (2005) reported that shortening the dry period from 56 to 28 d increased milk protein percentage. However, they did not consider parity effect in their experiment. Santschi et al. (2011) reported a higher milk protein percentage during early lactation in primiparous cows on a 35 d dry period compared to those on a 60-d dry period, but this difference decreased as days of lactation increased. Similarly, Annen et al. (2004) demonstrated that, regardless of parity and dry period length, milk protein percentage was higher in the first month of lactation but the difference declined over the 4-month period following calving.

Milk Lactose and Other Solids

There are very few studies that have reported the effect of dry period length and parity on milk lactose percentage and yield. Santschi et al. (2011) reported no effect of dry period length on milk lactose percentage. However, they observed that the interaction between treatment and parity was significant. Remond et al. (1992) reported that cows with 60-d dry period tended to have higher lactose yields compared to cows with no dry period. Rastani et al. (2005) also reported lower milk lactose yield as the dry period was shortened from 59 to 28 d. Santschi et al. (2011) reported no effect of dry period length on milk lactose yield in multiparous cows. However, in their study, second lactation cows with a 60-d dry period had a greater milk LOS yield compared to cows with 35-d dry period. In our study, cows on the SHORT treatment had higher blood glucose (Khazanehei et al., 2015-b). Thus, a higher availability of glucose but lower LOS percentage in cows on the SHORT treatment compared to cows on the CONV treatment may suggest a lower uptake of glucose by the mammary gland in cows on the SHORT treatment. The reason behind that is not clear.

Somatic Cell Counts

The higher milk SCC in cows on the SHORT treatment compared to the CONV treatment is in agreement with Remond et al. (1997) who reported that cows with a short or no dry period tended to have higher milk SCC. It has been suggested that an inadequate time of antibiotic therapy at drying off results in higher SCC in the subsequent lactation (Pezeshki et al. 2007). In contrast, other studies reported no effect of dry period length on milk SCC (Enevoldsen and Sorensen, 1992; Remond et al. 1992; Gulay et al. 2003; Annen et al. 2004; Rastani et al. 2005; Pezeshki et al. 2007; Watters et al. 2008; Santschi et al. 2011). However, with regards to parity, Pezeshki et al. (2007) demonstrated that multiparous cows with a 42-d dry period had higher milk SCC compared to cows with 56-d and 35-d dry periods. Santschi et al. (2011) reported that although shortening the dry period to 35 d had no effect on milk SCC in second-lactation cows, third-lactation cows tended to have lower SCC compared to cows with 60-d dry period. There are different recommended ranges to define low, medium and high level of SCC in individual cows. The low concentration of SCC has been suggested from <150,000 to 264,000 cells/mL and the defined range for high concentration of SCC is from >200,000 to >700,000 cells/mL (Bareille et al. 2000; De Vliegher et al. 2004; Svensson et al. 2006; Nyman et al. 2009). Mastitis is a common disease in dairy cows and can result in milk production loss (Hortet and Seegers, 1998). Lower milk production in first 3 weeks of lactation in PAR 3+ cows on the SHORT treatment may be associated with high SCC in these cows. Alternately, it has been reported that SCC level in non-infected mammary gland is associated with milk production and as milk yield increases the SCC decreases (Green et al. 2006). Therefore it is not possible to conclude that lower milk yields in PAR 3+ cows on the SHORT treatment resulted in high SCC in this group or it is as a result of combination of high milk SCC and lower feed intake.

CONCLUSIONS

Compared to a 60-d dry period with separate far-off and close-up periods, a 40-d dry period during which only a close up diet was fed reduced DMI, fat corrected milk yield and milk protein yield in early lactation for the third-parity and older cows, but not for the second-parity cows. These differences between parity groups were most likely due to higher fat deposition in adipose tissues and associated problems in the third-parity and older cows compared to the second-parity cows due to the feeding of 19 d longer close-up diet in the SHORT treatment. Based on these results we recommend implementing a 40-d dry period with our close-up diet only for parity 2 cows, as this may be detrimental due to excessive energy intake for mature third-parity and older cows.

CHAPTER THREE

MANUSCRIPT II

Effects of dry period management and parity on rumen fermentation, blood metabolites, and liver triacylglyceride in dairy cows

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3.1 ABSTRACT

The effects of dry period management on rumen fermentation, blood metabolites and liver TAG were determined in 11 second-parity (PAR2) and 15 third-parity and older (PAR3+) cows. Cows were paired and randomly assigned to two treatments including a short 40-d dry period (SHORT) with only a close-up diet (1.43 Mcal/kg DM), or 2) a conventional 60-d dry period (CONV) with 39 d far-off diet (1.28 Mcal/ kg DM) and 21 d close-up diet (1.43 Mcal/kg DM) in a randomized block design. Concentrations of total VFA, ammonia, and lactate in rumen fluid, and also concentrations of lactate, beta hydroxybutyric acid (BHBA), urea and insulin in plasma were not affected by treatment. Through the 3 wk after calving, cows on the SHORT treatment had higher blood NEFA and glucose, and at 1 wk after calving these cows had a higher liver TAG than cows on the CONV treatment. Also, during this time PAR3+ cows had higher serum NEFA compared to PAR2 cows. Results suggest that the SHORT treatment resulted in more lipolysis, TAG accumulation in the liver and glucose sparing in early lactation compared to the CONV treatment and that this effect was greater in PAR3+ cows than in PAR2 cows.

Key words: Dry period, dairy cows, blood metabolites, rumen fermentation, liver

3.2 INTRODUCTION

The transition period is a challenging phase as a consequence of metabolic and physiologic adaptations that cows undergo during this time. The high energy demand during early lactation commonly results in a negative energy balance, lipolysis, ketogenesis and fat accumulation in the liver, and, therefore, in reduced metabolic health (Weber et al. 2013). Dry period management affects metabolic health in early lactation (Janovick et al., 2011, van Knegsel et al., 2013). A short dry period between 28 to 35 d with only one diet may reduce the stresses of switching diets and improve rumen papillae development, feed intake, and, consequently, attenuate the negative energy balance in early lactation compared to a 60-d dry period with a far-off and a close-up diet (Rastani et al. 2005; Jolicoeur et al. 2014). In agreement, Shoshani et al. (2014) observed that concentrations of NEFA in blood at 1 wk after calving were lower after a 40-d dry period compared to after a 60-d dry period. Jolicoeur et al. (2014) also found that cows with a 35-d dry period had lower plasma concentrations of NEFA and BHBA compared to cows with 63-d dry period after calving. Parity of dairy cows also affects the required length of the dry period. Collier et al. (2012) reported that the required length of dry period for the first parity cows is 60 d, while for the second parity and older cows it can be reduced to 30 d with no negative effect on production and health. Conversely, Atashi et al. (2013) showed that third parity and older cows need longer dry periods compared to younger animals. Khazanehei et al. (2015-a) suggested that this difference between parity groups might be due to differences in growth and fat deposition between the first and second parity cows compared to the older cows.

We hypothesized that shortening the dry period to 40 d and using only a close-up diet throughout this short dry period improves energy availability, decreases lipolysis in adipose tissues and ketogenesis, and also reduces accumulation of triacylglyceride (TAG) in the liver of dairy cows during early lactation compared to a 60-d dry period with a far-off and a close-up diet. We also hypothesized that these changes are affected by parity. The objective of this research was to determine whether a 40-d dry period during which only a close-up diet is fed improves metabolic health and energy availability regards to rumen fermentation, plasma concentrations of glucose, insulin, BHBA, non-esterified fatty acids (NEFA), urea, and lactate and hepatic concentration of TAG compared with a 60-d dry period with separate far-off and close-up diets, and whether the interaction between parity and dry cow management affects these measures.

3.3 MATERIAL AND METHODS

Animals and experiment design

All procedures were conducted at the Glenlea Dairy Research Unit at the University of Manitoba in accordance with the Canadian Council for Animal Care guidelines (CCAC, 1993). Treatments and experimental design were as previously described (Khazanehei et al. 2015a). Briefly, twenty-six Holstein dairy cows (11 parity 2 and 15 parity 3 and later during their dry period) participated in the study. Cows were paired based on expected calving date and randomly assigned to two treatments within each pair. Treatments included a conventional 60-d dry period (CONV) with 39 d of feeding a far-off diet and 21 d of feeding a close-up diet and a short 40-d dry period (SHORT) with only a close-up diet. A common lactation diet was fed to all cows after calving. The compositions of diets are shown on Table 3.1. In total, 5 and 6 PAR 2 cows received the SHORT and CONV treatments, respectively, and 8 and 7 PAR 3+ cows received the SHORT and CONV treatments, respectively. Milk production was not considered as a grouping factor since there was no treatment difference in the milk production during the previous lactation (11104 kg in the SHORT treatment vs. 10612 kg in the CONV treatment; $P>0.1$). Average body weights of cows at the beginning of study on the CONV and SHORT treatments were 737 ± 60 and 782 ± 69 kg (mean ± SD), respectively. Body condition scores were between 2.75 and 4.00 with an average of 3.36 ± 0.30 and 3.44 ± 0.42 for cows on the CONV and SHORT treatments, respectively, on a 1 to 5 scale (Edmonson et al. 1989).

Cows were dried off gradually within four days by decreasing and switching feed to a lower concentrate diet, and reducing milking frequency. Cefa-Dri (cephapirin benzathine Boehringer Ingelheim Canada, Burlington, ON) was used as intramammary infusion in dry cows. Actual dry period lengths for the SHORT treatment were 34 ± 6 d (mean ± SD) with a minimum of 23 d and a maximum of 44 d and for the CONV treatment they were 59 ± 4 d (mean ± SD) with the minimum of 53 d and a maximum of 68 d. These variations in dry period length were caused by cows calving earlier or later than expected.

Table 3.1. Nutrient composition of far-off, close-up, and lactation diets (mean ± SD)

Item	Nutrient Composition[1]		
	Far-off	Close-up	Lactation
Crude protein, %	14.8±0.4	14.9±1.9	17.7±1.3
Neutral detergent fiber, %	50.2±1.1	38.1±7.4	31.2±5.6
Acid detergent fiber, %	32.2±1.2	23.2±5.1	19.3±3.3
NFC[2], %	26.2±1.6	36.2±6.1	39.4±3.9
Crude fat, %	1.7±0.3	3.3±1.0	4.2±0.7
Calcium, %	0.6±0.1	0.8±0.2	1.0±0.1
Phosphorous, %	0.3±0.0	0.4±0.1	0.4±0.0
Magnesium, %	0.2±0.0	0.4±0.1	0.4±0.1
Potassium, %	2.1±0.1	1.7±0.4	1.5±0.1
Ash, %	7.1±1.0	7.8±0.8	7.5±0.7
Net Energy for lactation, Mcal/kg	1.28	1.39	1.68

[1] Far-off diet ingredients (% of DM): timothy hay, 89.5; soybean-meal, 8.2; mineral, 2.2; forage to concentrate ratio, 90 to 10; Close-up diet ingredients (% of DM): timothy hay, 43.0; barley silage, 25.3; Transrite, 31.6; forage to concentrate ratio, 68 to 32; Lactation diet ingredients (% of DM): mixed hay, 20.4; corn silage, 32.2; roasted soybeans, 4.4; Energy supplement, 39.5; protein supplement, 2.2; forage to ratio concentrate ratio, 53 to 47.

[2] Non fiber carbohydrate= 100 - (NDF% + CP % + Crude fat % + Ash %).

Sample collections and analyses

Blood samples were taken from the coccygeal vein 5 h after feeding on a weekly basis, beginning 3 wks before calving until 9 wks after calving. Two 10-mL evacuated tubes, one coated with freeze-dried Sodium Heparin (143 USP Units) and the other one coated with only silicone (BD, Franklin Lakes, NJ), were used at each sampling time for plasma and serum collection, respectively. Plasma tubes were centrifuged at 1,500 × g for 15 min at 4°C immediately after collection. Serum tubes were kept at room temperature for 30 min to clot and were subsequently centrifuged under the same conditions as plasma tubes. After centrifuging, plasma and serum were transferred into 2-mL tubes and stored at −20 °C for future analysis. Commercial kits were used for the analysis of serum NEFA (HR Series NEFA-HR (2), code no. 999-34691, Wako Pure Chemical Industries, Ltd; Chuo-Ku Osaka 540-8605 Japan), BHBA using Roche Cobas 6000 c501 automated chemistry analyzer (Roche Canada, Laval, QC, Canada) and insulin using Mercodia bovine insulin ELISA (code no. 10-1201-01, Mercodia, Uppsala, Sweden). Plasma glucose, lactate and urea were determined using Nova Stat profile M blood gas and electrolyte analyzer (Nova Biomedical Corporation, Waltham, MA).

Rumen samples were taken weekly from 3 wk before until 9 wk after calving at 5 h after feed delivery, at the same time as blood samples, using a stomach tube (Bramley et al. 2008). The pH of rumen samples was determined immediately after sampling using an Accumet Basic 15 pH meter (Thermo Fisher Scientific Inc. Waltham, MA). Approximately 500 mL of rumen fluid was collected, mixed thoroughly and strained through four layers of cheesecloth. Subsequently, rumen fluid was transferred into two 15 mL tubes and stored at –20 °C for analysis of volatile fatty acids (VFA), lactate and ammonia nitrogen. The analysis of VFA was conducted by gas chromatography (model 3900 Star, Varian, Walnut Creek, CA) as described by Bhandari et al. (2007). Briefly, after the samples were thawed at room temperature, 3 mL of subsample was transferred into test tubes and mixed thoroughly with 0.6 mL of 25% metaphosphoric acid. Subsequently, 0.24 mL of 25% sodium hydroxide and 0.384 mL of 0.3 M oxalic acid were added to the test tubes and the tubes were vortexed. The mixture in the test tubes were then centrifuged at 3,000 × g for 20 min, and 2 mL of supernatant was transferred into vials for VFA analysis. Rumen ammonia was determined using colorimetric method described by Novozamsky et al. (1974). Rumen D and L lactate were measured using EnzymPlus D/L-Lactic Acid kits (Biocontrol, US).

Liver biopsies were performed according to the University of Manitoba Veterinary Services guidelines at -3, +1 and +4 wk, relative to calving. Briefly, the animal was restrained in squeeze chute and Anafen and Excenel RTU were administrated according to the label directions. The biopsy location was determined using a hypothetical line drawn from the hook bone down to the elbow and between the 11th and 12th rib area. An area of 15 cm^2 was clipped, sterilized, and anesthetized using 4 cc Lidocaine. A 1.5 to 2 cm horizontal incision was made and liver samples were taken using a biopsy trocar. The samples were then placed in collection vials and stored at –80 °C.

Statistical analyses

Data measured over time were subjected to statistical analysis using the REPEATED statement in the MIXED procedure of SAS (Littell et al., 1996). Differences in variables across periods (pre- or post-partum) were tested in a randomized block design (Littell et al. 1996). For each variable analyzed, cow nested within treatment and block were considered random effects. The statistical model included treatment, parity, week, interactions of treatment and parity, treatment and week, parity and week, and treatment, parity and week. When week intervals were not equally spaced, spatial exponential, spatial Gaussian, spatial linear, spatial linear log, spatial power, and spatial spherical covariance structures were compared. When week intervals were equally spaced, variance components, compound symmetry, heterogeneous compound symmetry, unstructured, first-order autoregressive, heterogeneous first-order autoregressive, toeplitz, and first-order ante-dependence covariance structure were compared. The covariance structure with the smallest Akaike information criterion was used (Littell et al. 1996). When interactions between treatment, parity and week were significant, the SLICE option in LSMEANS statement was used to help explanation of changes within weeks. Blood serum NEFA values were BOXCOX transformed to normalize the residuals. Statistical differences were considered significant at $P<0.05$ and tendencies towards significance at $0.05 < P < 0.1$ were discussed.

3.4 RESULTS

Rumen variables

Treatment and interaction between treatment and parity had no effect on total VFA concentrations in the rumen fluid across 3 wk before calving and also 9 wk after calving. However, during these periods, total VFA concentration were affected by parity, as cows in PAR 2 had higher total rumen VFA compared to cows in parity 3+ ($P=0.03$ and $P=0.06$, respectively; Table 3.2).

Rumen acetic acid concentrations were not affected by treatment, parity and their interaction across 3-wk period before and 9-wk period after calving (Table 3.2).

Concentrations of propionic acid were not affected by treatment, parity or their interaction across 3 wk before calving, however, across 9 wk after calving rumen propionate concentrations were affected by interaction between treatment and parity as cows in PAR 2 on the SHORT treatment had higher rumen propionic acid concentrations compared to cows in PAR 3+ on the same treatment and cows in PAR 2 on the CONV treatment (*P*=0.01; Table 3.2). During this time, there was no difference between PAR 3+ cows on the CONV treatment and others (*P*>0.10; Table 3.2).

The ratio of acetic acid to propionic acid (A: P) was not affected by treatment, parity and their interaction across 3 wk before calving (Table 3.2), however, this ratio was affected by the interaction between treatment and parity across 9 wk after calving as PAR 2 cows on the SHORT treatment had a lower A: P ratio compared to PAR 3+ cows on the same treatment and PAR 2 cows on the CONV treatment (*P*=0.03). During this time, there was no difference between PAR 3+ cows on the CONV treatment and others (*P*>0.25; Table 3.2).

Concentrations of rumen butyric acid and other VFAs (Including iso-butyric acid, iso-valeric acid and valeric acid) were not affected by treatment, parity and their interaction across 3 wk before (*P*>0.1) and also across 9 wk after calving (*P*>0.1; Table 3.2).

Concentrations of rumen ammonia across the 3 wk before calving were not affected by treatment, parity and their interaction. However, across 9 wk after calving cows on the CONV treatment tended to have higher rumen ammonia compared to cows on the SHORT treatment (*P*=0.09; Table 3.2).

Concentrations of D- and L-lactate in the rumen across 3 wk before calving and also after calving were not affected by treatment, parity and their interaction (*P*>0.1; Table 3.2). Rumen pH values across 3 wk before calving were affected by parity (*P*=0.05), as cows in PAR 3+ had higher rumen pH values compared to cows in PAR 2 (Table 3.2). Rumen pH values across 9 wk after calving were not affected by parity, treatment or their interactions.

Table 3.2. Effect of dry period management and parity on rumen parameters across pre- and postpartum.

	Treatment[1]				SED[2]	*P*-value		
	SHORT		CONV					
Items	PAR 2	PAR 3+	PAR 2	PAR 3+		Trt	Parity	Trt × Parity
Prepartum								
Total VFA (mmol/L)	112.6	101.2	113.2	98.0	7.81	0.79	0.03	0.75
Acetate (%)	68.3	68.4	68.3	67.9	1.13	0.71	0.85	0.74
Propionate (%)	17.0	17.4	17.3	17.4	0.73	0.71	0.60	0.84
A: P	4.1	4.0	4.1	3.9	0.22	0.78	0.46	0.85
Butyrate (%)	11.7	10.8	11.3	11.4	0.58	0.84	0.39	0.26
Other VFAs[3] (%)	3.2	3.3	3.2	3.1	0.50	0.82	0.93	0.79
Ammonia (mg/mL)	8.7	9.9	11.4	10.1	2.40	0.41	0.97	0.48
D-Lactate (mg/dl)	1.6	2.5	2.2	3.1	1.90	0.63	0.57	0.96
L-Lactate (mg/dl)	1.1	1.5	0.9	2.1	1.18	0.77	0.42	0.69
pH	6.4	6.5	6.4	6.6	0.09	0.60	0.05	0.22
Postpartum								
Total VFA (mmol/L)	121.8	109.5	122.8	116.6	6.76	0.33	0.06	0.56
Acetate (%)	64.4	65.3	65.8	64.3	1.49	0.84	0.75	0.25
Propionate (%)	23.4^{a}	20.2^{b}	20.6^{b}	22.1^{ab}	1.12	0.52	0.31	0.01
A: P	2.8^{a}	3.4^{b}	3.3^{b}	3.1^{ab}	0.22	0.48	0.33	0.03
Butyrate (%)	10.5	10.8	10.6	11.2	0.37	0.51	0.22	0.78
Other VFAs[3] (%)	2.7	3.1	2.8	2.8	0.22	0.71	0.16	0.21
Ammonia (mg/mL)	9.3	11.4	12.0	11.9	1.30	0.09	0.30	0.24
D-Lactate (mg/dl)	3.8	5.1	9.0	6.7	4.27	0.45	0.91	0.69
L-Lactate (mg/dl)	2.1	3.2	4.1	5.7	2.73	0.44	0.63	0.92
pH	6.1	6.2	6.1	6.2	0.10	1.00	0.22	0.37

[1] Treatment consisted of a short 40-d dry period with a close-up diet throughout entire dry period (SHORT) and a conventional 60-d dry period with separate far-off and close-up diets (CONV). The PAR 2 shows cows in parity 2 and PAR 3+ shows cows in parity 3 and more.

[2] SED: Standard error of difference.

[3] Other VFAs including iso-butyric acid, valeric acid, iso-valeric acid.

Blood variables

Plasma glucose concentrations across 3 wk before calving and also across 3 wk after calving were affected by parity, as PAR 3+ cows had higher concentrations of plasma glucose compared to PAR 2 cows ($P=0.03$ and $P<0.01$, respectively). In addition, plasma glucose concentrations across 3 wk after calving were affected by treatment as cows on the SHORT treatment ($P=0.03$) had higher plasma glucose than cows on the CONV treatment (Table 3.3). Plasma lactate concentrations across 3 wk before calving and also across 9 wk after calving were not affected by parity, treatment or their interactions (Table 3.3).

Table 3.3. Effect of dry period management and parity on blood parameters and liver TAG across pre- and postpartum.

	Treatment[1]				SED[2]	*P*-value		
	SHORT		CONV					
Items	PAR 2	PAR 3+	PAR 2	PAR 3+		Trt	Parity	Trt × Parity
Prepartum								
Glucose (mg/dL)	81.0	86.0	72.2	86.1	5.48	0.22	0.03	0.29
Lactate (mmol/L)	1.4	1.5	1.5	1.6	0.35	0.70	0.52	0.90
NEFA (mmol/L)*	0.1	0.2	0.1	0.2	0.06	0.21	0.14	0.96
BHBA (µmol/L)	464.0	640.0	613.0	489.0	67	0.98	0.66	0.04
Urea (mmol/L)	6.3	5.2	5.9	5.6	0.63	0.95	0.12	0.33
Insulin (µg/L)	1.0	0.5	0.5	0.6	0.20	0.15	0.22	0.06
Liver TAG (%)	0.2	0.4	0.3	0.2	0.06	0.96	0.57	0.95
Postpartum								
Glucose (mg/dL)	61.3	80.9	58.7	69.1	4.97	0.03	<0.01	0.24
Lactate (mmol/L)	1.3	1.2	1.0	1.3	0.34	0.65	0.71	0.49
NEFA (mmol/L)*	0.2	0.3	0.2	0.2	0.05	0.06	0.01	0.91
BHBA (µmol/L)	1041.0	1120.0	1058.0	860.0	275	0.51	0.77	0.50
Urea (mmol/L)	5.6	5.4	5.9	5.2	0.51	0.95	0.23	0.51
Insulin (µg/L)	0.3	0.3	0.3	0.2	0.06	1.00	0.34	0.19
Liver TAG (%)*	11.7	9.2	5.6	10.0	3.80	0.24	0.91	0.21

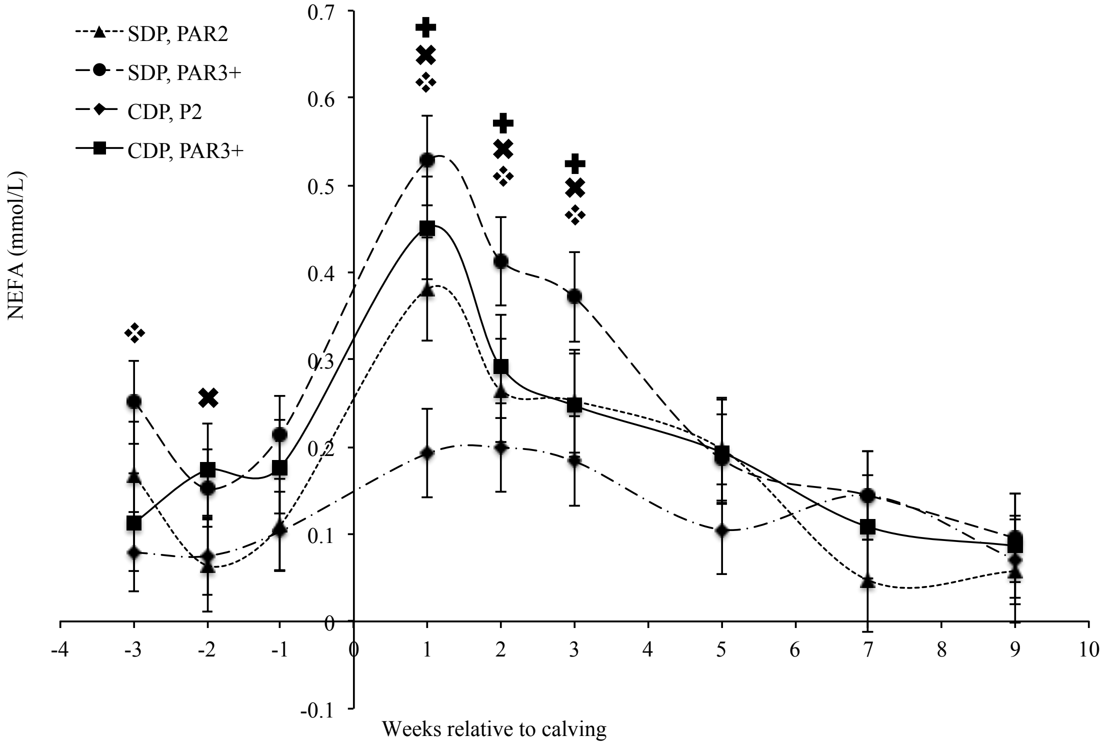
[1] Treatment consisted of a short 40-d dry period with a close-up diet throughout entire dry period (SHORT) and a conventional 60-d dry period with separate far-off and close-up diets (CONV). The PAR 2 shows cows in parity 2 and PAR 3+ shows cows in parity 3 and more.

[2] SED: Standard error of difference

* The interaction between treatment, parity and week was significant.

Serum NEFA concentrations were not affected by parity, treatment and their interactions across 3 wk before calving. However, across 9 wk after calving, these concentrations were affected by treatment and parity. Cows on the SHORT treatment tended to have higher serum NEFA concentrations compared to the cows on the CONV treatment (*P*=0.06). Also during this time, PAR 3+ cows had higher serum NEFA concentrations than PAR 2 cows (*P*=0.01; Table 3.3). The interaction between treatment, parity and week tended to be significant for serum NEFA across 3 wk before and 9 wk after calving (*P*<0.1). Therefore, the slice option was used to compare the effect of treatment, parity and their interaction within each week on serum concentrations of NEFA (Fig. 3.1). At wk −3, +1, +2 and +3, relative to calving, cows on the SHORT had higher serum NEFA compared to cows on the CONV treatment (*P*<0.05). In addition, at wk −2, +1, +2 and +3, relative to calving, cows in PAR 3+ had higher serum NEFA compared to cows in PAR 2. Serum concentrations of NEFA at wk +1, +2, and +3, relative to calving, were also higher in PAR 3+ cows on the SHORT treatment compared to PAR 2 on the same treatment and also on the CONV treatment (*P*<0.1). Concentrations of NEFA were not affected by treatment, parity and their interactions at wk +5, +7 and +9, relative to calving (Fig. 3.1).

Fig. 3.1. Effect of the SHORT or CONV treatment and parity on serum concentration of NEFA. Symbols are: significant effects of treatment (❖) parity (✚) and their interactions (✖).



The interaction between treatment and parity was significant for serum BHBA across 3 wk before calving (Table 3.3). Cows in PAR 2 on the SHORT treatment and cows in PAR 3+ on the CONV treatment had lower BHBA concentrations compared to other cows (*P* = 0.04). Concentrations of serum BHBA were not affected by treatment, parity, or their interactions across 9 wk after calving (Table 3.3).

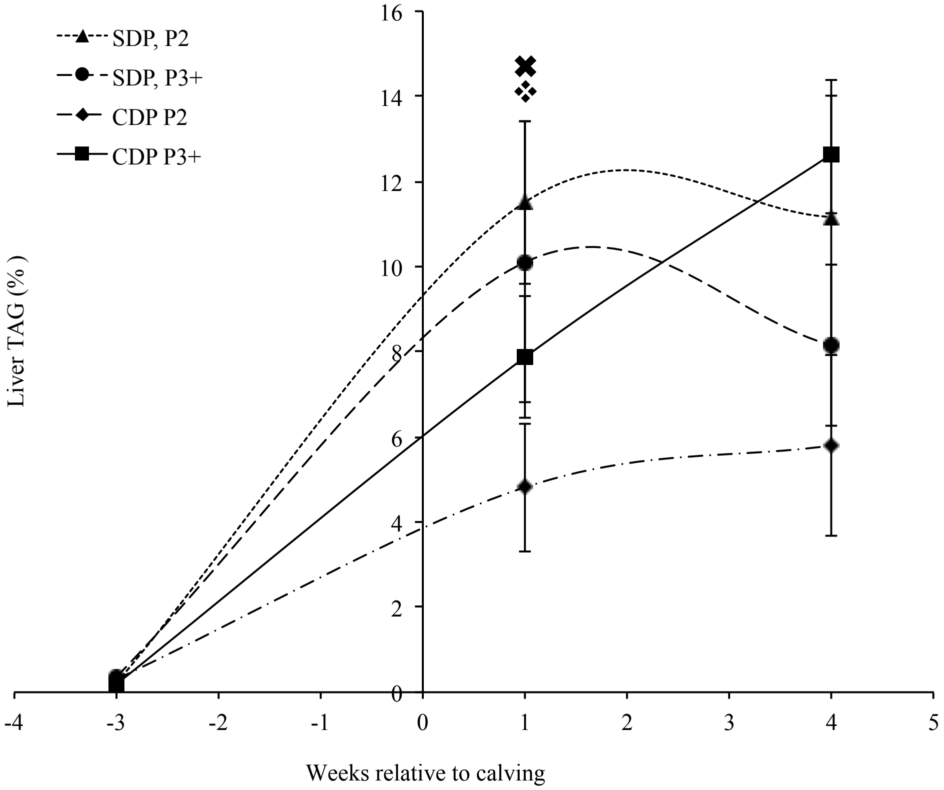
Plasma urea concentrations were not affected by treatment, parity and their interactions across 3 wk before and across 9 wk after calving (Table 3.3).

Concentrations of serum insulin across 3 wk before calving were affected by interaction between treatment and parity, as cows in PAR 2 on the SHORT tended to have higher serum insulin compared to the other cows (*P*=0.06).

Liver variables

There was no effect of treatment, parity and their interactions on liver TAG at wk −3 and +4, relative to calving (Fig. 3.2). However, liver TAG was affected by treatment and interaction of treatment and parity at the first week after calving. Cows on the SHORT treatment had higher liver TAG compared to cows on the CONV treatment (*P*=0.02). In addition, both PAR 2 and PAR 3+ cows on the SHORT treatment tended to have higher liver TAG compared to cows in PAR 2 on the CONV treatment (*P*<0.1).

Fig. 3.2. Effect of the SHORT or the CONV and parity on liver TAG. Symbols are: significant effects of treatment (❖) and interactions of treatment and parity (✖).



3.5 DISCUSSION

The effects of treatment on rumen concentrations of total VFA and individual VFA from 3 wk before calving until 9 wk after calving were limited. In a parallel study, Khazanehei et al. (2015a) reported that the dry matter intakes of cows during this period were also not affected by treatment. As the diet fed to these cows did not differ between the treatments during this period, this absence of a feed intake effect may explain why treatment did not affect the concentrations of VFA in the rumen. In contrast, Jolicoeur et al. (2014) reported that cows with a 63-d dry period had higher rumen VFA compared to cows with a 35-d dry period. They suggested that this higher VFA could be as a result of negative impact of switching diets during a 65-d dry period on rumen papillae and its absorptive ability. A difference between our study and that of Jolicoeur et al. (2014) was that the lactation diet in our study contained more energy and further the rumen fluid concentrations of total VFA after calving were higher. These differences may have resulted in a relatively higher growth of rumen papillae in our study that removed the effect that pre-partum feeding may have had on rumen papillae development.

In our study, the cows on the SHORT treatment had received the close-up diet for an average of 34 d, whereas the cows on the CONV received the close-up diet for 21 d and received the lower energy far-off diet for 39 d prior. This could have resulted in relatively more developed rumen papillae, a higher absorption capacity for VFA, and possibly lower VFA concentrations in the rumen in cows on the SHORT treatment during early lactation (Dirksen et al. 1985; Xu, 1999). Our results do not confirm this, which may be as a result of the high-energy lactation diet that allowed for very rapid growth of rumen papillae in early lactation.

As the dry matter intake, both absolute and as a percentage of body weight, and the diets did not differ between parity groups (Khazanehei et al. 2015a) the higher concentrations of total VFA in the PAR 2 group during early lactation are difficult to explain. Differences in rumen volume and absorption surface area between the parity groups could have played a role in this (Dado and Allen, 1994), but these parameters were not determined. Studies have shown that, even if dry matter intakes do not differ, multiparous cows eat faster and larger portions of feed in each meal compared to primiparous cows (Dado and Allen, 1994; Grant and Albright, 1995; Bowman et al. 2003). Also, multiparous cows are more efficient in rumination and spend less time on same amount of feed for chewing compared to primiparous cows (Bowman et al. 2003). Eating large portions of feed in a short period of time and also efficient chewing may increase the ruminal passage rate and, consequently, higher clearance of VFA from rumen through this route. This may partly explain lower total VFA in PAR 3+ cows compared to PAR 2 cows in our study. In agreement with the VFA data, the rumen pH of the PAR 3+ cows was higher than those of the PAR 2 cows across the 3 wk before calving. Our results are in contrast to those of Odensten et al. (2005) who reported that the concentrations of total VFA before calving were lower in primiparous compared to multiparous cows. This discrepancy is probably due to the exclusion of primiparous cows in our study, which only allowed comparisons between second parity and older cows.

Following calving, cows in PAR 2 on the SHORT treatment had a lower A:P ratio in rumen fluid than the other cows. This effect was mainly due to a higher concentration of propionate, and could have resulted in a higher energy availability to the cows (Russell, 1998).

Our results regarding plasma glucose are in agreement with Rastani et al. (2005), who reported higher plasma glucose concentrations after calving in cows with a 28-d dry period compared to a 56-d dry period. However, the higher plasma glucose in the cows on the SHORT treatment across the 3 wk after calving, was not likely due to a higher supply of propionate for gluconeogenesis (Huntington et al. 2006), as the concentrations of total VFA and the molar proportion of propionate were not affected by treatment during that time. It is also not likely that this effect was mediated by insulin, as insulin concentrations in the plasma did not differ between treatments and parity groups. A more probable explanation is that less glucose was used for milk yield due to a lower milk production of cows, and especially in PAR 3+ cows, on this treatment (Khazanehei et al. 2015a). Also, as cows on the SHORT treatment had higher serum NEFA concentrations in early lactation, a higher NEFA supply could have led to glucose sparing (Grummer and Carroll, 1991). The parity effect on plasma glucose may also be explained by this glucose sparing effect, as the PAR 3+ cows had higher serum NEFA concentrations after calving compared with younger cows.

A reduction in feed intake, along with hormonal changes during the last few days before parturition, increased required energy for the gravid uterus, and uterine contractions shortly before calving initiate mobilization of NEFA from adipose tissues (McNamara and Cronje, 2000). Serum NEFA concentrations were higher after calving in cows on the SHORT treatment than in cows on the CONV treatment. This is in contrast with Jolicoeur et al. (2014) who reported that cows with a 35-d dry period had lower concentrations of NEFA in plasma after calving compared to a 60-d dry period. This contrast may be explained by the relatively high energy content of our lactations diet that could have negated treatment effects on NEFA and lipolysis in early lactation. After calving, mobilization of fatty acids from adipose tissues to liver and mammary glands increases dramatically to compensate for the increase in the energy required for milk production and for milk fat synthesis (McNamara and Cronje, 2000). Esterification rate in adipose tissues increases in response to glucose and insulin (Rukkwamsuk et al. 1999). However, cows with higher deposited fat are less responsive to these signals, which results in higher mobilization of NEFA (Rukkwamsuk et al., 1999). Fat deposition in adipose tissue was not measured in our study. However, cows on the SHORT treatment received the close-up diet 19 d longer than the cows on the CONV treatment, which likely resulted in higher fat deposition in the SHORT treatment cows. Regardless of dry period management, PAR 3+ cows had a higher lipolysis as shown by higher NEFA concentrations compared to PAR 2 cows. In agreement, differences in NEFA concentrations between primiparous and multiparous cows have been attributed to differences in fat deposition between these parity groups (Vandehaar et al., 1999; Grummer et al., 2004). Dairy cows reach the mature size in their third parity, and before this time they are still growing (NRC, 2001). Hence, this parity effect may be explained by higher energy deposit in adipose tissue than in lean muscle tissue in the more mature PAR 3+ cows leading to increased lipolysis and associated problems in early lactation (Demment and Van Soest, 1985; Owens et al. 1993; McNamara and Cronje, 2000; NRC, 2001).

In dairy cows, the liver has a limited capacity to oxidize fatty acids or export them as very low-density lipoproteins (Van den Top et al. 1995). Fatty liver occurs when this capacity is exceeded, and the uptake of fatty acids from the blood becomes higher than the oxidation and export of fatty acids (Grummer, 1993). The liver TAG contents, therefore, reflect the concentration of NEFA in serum (Vazquezanon et al. 1994; Bobe et al. 2004), which is confirmed by our study. The higher liver TAG in cows on the SHORT treatment can, therefore, be explained by 19 d longer consumption of the close-up diet than the cows on the CONV treatment, which increased their prepartum energy intake. The normal concentration of liver TAG in dairy cows is less than 1% (on wet weight basis), however, during the first month of lactation due to mobilization of fatty acids to liver, the percentage of TAG can increase to 5%, in mild cases, or 10%, in moderate cases, or more than 10% in severe cases of fatty liver (Bobe et al. 2004). This shows that during the first week after calving, cows in our study, and especially those on the SHORT treatment experienced severe fatty acid accumulation in the liver. Accumulation of TAG in liver, such as that observed in our study may interfere with metabolic functions of liver such as reducing ureagenesis and gluconeogenesis (Strang et al. 1998), but our results do not confirm this as concentrations of urea and glucose in the plasma were not affected by dry period length during this time.

3.6 CONCLUSIONS

We hypothesized that applying a 40-d dry period management with a close-up diet during the entire dry period compared to a conventional 60-d dry period with separate far-off and close-up diet improves energy availability, decreases lipolysis in adipose tissues and ketogenesis, and also reduces accumulation of TAG in the liver in dairy cows in early lactation. In contrast, we observed that cows on the SHORT treatment had higher concentrations of NEFA and tended to have higher liver TAG immediately after calving compared to cows on the CONV treatment. These effects tended to be greater in third and higher parity cows compared to second parity cows. These differences between treatments are likely due to the higher energy intakes of the cows on the SHORT treatment compared to those on the CONV treatment between 40 and 21 d before calving, which may have led to comparatively more fat deposition in the cows on the SHORT treatment during the dry period and more lipolysis during early lactation. The higher plasma glucose concentrations after calving in cows on the SHORT treatment were likely due to comparatively lower milk yields and higher serum NEFA concentrations in these cows that contributed to glucose sparing. The difference between parity groups may be explained by relatively higher growth and lower fat deposition in second parity cows compared to older cows.

A short 40-d dry period with one close-up diet may be beneficial compared to a 60-d dry period with separate far-off and close up diets for second parity cows but not for older cows, especially when this results in excessive energy intakes during the dry period.

CHAPTER FOUR

MANUSCRIPT III

Hepatic gene expression in a 40-d dry period with single close-up diet compared to a conventional 60-d dry period

-effects on lipid and carbohydrate metabolism-

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4.1 ABSTRACT

Effects of dry period management on liver gene expression were determined in 11 second-parity (PAR2) and 15 third-parity and older (PAR3+) cows. Cows were paired based on expected calving date and randomly assigned to two treatments including a short 40-d dry period (SHORT) with only a close-up diet (containing 1.43 net energy for lactation (NEL) Mcal/kg DM), and a conventional 60-d dry period (CONV) with 39 d far-off diet (containing NEL1.28 Mcal/ kg DM) and 21 d close-up diet (containing 1.43 Mcal/kg DM NEL) in a randomized block design. Liver biopsies were obtained at week -3, 1 and 4 relative to parturition. Differentially expressed genes were analyzed by Affymetrix microarray and considered significant at an FDR-adjusted $P = 0.1$ and a log 2 fold-change greater than 1, as determined with FlexArray 1.6.1. The gene networks were determined using Ingenuity Pathway Analysis (IPA). Expression of key genes involved in β-oxidation such as ACSL1, CPT1B, CPT2, and ACADVL increased at wk 1 after calving compared to wk 3 before calving in both treatments. Other genes involved in β-oxidation, such as CD36, CYC and ACOX2, were upregulated during this period in the CONV treatment only. This probably resulted in a lower hepatic β-oxidation capacity in the SHORT treatment compared to the CONV treatment. In addition, during this period, the expression of DGAT, a key gene in the triglyceride synthesis, increased in the SHORT treatment while remained unchanged in the CONV treatment. The expression patterns of genes involved in gluconeogenesis showed a higher capacity at first week after calving compared to 3 wks before calving in the SHORT treatment compared to the CONV treatment as G6PC, a gene involved in the last step of gluconeogenesis, and were upregulated only in the SHORT treatment. However, probably due to absence of a change in the expression of PCK, a key gene in converting oxaloacetate to pyruvate, the synthesized oxaloacetate was used to increase the capacity of the

tricarboxylic acid cycle for oxidation of acetyl-CoA. In conclusion, the lower capacity of β-oxidation and higher capacity of triglyceride synthesis in the SHORT treatment resulted in accumulation of triglyceride in the liver in this group compared to the CONV treatment.

4.2 Introduction

We demonstrated that dry period management is able to influence energy status (Khazanehei, et al. 2015a), rumen fermentation and blood parameters of dairy cows (Khazanehei, et al. 2015b) during the pre- and post-calving periods. It has been shown that physiological stage (e.g. pregnancy, production), energy status and energy intake of dairy cow have large impacts on hepatic gene expression (Loor et al. 2005, Loor, 2010, McCarthy et al. 2010). A negative energy balance in early lactation causes mobilization of non-esterified fatty acids (NEFA) from adipose tissues to the liver. Mobilized long chain fatty acids (LCFA) are able to act as ligands and activate key genes involved in lipid and carbohydrate metabolism such as peroxisome proliferative-activated receptor-γ coactivator 1 α (PPARGC1A), also known as PGC-1 α, peroxisome proliferative-activated receptor-γ (PPARγ), peroxisome proliferative-activated receptor-α (PPARα) and their target genes to regulate energy homeostasis in body. The LCFAs in the liver can go through different pathways such as β-oxidation, cholesterol and triacylglyceride (TAG) biosynthesis, or are exported from the liver as very low-density lipoproteins (VLDL) (Drackley, 1999). Dairy cows have a low inherited ability to export the TAG from liver via VLDL. Therefore, during periods of negative energy balance when excess amounts of NEFA transfer to the liver, not all LCFAs can be oxidized and, consequently, accumulate as triacylglycerol (TAG) in hepatocytes and result in fatty liver (Grummer, 1993, Bobe et al. 2004). The Liver is the main organ for gluconeogenesis which provides required glucose for milk lactose, which is a determinant factor in milk production. It has been suggested that the liver should adapt to the high-demand metabolism during early lactation since it plays the central role in regulation of energy homeostasis in dairy cows. The dry period is a critical time for dairy cows and it has been shown that dry period management can impact their health and production after calving. Our hypothesis was that a short 40-d dry period with a single close-up diet stimulates expression of genes and accelerates liver adaptation to higher lipid metabolism and also more gluconeogenesis compared to a 60-d dry period with separate far-off and close-up diets. Our objective was to determine whether the 40-d dry period with single close-up diet increases the expression of genes involved in hepatic β-oxidation and gluconeogenesis compared to the conventional 60-d dry period.

4.3 Material and Methods

Animals, experiment design, treatments and liver biopsy

All procedures were conducted at the Glenlea Dairy Research Unit at the University of Manitoba, in accordance with the Canadian Council for Animal Care guidelines (Olfert et al. 1993). Treatments and experimental design are as previously described (Khazanehei et al. 2015a). Briefly, twenty-six Holstein dairy cows (11 second parity and 15 third parity and older) participated in the study. Cows were paired based on expected calving date and randomly assigned to two treatments within each pair. At the beginning of experiment, cows on the CONV and SHORT treatments had body weights of 737 ± 60 and 782 ± 69 kg (mean ± SD), respectively and body condition scores between 2.75 and 4 with average of 3.36 ± 0.11 and 3.40 ± 0.11, respectively, on a 1 to 5 scale (Edmonson et al. 1989). Treatments were a 60-d dry period (CONV) with separate 39-d far-off and 21-d close-up diets and a 40-d dry period (SHORT) with only a close-up diet. A common diet was fed to all cows after calving. On average, CP, NDF and NEL for far-off diet were 14.8, 50.2 (percentage of DM), and 1.28 (Mcal NE_L/kg DM), for close-up diet were 14.9, 38.1 (percentage of DM), and 1.39 (Mcal NE_L/kg DM) and for lactation diet were 17.7, 31.2 (percentage of DM) and 1.68 (Mcal NE_L/kg DM) respectively. The compositions of diets are shown on Table 4.1. Actual dry period lengths for SHORT treatment were 34 ± 6 d (mean ± SD) with a minimum of 23 d and maximum of 44 d and for CONV they were 59 ± 4 d (mean ± SD) with a minimum of 53 d and max of 68 d. The variations in dry period length were due to earlier or later calving than the expected date.

Liver biopsies were collected according to the University of Manitoba Veterinary Services guidelines at -3, +1 and +4 wk, relative to calving. Briefly, the animal was restrained in squeeze chute and Anafen (as an anti-inflammatory agent) and Excenel RTU (as an antibiotic agent) were administrated according to the label directions. The biopsy location was determined by hypothetical line drawn from the hook bone down to the elbow and between the 11th and 12th rib area. An area of 15 cm square was clipped, sterilized, and anesthetized by 4 cc lidocine. Using the scalpel, a 1.5 to 2 cm horizontal incision was made and a liver sample was taken using biopsy trocar. The samples were then placed in collection vials containing RNase inhibitor and kept in – 80 °C.

Table 4.1. Nutrient composition of far-off, close-up, and lactation diets (mean ± SD)

Item	Nutrient Composition[1]		
	Far-off	Close-up	Lactation
Crude protein, %	14.8±0.4	14.9±1.9	17.7±1.3
Neutral detergent fiber, %	50.2±1.1	38.1±7.4	31.2±5.6
Acid detergent fiber, %	32.2±1.2	23.2±5.1	19.3±3.3
NFC[2], %	26.2±1.6	36.2±6.1	39.4±3.9
Crude fat, %	1.7±0.3	3.3±1.0	4.2±0.7
Calcium, %	0.6±0.1	0.8±0.2	1.0±0.1
Phosphorous, %	0.3±0.0	0.4±0.1	0.4±0.0
Magnesium, %	0.2±0.0	0.4±0.1	0.4±0.1
Potassium, %	2.1±0.1	1.7±0.4	1.5±0.1
Ash, %	7.1±1.0	7.8±0.8	7.5±0.7
Net Energy for lactation, Mcal/kg	1.28	1.39	1.68

[1] Far-off diet ingredients (% of DM): timothy hay, 89.5; soybean-meal, 8.2; mineral, 2.2; forage to concentrate ratio, 90 to 10; Close-up diet ingredients (% of DM): timothy hay, 43.0; barley silage, 25.3; Transrite, 31.6; forage to concentrate ratio, 68 to 32; Lactation diet ingredients (% of DM): mixed hay, 20.4; corn silage, 32.2; roasted soybeans, 4.4; Energy supplement, 39.5; protein supplement, 2.2; forage to ratio concentrate ratio, 43 to 47.

[2] Non fiber carbohydrate= 100 - (NDF% + CP % + Crude fat % + Ash %)

RNA extractions, quality check, and sample preparations

High quality RNA from liver tissue samples was extracted using TRIzol Reagent (Invitrogen life technologies, P/N 15596-018), RNeasy Mini Kit (QIAGEN, P/N 74104) and RNase-Free DNase Set (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Briefly, the liver tissue samples were thawed at ambient room temperature, weighted (50 mg) and placed in 1 ml of ice-cold TRIzol reagent. Subsequently, samples were homogenized using a bead beater (Mini-Beadbeater, BioSpec products) followed by centrifuging at 12000 g for 10 minutes to remove the fat. The RNA was then separated by adding chloroform to the mixture. The RNeasy kit was used to purify RNA from remaining animal cells and tissues, crude RNA preps and enzymatic reactions. In order to obtain high quality RNA suitable to use in GeneChip expression probe arrays, the DNA was removed from the mixture using RNase-Free DNase Set kit and was placed in -80 °C for future use. The concentration of RNA was quantified before freezing using Nanodrop (2000, Thermo Scientific, USA). The A_{260}/A_{280} ratio of measured RNA ranged from 1.8 to 2.1, which is an indication of RNA that the RNA was successfully purified. .

Microarray

The GeneChip Bovine Genome Array (Affymetrix. P/N 900563) was acquired to monitor almost 23000 bovine transcripts. Due to limited resources, we were not able to use all samples for GeneChip expression analysis. Therefore, samples within each time-point and treatment group were categorized and pooled according to their TAG content (mild, moderate and sever). In total 54 samples, 27 from each treatment, 9 from each sampling time were used, and then 3 samples from each time point (including mild, moderate and sever) were pooled together to make 18 pool-samples including 3 replicates for each time point. All the steps of hybridization, wash, stain and scan were followed according to Affymetrix GeneChip protocols. The quality assessment of the array images was done using GeneChip Operating System software (Affymetrix GeneChip protocol).

Real-time RT-PCR

The relative expressions of 13 candidate genes were measured using StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) with SYBR Green technology to verify the microarray results. The isolated RNA used in microarray analysis was also utilized in a relative expression study of 13 candidate genes. The first strand cDNA synthesis was carried out according to the SuperScript III Reverse Transcriptase kit protocol (Invitrogen, Carlsbad, CA),and RNA complementary to the cDNA was removed using E. Coli RNase H (Invitrogen, Carlsbad, CA). The cDNA was then diluted to 5 ng/µl and stored in -80 °C until used in the polymerase chain reaction. A threefold standard curve was made using pooled cDNA from all samples. All samples, a six-point relative standard curve and the non-template control were run in triplicate. Each reaction mixture in the real-time PCR contained 7.5 µl of 1× Power SYBR Green master mix (Applied Biosystems, Foster City, CA), 0.4 µl of each 10 µM forward and reverse primers of the target gene, 4 µl of cDNA and 11.3 µl of DNase/RNase-free water in a total volume of 20 µl in a MicroAmp Fast Optical 96-Well reaction plate (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the following conditions: 10 min at 95 °C, 40 cycle of 15 s at 95 °C and 1 min at 59.9 °C. Melting curve analysis was performed after amplification, and the fluorescent signals were continuously collected in 15 s increments up to 95 °C. All 13 pairs of genes of interest including a reference gene were designed using Primer Express Software V3.0 (Applied Biosystems, Foster City, CA). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, Iowa, USA). The Sequences were used are listed in Table 4.2.

Table 4.2. Primers used for the relative quantification of target cDNAs

Gene	Primer	Sequence	Length (bp)	Reference
ACSL1	ACSL1-F	GCTTGTTGATGTGGAAGAAATGAA	24	This paper
	ACSL1-R	GCAACCAGCCGTCTTTATCC	20	This paper
ACTB	ACTB-F	CGCAGAAAACGAGATGAGATTG	22	This paper
	ACTB-R	CTGTCACCTTCACCGTTCCA	20	This paper
ANGPTL4	ANGPTL4-F	AGGAAGAGGCTGCCCAAGAT	20	This paper
	ANGPTL4-R	CCCTCTCTCCCTCTTCAAACAG	22	This paper
APOA1	APOA1-F	CCGTGTATGTGGAAGCAATCA	21	This paper
	APOA1-R	CCCAGTTGTCCAGGAGTTTCA	21	This paper
APOA4	APOA4-F	CCACCGTGATTTGGGACTACTT	22	This paper
	APOA4-R	CGTCCGTGTAGGTGCTCACTT	21	This paper
CPT1B	CPT1B-F	CTACCACGGTTGGATGTTTGAG	22	This paper
	CPT1B-R	CAGCAAGTGTTCCACGGATTC	21	This paper
CYCS	CYCS-F	GAAGTGTGCCCAGTGCCATA	20	This paper
	CYCS-R	TGACCTGTCTTTCGTCCAAACA	22	This paper
ELOVL5	ELOVL5-F	GCCCATTTTGCTTTCCACTTAG	22	This paper
	ELOVL5-R	TCCCCGCACTGCTCATCTAT	20	This paper
GHR	GHR-F	GCCAGCAGCCCAGTGTTATC	20	This paper
	GHR-R	TGTCGCTTACCTGGGCATAAA	21	This paper
G6PC	G6PC-F	TGCTCATTTTCCCCATCAAGTT	22	This paper
	G6PC-R	GACCCCCAGCCACTTTAACA	20	This paper
IGF-1	IGF-1-F	CCATCTCCCTGGATTTCTTTTTG	23	This paper
	IGF-1-R	AGAGATGCGAGGAGGATGTGA	21	This paper
LDHA	LDHA-F	CAGCTCGCTTCCGTTATCTCA	21	This paper
	LDHA-R	TCAGGGAGACACCAGCAACA	20	This paper
PC	PC-F	TGCCCACAGCTTCAACAAAC	20	This paper
	PC-R	TCGCAGAACTTGAAGACCACAT	22	This paper
PPARGC1A	PPARGC1A-F	AAAAGCCACAAAGACGTCCG	20	This paper
	PPARGC1A-R	TCTGCTGCTGTTCCGGTTCT	20	This paper

Statistical analyses

Data from each array were imported into FlexArray software package (1.6.1.1) for statistical analysis. All data were normalized for background noise by the RMA algorithm, and then statistically analyzed by the EB (Wright and Simon) method. The FDR correction was applied using the Benjamin Hochberg algorithm (Benjamini and Hochberg, 1995). A cut-off point was selected, such that genes with a corrected *P* value smaller or equal to 0.1 and 2-fold log change greater than 1 were picked for further analyses.

The relative gene expression was calculated using relative expression software tool (REST) (Pfaffl et al. 2002). The software considers the differences in reaction efficiencies between actin beta (ACTB) as reference gene and the gene of interest. It also takes the reference gene normalization into account and uses randomization methods to overcome the difficulties of statistical analyses.

4.4 Results

In total, 9 comparisons between the time points within and between the treatments were made to determine the effect of dry period management on the liver gene expression throughout the transition period. The wk -3 vs. wk +1, wk -3 vs. wk +4, wk +1 vs. wk +4 within each treatment. Cross comparisons of each sampling time between treatments were also made. After applying the cut-off point of adjusted $P \leq 0.1$ and log 2-fold ≥ 1 for each comparison using FlexArray software, there was no difference in gene expression between the treatments at wk 3 pre-calving and at wk 1 and 4 post-calving. The comparisons between sampling times within the SHORT treatment revealed that 237 genes were differentially expressed more than 2-fold in wk 1 post-calving compared to wk 3 pre-calving. When the wk 3 pre-calving was compared with the wk 4 post-calving, there were 8 genes that were expressed differently. Also, comparisons between wk 1 and 4 post-calving showed that there were 84 genes that were expressed more than 2-fold, among which, 64 genes were down-regulated and 20 genes were upregulated. The comparisons between weeks within the CONV treatment showed that there were 174 genes that were expressed more than 2 fold differently when the wk 1 post-calving was compared to the wk 3 pre-calving. Among these, 67 genes were down-regulated and 107 genes were upregulated. In addition, there were 18 genes that were down-regulated and 36 genes upregulated when wk 4 post-calving was compared to the wk 3 pre-calving. Also, 2 genes were down-regulated at wk 4 compared to wk 1 post-calving.

Gene Network Analysis within treatments across transition period

Lipid metabolism

The major changes in liver gene expression occurred at wk 1 after calving compared to 3 wks before calving (Tables 4.3 and 4.6). The pathway analyses of differentially expressed genes revealed that genes involved in lipid synthesis and transport, such as APOA4 (🡅), APOA1 (🡅), CREB3L3 (🡅), APOC2 (🡅), ASCL1 (🡅), PPARGC1A (🡅), GHR(🡇), IGF1 (🡇), IGFBP2 (🡅), IGFBP3 (🡇), ELOVL5 (🡅), PLIN2 (🡅), and THRSP (🡇) were expressed differently in both treatments at wk 1 after calving compared to the 3 wk before calving. In addition within this network, there were 4 genes including, FADS2 (🡇), IGFBP1 (🡅), LPIN1 (🡅), ME1 (🡅), and SCD (🡇) that were expressed differently only in the CONV treatment and 4 genes including CYP2E1 (🡇), DGAT (🡅), and FDPS (🡅) were expressed only in the SHORT treatment. The pathway analyses also revealed that genes involved in lipid oxidation pathways, including APOA1 (🡅), ACADVL (🡅), ADIPOR2 (🡅), ACSL1 (🡅), CPT1B (🡅), CPT2 (🡅), MLYCD

(↑), PPARGC1A (↑) and SLC22A5 (↑) expressed differently in both treatments when wk 1 postpartum was compared to wk 3 prepartum (Tables 4.3 and 4.6). During this time, 6 genes including ACOX2 (↑), LPIN1 (↑), CYC1 (↑), CROT (↑), CD36 (↑) and SCD (↓) expressed differently only in the CONV treatment, and 2 genes including CYP2E1 (↓) and CRAT (↑) expressed differently only in the SHORT treatment (Tables 4.3 and 4.6). Comparisons between wk 1 and 4 showed that genes involved in lipid metabolism expressed differentially only in the SHORT treatment, but remained unchanged in the CONV treatment. In this regard, genes involved in lipid synthesis including THRSP (↑), SCD (↑), FADS2 (↑), APOA1 (↓), CREB3L3 (↓), ELOVL5 (↓), IGFBP1 (↓) and PPARGC1A (↓) and genes involved in lipid oxidation including APOA1 (↓), CRAT (↓), CROT (↓), PPARGC1A (↓), and SCD (↑) expressed differently only in the SHORT treatment (Tables 4.4 and 4.7). Comparisons between wk 4 postpartum and wk 3 prepartum showed that, APOA1 (↑), APOA4 (↑), GHR (↓), ASCL1 (↑), IGF1 (↓), IGFBP3 (↓) and PPARGC1A (↓ in the SHORT treatment; ↑ in the CONV treatment) expressed differently in both treatments (Tables 4.5 and 4.8). In the CONV treatment, 3 genes involved in lipid synthesis including ELOVL5 (↑) and IGFBP2 (↑), 1 gene involved in lipid secretion including APOC2 (↑) and 4 genes involved in lipid oxidation including ACADVL (↑), CD36 (↑), CPT1B (↑) and SLC22A5 (↑) were expressed differently while they remained unchanged in the SHORT treatment (Tables 4.5 and 4.8).

Table 4.3 Comparison of liver gene expression between wk +1 and -3 within the SHORT treatment

Gene	Prediction based on expression direction	Microarray[1] (Fold change)	Real-time[2] (Fold change)	Function
-----------------------------------Lipid synthesis--------------------------------				
APOA1	Increased	UP (2.831)	UP (17.119)	Increases
APOA4	Increased	UP (4.184)	UP (18.758)	Increases
APOC2	Increased	UP (1.294)	-	Increases
ASCL1	Increased	UP (1.214)	UP (2.727)	Increases
CREB3L3	Increased	UP (1.441)	-	Increases
DGAT	Increased	UP (0.380)	-	Increases
PPARGC1A	Increased	UP (1.036)	1.003	Increases
FDPS	Increased	UP (1.025)	-	Increases
IGFBP1	No change	1.153	-	Increases
IGFBP2	Increased	UP (1.489)	-	Increases
IGFBP3	Decreased	DOWN (-0.758)	-	Increases
ELOVL5	Decreased	UP (1.089)	UP (1.673)	Decreases
GHR	Increased	DOWN (-1.126)	DOWN (0.325)	Decreases
CYP2E1	Decreased	DOWN (-1.243)	-	Increases
ME1	No Change	1.856	-	Increases
IGF1	Decreased	DOWN (-1.710)	DOWN (0.20)	Increases
THRSP	Decreased	DOWN (-2.229)	-	Increases
CYP26A1	Decreased	DOWN (-2.702)	-	Increases
PLIN2	Increased	UP (1.475)	-	Increases
SREBP1	No change	-0.047	-	Increases
-----------------------------------Lipid secretion--------------------------------				
APOC2	Increased	UP (1.294)	-	Increases
APOA1	Increased	UP (2.831)	UP (17.119)	Increases
APOA4	Increased	UP (4.184)	UP (18.758)	Increases
ABCA1	No change	-0.391	-	Increases
PLIN2	Increased	UP (1.475)	-	Increases
SCD	No change	-0.816	-	Increases
-----------------------------------Lipid oxidation--------------------------------				
APOA1	Increased	UP (2.831)	UP (17.119)	Increases
ACADVL	Increased	UP (0.931)	-	Increases
ADIPOR2	Increased	UP (1.329)	-	Increases
ACOX2	No change	0.312	-	Increases
ACSL1	Increased	UP (1.214)	-	Increases
CD36	No change	0.429	-	Increases
CRAT	Increased	UP (0.492)	-	Increases
CROT	No change	0.621	-	Increases
PPARGC1A	Increased	UP (1.036)	1.003	Increases
AOX1	Decreased	DOWN (-1.026)	-	Increases
CYP2E1	Decreased	DOWN (-1.243)	-	Increases
CYP2U1	Decreased	DOWN (-1.266)	-	Increases
CPT1B	Increased	UP (2.265)	UP (5.181)	Increases

CPT2	Increased	UP (0.458)	-	Increases
ELOVL5	Increased	UP (1.089)	UP (1.673)	Increases
LPIN1	No change	-0.172	-	Increases
MLYCD	Increased	UP (0.896)	-	Increases
SLC22A5	Increased	UP (1.275)	-	Increases
CYC1	No change	Up (0.554)	1.335	Increases
------------------------------------ Hepatokines --------------------------------				
ANGPTL4	Increased	UP (2.086)	UP (9.241)	Increases

[1] When genes were significantly up- or down regulated it was shown by UP or DOWN, respectively.

[2] When expression of genes was not determined by real-time PCR it was shown by (-).

Table 4.4 Comparison of liver gene expression between wk +4 and +1 within the SHORT treatment

Gene	Prediction based on expression direction	Microarray [1] (Fold change)	Real-time[2] (Fold change)	Function
------------------------------------Lipid synthesis--------------------------------				
THRSP	Increased	UP (2.059)	-	Increases
SCD	Increased	UP (1.631)	-	Increases
CYP26A1	Increased	UP (1.381)	-	Increases
ABCG8	Increased	UP (1.115)	-	Increases
DGAT	No change	-0.043	-	Increases
FADS2	Increased	UP (1.007)	-	Increases
APOA1	Decreased	DOWN (-1.038)	DOWN (0.148)	Increase
APOA4	Decreased	DOWN (-1.905)	0.578	Increase
CREB3L3	Decreased	DOWN (-1.282)	-	Increase
GHR	No change	0.136	0.729	Decreases
ELOVL5	Increased	-0.163	DOWN (0.711)	Decreases
ASCL1	Increased	-0.475	UP (2.727)	Increases
IGF1	No change	0.879	1.3.43	Increases
IGFBP1	Decreased	DOWN (-1.471)	-	Increases
IGFBP2	No change	-0.785	-	Increases
IGFBP3	No change	0.063	-	Increases
ME1	No change	-1.313	-	Increases
PLIN2	No change	-923	-	Increases
PPARGC1A	Decreased	-0.466	DOWN (0.355)	Increases
SREBP1	No change	-0.053	-	Increases
------------------------------------Lipid secretion--------------------------------				
APOC2	Decreased	DOWN (-0.616)	-	Increase
APOA1	Decreased	DOWN (-1.038)	DOWN (0.148)	Increase
APOA4	Decreased	DOWN (-1.905)	0.578	Increase
APOA5	No change	-0.410	-	
ABCA1	No change	-0.299	-	Increases
PLIN2	No change	-923	-	Increases
SCD	Increased	UP (1.631)	-	Increases
------------------------------------Lipid oxidation--------------------------------				
ACADVL	Decreased	DOWN (-0.677)	-	Increases
ADIPOR2	No change	-0.547	-	Increases
APOA1	Decreased	DOWN (-1.038)	DOWN (0.148)	Increases
ACOX2	No change	0.252	-	Increases
CD36	No change	0.086	-	Increases
CPT1B	Decreased	DOWN (-1.840)	DOWN (0.285)	Increases
CPT2	No change	-0.293	-	Increases
CRAT		DOWN (-0.401)	-	
CROT		DOWN (-0.593)	-	
SCD	Decreased	UP (1.631)	-	Decreases
CYP2C9	Increased	UP (1.438)	-	Increases
CYP2C19	Increased	UP (1.099)	-	Increases

CYP2U1		0.489	-	Increases
CYC1	No change	-0.381	0.571	Increases
PPARGC1A	Decreased	-0.466	DOWN (0.355)	Increases
SLC22A5	No change	-0.738	-	Increases
		------------------------------------ Hepatokines ---------------------------------		
ANGPTL4	Decreased	-1.713	DOWN (0.125)	Increases

[1] When genes were significantly up- or down regulated it was shown by UP or DOWN, respectively.

[2] When expression of genes was not determined by real-time PCR it was shown by (-).

Table 4.5 Comparison of liver gene expression between wk +4 and -3 within the SHORT treatment

Gene	Prediction based on expression direction	Microarray [1] (Fold change)	Real-time[2] (Fold change)	Function
Lipid synthesis				
APOA1	Increased	UP (1.793)	UP (2.532)	Increases
APOA4	Increased	2.279	UP (10.838)	Increases
DGAT	No change	-0.033	-	Increases
ELOVL5	No change	0.926	1.189	Decreases
FABP5	Increased	UP (1.599)	-	Increases
GHR	Decreases	-0.714	DOWN (0.237)	Decreases
ASCL1	Increased	UP (0.739)	UP (1.227)	Increases
IGF1	Decreased	-0.831	DOWN (0.269)	Increases
IGFBP1	No change	0.059	-	Increases
IGFBP2	No change	0.704	-	Increases
IGFBP3	Decreased	DOWN (-0.695)	-	Increases
ME1	No change	0.543	-	Increases
PPARGC1A	Decreased	0.265	DOWN (0.356)	Increases
PLIN2	No change	0.552	-	Increases
SREBP1	No change	-0.100	-	Increases
Lipid secretion				
APOC2	No change	0.676	-	Increases
APOA1	Increased	UP (1.793)	UP (2.532)	Increases
APOA4	Increased	2.279	UP (10.838)	Increases
APOA5	No change	0.174	-	Increases
ABCA1	No change	-0.338	-	Increases
PLIN2	No change	0.552	-	Increases
SCD	No change	0.815	-	Increases
Lipid oxidation				
ACADVL	No change	0.253	-	Increases
ADIPOR2	No change	0.782	-	Increases
APOA1	Increased	UP (1.793)	UP (2.532)	Increases
ACOX2	No change	0.648	-	Increases
CD36	No change	0.515	-	Increases
CPT1B	No change	0.424	1.475	Increases
CPT2	No change	0.165	-	Increases
CRAT	No change	0.092	-	Increases
CROT	No change	0.029	-	Increases
CYC1	No change	0.174	0.763	Increases
PPARGC1A	Decreased	0.265	DOWN (0.356)	Increases
SLC22A5	No change	0.537	-	Increases
Hepatokines				
ANGPTL4	No change	0.372	1.156	Increases

[1] When genes were significantly up- or down regulated it was shown by UP or DOWN, respectively.

[2] When expression of genes was not determined by real-time PCR it was shown by (-).

Table 4.6 Comparison of liver gene expression between wk +1 and -3 within the CONV treatment

Gene	Prediction based on expression direction	Microarray[1] (Fold change)	Real-time[2] (Fold change)	Function
------------------------------------Lipid synthesis--------------------------------				
APOA1	Increased	UP (2.647)	UP (6.079)	Increases
APOA4	Increased	UP (4.122)	UP (11.802)	Increases
APOC2	Increased	UP (1.078)	-	Increases
ASCL1	Increased	UP (1.174)	UP (3.145)	Increases
CREB3L3	Increased	UP (1.365)	-	Increases
DGAT	No change	-0.061	-	Increases
ELOVL5	Decreased	UP (1.160)	UP (3.143)	Decreases
FADS2	Decreased	DOWN (-1.648)	-	Decreases
GHR	Increased	DOWN (-1.084)	DOWN (0.399)	Decreases
IGFBP1	Increased	UP (1.781)	-	Increases
IGFBP2	Increases	UP (1.496)	-	Increases
IGFBP3	Decreased	DOWN (-0.908)	-	Increases
IGF1	Decreased	DOWN (-2.066)	DOWN (0.245)	Increases
LPIN1	Increased	UP (1.256)	-	Increases
ME1	Increased	UP (1.297)	-	Increases
PPARGC1A	Increased	UP (1.036)	UP (1.399)	Increases
PLIN2	Increased	UP (1.392)	-	Increases
SCD	Decreased	DOWN (-1.787)	-	Increases
THRSP	Decreased	DOWN (-2.433)	-	Increases
SREBP1	No change	-0.228	-	Increases
------------------------------------Lipid secretion--------------------------------				
APOC2	Increased	UP (1.078)	-	Increases
APOA1	Increased	UP (2.647)	UP (6.079)	Increases
APOA4	Increased	UP (4.122)	UP (11.802)	Increases
APOA5	Increased	UP (0.601)	-	Increases
ABCA1	Decreased	DOWN (-0.474)	-	Increases
PLIN2	Increased	UP (1.392)	-	Increases
SCD	Decreased	DOWN (-1.787)	-	Increases
------------------------------------Lipid oxidation--------------------------------				
ACADVL	Increased	UP (0.838)	-	Increases
ADIPOR2	Increased	UP (1.029)	-	Increases
APOA1	Increased	UP (2.647)	UP (6.079)	Increases
ACOX2	Increases	UP (0.470)	-	Increases
LPIN1	Increased	UP (1.258)	-	Increases
ACSL1	Increased	UP (1.174)	UP (3.145)	Increases
CD36	Increased	UP (0.539)	-	Increases
CYC1	Increased	0.343	UP (1.867)	Increases
CRAT	No change	0.296	-	Increases
CROT	Increases	UP (0.635)	-	Increases
PPARGC1A	Increased	UP (1.038)	1.399	Increases
CPT1B	Increased	UP (2.615)	UP (7.414)	Increases

CPT2	Increased	UP (0.558)	-	Increases
ELOVL5	Increased	UP (1.160)	-	Increases
MLYCD	Increased	UP (0.876)	-	Increases
SCD	Increased	DOWN (-1.787)	-	Decreases
SLC22A5	Increased	UP (1.273)	-	Increases
------------------------------------ Hepatokines---------------------------------				
ANGPTL4	Increased	UP (1.677)	5.204	Increases

[1] When genes were significantly up- or down regulated it was shown by UP or DOWN, respectively.

[2] When expression of genes was not determined by real-time PCR it was shown by (-).

Table 4.7 Comparison of liver gene expression between wk +4 and +1 within the CONV treatment

Gene	Prediction based on expression direction	Microarray [1] (Fold change)	Real-time[2] (Fold change)	Function
	------------------------------------Lipid synthesis---------------------------------			
GHR	Decreased	0.169	UP (2.505)	Decreases
ACSL1	Decreased	-0.636	DOWN (0.60)	Increases
APOA1	No change	-0.515	0.892	Increases
APOA4	Decreased	-1.223	DOWN (0.144)	Increases
CREB3L3	No change	-0.692	-	Increases
DGAT	No change	0.164	-	Increases
ELOVL5	No change	-0.596	0.906	Decreases
FADS2	No change	0.998	-	Increases
IGF-1	Increased	0.680	UP (2.477)	Increases
IGFBP1	No change	-1.134	-	Increases
IGFBP2	No change	-0.193	-	Increases
IGFBP3	No change	0.296	-	Increases
ME1	No change	-1.217	-	Increases
PLIN2	No change	-0.923	-	Increases
PPARGC1A	No change	-0.379	1.227	Increases
SCD	No change	0.827	-	Increases
SREBP1	No change	0.276	-	Increases
THRSP	No change	1.091	-	Increases
	------------------------------------Lipid secretion--------------------------------			
APOC2	No change	-0.284		Increases
APOA1	No change	-0.515	0.892	Increases
APOA4	Decreased	-1.223	DOWN (0.144)	Increases
APOA5	No change	-0.127	-	Increases
ABCA1	No change	0.091	-	Increases
PLIN2	No change	-0.923	-	Increases
SCD	No change	0.827	-	Increases
	------------------------------------Lipid oxidation---------------------------------			
ACADVL	Decreased	-0.382	-	Increases
ACSL1	Decreased	-0.636	DOWN (0.60)	Increases
ACOX2	No change	-0.188	-	Increases
ADIPOR2	No change	-0.558	-	Increases
APOA1	No change	-0.515	0.892	Increases
CD36	No change	0.149	-	Increases
CPT1B	Decreased	-1.460	DOWN (0.283)	Increases
CPT2	No change	-0.246	-	Increases
CRAT	No change	-0.106	-	Increases
CROT	No change	-0.507	-	Increases
CYC1	Decreased	-0.084	DOWN (0.589)	Increases
CYP2U1	No change	0.303	-	Increases
PPARGC1A	No change	-0.379	1.227	Increases
SLC22A5	No change	-0.298	-	Increases

------------------------------------ Hepatokines ---------------------------------				
ANGPTL4	DOWN	-0.549	DOWN (0.621)	Increases

[1] When genes were significantly up- or down regulated it was shown by UP or DOWN, respectively.

[2] When expression of genes was not determined by real-time PCR it was shown by (-).

Table 4.8 Comparison of liver gene expression between wk +4 and -3 within the CONV treatment

Gene	Prediction based on expression direction	Microarray[1] (Fold change)	Real-time[2] (Fold change)	Function
Lipid synthesis				
APOA1	Increased	UP (2.133)	UP (5.424)	Increases
APOA4	Increased	UP (2.894)	1.705	Increases
ACSL1	Increased	0.538	UP (1.9)	Increases
MYC	Increased	UP (1.122)	-	Increases
DGAT	No change	0.253	-	Increases
ELOVL5	Decreased	0.563	UP (2.847)	Decreases
IGF1	Decreased	DOWN (-1.386)	DOWN (0.606)	Increases
IGFBP1	No change	0.647	-	Increases
IGFBP2	Increased	UP (1.304)	-	Increases
IGFBP3	Decreased	DOWN (-0.612)	-	Increases
FABP5	Increased	UP (2.016)	-	Increases
GHR	No change	0.017	0.99	Decreases
ME1	No change	0.080	-	Increases
PLIN2	No change	0.469	-	Increases
PPARGC1A	Increased	0.557	UP (1.716)	Increases
SREBP1	No change	0.048	-	Increases
Lipid secretion				
APOC2	Increased	UP (0.794)	-	Increases
APOA1	Increased	UP (2.133)	UP (5.424)	Increases
APOA4	Increased	UP (2.894)	1.705	Increases
APOA5	No change	0.474	-	Increases
ABCA1	No change	-0.382	-	Increases
PLIN2	No change	0.469	-	Increases
SCD	No change	-0.960	-	Increases
Lipid oxidation				
ACADVL	Increased	UP (0.456)	-	Increases
ACOX2	Increased	UP (0.282)	-	Increases
ADIPOR2	No change	0.471	-	Increases
APOA1	Increased	UP (2.133)	UP (5.424)	Increases
ACSL1	Increased	0.538	UP (1.900)	Increases
CD36	Increased	UP (0.688)	-	Increases
CPT1B	Increased	UP (1.155)	UP (2.101)	Increases
CPT2	No change	0.312	-	
CRAT	No change	0.190	-	Increases
CROT	No change	0.128	-	Increases
CYC1	No change	0.259	1.1	Increases
CYP2U1	Decreased	DOWN (-0.807)	-	Increases
PPARGC1A	Increased	0.557	UP (1.716)	Increases
SLC22A5	Increased	UP (0.975)	-	Increases
Hepatokines				
ANGPTL4	Increased	UP (1.128)	UP (3.229)	Increases

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[1] When genes were significantly up- or down regulated it was shown by UP or DOWN, respectively.

[2] When expression of genes was not determined by real-time PCR it was shown by (-).

Carbohydrate metabolism

The greatest change in expression of genes involved in carbohydrate metabolism occurred at wk 1 after calving compared to wk 3 before calving (Tables 4.9 and 4.12). During this time, 6 genes involved in gluconeogenesis, including ADIPOR2 (↑), ALDOA (↑), FBP1 (↑), CREB3L3 (↑), PC (↑) and PPARGC1A (↑) expressed differently in both treatments, while genes GNMT (↑) and G6PC (↑) were only expressed differentially in the SHORT treatment (Tables 4.9 and 4.12). In addition, genes involved in glycolysis, including ALDOA (↑), GK (↑), LDHA (↑) and BRP44L (↑) expressed differently in both treatments (Tables 4.9 and 4.12). The PPP1R3C (↓), IGF1 (↓), GYS2 (↑) and GK (↑) genes, which are involved in glycogenesis, expressed differently in both treatments. Comparisons between wk 4 and wk 1 of postpartum showed that genes involved in gluconeogenesis, such as PC (↓), PCK2 (↓), SCD (↑), GNMT (↓), CREB3L3 (↓), and IGFBP1 (↓) expressed differently only in the SHORT treatment (Tables 4.10). In addition, the GK (↓), IGFBP1 (↓), PPP1R3C (↑) and UGP2 (↑) genes that are involved in glycolysis and glycogenesis expressed differently only in the SHORT treatment (Table 4.10). Gene network analyses showed that genes involved in carbohydrate metabolism, including PPARGC1A (↓), BRP44L (↑), LDHA (↑) and expressed differently in both treatments when wk 4 postpartum compared with wk 3 prepartum (Tables 4.11 and 4.14). The PC (↑) and IGF1 (↓) were expressed differently only in the CONV treatment and G6PC was the only gene that expressed differently in the SHORT treatment (Tables 4.11 and 4.14). Other genes involved in carbohydrate metabolism such as ADIPOR1, ALDOA, GK, PFKL, GYS2, IGFBP1, PP1R3C and UGP2 remained unchanged in both treatments when 4 wk postpartum was compared with 3 wk prepartum (Tables 4.11 and 4.14).

Table 4.9. Comparison of liver gene expression between wk +1 and -3 within the SHORT treatment

Gene	Prediction based on expression direction	Microarray [1] (Fold change)	Real-time[2] (Fold change)	Function
------------------------------------Gluconeogenesis--------------------------------				
ALDOA	Increased	UP (0.710)	-	Increases
FBP1	Increased	UP (0.679)	-	Increases
PC	Increased	UP (2.405)	UP (6.521)	Increases
G6PC	Increased	UP (1.041)	1.208	Increases
PCK1	No change	0.329	-	Increases
PCK2	No change	0.290	-	Increases
PPARGC1A	Increased	UP (1.036)	1.003	Increases
CREB3L3	Increased	UP (1.441)	-	Increases
GNMT	Increased	UP (1.091)	-	Increases
ADIPOR2	Decreased	UP (1.329)	-	Decreases
------------------------------------Glycolysis--------------------------------				
ALDOA	Increased	UP (0.710)	-	Increases
GK	Increased	UP (1.977)	-	Increases
LDHA	Increased	UP (2.079)	UP (3.391)	Increases
PFKL	No change	0.113	-	Increases
BRP44L	Increased	UP (1.500)	-	Increases
------------------------------------Glycogenesis--------------------------------				
GK	Increased	UP (1.977)	-	Increases
GYS2	Increased	UP (0.713)	-	Increases
IGF1	Decreased	DOWN (-1.710)	DOWN (0.2)	Increases
IGFBP1	No change	1.530	-	Increases
PPP1R3C	Decreased	DOWN (-2.033)	-	Increases
UGP2	Increased	UP (0.655)	-	Increases

[1] When genes were significantly up- or down regulated it was shown by UP or DOWN, respectively.

[2] When expression of genes was not determined by real-time PCR it was shown by (-).

Table 4.10. Comparison of liver gene expression between wk +4 and +1 within the SHORT treatment

Gene	Prediction based on expression direction	Microarray [1] (Fold change)	Real-time[2] (Fold change)	Function
		------------------------------------Gluconeogenesis--------------------------------		
ADIPOR2	No change	-0.547	-	Decreases
ALDOA	No change	-0.367	-	Increases
FBP1	No change	-0.487	-	Increases
PCK1	No change	0.053	-	Increases
PCK2	Decreased	DOWN (-0.481)	-	Increases
SCD	Increased	UP (1.631)	-	Increases
GNMT	Decreased	DOWN (-1.258)	-	Increases
CREB3L3	Decreased	DOWN (-1.282)	-	Increases
IGFBP1	Decreased	DOWN (-1.471)	-	Increases
PC	Decreased	DOWN (-1.594)	DOWN (0.241)	Increases
PPARGC1A	Decreased	-0.466	DOWN (0.355)	Increases
G6PC	Decreased	-0.219	DOWN (0.238)	Increases
		------------------------------------Glycolysis--------------------------------		
ALDOA	No change	-0.367	-	Increases
GK	Decreased	DOWN (-1.394)	-	Increases
LDHA	Decreased	DOWN (-0.938)	DOWN (0.402)	Increases
PFKL	No change	0.113	-	Increases
BRP44L	No change	-0.405	-	Increases
		------------------------------------Glycogenesis--------------------------------		
GK	Decreased	DOWN (-1.394)	-	Increases
GYS2	No change	-0.770	-	Increases
IGFBP1	Decreased	DOWN (-1.471)	-	Increases
IGF1	No change	0.879	1.343	Increases
PPP1R3C	Increased	UP (1.253)	-	Increases
UGP2	Increased	UP (0.655)	-	Increases

[1] When genes were significantly up- or down regulated it was shown by UP or DOWN, respectively.

[2] When expression of genes was not determined by real-time PCR it was shown by (-).

Table 4.11. Comparison of liver gene expression between wk +4 and -3 within the SHORT treatment

Gene	Prediction based on expression direction	Microarray [1] (Fold change)	Real-time[2] (Fold change)	Function
------------Gluconeogenesis------------				
ADIPOR2	No change	0.782	-	Decreases
ALDOA	No change	0.343	-	Increases
PC	Increased	0.811	UP (1.569)	Increases
PPARGC1A	Decreased	0.265	DOWN (0.356)	Increases
G6PC	Decreased	0.527	DOWN (0.288)	Increases
------------Glycolysis------------				
ALDOA	No change	0.343	-	Increases
BRP44L	Increased	UP (1.091)	-	Increases
GK	No change	0.582	-	Increases
PFKL	No change	-0.089	-	Increases
LDHA	Increased	UP (1-140)	UP (1.364)	Increases
------------Glycogenesis------------				
G6PC	Increased	0.527	DOWN (0.288)	Decreases
GK	No change	0.582	-	Increases
GYS2	No change	-0.057	-	Increases
IGF1	Decreased	-0.831	DOWN (0.269)	Increases
IGFBP1	No change	0.704	-	Increases
PPP1R3C	No change	-0.701	-	Increases
UGP2	No change	0.009	-	Increases

[1] When genes were significantly up- or down regulated it was shown by UP or DOWN, respectively.

[2] When expression of genes was not determined by real-time PCR it was shown by (-).

Table 4.12. Comparison of liver gene expression between wk +1 and -3 within the CONV treatment

Gene	Prediction based on expression direction	Microarray [1] (Fold change)	Real-time[2] (Fold change)	Function
------------------------------------Gluconeogenesis--------------------------------				
ADIPOR2	Decreased	UP (1.029)	-	Decreases
ALDOA	Increased	UP (0.548)	-	Increases
FBP1	Increased	UP (0.74)	-	Increases
PCK1	No change	0.153	-	Increases
PCK2	No change	0.094	-	Increases
IGFBP1	Increased	UP (1.781)	-	Increases
PC	Increased	UP (1.731)	UP (4.155)	Increases
CREB3L3	Increased	UP (1.365)	-	Increases
PPARGC1A	Increased	UP (1.038)	UP (1.399)	Increases
ADIPOR2	Decreased	UP (1.029)	-	Decreases
G6PC	No change	0.880	1.01	Increases
GNMT	No change	0.430		Increases
------------------------------------Glycolysis--------------------------------				
ALDOA	Increased	UP (0.548)	-	Increases
BRP44L	Increased	UP (1.306)	-	Increases
GK	Increased	UP (2.328)	-	Increases
LDHA	Increased	UP (1.831)	UP (3.084)	Increases
PFKL	No change	0.005	-	Increases
------------------------------------Glycogenesis--------------------------------				
GK	Increased	UP (2.328)	-	Increases
G6PC	No change	0.880	1.01	Decreases
GYS2	Increased	UP (0.921)	-	Increases
IGF1	Decreased	DOWN (-2.066)	DOWN (0.245)	Increases
IGFBP1	Decreased	UP (1.781)	-	Decreases
PPP1R3C	Decreased	DOWN (-1.613)	-	Increases
UGP2	No change	0.393	-	Increases

[1] When genes were significantly up- or down regulated it was shown by UP or DOWN, respectively.

[2] When expression of genes was not determined by real-time PCR it was shown by (-).

Table 4.13. Comparison of liver gene expression between wk +4 and +1 within the CONV treatment

Gene	Prediction based on expression direction	Microarray [1] (Fold change)	Real-time[2] (Fold change)	Function
------------------------------------Gluconeogenesis--------------------------------				
ADIPOR2	No change	-0.558	-	Decreases
ALDOA	No change	-0.246	-	Increases
CREB3L3	No change	-0.692	-	Increases
GNMT	No change	-0.360	-	Increases
G6PC	Increased	-0.014	UP (1.345)	Increases
FBP1	No change	-0.408	-	Increases
IGF1	No change	0.680	-	Increases
IGFBP1	No change	-1.134	-	Increases
PCK1	No change	0.123	-	Increases
PCK2	No change	-0.161	-	Increases
PC	No change	0.085	0.815	Increases
PPARGC1A	No change	-0.482	1.277	Increases
SCD	No change	0.827	-	Increases
------------------------------------Glycolysis--------------------------------				
ALDOA	No change	-0.246	-	Increases
BRP44L	No change	-0.417	-	Increases
GK	No change	-1.505	-	Increases
LDHA	Decreased	-0.936	DOWN (0.664)	Increases
PFKL	No change	0.022	-	Increases
------------------------------------Glycogenesis--------------------------------				
GK	No change	-1.505	-	Increases
GYS2	No change	-0.648	-	Increases
IGF1	Increased	0.680	UP (2.477)	Increases
IGFBP1	No change	-1.134	-	Increases
PPP1R3C	No change	0.447	-	Increases
UGP2	No change	-0.423	-	Increases

[1] When genes were significantly up- or down regulated it was shown by UP or DOWN, respectively.

[2] When expression of genes was not determined by real-time PCR it was shown by (-).

Table 4.14. Comparison of liver gene expression between wk +4 and -3 within the CONV treatment

Gene	Prediction based on expression direction	Microarray [1] (Fold change)	Real-time[2] (Fold change)	Function
------------------------------------Gluconeogenesis--------------------------------				
ADIPOR2	No change	0.471	-	Decreases
ALDOA	No change	0.301	-	Increases
CREB3L3	Increased	UP (0.673)	-	Increases
GNMT	No change	-0.027	-	Increases
G6PC	No change	0.455	1.358	Increases
FBP1	No change	0.194	-	Increases
IGF1	Decreased	DOWN (-0.680)	DOWN (0.606)	Increases
IGFBP1	Increased	UP (1.304)	-	Increases
PCK1	No change	0.276	-	Increases
PCK2	No change	-0.067	-	Increases
PC	Increased	UP (1.158)	UP (3.385)	Increases
PPARGC1A	Increased	0.557	UP (1.716)	Increases
SCD	No change	-0.960	-	Increases
------------------------------------Glycolysis--------------------------------				
ALDOA	No change	0.301	-	Increases
BRP44L	Increased	UP (0.888)	-	Increases
GK	No change	0.823	-	Increases
LDHA	Increased	UP (0.896)	UP (1.986)	Increases
PFKL	No change	0.027	-	Increases
------------------------------------Glycogenesis--------------------------------				
GK	No change	0.823	-	Increases
GYS2	No change	0.273	-	Increases
IGF1	Decreased	DOWN (-1.386)	DOWN (0.606)	Increases
IGFBP1	No change	0.647	-	Increases
PPP1R3C	No change	-1.046	-	Increases
UGP2	No change	-0.030	-	Increases

[1] When genes were significantly up- or down regulated it was shown by UP or DOWN, respectively.

[2] When expression of genes was not determined by real-time PCR it was shown by (-).

Transcript evaluation

Information about the target genes is illustrated in Table 4.2. Thirteen genes, including ACSL1, ANGPTL4, APOA1, APOA4, CPT1B, CYC1, ELOVL5, GHR, G6PC, IGF-1, LDHA, PC, and PPARGC1A were chosen based on their key functions in the carbohydrate and lipid metabolism to verify the microarray expression profile using real-time PCR.

Comparison between wk +1 and wk -3 in both treatments revealed that ACSL1, ANGPTL4, APOA1, APOA4, CPT1B, ELOVL5, PC were upregulated, while GHR and IGF-1 were down regulated in both microarray and real-time PCR. The LDHA showed upregulation only in real-time PCR. The G6PC and PPARGC1A genes were unchanged in both methods. The real-time results showed higher fold-change compared to the microarray results. The only difference between the two treatments was the expression of CYC1, which only upregulated in the CONV treatment but remained unchanged in the SHORT treatment.

Comparison between wk +4 and wk +1 in the SHORT treatment revealed that the APOA1, ANGPTL4, CPT1B, and PC genes were down regulated, and the CYC1, GHR, and IGF-1 genes remained unchanged with using both methods. The ELOVL5, G6PC, LDHA, and PPARGC1A genes were down-regulated, and ACSL1 upregulated with the real-time and remained unchanged with microarray. The APOA4 down regulated only in the microarray.

Comparison between wk +4 and wk +1 in the CONV treatment revealed that ACSL1, ANGPTL4, APOA4, CYC1, CPT1B, and LDHA were down-regulated while GHR, G6PC, IGF-1, were upregulated with the real-time PCR. The APOA1, ELOVL5, PC, and PPARGC1A showed no change in both real-time PCR and microarray.

Comparison between wk +4 and wk -3 in the SHORT treatment revealed that APOA1 and LDHA were upregulated with both real-time PCR and microarray analysis. The APOA4, ASCL1, and PC genes showed upregulations, and GHR, IGF-1, G6PC and PPARGC1A showed down-regulations only in the real-time PCR, and not with microarray analysis. The expressions of the ANGPTL4, CPT1B, CYC1, and ELOVL5 genes remained unchanged when examined by both methods.

Comparisons between wk +4 and wk -3 in the CONV treatment revealed that APOA1, ANGPTL4 were upregulated, while IGF-1 was down regulated with both methods. The APOA4 was only upregulated in the microarray. The ACSL1, CPT1B, ELOVL5, G6PC, LDHA, PC and PPARGC1A were upregulated only in the real-time PCR. The CYC1 and GHR remained unchanged when examined by both methods.

4.5 Discussion

Dry period management and liver metabolism adaptation

A large network of genes regulates lipid and carbohydrate metabolism in the liver. During late gestation and early lactation, dairy cows undergo huge physiological changes that have extensive impacts on liver gene expression (Loor, 2010, McCarthy et al. 2010, Khan et al. 2014, Shahzad et al. 2014). Khazanehei et al. (2015b) reported a higher concentration of liver TAG in cows that experienced a 40-d dry period during which a single close-up diet has been consumed compared to a conventional 60-d dry period with separate far-off and close-up diets. Our microarray study showed differentially expression of some key nuclear receptors and their target genes. This study also revealed some genes that uniquely expressed differently in either of treatments. The PGC-1 α gene has been shown to be a key player in many physiological processes in the body, such as gluconeogenesis, mitochondrial biogenesis, and fatty acid oxidation (Puigserver and Spiegelman, 2003, Wu et al. 2003). In agreement, in our study PGC-1 α was upregulated in wk 1 of lactation compared to 3 wk before calving in both treatments, probably in response to the high metabolism demands during this time. The PGC-1 α gene regulates several nuclear receptors such as PPARγ and PPARα. The interaction between PGC-1 α and PPARγ or PPARγ is ligand-dependent, which means that the binding of PGC-1 α to PPARα or PPARγ is dependent on endogenous ligands, such as fatty acids (Rosen and Spiegelman, 2001). Lipin1 (LPIN1) is another gene associated with co-activation of PPARα and PPARγ (Thering et al. 2009, Kim et al. 2013), and its expression is induced by PGC-1 α (Finck et al. 2006). The PPARα is essential for adaptation of liver to increased β-oxidation, ketogenesis, gluconeogenesis, and lower energy expenditure (Khan et al. 2014, Nakamura et al. 2014). The PPARγ also plays an essential role in adipogenesis, insulin sensitivity, and glucose homeostasis (Rosen and Spiegelman, 2001). The Somatotropic axis also plays an important role in adaptation of liver metabolism during the transition period. A key hormone involved in somatotropic axis is growth hormone (GH), which is produced in the anterior pituitary of brain and binds to hepatic growth hormone receptor (GHR), and results in synthesis of IGF-1 (Inagaki et al. 2008). This GH can increase gluconeogenesis and decrease lipogenesis directly in the liver. This gene also increases the uptake of NEFA by mediating of the IGF-1 and IGF-binding protein (IGFBP) (Renaville et al. 2002). The decreased expression of GHR and IGF-1 at wk 1 after calving compared to 3 wk before calving in both treatments is in agreement with others who reported a reduced expression of these genes during first wk of lactation, when dairy cows cope with a negative energy balance (McCabe et al. 2012; Khan et al. 2014). It has been shown that the energy balance has a positive correlation with circulating IGF-I and negative correlation with NEFA concentration during the first 6 wks of lactation (Beam and Butler, 1998). Deletion of hepatic GHR and IGF-1 genes in mice resulted in higher circulation of GH due to the lack of inhibitory effects of IGF-1 (Liu et al.

2000). The antagonist effects of GH on insulin activity (Fan et al. 2009) suggest higher lipolysis activity, as reported by Khazanehei et al. (2015b). IGFBP binds to IGF-1 and facilitates transportation and its interaction with receptors in peripheral tissues. Insulin and IGF-1 have inhibitory effects on IGFBP1 (Kelley et al. 1996) and has been suggested to have similar effects on IGFBP2 (Gross et al. 2011). The lower concentration of insulin (Khazanehei et al. 2015b) and IGF-1 in both treatments in our study may explain the higher expression of IGFBP1 and IGFBP2 during the first wk of lactation compared to wk 3 before calving. IGFBP3 has been suggested to control the insulin-like activity of IGF-1 more effectively during the catabolic state of negative energy balance (Gross et al. 2011). The expression and circulation of encoded protein by IGFBP3 decreases during a negative energy balance, and its affinity to IGF-1 is reduced due to the specific activity of an IGFBP3 protease (Breier, 1999). This is in agreement with our observation that the expression of IGFBP3 decreased in both treatments at wk 1 and wk 4 of lactation compared to 3 wk prepartum.

Lipid Metabolism

Hepatic β-oxidation of fatty acids

It has been suggested that LCFAs perform as endogenous ligands for the activation of PPARα and PPARγ (Rosen and Spiegelman, 2001, Bionaz et al. 2013, Khan et al. 2014). Around calving, fatty acids are mobilized from adipose tissues to the liver, as a result of negative energy balance, and may act as ligands for PPARs. This then induces a network of their target genes and adapts the liver to a higher oxidation of fatty acids in mitochondria, as well as in the peroxisome (Bionaz et al. 2013; Khan et al. 2014). Khazanehei et al. (2015a) reported that cows on the SHORT and CONV treatments experienced negative energy balance and increased blood NEFA at the first wk after calving, while at this time cows on the SHORT treatment had higher blood NEFA and liver TAG concentrations compared to the CONV treatment. The lower liver TAG in the CONV treatment at this time can be explained by higher expressions of PGC-1 α and LPIN1 in the liver of cows in this group, which induced a signaling pathway, and increased the expression of genes involved in fatty acid oxidation. In our study, the expression of PPARα did not change in either of treatment during the post-calving period compared to pre-calving. This is in agreement with others who reported higher expression of PPARα target genes such as carnitine palmitoyltransferase 1 (CPT1), acyl-CoA dehydrogenase, very long chain (ACADVL), Acyl-CoA oxidase 2 (ACOX2), carnitine octanoyltransferase (CROT), peroxisomal carnitine acetyltransferase (CRAT), and malonyl-CoA decarboxylase (MLYCD) after calving, without a change in the expression of this nuclear receptor (Carriquiry et al. 2009; van Dorland et al. 2009; Khan et al. 2014). The β-oxidation occurs in mitochondria and peroxisomes, and although its mechanism is similar between these two organelles, mitochondria and peroxisomes are different in their substrate specificity and also in transporting the products across their membrane (Violante et al. 2013). The oxidation of all short- and medium-chain fatty acids and most of the long-chain fatty acids occur in mitochondria, while peroxisomes are responsible for oxidation of dicarboxylic fatty acids, very long-chain fatty acids, bile acids and branched-chain fatty acids (Violante et al. 2013).

Acyl-CoA synthetase, long chain 1 (ACSL1), CPT1B, and ACADVL are 3 key genes involved in β-oxidation that were upregulated in our study at the first wk after calving compared to 3 wk before calving (Figure 4.1). Fatty acids that have been mobilized to the liver need to be activated by ACSL1 to be transferred to the mitochondria by CPT1 (Voet and Voet, 1995). The CPT1 subfamily has three members termed CPT1A, CPT1B, and CPT1C. The last one expresses in the brain, while the two others express in a wide variety of tissues. CPT1A and CPT1B are known as the liver and muscle types, respectively, because they were found first in these tissues (Price et al. 2003). There are only a limited number of studies that have reported the expression of CPT1B in the liver (Price et al. 2003), while in most of studies the expression of CPT1A has been reported as the hepatic isoform (Loor et al. 2005; Weber et al. 2013; Selim et al. 2014). Northern blot studies in the ovine liver have shown that CPT1B transcripts are less tissue restricted, compared to CPT1A (Price et al. 2003). The difference between these isomers is related to their sensitivity to malonyl-CoA. However, studies in sheep have shown that the sensitivity of CPT1B and CPT1A to malonyl-CoA in liver is similar to that in muscle (Bartelds et al. 2000).

The first step of the β-oxidation pathway occurs in inner membrane of mitochondria, where ACADVL forms a trans-αβ double bond in long fatty acids (Khan et al. 2014). MLYCD is another gene that catalyzes the decarboxylation of cytosolic malonyl-CoA and increases fatty acid oxidation (Lee et al. 2004). This gene is activated by PPARα, and its upregulation increases the CPT1 activity and reduces accumulation of fat in hepatocytes by reducing the availability of malonyl-CoA (Derdak et al. 2013, Khan et al. 2014). The expression of MLYCD increased in both treatments at wk 1 compared to 3 wk before calving, which may be due to increased hepatic fatty oxidation, however, this expression remained unchanged between wk +4 and +1, relative to calving. In agreement, Khan et al. (2014) reported a higher expression of MLYCD after calving in overfed cows. Cytochrome c, encoded by the CYC1 gene, is an essential carrier of electrons between the complexes III and IV in the electron transport chain in mitochondria (Simon et al. 2013). This gene is involved in hydroxylation of fatty acids and also apoptosis. Upregulation of CYC1 in the CONV treatment at first wk of lactation along with high amount of mobilized NEFA to the liver at this time, probably aids fatty acid oxidation, while the absence of a change in the expression of CYC1 in the SHORT treatment at this time probably reduced the capacity of the liver for fatty acid oxidation.

Excess mobilization of NEFA results in transporting very long-chain and branched-chain fatty acids to peroxisomes. Fatty acids are transported to the peroxisomes as acyl-CoA esters by ATP-binding cassette (ABC) to undergo β-oxidation. The end products of acyl-CoA β-oxidation in peroxisomes are medium-chain fatty acids, which after conversion to acylcarnitine, by CROT or CRAT, are transferred to mitochondria for further oxidation (Violante et al. 2013). In agreement, the expression of CRAT in the SHORT treatment and CROT in the CONV treatment in our study increased in first wk of lactation compared to 3 wk before calving. Why only one of these genes upregulated in each treatment is not apparent. ACOX2 also modulates the β-oxidation of branched fatty acids in peroxisomes. Increased expression of this gene at the first wk of lactation in the CONV treatment may increase the capacity of the liver for oxidation of fatty acids; while the absence of a difference in the expression of ACOX2 in the SHORT treatment may have a negative impact on fatty acid oxidation. It has been suggested that a higher expression of ELOVL5 increases the expression of genes involved in peroxisomal β-oxidation by biotransformation of potentially damaging fatty acids, such as palmitate, stearate, and oleate, into unsaturated long chain fatty acids (Hall et al. 2010). Adipose tissue also contributes to energy homeostasis in the liver by the secretion of adipokines, such as adiponectin. Adiponectin receptor 2 (ADIPOR2) is the hepatic receptor for adiponectin, which act as a mediator to increase AMP-activated protein kinase (AMPK) activity, PPARα and, eventually, fatty acid β-oxidation (Loor et al. 2006). A higher expression of ADIPOR2 in the liver after calving has been reported previously (Loor et al. 2006). In agreement, in our study the expression of this gene was upregulated in both treatments at the first wk of lactation compared to 3 wk before calving, which probably increased the capacity of the liver for higher fatty acid oxidation.

Among the cytochrome p450 hydroxylases isoforms, CYP2E1 has a high NADPH oxidase activity that is weakly coupled to the NADPH–cytochrome P450 reductase. As a result, CYP2E1 is able to efficiently start the NADPH-dependant lipid peroxidation in the liver (Alanazi et al. 2012). Therefore, down-regulation of this gene at the first wk after calving compared to the 3 wk before calving in the SHORT treatment may reduce the capacity of the liver to oxidize the mobilized fat. The expression of CYP2E1 remained unchanged between wk +1 and -3 in the CONV treatment, which may potentially cause higher fatty acids oxidation compared to the SHORT treatment. The CYP2U1 is another member of the cytochrome p450 family that is expressed in the liver, brain, lunge and some other tissues in the human and the mouse (Siller et al. 2014). To the knowledge of the authors, there is no report of expression of CYP2U1 in the bovine liver. Our microarray study showed the down-regulation of this gene in both treatments at the first wk after calving compared to wk 3 before calving. It has been reported that the CYP2U1 gene is involved in ω and ω-1 hydroxylations of fatty acids, and also oxidation of endogenous N-arachidonoylserotonin. This may regulate the arachidonic acid or other fatty acids signaling pathways (Siller et al. 2014).

Khazanehei et al. (2015b) reported lower liver TAG in the CONV treatment compared to the SHORT treatment at first week after calving. This is probably due to a higher hepatic β-oxidation capacity of the CONV treatment as a result of higher expression of genes involved in β-oxidation, such as CPT1B, ACOX2, CYC1, LPIN1 and CYP2E1.

Hepatic lipogenesis

The expression of genes within the PPARγ network, such as thyroid hormone responsive (THRSP) and stearoyl-CoA desaturase (SCD, also known as delta9-desaturase), which are involved in anabolic pathways in subcutaneous adipose tissues, have been reported to decrease 14 d after calving compared to 30 d before calving (Loor, 2010). In agreement, we observed down-regulation of THRSP in our microarray study at 1 wk after calving compared to 3 wk before calving in both treatments. The protein encoded by the SCD gene is involved in fatty acid biosynthesis, mainly oleic acid, which is essential for the biosynthesis and export of very low-density lipoprotein (VLDL) from the liver (Legrand et al. 1997). The expression of SCD decreased in the CONV treatment, while it remained unchanged in the SHORT treatment. The expression of very long chain fatty acid 5 (ELOVL5) increased in both treatments after calving. The protein encoded by the ELOVL5 gene is a rate-limiting enzyme, and responsible for the condensation of fatty acyl-CoA and malonyl-CoA, which are necessary in the first step of fatty acid elongation in the endoplasmic reticulum. Studies have shown that the production of arachidonic acid (C20:4, n-6) and docosahexaenoic acid (DHA, C22:6, n-3) increase with higher expression of ELOVL5. On the other hand, these fatty acids result in inactivation of sterol regulatory element-binding protein 1 (SREBP1) and its target genes, and lower fatty acids and triglyceride synthesis in hepatocytes (Moon et al. 2009). In agreement, the expression of SREBP1 remained unchanged in both treatments at first wk of lactation compared to 3 wk before calving, which was probably due to the high mobilization of NEFA to the liver, as described by Khazanehei et al. (2015b). This negative feed back loop is probably one of the mechanisms to reduce the uptake and accumulation of fatty acids in the liver. The fatty acid desaturase 2 (FADS2), known previously as delta6-desaturase, is responsible for the desaturation of α-linoleic acid (C18:3) and linoleic acid in the liver and adipose tissues that eventually are utilized, by mediation of FADS1, for the synthesis of arachidonic acid (C20:4) and eicosapentaenoic acid (C20:5) (Jacobi et al. 2011). In our study, the expression of FADS2 did not change between 3 wk before and 1 wk after calving in the SHORT treatment. However, this gene upregulated in wk +4. In the other hand, in cows on the CONV treatment, FADS2 was down-regulated in wk 1 after calving compared to the 3 wk before calving. However there was no difference between wk +4 and wk +1. This is in agreement with McCabe et al. (2012), who reported that the FADS2 was down regulated in cows with a severe negative energy balance (McCabe et al. 2012). Diacylglycerol O-acyltransferase (DGAT) catalyzes the final step of TAG synthesis. The expression of this gene increased only in the SHORT treatment, probably in response to a higher mobilization of NEFA to liver in this treatment, compared to the CONV treatment. A higher mobilization of NEFA (Khazanehei et al. 2015b) along with higher expression of DGAT and lower expression of ELOVL5, which reduces the inhibitory effect of ELOVL5 on the uptake of fatty acids and TAG synthesis, in the SHORT treatment, probably increased the TAG synthesis in the liver of this group compared to the CONV treatment.

Hepatic export of fatty acids

Protein encoded by apolipoprotein C-II (APOC2) gene is an essential component of very low-density lipoprotein (VLDL) that is responsible for exporting TAG from the liver. APOC2 and apolipoprotein A-IV (APOA4) activate lipoprotein lipase (LPL) that is responsible for hydrolyzing chylomicrons and VLDL-TAG and facilitates their transportation to the peripheral tissues (Lee, 2012). The higher expression between 6 to 18 2-fold log change of APOC2 and APOA4 in both treatments at first wk of calving compared to the 3 wk before calving showed the attempt of the liver for the reduction of the TAG content. However, it seemed this increased expression was not able to eliminate accumulation of TAG during this period. As mentioned above, SCD is responsible for the biosynthesis of oleic acid, which is essential for formation of VLDL in liver. The lower expression of SCD in the first and fourth wk of lactation compared to the 3 wk before calving in the CONV treatment probably reduced the capacity of the liver for exporting VLDL. While in the SHORT treatment, the expression of SCD remained unchanged between wk +1 and -3, it increased in wk +4 compared to wk +1, relative to calving. A lower expression of SCD has also been reported during first 35-d of lactation compared to prepartum (Bionaz et al. 2007). The proteins encoded by the APOA5 and PLIN2 genes are important components of several lipoproteins, such as VLDL and HDL. In our microarray study, the expression of PLIN2 increased in both treatments, however, the expression of APOA5 increased in the first wk of lactation only in the CONV treatment, and remained unchanged in the SHORT treatment. This may result in reducing the hepatic capacity of cows on the SHORT treatment to export the TAG via VLDL and HDL. The most abundant protein constituent of HDL is APOA, which is essential for the formation of HDL. Other components of HDL are phospholipids, cholesterol, esterified cholesterol and triacylglyceride (Forti and Diament, 2006). The PPARγ/LXRs heterodimers increase the expression of apolipoprotein A-I (APOA1) and ATP-binding cassette transporter A1 (ABCA1), two key genes in cholesterol homeostasis. These result in an increased cholesterol efflux as high-density lipoprotein (HDL) from the liver (Iio et al. 2012). In our study, the expression of APOA1 increased in both treatments after calving by 17-fold in the SHORT treatment and by a 6-fold in the CONV treatment. The expression of ABCA1 remained unchanged in the first wk of lactation in the SHORT treatment, while it was downregulated in the CONV treatment. Murine hepatocyte studies have shown that a higher expression of APOA1, along with ABCA1 increases the HDL secretions from the liver, which can circulate into peripheral tissues (Sahoo et al. 2004). These hepatic ABCA1-generated HDLs can then interact with ABCA1 on cells, increase in size, and act as the carrier of cholesterol from these cells (Francone et al. 2003). The produced HDLs can either return to the liver, and be excreted out of the body through bile acids, or be delivered to adrenal glands, ovaries, and testes to be used for the synthesis of steroid hormones (Sahoo et al. 2004). Expression patterns of APOA1, APOA4, APOC2, ABCA1 and SCD genes in our study suggest that during the negative energy balance that coincides with a high mobilization of NEFA to the liver, an alternative pathway for exporting the TAG and cholesterol from liver is through HDL. Although, gene expression patterns in cows on the SHORT treatment showed a higher capacity of liver for the export of TAG through VLDL, the expression of other components of this macromolecule such as APOA5 and APOB remained unchanged. This probably led to low VLDL synthesis and TAG export from liver.

Hepatokines

The bovine hepatic angiopoietin-like 4 (ANGPTL4) is responsible for the regulation of concentrations of plasma cholesterol, triglyceride, and NEFA (McCabe et al. 2012). Upregulation of ANGPTL4 has an inhibitory effect on lipoprotein lipase (LPL) activity in adipose tissues that results in a lower absorption of VLDL-TAG and a higher lipolysis (McCabe et al. 2012). The higher plasma concentrations of NEFA at first week after calving in both treatments in our study is likely due to the upregulation of ANGPTL4 in both groups at this time. In addition, a higher upregulation of ANGPTL4 in the liver of cows on the SHORT treatment (9.24 2-fold log-change) compared to the CONV treatment (5.204 2-fold log-change) resulted in significantly higher concentrations of NEFA in the SHORT compared to the CONV treatment. This is in agreement with others who reported a higher expression of ANGPTL4 in early lactation when cows experience high negative energy balance (McCabe et al. 2012; Khan et al. 2014).

Carbohydrate metabolism

Glucose absorption in ruminants is very limited due to extensive microbial fermentation in rumen. The liver is the main organ for gluconeogenesis, and provides 80 to 90% of required glucose. This occurs through two processes, i.e., gluconeogenesis or glycogenolysis (Danfaer et al. 1995). The later pathway is responsible for the short fast state; however, in the long-term state, gluconeogenesis is the main pathway for the production of glucose. Although, the major precursor for gluconeogenesis in ruminants is propionate, as a result of dramatically increased glucose demands of mammary glands during early lactation, other substrates, such as lactate (mostly), glycerol, and amino acids, are also used for gluconeogenesis (Overton et al. 1999; Larsen and Kristensen, 2013).

Gluconeogenesis

There are three enzymes that regulate the rate of gluconeogenesis (Figure 4.1), i.e., phosphoenolpyruvate carboxykinase (PCK), fructose-1,6-bisphosphatase (FBP), and glucose-6-phosphatase (G6PC). A fasting state induces the cyclic-AMP-responsive-element-binding protein H (CREB-H) encoded by the cAMP responsive element binding protein 3-like 3 (CREB3L3) gene, which activates PGC-1α (Lee et al. 2010). It has been suggested that induction of PGC-1α increases the expression of the PCK, FBP and G6PC genes (Puigserver and

Spiegelman, 2003). In agreement, upregulation of CREB3L3 in our microarray study probably resulted in an increased expression of G6PC and FBP in both treatments at the first wk of lactation. However, it is not apparent why there was no change in expression of PCK at this time. The aldolase A, fructose-bisphosphate (ALDOA) gene catalyzes the conversion of fructose-1,6-biphosphate to glyceraldehyde 3-phosphate and dihydroacetone phosphate. This gene functions both in glycolysis and gluconeogenesis, and its upregulation in both treatments may end in either pathways. Studies with mice have shown that similar to LCFA, glucose acts as a ligand for the activation of PPARα. The same activity has been suggested in ruminants (Bionaz et al. 2013). Activation of PPARα by both long chain fatty acid and glucose shows the regulatory effects of these ligands on lipid or carbohydrate metabolism. The combination of fatty acids mimicking the NEFA composition around calving has shown that increased the expression of pyruvate carboxylase (PC) in dairy cows (White et al. 2012) that suggested the regulatory effect of PPARα. The protein encoded by pyruvate carboxylase (PC) gene is essential for synthesis of oxaloacetate, which is the main carbon supplier for both gluconeogenesis and the tricarboxylic acid cycle (Jitrapakdee et al. 2006). Activity of the PCK1 (cytosolic PCK) and PCK2 (mitochondrial PCK) genes regulates the pathway for which oxaloacetate is used. Upregulation of PC along with PCK1 and 2 increases gluconeogenesis, as higher expression of PCK1 increases the glucose production from amino acids, whereas upregulation of PCK2 causes lactate to be used for glucose production. In the other hand, upregulation of PC without a change in PCK isoforms results in utilization of oxaloacetate for the increase of the oxidative capacity of the tricarboxylic acid cycle (White et al. 2012). Upregulation of G6PC, FBP1, ALDOA, and PC in the SHORT treatment during the first week of lactation may reflect a higher capacity of the liver for gluconeogenesis in this treatment, compared to the CONV treatment during which only

FBP1, ALDOA and PC were upregulated. In addition, the expression of solute carrier family-2 (SLC2A2) gene that its encoded protein facilitates the transportation of glucose through plasma membrane was down-regulated in wk 1 after calving in the CONV treatment, while was remained unchanged in the SHORT treatment. This may also show the higher hepatic capacity of SHORT treatment for gluconeogenesis. As mentioned above, since both isoforms of PCK remained unchanged during the first week of calving in both treatments, the produced oxaloacetate probably had been used to increase the oxidative capacity of the tricarboxylic acid cycle, rather than to be used for gluconeogenesis. A higher expression of PC also has been reported by others at first wk post-calving compared to the 14 wk before calving (Loor et al. 2006). Glycine N-methyltransferase (GNMT) is another gene that has been shown to be involved in the regulation of gluconeogenesis by affecting FBP1, PCK, and G6PC transporter (Liu et al. 2007). Growth hormone is responsible for regulating of GNMT in mice. However, the mechanisms through which GNMT regulates the FBP1, PCK, and G6PC transporters are not known (Liu et al. 2007). It has been reported that PPARγ induces adiponectin expression that activates the AMPK and, consequently, reduces the expression of gluconeogenic enzymes, such as PCK and G6PC in humans (Yamauchi et al. 2007, Nakamura et al. 2014). In our microarray study, the hepatic ADIPOR2 gene upregulated at first wk of lactation in both treatments compared to 3 wk before calving. There should be a controlling mechanism that balances the adiponectin and CREB-H effects on gluconeogenic enzymes. This may explain why although CREB-H upregulated after calving, there was no change in expression of PCK in both treatments, and why the expression of G6PC in the CONV treatment remained unchanged. . The protein encoded by the lactate dehydrogenase A (LDHA) gene catalyzes the conversion of lactate to pyruvate that can go through gluconeogenesis pathway or tricarboxylic acid cycle.

Expression of this gene increased in both treatments and may provide extra source of pyruvate in hepatocytes.

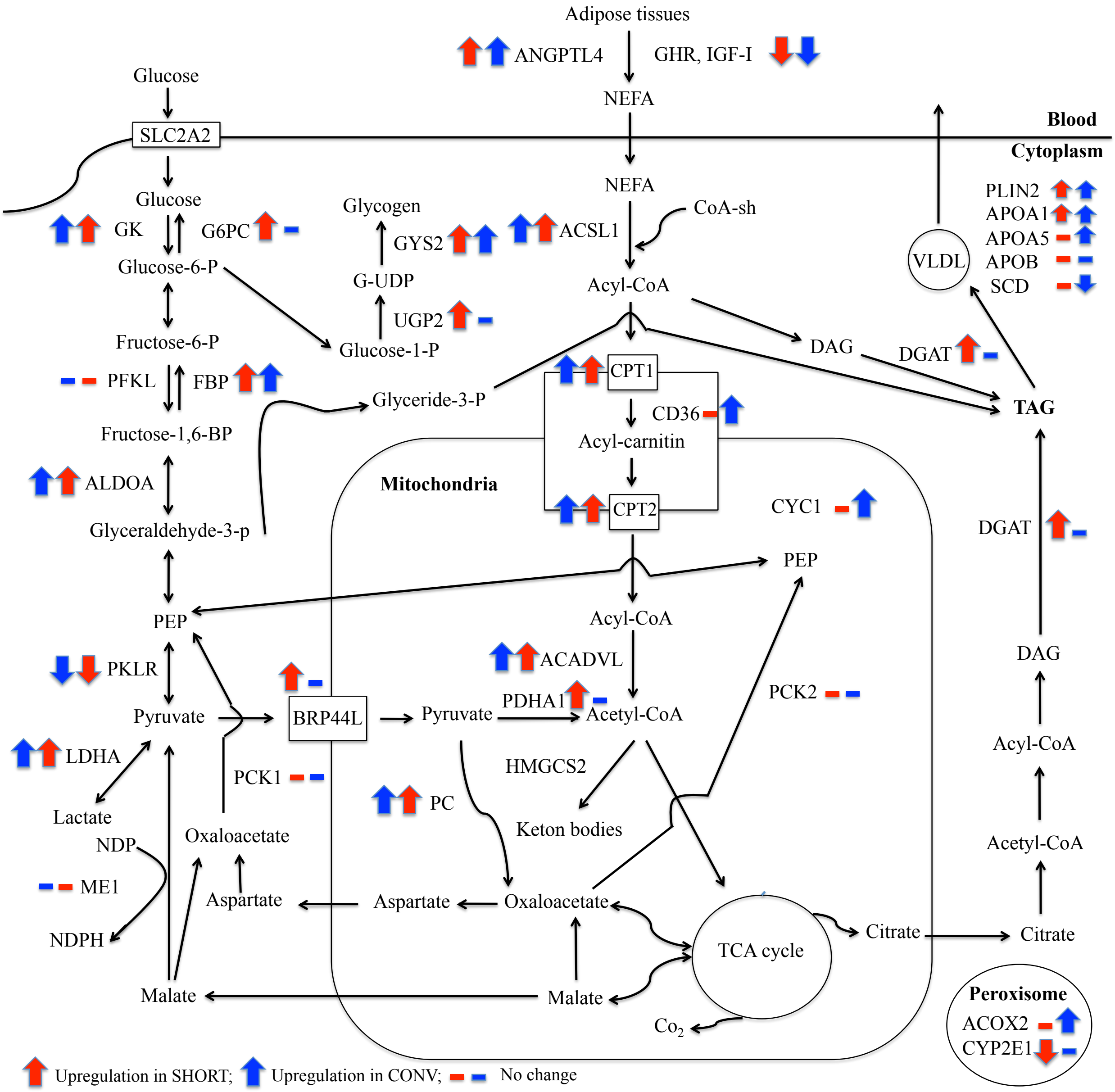
Glycolysis

Induction of PPARγ results in activation of glucokinase (GK), which is responsible for the phosphorylation of glucose and the formation of glucose-6-phosphate. This is the first step for both the glycolysis and glycogenesis pathways. The GK serves as a glucose sensor, and plays an important role in switching the hepatic carbohydrate metabolism during fed and fasting states (Nakamura et al. 2014). In our microarray study, expression of GK increased in both treatments at first wk of lactation compared to at 3 wk before calving. This probably resulted in formation of G6P. The protein encoded by phosphofructokinase (PFKL), which catalyzes the conversion of fructose-6-phosphate (F6P) to fructose-1-6-biphosphate, remained unchanged in both treatments between first wk after calving and the 3 wk before calving. This is the first rate-limiting step in the glycolysis pathway in both treatments. As mentioned above, ALDOA catalyzes the next step of glycolysis that produces glyceraldehyde 3-phosphate and dihydroacetone phosphate. This is a common step in both glycolysis and gluconeogenesis, and its upregulation in both treatments may result in either pathway. The produced glyceraldehyde 3-phosphate in this step can enter to the lipogenesis pathway, and be converted to triglyceride, or continue in glycolysis pathway and convert to phosphoenolpyruvate. In the last step of glycolysis, pyruvate kinase (PKLR) catalyzes the synthesis of pyruvate in hepatocyte cytosol. The expression of PKLR decreased in both treatments in wk 1 after calving compared to wk 3 before calving, and may be another rate-limiting step in the glycolysis pathway in both treatments. Cytosolic pyruvate then is transferred to the mitochondria by mediation of BRP44L, a pyruvate carrier, whose expression increased in both treatments in wk 1 after calving compared to 3 wk before calving. Pyruvate dehydrogenase (PDHA1) catalyzes the conversion of pyruvate to acetyl CoA in mitochondria. The expression of PDHA1 increased in first week of lactation in the SHORT treatment, however, remained unchanged in the CONV treatment. The increased expression of PDHA1 in the SHORT treatment resulted in increased synthesis of acetyl CoA that can go to the TCA cycle. The pyruvate that enters mitochondria also can be converted to oxaloacetate by mediation of PC, and can either entered to TCA cycle or be exported to the cytosol. Since the capacity of converting the produced pyruvate to acetyl CoA in the CONV treatment is limited, probably it underwent through synthesis of oxaloacetate. The oxaloacetate that was transferred to the cytosol could then be converted to malat and by mediation of malic enzyme 1 (ME1) to pyruvate. The activity of ME1 is essential for the production of producing the required NADPH for cytosolic fatty acid synthesis (Zheng et al. 2012). In agreement, the expression of ME1 increased in the CONV treatment, while remained unchanged in the SHORT treatment. In addition, cytosolic pyruvate can be converted to lactate by LDHA enzyme that upregulated in both treatments at first wk after calving compared to the 3 wk before calving.

Glycogenesis

As mentioned above, GK is responsible for catalyzing the first step of glycogenesis by the formation of glucose-6-phosphate. Other key genes in glycogenesis are UDP-glucose pyrophosphorylase 2 (UGP2) and glycogen synthase 2 (GYS2), which are responsible for catalyzing the conversion of glucose 1-phosphate to UDP-glucose and then to glycogen (n+1). In agreement, all three genes upregulated in the SHORT treatment in first week of lactation compared to 3 wk before calving in our microarray study, which interestingly, showed the high capacity of glycogenesis at the time of the negative energy balance. In the CONV treatment, only the expression of GK and GYS2 increased, and although UGP2 upregulated numerically, its expression remained statistically unchanged, which probably reduced the capacity of the liver for glycogenesis in this treatment. Glycogenesis during this time may be explained by higher expression of genes involved in gluconeogenesis, such as ALDOA and FBP that probably increased the synthesis of glucose-6-phsphate. However because of the absence of a change in the expression of plasma membrane glucose carrier (SLC2A2), some portion of it went through the glycogenesis pathway. Studies have shown that there are links between glycogen and lipid metabolism (Lu et al. 2014). A high-fat diet increases the glycogen synthesis by inducing the expression of protein phosphatase 1, regulatory subunit 3C (PPP1R3C, also known as PTG). The promoter of PTG is activated by a mechanistic target of rapamycin complex 1 (mTORC1) and its target transcription factor SREBP1. Insulin also has regulatory effects on the SREBP1 gene (Lu et al. 2014). It has been shown that there is an interaction between PTG and lipogenesis, since deletion of the PTG not only prevents the glycogen accumulation in liver but also down regulates the expression of the lipogenic genes (Lu et al. 2014). In our study, the high mobilization of fat to the liver at first week of lactation probably simulated the same situation. However, probably due to the low concentration of insulin in early lactation, fatty acids had no stimulatory effect on SREBP1 and, consequently, resulted in lower expression of PTG in both treatments in first week after calving compared to 3 wk before calving. As mentioned above, IGF-1 has insulin like effects, and increases glycogenesis and IGFBP facilitate IGF-1 transportation. Down-regulation of IGF-1 in both treatments after calving reflects the lower capacity of liver for glycogenesis.

Figure 4.1. Effect of the SHORT and CONV treatments on hepatic expression of genes involved in carbohydrate and lipid metabolism at wk 1 after calving compared to 3 wk before calving.



4.6 Conclusion

Expression of key genes involved in β-oxidation, such as ACSL1, CPT1B, CPT2, and ACADVL, increased in the first week after calving compared to 3 wk before calving in both treatments. Other genes involved in β-oxidation, such as CD36, CYC and ACOX2 upregulated during this time in the CONV treatment, while they remained unchanged in the SHORT treatment. This, probably, resulted in a lower hepatic β-oxidation capacity in the SHORT treatment compared to the CONV treatment. In addition, at first week after calving compared to 3 wks before calving, the expression of DGAT, a key gene in triglyceride synthesis, increased in the SHORT treatment, while remained unchanged in the CONV treatment. Moreover, expression patterns of genes involved in VLDL synthesis in both treatments showed the limited capacity of the liver to export the triglyceride from the liver. In summary, the lower capacity of β-oxidation and higher triglyceride synthesis in the SHORT treatment, along with low VLDL synthesis capacity, probably resulted in accumulation of triglyceride in the liver in this treatment compared to the CONV treatment. The expression patterns of genes involved in gluconeogenesis showed a higher capacity of the SHORT treatment compared to the CONV treatment. However, probably because of the absence of differences in expressions of PCK1 and 2, synthesized oxaloacetate was used for increasing the capacity of the tricarboxylic acid cycle. Also, glycolysis was reduced in both treatments after calving, while the capacity of glycogenesis increased. In conclusion, we observed differential hepatic gene expression in early lactation, probably as a result of high metabolism demands during this time. However, we did not observe beneficial effects in applying a SHORT dry period with a single close-up diet on the adaptation of hepatic metabolism compared to a conventional 60-d dry period with separate far-off and close-up diets.

CHAPTER FIVE

MANUSCRIPT IV

Exploring the effects of a 40 day dry period with a single close-up diet compared to a 60 day dry period with far-off and close-up diets on rumen microbiome

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5.1 ABSTRACT

Effects of dry period management on the rumen microbiome in the liquid phase were determined in 8 second-parity (PAR2) and 8 third-parity and older (PAR3+) cows. Cows were paired based on expected calving date and randomly assigned to two treatments, including a short 40-d dry period (SHORT) with only a close-up diet (1.43 Mcal NEl/kg DM), and a conventional 60-d dry period (CONV) with 39 d far-off diet (1.28 Mcal NEl/ kg DM) and 21 d close-up diet (1.43 Mcal NEl/kg DM) in a randomized block design. Rumen samples were taken weekly from 2 wk before until 7 wk after calving at 5 h after feed delivery, using a stomach tube. Our study showed differences between the SHORT and CONV treatment on rumen microbiota during the dry period, as the SHORT treatment increased the relative abundance of Firmicutes and reduced the relative abundance of Bacteroidetes compared to the CONV treatment. Results also demonstrated that the SHORT treatment reduced the shifts of rumen microbiota during the transition period. Our data proposes a core function in the rumen that remains similar during the transition period even when the composition changes. Moreover, the individual differences were detected between animals within the same diet and treatment, probably due to differences in physiology and genetics, which suggests a core function between animals in addition to a core function between pre- and post-calving period.

5.2 Introduction

The rumen is an ecological community of different protozoal, archaeal, fungal and, more importantly, bacterial species that symbiotically work together to ferment the plant materials (Brulc et al. 2009). The Beginning of the rumen microbial research goes back to 1950s when microbes were characterizing in anaerobic culture-based systems (Staley et al., 1985). The major limitation of the culture-based studies was the fact that less than 1 percent of rumen bacteria could be detected by this method (Staley et al. 1985). Recently, acquiring new techniques, such as next generation sequencing and analytical methods, has led to identification of a large portion of rumen microbiome at different taxonomy levels (Vetrovsky and Baldrian, 2013, Pitta et al. 2014b). This aids in a better understanding of relationships between the rumen microbiota and animal physiology, health and production (Jami et al. 2014). This relationship is a two-way interaction between host and microbiome. Variations in the microbial profile between animals of the same age and diet suggest that different genetic merit and physiology of the host can affect microbiome profile (Backhed et al. 2005, Pitta et al. 2010). On the other hand, factors such as antibiotics and diet that affect rumen microbiota profile, can also change animal physiology, and affect health and production (Lettat and Benchaar, 2013, Jami et al. 2014). Therefore, practicing appropriate nutrition management can manipulate the rumen microbiome in order to improve animal health and production, and reduce the susceptibility of the animal to metabolic disorders. A conventional 60-d dry period management has been used by dairy industry for the past few decades. However, due to high adaptation stress associated with this management, recent studies proposed a 40-d dry period with only a single close-up diet to reduce the number of adaptation that rumen microbiome has to bear and, consequently, improve the production and fertility of dairy cows (Rastani et al. 2005, Shoshani et al. 2014). We hypothesized that shortening the dry period to 40 days and only using a close-up diet during this period reduces the compositional and functional shifts in the rumen microbiome during the transition from prepartum to postpartum compared to a conventional 60-d dry period, during which far-off and close-up diets are fed for 39 and 21 days, respectively. . The objectives of this study were 1) to compare the effects of a 40-d dry period management with a single close-up diet and a conventional 60-d dry period with separate far-off and close-up diets on the rumen microbiome during the pre- and post-calving period and their shifts during this time. 2) To characterize the rumen microbiota profile during the transition period within each treatment. 3) To describe the rumen microbiome in cows with different parities. 4) To explore the functionality of rumen microbiota in the two treatments and during the transition period.

5.3 Materials and Methods

Animals and Experiment Design

The study was conducted at the Glenlea Research Station, University of Manitoba. It was approved by the University of Manitoba Animal Care Committee, and followed the guidelines of the Canadian Council for Animal Care (CCAC, 1993). Twenty-six Holstein dairy cows (11 second parity in the subsequent lactation (PAR 2) and 15 third and higher parity (PAR 3+) in the subsequent lactation) were paired based on expected calving date and randomly assigned to two treatments within each pair. Cows were housed in tie-stalls during the entire experiment. Treatments included a conventional 60-d dry period with separate 39-d far-off and 21-d close-up period (CONV) and a short 40-d dry period during which only a close-up diet was fed (SHORT). The diet fed during lactation did not differ between treatments. Nutrient and ingredient compositions of diets and feeds are presented in Table 5.1 and 5.2. At the beginning of experiment, cows on the CONV and SHORT treatments had body weights of 737 ± 60 and 782 ± 69 kg (mean ± SD), respectively and body condition scores between 2.75 and 4 with average of 3.36 ± 0.11 and 3.40 ± 0.11, respectively, on a 1 to 5 scale (Edmonson et al. 1989). Actual dry period lengths for SHORT treatment were 34 ± 6 d (mean ± SD) with a minimum of 23 d and maximum of 44 d and for CONV treatment were 59 ± 4 d (mean ± SD) with a minimum of 53 d and max of 68 d. Diets were fed as total mixed rations (TMR) and were provided *ad libitum* twice a day at 900 and 1500, and cows had unlimited access to water.

Table 5.1. Nutrient composition of far-off, close-up, and lactation diets (mean ± SD)

Item	Nutrient Composition[1]		
	Far-off	Close-up	Lactation
Crude protein, %	14.8±0.4	14.9±1.9	17.7±1.3
Neutral detergent fiber, %	50.2±1.1	38.1±7.4	31.2±5.6
Acid detergent fiber, %	32.2±1.2	23.2±5.1	19.3±3.3
NFC[2], %	26.2±1.6	36.2±6.1	39.4±3.9
Crude fat, %	1.7±0.3	3.3±1.0	4.2±0.7
Calcium, %	0.6±0.1	0.8±0.2	1.0±0.1
Phosphorous, %	0.3±0.0	0.4±0.1	0.4±0.0
Magnesium, %	0.2±0.0	0.4±0.1	0.4±0.1
Potassium, %	2.1±0.1	1.7±0.4	1.5±0.1
Ash, %	7.1±1.0	7.8±0.8	7.5±0.7
Net Energy for lactation, Mcal/kg	1.28	1.39	1.68

[1] Far-off diet ingredients (% of DM): timothy hay, 89.5; soybean-meal, 8.2; mineral, 2.2; forage to concentrate ratio, 90 to 10; Close-up diet ingredients (% of DM): timothy hay, 43.0; barley silage, 25.3; Transrite, 31.6; forage to concentrate ratio, 68 to 32; Lactation diet ingredients (% of DM): mixed hay, 20.4; corn silage, 32.2; roasted soybeans, 4.4; Energy supplement, 39.5; protein supplement, 2.2; forage to ratio concentrate ratio, 53 to 47.

[2] Non fiber carbohydrate= 100 - (NDF% + CP % + Crude fat % + Ash %)

Table 5.2. Ingredient composition of experimental diets (dry matter basis)

Item	Diets		
	Far-off	Close-up	Lactation
	Ingredients, % of DM		
Timothy hay	89.5	43.0	-
Mixed hay	-	-	20.4
Barley silage	-	25.3	-
Corn silage	-	-	32.2
Roasted soybeans	-	-	4.4
Soybean-Meal	8.2	-	-
Energy supplement	-	-	39.5
Protein supplement	-	-	2.2
Transrite	-	31.6	-
Mineral	2.2	-	-
Forage: Concentrate	90:10	68:32	53:47

Sample collections and bacterial DNA extraction

Rumen samples were taken in week -2, -1, 1, 2, and 7, relative to calving, at 5 hr after feeding. Approximately 500 mL of rumen fluid were collected by stomach tube, mixed thoroughly and strained through four layers of cheesecloth. Subsequently, rumen fluid was aliquoted into two 15 mL tubes and stored at -20°C for future microbial profile analyses.

Genomic DNA was extracted from 80 samples taken from 16 cows out of 26 cows that had all 5 sampling times. Frozen rumen fluid samples were thawed at room temperature, and they were thoroughly homogenized. A total of 3 ml from each sample was added to ZR Bashing-Bead lysis tubes using ZR Fecal DNA Kit (D6010, Zymo Research Corp., Orange, CA), and DNA was extracted according to the manufacturer's protocol. Concentrations of DNA were quantified using Nanodrop (2000, Thermo Scientific, USA) and the 260/280 ratios of all samples were checked to check the purity. To ensure the quality of DNA, the V1 and V2 regions of the of the 16S ribosomal RNA (rRNA) gene were amplified using universal bacterial primers, 27f (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342r (5'-CTGCTGCCTCCCGTAG-3'). Polymerase Chain Reaction (PCR) was performed using the following cycle conditions: an initial denaturation at 94°C for 60 s, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, elongation at 72°C for 2 min, and then a final elongation step at 72°C for 5 min. The PCR products were verified by agarose gel electrophoresis. The genomic DNA from those samples that did not meet the quantity and quality requirements were re-extracted and low quality samples were removed. In total 77 samples were used for the down stream process.

Library construction and Illumina sequencing

The genomic library was constructed as described by Derakhshani et al. (2014). Briefly, the V4 region of 16S rRNA gene was amplified using F515/R806 primers. Then, the ZR-96 DNA Clean-up Kit™ (ZYMO Research, CA, USA) was used to purify PCR products from primers, dNTPs and reaction components. In the next step, the concentration of PCR products from individual samples were quantified using Picogreen dsDNA (Invitrogen, NY, USA), were diluted to 200 ng and pooled to make the DNA library. The 15% of PhiX control library was spiked into the diluted 5 pM amplicon pool to minimize the unbalanced and biased base composition because of the low diversity of 16S rRNA libraries. Customized sequencing primers for read1 (5′-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3′), read2 (5′-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3′) and index read (5′-ATTAGAWACCCBDGTAGTCCGGCTGACTGACT-3′) were synthesized and purified by polyacrylamide gel electrophoresis (Integrated DNA Technologies, IA, USA), and added to the MiSeq Reagent Kit V2 (300-cycle) (Illumina, CA, USA). The 150 paired-end sequencing reaction was performed on a MiSeq platform (Illumina, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Canada.

Bioinformatics analyses

The output read1.fastq file was used for down stream analyses, using pipelines of the open source software package QIIME (Caporaso et al. 2010b). Demultiplexing, quality filtering and chimeric read removal was implemented as described by Derakhshani et al. (2014). Operational Taxonomic Units (OTU) were assigned to representative sequences using RDP classifier (Wang et al. 2007) and aligned with the Greengenes Core reference database (DeSantis et al. 2006) using PyNAST algorithms (Caporaso et al. 2010a). A phylogenetic tree was built with FastTree 2.1.3. (Price et al. 2009), for further comparisons between microbial communities. The α-diversity (within community diversity) and β-diversity (between samples diversity) was calculated using QIIME. The Chao 1 estimator of species richness was used for the generation of the alpha rarefaction curve with twenty sampling repetitions at each sampling depth. An even depth of approximately 4090 sequences per sample was used for the calculation of richness and diversity indices. The weighted Unifrac distances were used to calculate the β-diversity matrices. Subsequently, principal coordinate analysis (PCoA) was conducted on the resulting distance matrices to generate two-dimensional plots using PRIMER v6 software (Clarke and Gorley, 2006). Permutational multivariate analysis of variance (PERMANOVA) (Anderson et al. 2008) was performed to calculate *P*-values and test for significant differences in β-diversity among treatment and parity groups and across sampling time points.

Statistical analyses

To test the effects of treatments, parities and also time relative to calving, the partial least square discriminant analysis (PLS-DA; SIMCA P+ 13.0, Umetrics, Umea, Sweden) was performed on genus data. For this analysis, data were scaled using Unit Variance in SIMCA (Umetrics, Umea, Sweden). Cross-validation then was performed to determine the number of significant PLS components, and a permutation testing was conducted to validate the model. To avoid over parameterization of the model, variable influence on projection values (VIP) were estimated for each genus, and genera with $VIP < 0.50$ were removed from the final model. The R^2 estimate then was used to evaluate the goodness of fit and the Q^2 estimate was used to evaluate the predictive value of the model. The PLS-regression coefficients were used to identify genera that were most characteristic of each treatment group and the PLS-DA loading scatter plots were used to visualize the results.

The UNIVARIATE procedure of SAS (SAS 9.3, 2012) was used to test the normality of residuals for Alpha biodiversity data. Non-normally distributed data were log transformed and then used to evaluate the effect of sampling date (pre-/post-calving and also weekly) using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC). The effect of cow within treatment and block were treated as random factor and all pair-wise comparisons among the groups were tested using the Tukey studentized range adjustment.

Metagenome prediction of rumen microbiota

The functional prediction was calculated as described by Langille et al. (2013). Briefly, the QIIME software was used for producing the OTU table containing Greengenes identifiers. The biom format of the OTU table was then used as an input for online Galaxy version of PICRUST. The OTU table was normalized for multiple 16S copy numbers in order to acquire the true abundances of contained taxa. The metagenomics prediction was then calculated by considering the genome content of each OTU and the KEGG Orthology.

5.4 Results

Sequence Analysis

A total of 77 samples were used for sequencing. After demultiplexing according to primer and barcode sequences, separating bacterial sequences and denoising, a total of 928,424 reads were used for downstream analyses. The sample libraries ranged from 4,107 to 19,952 reads with an average of 12,057 reads per sample. Reads were binned into individual samples based on the barcode sequence, and complementary phylogenetic and taxon-based analysis methods were used to assign operational taxonomy units (OTUs) at 97% sequence identity. Subsequebntly, bacterial communities were compared across treatments, parities and sampling time points. A total of 27 phyla and 358 genera were identified which after removing rare bacteria (<0.1%), total of 10 phyla and 46 genera remained.

Overall rumen microbiota composition

Sixteen low abundance phyla (less than 0.1%), including Acidobacteria, Armatimonadetes, BRC1, Deferribacteres, Elusimicrobia, Fibrobacteres, Fusobacteria, GN02, Gemmatimonadetes, Planctomycetes, SR1, Synergistetes, TM6, TM7, Thermi, and WPS-2, were removed from downstream analyses. The remaining 10 abundant phyla were categorized into two groups, 1) relative abundance larger than 1% which included Actinobacteria, Bacteroidetes, Firmicutes, Spirochaetes, and Tenericutes, and which composed 4.16%, 31.04%, 53.82%, 2.10%, and 2.47% of total rumen bacteria, respectively (Figures 5.1 and 5.2); 2) those with relative abundances between 0.1 to 1%, which consisted of Chloroflexi, Cyanobacteria, Lentisphaerae, Proteobacteria, and Verrucomicrobia, and comprised 0.27%, 0.36%, 0.12%, 0.78% and 0.35% of total rumen bacteria, respectively (Figures 5.1 and 5.2). One phylum out of 27 was considered as "Other" (4.32%), which consisted of sequences that could not be aligned to the database.

Figure 5.1. The relative abundances of 10 predominant rumen bacteria phyla in the SHORT treatment across weeks before and after calving.

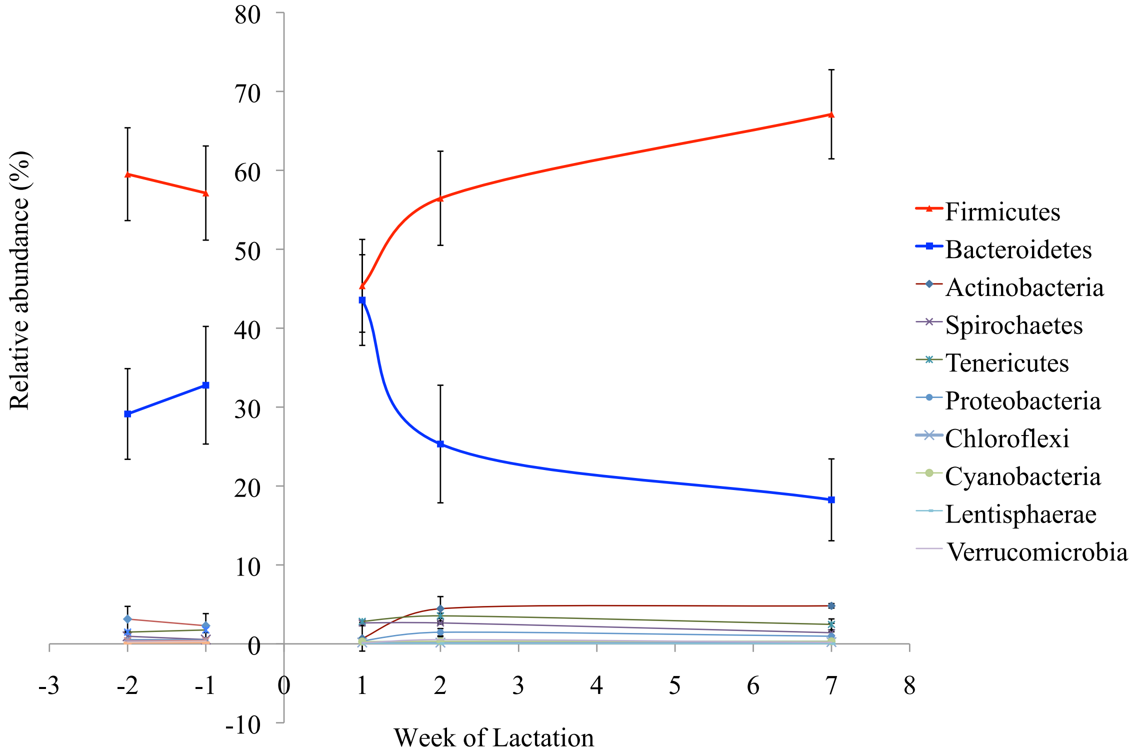
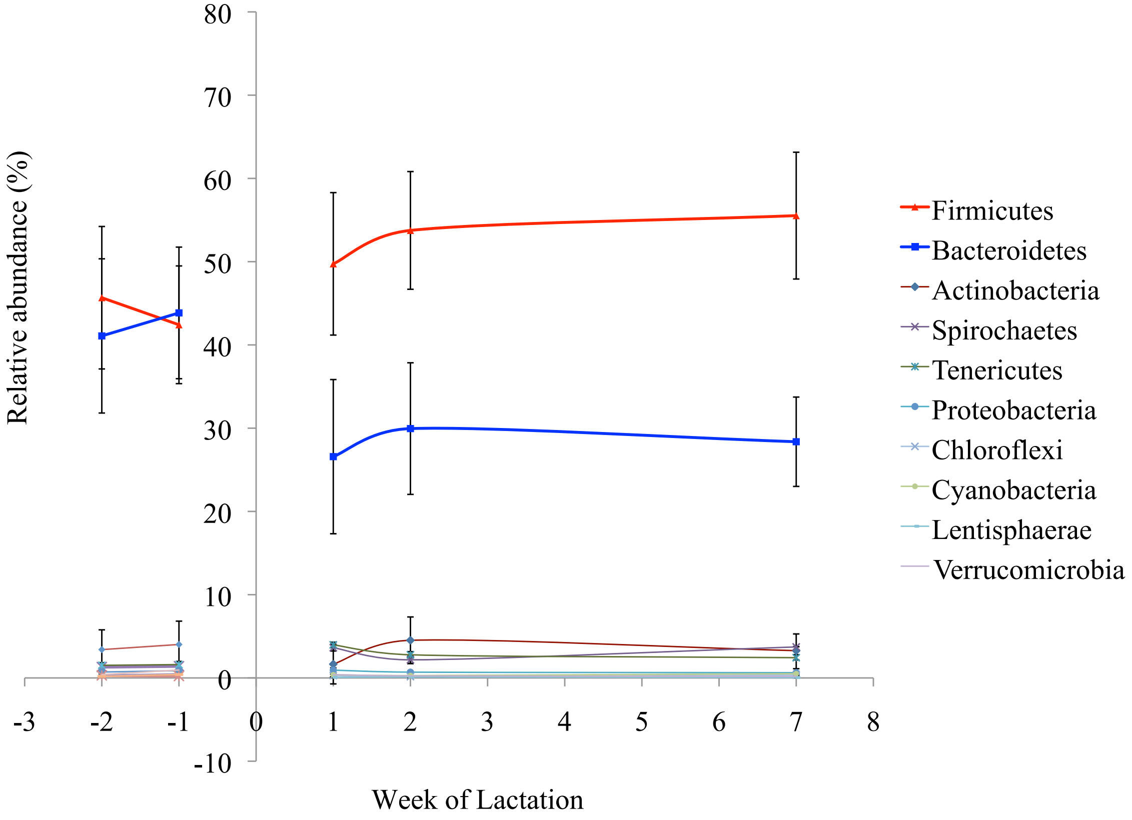


Figure 5.2. The relative abundances of 10 predominant rumen bacteria phyla in the CONV treatment across weeks before and after calving.



Alpha and β-diversity analyses

Effect of treatments on microbial α-diversity

Bacterial diversity between treatments was compared separately for the pre- and postcalving period, since the diets and physiology of the animals different between these two time periods. Bacterial α-diversity was evaluated by rarefaction analyses in 4,090-sequence subsets from each sample under different treatments during pre- and post-calving time. The rarefaction analysis showed that, probably, looking at larger number of sequences would have resulted in the identification of more phylotypes. The average Good's coverage of 82% in each treatment indicates that a large number of present bacteria in our samples were detected. The estimators of richness, the Chao1, and diversity, Shannon and Simpson indices, by treatments and during pre- and post-calving are presented in Table 5.3. The richness of microbiota before calving was affected by treatment as cows on the SHORT treatment had higher richness compared to cows on the CONV treatment. After calving, there was no difference in richness between the two treatments. The Shannon and Simpson as indices of diversity were not different between treatments before and after calving. We were also interested in variations in microbiome profile during the transition period within each treatment (Table 5.4). In both treatments the richness of the microbiome changed after calving. In the SHORT treatment, the Chao1 index indicated that richness was significantly higher before calving compared to after calving. The diversity estimators of Shannon and Simpson also showed the same trend, as microbiome diversity was higher before calving compared to after calving in this treatment. In contrast to the SHORT treatment, the rumen microbiome richness in the CONV treatment was significantly higher after calving compared to before calving. The Shannon and Simpson indices as diversity estimators were not different between before and after calving.

Table 5.3. Summary of comparisons between rumen microbiota DNA sequences of different treatments within pre or post-calving period.

Variables[2]		Mean results for indicated variables[1]				
		Observed Species	Goods-Coverage (%)	Richness[3]	Diversity[4]	
				Chao 1	Shannon	Simpson
Before	SHORT	1147	0.82	2930.51	8.26	0.986
calving	CONV	1080	0.83	2639.29	8.09	0.987
SEM				107.57	0.18	0.002
P-value				0.02	0.38	0.492
After	SHORT	1068	0.83	2676.35	8.09	0.984
calving	CONV	1149	0.82	2912.58	8.37	0.987
SEM				146.23	0.19	0.004
P-value				0.13	0.14	0.432

[1] Means are from statistical models based on 77 rumen samples across two treatments (8 cows per treatment).

[2] Dry period managements are SHORT treatment: 40-d dry period with one close-up diet; CONV treatment: 60-d dry period with two separate far-off and close-up diets.

[3] Based on Chao1 richness index.

[4] Based on Shannon and Simpson diversity estimators.

Table 5.4. Summary of comparisons between rumen microbiota DNA sequences of pre- and post-calving period within each treatment.

Variables[2]		Mean results for indicated variables[1]				
		Observed Species	Goods-Coverage (%)	Richness[3]	Diversity[4]	
				Chao 1	Shannon	Simpson
SHORT	Before	1147	0.82	2930.08	8.25	0.987
	After	1068	0.83	2676.59	7.95	0.981
SED				124.99	0.19	0.003
P-value				0.05	0.12	0.097
CONV	Before	1080	0.83	2648.77	8.19	0.984
	After	1147	0.82	2911.44	8.35	0.987
SED				143.73	0.19	0.004
P-value				0.08	0.41	0.431

[1] Means are from statistical models based on 77 rumen samples across two treatments (8 cows per treatment).

[2] Dry period managements are SHORT treatment: 40-d dry period with one close-up diet; CONV treatment: 60-d dry period with two separate far-off and close-up diets.

[3] Based on Chao1 richness index.

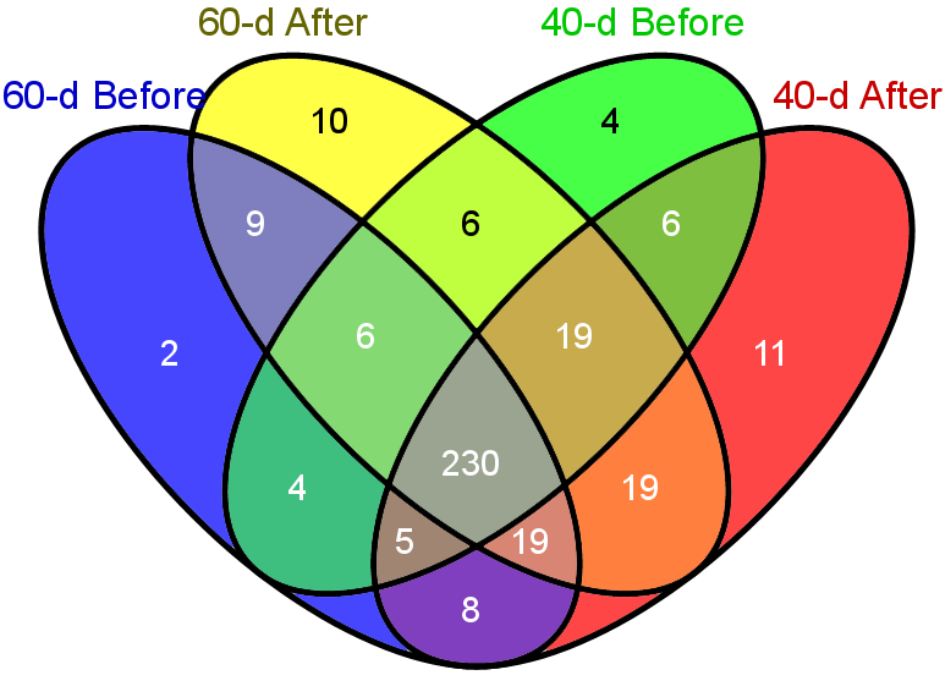
[4] Based on Shannon and Simpson diversity estimators.

Effect of treatments on microbial β-diversity

The diversity before and after calving within each treatment showed that there was a significant difference in this diversity between pre- and postpartum within the CONV treatment ($P<0.01$). However, there was no difference in bacterial diversity between pre- and postpartum within the SHORT treatment ($P<0.19$). In addition, community variation between weeks within CONV treatment were compared, and this showed that week +1 tended to be different from week -1 ($P < 0.08$). However, there was no significant difference among other weeks. The PERMANOVA and PCoA analyses also revealed that within the SHORT treatment weeks -1 vs. 2 and 1 vs. 7, were significantly different ($P<0.05$ and $P<0.03$, respectively), and weeks 1 vs. 2 tended to be different ($P < 0.09$). There was no significant difference between other weeks within the SHORT treatment. Additionally, we examined the similarity and differences between treatments within each sampling time. There was no difference between SHORT and CONV treatments within weeks -1, -2, +2 and +7. However, in first week after calving rumen microbiota β-diversity differed between treatments ($P<0.03$).

Phylogenetic analysis of sequenced samples resulted in identification of 358 genera. Most of these were common between treatments during the transition period, and some were specific to each of treatments during pre- or post-calving phase (Figure 5.3). Comparisons between pre- and post-calving in the SHORT treatment revealed 260 genera that were common between these two periods. There were also 20 and 57 specific genera associated with pre- and post-calving, respectively. The same comparison was conducted for the CONV treatment, which showed 264 common genera between pre- and post-calving period, and 19 unique genera associated with the pre-calving and 54 unique genera associated with the post-calving period.

Figure 5.3. Total number of specific and common genera between the 40-d (SHORT) and the 60-d (CONV) treatments during pre and postpartum.



Effect of parity on microbial α-diversity

We also examined at the effect of parity on shaping the rumen microbiome during the transition period within the two treatments (Tables 5.5 and 5.6). There was no difference in richness and diversity of rumen microbiota between PAR 2 and PAR 3+ cows during the pre- and also post-calving periods. In addition, microbiome richness and diversity were determined across the transition period for each parity within each treatment. The richness of the rumen microbiome in PAR 2 cows on both treatments tended ($P< 0.1$) to change after calving. In the SHORT treatment, PAR 2 cows had higher richness during pre-calving compared to post-calving. However, this trend switched in the CONV treatment, as microbiome richness was higher during post-calving compared to pre-calving. The Shannon and Simpson indices of diversity were not different between pre- and post-calving in PAR 2 cows. Also, the richness and diversity of the PAR 3+ cows in both treatments showed no difference in theses indices between the pre- and post-calving periods.

Table 5.5. Summary of comparisons between rumen microbiota DNA sequences of PAR 2 and PAR 3+ within pre- or post-calving period within each treatment.

Variables[2]		Mean results for indicated variables[1]				
		Observed Species	Goods-Coverage (%)	Richness[3]	Diversity[4]	
				Chao 1	Shannon	Simpson
		----------------------------------	SHORT Treatment	-------------------------------		
Before	PAR 2	1135	0.82	2969.13	8.22	0.987
calving	PAR 3+	1156	0.81	2922.20	8.28	0.986
SED				208.72	0.24	0.003
P-value				0.83	0.80	0.856
After	PAR 2	1067	0.83	2644.29	8.18	0.986
calving	PAR 3+	1069	0.83	2705.76	8.01	0.984
SED				194.71	0.27	0.008
P-value				0.76	0.53	0.789
		--------------------------------	CONV Treatment	-------------------------------		
Before	PAR 2	1077	0.83	2642.84	8.17	0.989
calving	PAR 3+	1098	0.83	2659.54	7.93	0.980
SED				231.10	0.46	0.007
P-value				0.94	0.62	0.227
After	PAR 2	1165	0.81	2971.19	8.42	0.988
calving	PAR 3+	1102	0.82	2739.83	8.14	0.983
SED				289.49	0.31	0.008
P-value				0.43	0.38	0.475

[1] Means are from statistical models based on 77 rumen samples across two treatments (8 cows per treatment).

[2] Parity groups are PAR 2: cows in second lactation (4 animals in the SHORT and 4 animals in the CONV treatments); PAR 3+: cows in third or more lactation (4 animals in the SHORT and 4 animals in the CONV treatments).

[3] Based on Chao1 richness index.

[4] Based on Shannon and Simpson diversity estimators.

Table 5.6. Summary of comparisons between rumen microbiota DNA sequences of pre- and post-calving within each parity within each treatment.

Variables[2]		Mean results for indicated variables[1]				
		Observed Species	Goods-Coverage (%)	Richness[3]	Diversity[4]	
				Chao 1	Shannon	Simpson
------------------------------------ SHORT Treatment ----------------------------						
PAR 2	Before calving	1139	0.82	2960.04	8.28	0.991
	After calving	1069	0.83	2644.29	8.17	0.986
SED				159.18	0.25	0.005
P-value				0.07	0.68	0.265
PAR 3+	Before calving	1156	0.81	2922.21	8.28	0.988
	After calving	1069	0.83	2705.76	7.90	0.983
SED				189.22	0.24	0.004
P-value				0.27	0.14	0.239
------------------------------------ CONV Treatment ----------------------------						
PAR 2	Before calving	1072	0.83	2644.48	8.42	0.989
	After calving	1165	0.81	2971.19	8.22	0.988
SED				176.73	0.23	0.005
P-value				0.08	0.39	0.773
PAR 3+	Before calving	1117	0.83	2737.94	8.05	0.981
	After calving	1100	0.82	2664.71	8.04	0.984
SED				229.10	0.39	0.011
P-value				0.76	0.98	0.820

[1] Means are from statistical models based on 77 rumen samples across two treatments (8 cows per treatment).

[2] Parity groups are PAR 2: cows in second lactation (4 animals in the SHORT and 4 animals in the CONV treatments); PAR 3+: cows in third or more lactation (4 animals in the SHORT and 4 animals in the CONV treatments).

[3] Based on Chao1 richness index.

[4] Based on Shannon and Simpson diversity estimators.

Effect of parity on microbial β-diversity

The results of PERMANOVA analyses showed no effect of parity on the rumen microbiome structure across weeks during the transition period within each treatment. In addition, there were no differences between PAR 2 and PAR 3+ within each treatment when compared during the combined pre- and post-calving periods.. Also, no difference between treatments was observed when the SHORT and CONV treatments were compared within individual sampling weeks, and within each parity. Moreover, there was no difference when parities were compared across weeks relative to calving, regardless of treatment. There was a significant difference between week 1 pre-calving compared to week 2 post-calving, when rumen microbiome communities were compared within PAR 2 cows, regardless of treatment ($P<0.05$). This comparison also showed that the microbiome in week 2 and 1 pre-calving tended to be different from that in week 2 and 7 post-calving ($P<0.1$ and $P<0.06$), respectively.

We were also interested in changes in rumen microbiota communities in each parity group across weeks within each treatment. This revealed no differences among weeks. In addition, comparisons between treatments within each sampling time for each parity group showed no significant differences, although, there was a trend between treatments at first week after calving in PAR 2 cows ($P<0.1$). The pair-wise test within the CONV treatment showed no difference rumen microbiota communities between parities during pre- and also post-calving. The same trend was observed within the SHORT treatment.

Discrimination analysis of rumen microbiota

The comparisons that were identified as significant through PERMANOVA were used for PLS-DA analyses using SIMCA. Generally, in week 1 after calving the populations of Actinobacteria and Firmicutes in the CONV treatment and that of Bacteroidetes in the SHORT treatment were higher. The Increased Firmicutes phylum was composed of *Clostridium*, Erysipelotrichales (o), RF39 (o), *Bulleidia*, Lachnospiraceae (f), Coriobacteriaceae (f), *Coprococcus*, Coriobacteriales (o), *Atopobium*, *Butyrivibrio*, and Bacillaceae (f). Actinobacteria was composed of Bifidobacteriaceae (f) and *Bifidobacterium*. In the SHORT treatment, the numbers of *Prevotella* and *Paludibacter* as members of Bacteroidetes were higher than those in the CONV treatment. The only genus from Firmicutes phylum that had higher abundance in the SHORT treatment was *Ruminococcus* (Figure 5.4).

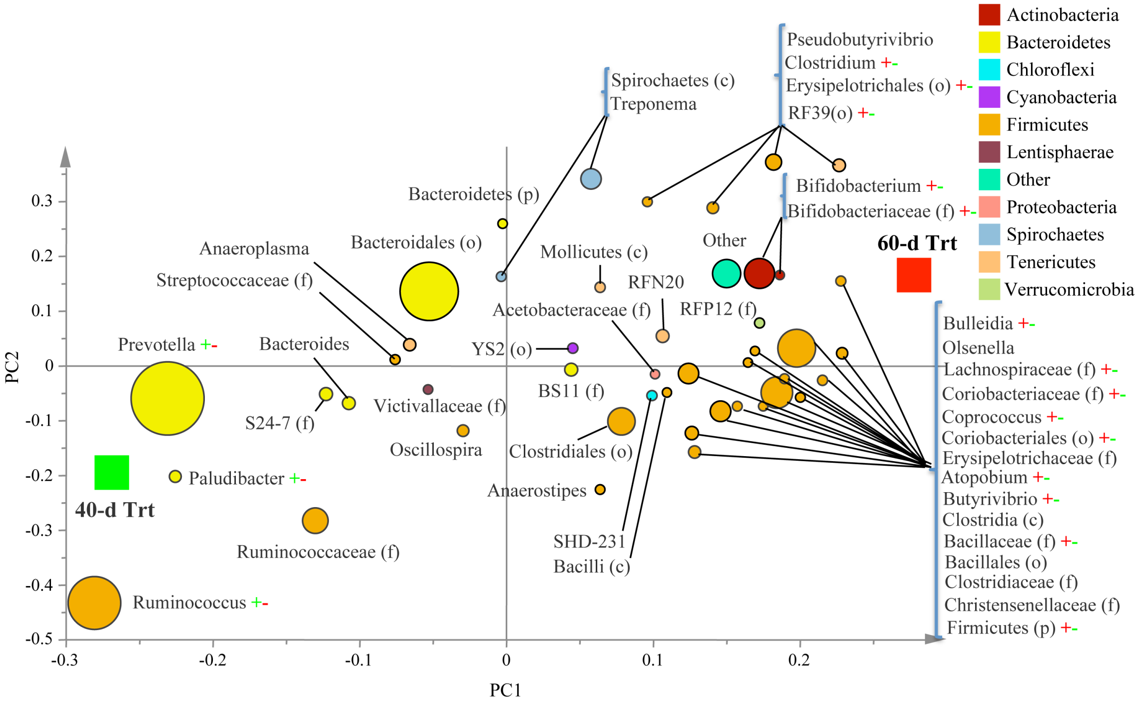


Figure 5.4. Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundances of bacterial genera in the rumen microbiota and their association with 40-d treatment (SHORT) and 60-d treatment (CONV) in the first week after calving. The presented genera are colored according to their corresponding phyla. The size of circles is indicative of taxa abundance. Taxa closer to 40-d or 60-d indicate positive association with either treatment group. The R^2 (= 0.94) and Q^2 (= 0.66) estimates were calculated based on three components. Some sequences could only be affiliated to phylum (P) class (C), order (O), or family (F) levels.

As revealed by PERMANOVA analyses, the microbial community profile was different between before and after calving within the CONV treatment (Figure. 5.2). The PLS-DA analyses showed that Firmicutes and Spirochaetaes increased after calving, while Bacteroidetes were higher before calving. At the lower phylogenic levels, Firmicutes consisted of the class Clostridia, orders Clostridiales and Erysipelotrichales, and genera *Clostridium* and *Olsenella.* Spirochaetaes consisted of the class Spirochaetes and genus *Treponema.* The Spirochaetaes, *RFN20* from Tenericutes and family of Bifidobacteriaceae from Actinobacteria also increased after calving compared to before calving in the CONV treatment, although not greatly, after calving. Before calving the numbers of *Victivallaceae* from the Lentisphaerae phylum, and *Prevotella* and *Paludibacter* from Bacteroidetes phylum were dominant (Figure 5.5).

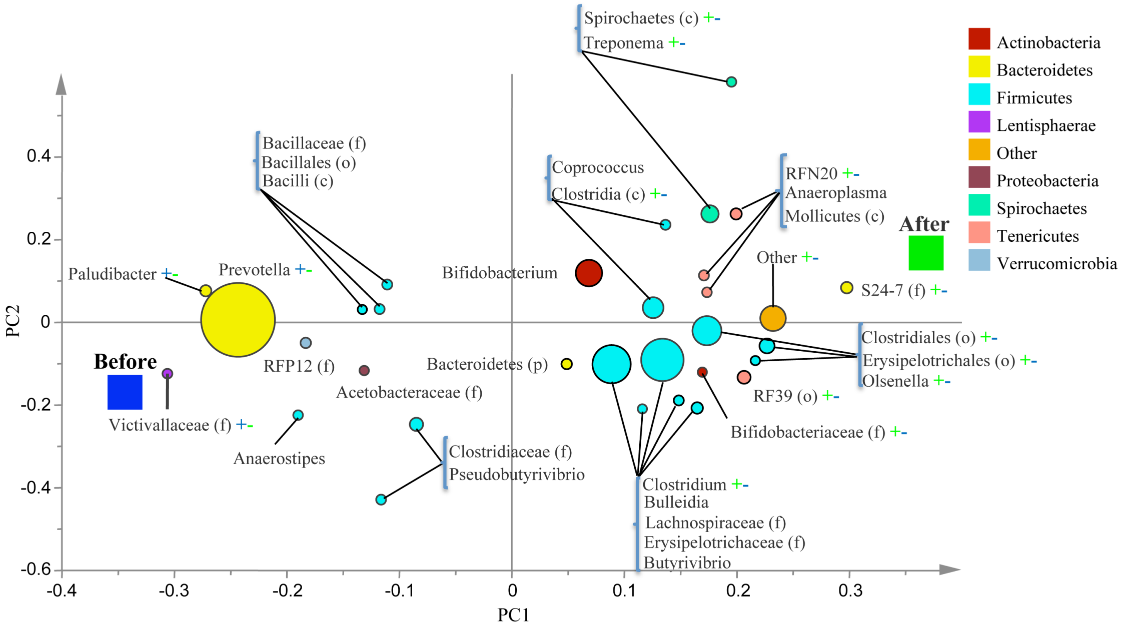


Figure 5.5. Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundances of bacterial genera in the rumen microbiota and their association with before and after calving within CONV treatment. The presented genera are colored according to their corresponding phyla. The size of circles is indicative of taxa abundance. Taxa closer to before or after indicate positive association with either time group. The R^2 (= 0.70) and Q^2 (= 0.31) estimates were calculated based on three components. Some sequences could only be affiliated to phylum (P) class (C), order (O), or family (F) levels.

Comparisons across weeks relative to calving revealed that the number of Firmicutes increased at the first week after calving compared to 1 week before calving in the CONV treatment (Figure. 5.3). On the other hand, the abundance of Bacteroidetes and Lentisphaerae were higher compared to other phyla at 1 week before compared to 1 week after calving.

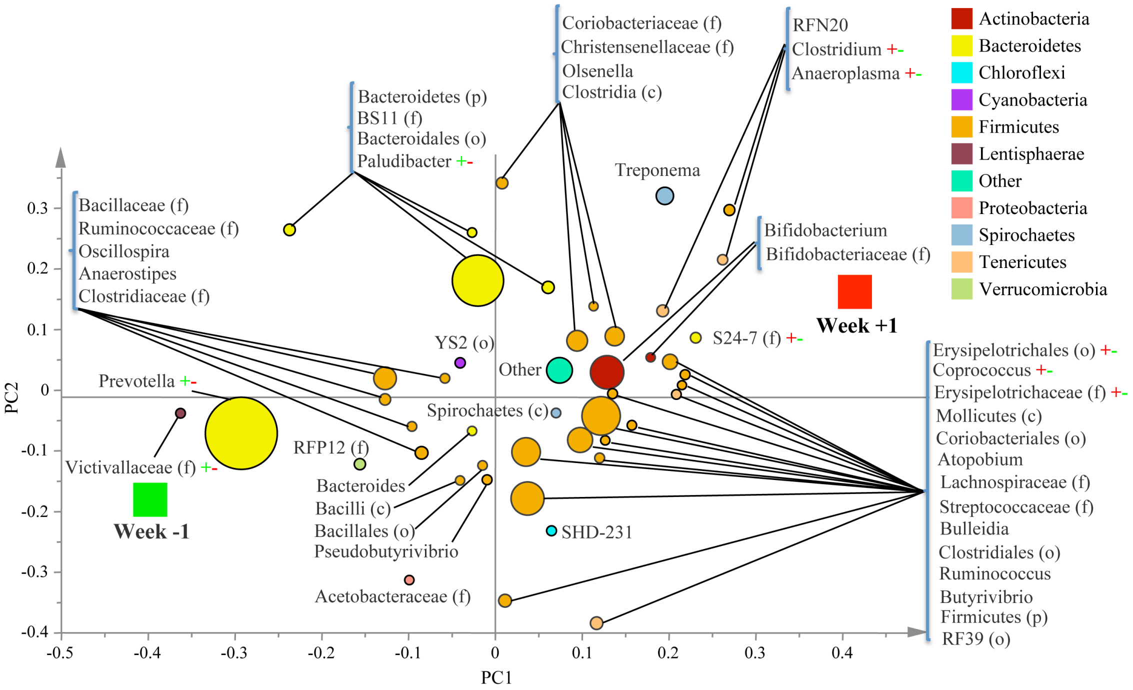


Figure 5.6. Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundances of bacterial genera in the rumen microbiota and their association with week +1 and week -1, relative to parturition, within CONV treatment. The presented genera are colored according to their corresponding phyla. The size of circles is indicative of taxa abundance. Taxa closer to week +1 or week -1 indicates positive association with either week. The R^2 (= 0.97) and Q^2 (= 0.37) estimates were calculated based on three components. Some sequences could only be affiliated to phylum (P) class (C), order (O), or family (F) levels.

Within the SHORT treatment, the abundances of Firmicutes, mostly, and Chloroflexi, Bacteroidetes and Lentisphaerae, in lesser extent, were higher at 1 week before compared to 2 weeks after calving (Figure. 5.7). The class Clostridia, order Coriobacteriales, families Christensenellaceae and Clostridiaceae, and genera *Bulleidia, Coprococcus,* and *Anaerostipes* from Firmicutes, SHD-231 from Chloroflexi, family Victivallaceae from Lentisphaerae were higher at 1 week before calving compared to 2 wks after calving. However, at 2 weeks after calving, the abundances some Firmicutes, such as *RFN20* and Erysipelotrichaceae (f), and Mollicutes (c) from Tenericutes were higher compared to week 1 before calving.

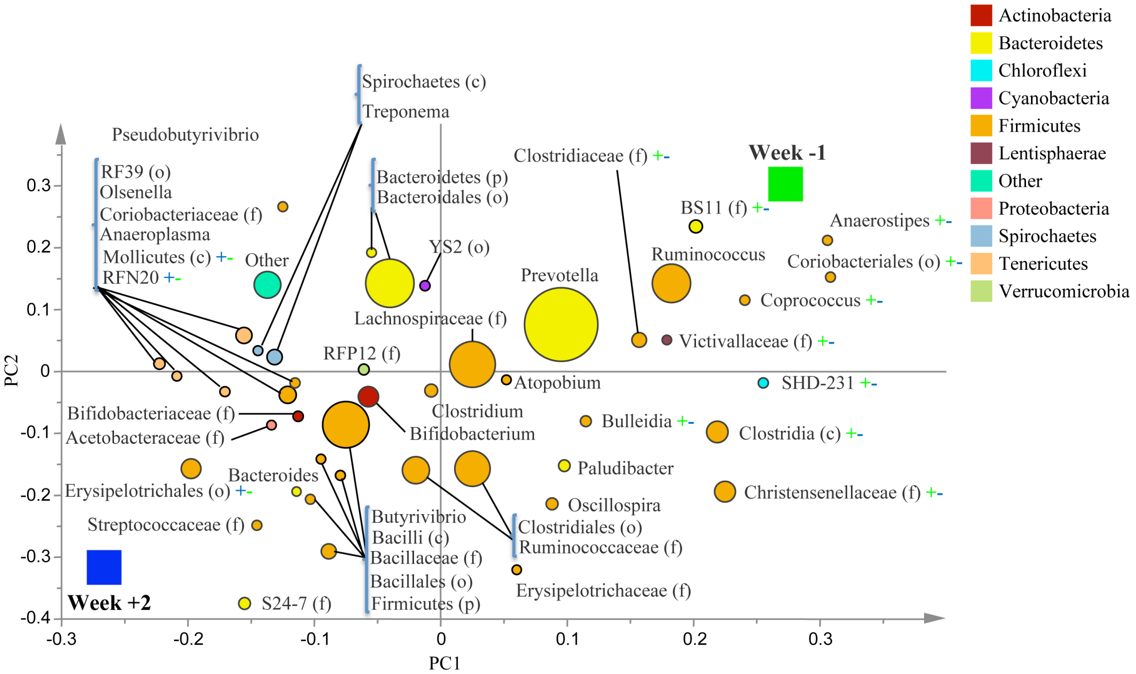


Figure 5.7. Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundances of bacterial genera in the rumen microbiota and their association with week +2 and week -1, relative to parturition, within SHORT treatment. The presented genera are colored according to their corresponding phyla. The size of circles is indicative of taxa abundance. Taxa closer to week +2 or week -1 indicate positive association with either week. The R^2 (= 0.93) and Q^2 (= 0.60) estimates were calculated based on three components. Some sequences could only be affiliated to phylum (P) class (C), order (O), or family (F) levels.

Also, comparisons within the SHORT treatment revealed that Firmicutes dominated at 7 week compared to 1 week after calving, while Bacteroidetes was more dominant at 1 week compared to 7 weeks after calving (Figure. 5.8). The abundance of Firmicutes such as Bacilli (c), Clostridia (c), Bacillales (o), Clostridiales (o), Coriobacteriales (o) and Erysipelotrichales (o), Clostridiaceae (f), Bacillaceae (f), Lachnospiraceae (f), Christensenellaceae (f), Coriobacteriaceae (f), and Erysipelotrichaceae (f), *Atopobium*, *Olsenella*, *Butyrivibrio*, and *Bulleidia* dominated at 7 weeks compared to 1 week after calving. Other important phyla at 7 week postpartum were Actinobacteria, including of the family Bifidobacteriaceae and the genus *Bifidobacterium,* and Tenericutes, which included of the genus of *RF39*. Comparing week 1 and week 7 after calving showed that the abundance of Bacteroidetes were about 25 percent higher during this time. The order Bacteroidales, family BS11 and genera *Prevotella* and *Paludibacter* were prominent among Bacteroidetes at wk 1 compared to wk 7. *Ruminococcus* was the only Firmicutes and Mollicutes (c) was only Tenericutes that were higher at week 1 compared to week 7 after calving.

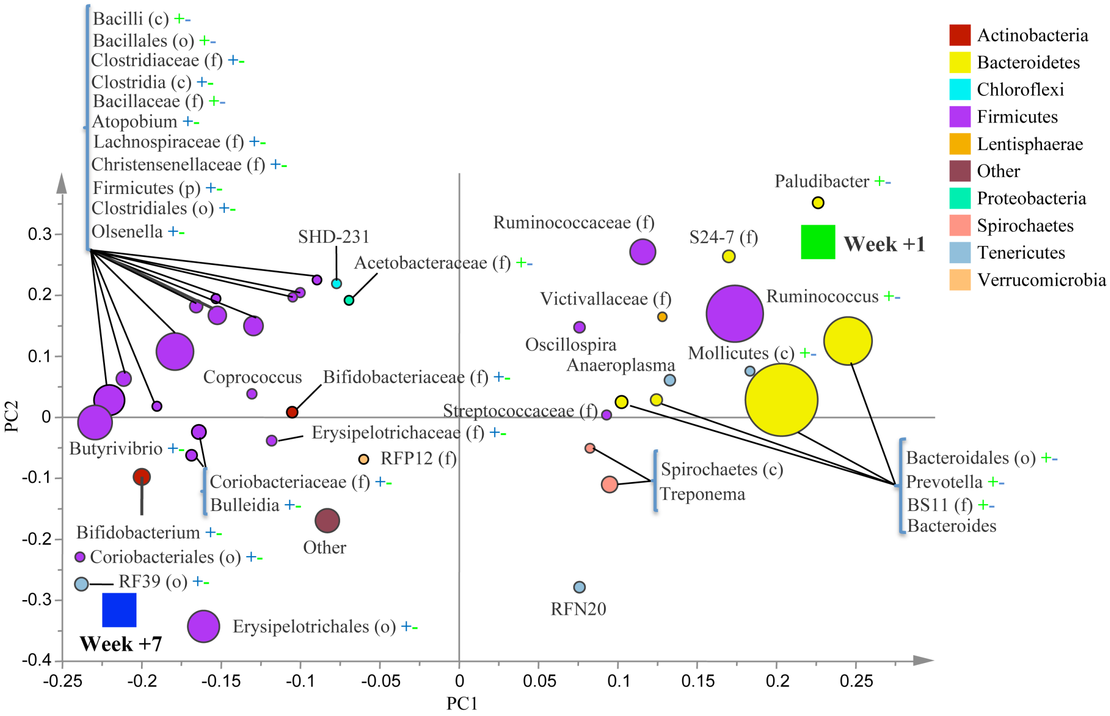
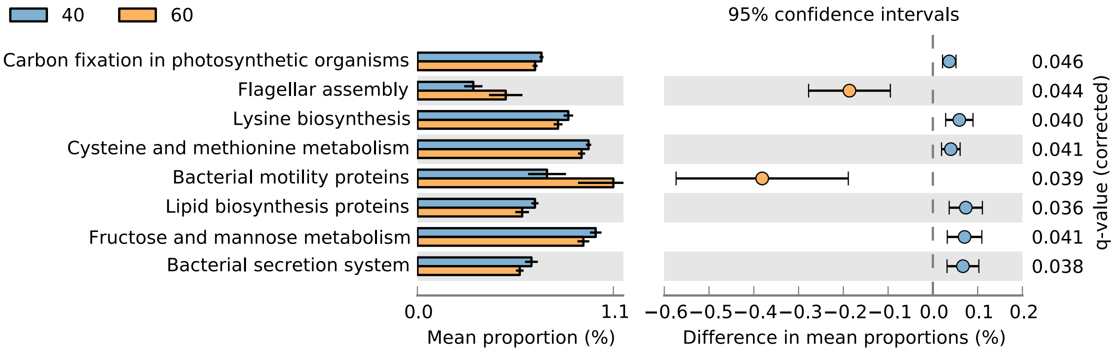


Figure 5.8. Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundances of bacterial genera in the rumen microbiota and their association with week +7 and week +1, relative to parturition, within SHORT treatment. The presented genera are colored according to their corresponding phyla. The size of circles is indicative of taxa abundance. Taxa closer to week +7 or week +1 indicate positive association with either week. The R^2 (= 0.82) and Q^2 (= 0.35) estimates were calculated based on three components. Some sequences could only be affiliated to phylum (P) class (C), order (O), or family (F) levels.

Metagenome Prediction of Rumen Microbiota

Different combinations of time relative to calving and treatment were examined to evaluate the functional differences between treatments during the pre- and post-calving period. There were no differences between treatments across weeks before and after calving. In addition, the microbial functions in both treatments did not change from pre-calving to post-calving. Moreover, there were no differences among weeks during the transition period within each treatment. There was only one functional difference in the first week of lactation in between the SHORT and CONV treatments (Figure 5.9). Rumen microbiota in the SHORT treatment had higher abundance genes involved in carbohydrate, amino acid, and lipid metabolism. In addition, they had a higher abundance of genes associated with the bacterial secretion systems. On the other hand, the abundance of genes associated with flagellar assembly and bacterial motility proteins were higher in the CONV treatment.

Figure 5.9. Functional prediction of rumen microbiota in first week after calving between the SHORT and CONV treatments.



5.5 Discussion

The use of recent breakthrough culture-independent techniques in gut microbiome studies have led to a better understanding of inter/intra microbial community relationships and also of microbial interactions with their environment and host (Nicholson et al. 2012). These advanced techniques are mostly used in human and mice, and less in domestic animal studies. These techniques were employed to analyze the samples collected to provide more in-depth information about presence of organisms and their function as a consequence of different management strategies during the dry period.

Overall rumen microbiota composition

The two predominant phyla of Firmicutes and Bacteroidetes observed in this study (Figure 5.1 and 5.2) were also reported in previous studies to have high abundances in rumen with that of Firmicutes exceeding that of Bacteroidetes (Ozutsumi et al. 2005, Tajima et al. 2007, Brulc et al. 2009). In contrast to our results, Pitta et al. (2014) reported a higher abundance of Bacteroidetes compared to that of Firmicutes in rumen when three levels of concentrate to forage ratio diets were fed.(0:100, 25:75 and 50:50). In another study, Pitta et al. (Pitta et al. 2010) demonstrated that in the liquid and solid rumen digesta of forage-fed steers, the abundance of Bacteroidetes was greater than that of Firmicutes, and both abundances were greater than that of the other phyla. It has been demonstrated that different factors, such as diet, age, animal, sampling method, the fraction (liquid or solid) of rumen digesta analyzed, the DNA/RNA extraction procedures, the primer used for amplification of variable regions, targeted variable region, differences in the copy number of the 16Sr RNA, and variation in sequencing, can affect results of microbiome studies (Brulc et al. 2009; Lodge-Ivey et al. 2009; Pitta et al. 2010; Mohammed et al. 2012; Vetrovsky and Baldrian, 2013; Fliegerova et al. 2014; Jami et al. 2014; Pitta et al. 2014b). Despite of these differences, a higher prevalence of Bacteroidetes in high fiber diets and higher prevalence of Firmicutes in high concentrate diets have been reported (Ozutsumi et al. 2005; Tajima et al. 2007; Brulc et al. 2009). The ratio of Firmicutes to Bacteroidetes affects the harvest of energy from the feed, and therefore, energy availability and is strongly correlated with milk fat yield in dairy cows (Jami et al. 2014). Firmicutes are more efficient in extracting energy from the feed materials than Bacteroidetes (Ley et al. 2006; Turnbaugh et al. 2006). In our study, the increased abundance of this phylum compared to that of other bacterial phyla involved in carbohydrate metabolism following the switch to a higher concentrate diet during the close-up and after calving, suggests a symbiotic adaptation between Firmicutes and the host. This adaptation is symbiotic as extracting more energy from the feed during the transition period is critical for dairy cows, as their energy requirements increase tremendously after calving. A higher abundance of Firmicutes compared to Bacteroidetes and other phyla before calving in the SHORT treatment compared to the CONV treatment is probably due to the consumption of close-up diet in this group for 40 d compared to only 21 days in the CONV treatment. It has been suggested that a conventional 60-d dry period with separate far-off and close-up period increases the adaptation stress since the diet changes 3 time, i.e. from the lactation diet to far-off diet, from the far-off to the close-up diet, and again from the close-up to the lactation diet (Rastani et al. 2005). In our study, offering the close-up diet during the entire dry period shaped the rumen microbiota structure similar to what was observed in postpartum cows after the consumption of lactation diet. This finding confirmed our hypothesis that providing a 40-d dry period with a single close-up diet reduces the microbial shifts and reduces adaptation stress during the transition period. The diet provided during the far-off period contains high forage and low concentrate, which affects the rumen microbiota composition and may induce an extra stress to animal, as readapting to the lactation diet is needed. The higher number of Bacteroidetes before calving in the CONV treatment is probably a carryover effect of feeding the forage-base diet of far-off phase as reported previously by others (Pitta et al. 2010). Although, the analysis of the samples collected before calving were not able to show clear shifting trends of Bacteroidetes and Firmicutes from the far-off to the close-up diet in the CONV treatment, it shows that the abundance of Bacteroidetes is diminishing and that of Firmicutes is increasing after introducing the close-up diet.

The weekly comparisons of communities between treatments showed a difference between the SHORT treatment and the CONV treatment during first week after calving. This difference was mostly due to a lower abundance of Firmicutes and a higher abundance of Bacteroidetes in the SHORT treatment compared to the CONV treatment. The first week after calving is the most challenging time for dairy cows, during which they are faced with negative energy balance and metabolic disorders. The sudden change of rumen microbiome profile in the SHORT treatment at the first week after calving suggests that there is a relationship between animal stress and gut microbiome. In agreement, microbial endocrinology studies have also shown that rumen microbiota growth changes greatly in response to host stress hormones such as the catecholamine stress hormones noradrenaline and adrenaline (Freestone and Lyte, 2010), however, we were not able to confirm this, due to lack of stress hormones measurements. It is also possible that this change at first week after calving on the SHORT treatment was as a result of normal hormonal change during this time or of introducing the lactation diet.

Richness and diversity

Treatments and their impacts on rumen microbiome

During the prepartum period, bacterial richness in rumen was significantly higher in the SHORT treatment compared to the CONV treatment (Table 5.3). This higher bacteria richness in the SHORT treatment before calving was probably due to an increase in available fermentable substrates, which stimulated the growth of bacteria specially Firmicutes (Goad et al. 1998, Fernando et al. 2010) and prepared the rumen for the lactation diet. After calving, due to receiving the same type of diet, the richness of rumen microbiome was similar between the two treatments (Table 5.3). The similar Shannon and Simpson indices in the two treatments during the pre- and post-calving periods (Table 5.3) show that the diversity of the SHORT and CONV treatments are similar. However, this cannot be translated into they have similar compositions of rumen microbiota. Comparisons of the richness and diversity between pre- and post-calving period within each treatment showed that the Simpson diversity index of rumen microbiome tended be reduced in the SHORT treatment after calving, while there was no change in the Shannon index (Table 5.4). The Simpson index is associated with the diversity of low abundant bacteria, while the Shannon index is associated with high abundant bacteria (Hill, 1973). The lower Simpson diversity may not be translated into vanishing of some bacteria taxa and, as shown by a lower richness, as the reduction in the Simpson index is probably due to reductions in the abundances of some rare bacteria in response to the lactation diet to a point that they were not detectable by the techniques that we used. In contrast, in the CONV treatment, the richness of rumen bacteria increased after calving, probably in response to the lactation diet with a high inclusion of highly fermentable carbohydrate (Table 5.4).

In order to get a better understanding of diversity of the rumen microbiome during the transition period within the two treatments, taxonomy analyses of sequenced samples were compared to detect the shared taxa. Comparisons between pre- and post-calving periods of the identified taxa revealed a high number of common bacteria between these two periods (Figure 5.3). The high number of common bacteria between pre- and post-calving in both treatments, regardless of dry period management, may suggest that: 1) there is a core microbiome that remains similar between the pre- and post calving periods, which may be capable of changing the prevalence of specific taxa based on available nutrients, or 2) the microbiota are able to change their functions according to the diet. In agreement, Fernando et al. (2010) reported a similar number of OTUs in hay-fed and grain-fed steers and suggested that although microbial diversity is similar between these two diets, the abundance of microbial taxa changes in favor of those species that digest fiber or grain.

Parity impacts on rumen microbiome

There are only a limited numbers of studies that considered the parity effect on shaping the rumen microbiome profile during the transition period (Pitta et al. 2014a). To the best of our knowledge, there is no report of the interaction effect of parity and a short dry period management during the transition period. The results of microbiome community analyses in our study showed no difference between parities during pre- or post-calving time within each treatment. The same trend was also observed in rumen microbial richness and diversity, when PAR 2 and PAR 3+ cows were compared within treatment (Table 5.5). In contrast to our results, Pitta et al. (2014a) reported that the diversity of the rumen microbiome across the transition period is higher in multiparous compared to primiparous cows. However, they did not determine the effect of parity during pre- and post calving period, and only compared primiparous and multiparous cows across the entire transition period. The discrepancy between the two studies is probably due to differences in parity of the cows as in our study, PAR 2 and PAR 3+ cows were compared, while Pitta et al. (2014a) compared primiparous and multiparous cows. Another difference between the two studies can be that the diet during pre- and post-calving periods as the ratio of forage to concentrate was different. The forage to concentrate ratio in pre- and post-calving diets in the Pitta et al (2014a) study were 80 to 20 and 50 to 50, respectively, whereas, this ratio in our study in pre- and post-calving diet were 68 to 32 and 53 to 47, respectively.

Examining at the microbial community, regardless of treatments, within each parity group across the transition period, showed differences in PAR 2 cows between pre- and post-calving. This was also observed in the alpha diversity analysis, as rumen microbiome richness of PAR 2 cows in both treatments tended ($P < 0.10$) to be different between pre- and post-calving (Table 5.6). Interestingly, this trend was similar to the treatment trends across the transition period (Table 5.4), which suggests that differences in microbiome richness in both treatments were associated mostly with the PAR 2 cows, rather than with the PAR 3+ cows. These results suggest that the rumen microbiomes of PAR 2 cows are more responsive to changes in diet compared to PAR 3+ cows, which had similar richness during pre- and post-calving.

Composition of rumen microbiota

The purpose of a 40-d dry period with only a single close-up diet was to prepare rumen for the lactation diet and reduce the adaptation stress of switching diets. Examination at the phylum level revealed that after calving and in response to the lactation diet with high-grain content, the abundances of Firmicutes increased and Bacteroidetes decreased in both treatments. Therefore, to prepare the rumen microbiota for the lactation diet, the abundances of these two major phyla during the dry period should follow a similar trend as lactation period. Providing a close-up diet for 40 days in the SHORT treatment compared providing this diet for only 21 d in the CONV treatment affected the rumen post-calving. The fact that there was no difference in the rumen microbiota composition between pre- and post-calving in the SHORT treatment can suggest that a 40-d dry period during which a single close-up diet was fed was able to make the rumen profile similar to that during the post-calving period, and prepare the rumen microbiota for the lactation diet. On the other hand, in the CONV treatment, the microbiota composition before calving was different from after calving. This difference suggests that the variations in the profile of the rumen microbiota is a result of the far-off diet. In order to get a better understanding of the microbiota adaptation within each treatment during the transition period, the lower taxonomy level was also investigated. The most abundant taxa across two weeks before calving in the CONV treatment were *Prevotella, Paludibacter* and Victivallaceae. The *Prevotella* belongs to the Bacteroidetes and it has been reported that it is the most abundant genus in rumen, accounting for 40 % to 69 % of total bacterial 16S rDNA gene sequences (Pitta et al. 2010, Wu et al. 2011, Jami and Mizrahi, 2012a, Pitta et al. 2014b). The high presence of *Prevotella in rumen digesta* has been reported when diets with different ratios of forage to concentrate are fed, which suggests various metabolic functions of species belonging to this genus (Kocherginskaya et al. 2001, Jami and Mizrahi, 2012a, Pitta et al. 2014b). *Paludibacter* is a gram-negative bacterium, a genus from phylum Bacteroidetes, which is isolated from plant residuals, and is able to ferment different types of sugar and produce acetate, propionate, and in lesser extent succinate as the fermentation end products (Ueki et al. 2006). Victivallaceae belongs to the phylum Lentisphaerae and has only one identified genus called *Victivallis*, which is known for its capability of degrading cellobiose (Zoetendal et al. 2003). Studies have suggested that different ratios of concentrate to forage in diet hinder or promote the growth of some specific bacteria or stimulate specific metabolic functions. For instance the higher abundance of *Prevotella* coupled with higher hemicellulose degradation has been reported when the ratio of concentrate to forage increased from 0 to 50% (Pitta et al. 2014a). Therefore, providing the concentrate during the close-up period in the CONV treatment, may promote the growth of *Prevotella, Paludibacter* and Victivallaceae before calving, and it may reduce the abundance of the other Bacteroidetes. After calving in the CONV treatment, *Treponema, RFN20, RF39, Clostridium, Olsenella* and Bifidobacteriaceae were increased. The Genus *Treponema* belongs to the phylum Spirochaetes, and is a gram-negative bacterium, which uses carbohydrates, amino acids and short chain fatty acids as energy sources. The RFN20 and RF39 belong to phylum Tenericutes, and has a wall-free cytoplasmic membrane. Most of the species in this phylum have not been cultured yet. However, the cultivated ones are known for their growth requirements of sterols (such as cholesterols) and fatty acids. Some species in the genus *Clostridium* are known for their capability for starch degradation and carbohydrate metabolism. The strains of genus *Olsenella* were characterized as acidotolerant and peptidolytic lactic acid utilizing bacteria (Kraatz et al. 2011). The family Bifidobacteriaceae also increased after calving in the CONV treatment. Some strains of this family are used as a probiotic due to the unique fructose-6-phosphate phosphoketolase that is associated with carbohydrate metabolism. Thus, the higher abundance of these taxa is probably due to the response to higher availability of fermentable carbohydrate and protein in the lactation diet. While the duration of the close-up diets in the SHORT treatment was longer than the one in the CONV treatment, there was no difference in rumen microbiota composition between pre- and post-calving in the SHORT treatment.

Dissimilarity of microbial communities between individual animals

The PERMANOVA analyses in this study revealed that microbial communities tended to be different among animals (*P*=0.08). The variations in rumen microbiome profile between animals have been reported in many studies (Brulc et al. 2009; Li et al. 2009; Jami and Mizrahi, 2012b; Mohammed et al. 2012; Jami et al. 2014; Khiaosa-ard and Zebeli, 2014). This dissimilarity of microbiome profile between individuals is probably as a result of differences in physiology of animals or intra-interactions of gut microbiome or inter-interaction of microbiome with the host (Li et al. 2009; Jami et al. 2014; Walker et al. 2014). Differences in rumen microbiome composition between animals with the same diet and within the same treatment suggest that there might be a core function that is similar between animals.

Prediction of the functionality of rumen microbiota

The comparison of the functional prediction results in the SHORT treatment between pre- and post-calving periods showed no difference between these two periods. However, despite of the difference in microbial composition between pre- and post-calving in cows on the CONV treatment, no functional differences were observed between these two time periods. This suggests that, although microbial composition changes during the transition period, the functionality of community can stay the same as a result of similar available nutrients. It has been suggested that the core microbiome is responsible for keeping the important functions (Loudon et al. 2014). However, in another study, it has been suggested that, although there was no core microbiome, a core function is present (Ravel et al. 2011). These findings show the importance of a core function rather than a core microbiome. We compared the microbiome communities and their functions between weeks during the transition period in order to develop a deeper understanding of the relationships between microbial composition and its function. Community analyses between weeks in the SHORT treatment showed that the microbial profile changed at first week post-calving in comparison with week 2 and 7 of lactation. Interestingly, the changes in abundance of bacteria did not change the function of community, and there was no difference in microbiome community functions between the first week after calving and the other sampling times. Also, in the CONV treatment, although there was a tendency of difference in communities between week 1 pre-calving and week 1 post-calving, no difference was observed in microbial function across these weeks. These results are another evidence that the function of the community may remain similar, while the composition changes. The functional comparisons between treatments within each week showed that these functionalities were similar, except for week 1 after calving. Community analyses between treatments within each week also revealed that the community profile at first week after calving was different between treatments. Although, the main reason for this difference is not apparent, it may be due to the effects of the dry period management. Since both treatments were receiving the close-up diet during the last 3 weeks of gestation, this difference in composition might be the carryover effect of longer feeding of the close-up diets to cows in the SHORT treatment. Another reason for the change in microbiota profile might be interactions between the host and its gut microbiome in the first week of lactation, when these cows faced metabolic challenges. However, we were not able to confirm this, since we did not measure the plasma stress hormones of dairy cows.

5.6 Conclusion

Our study clearly discriminates between the rumen microbiota of the SHORT and the CONV treatments during the pre-calving period, and shows that consumption of close-up diet for a longer time increases the relative abundance of Firmicutes, and reduces the relative abundance of Bacteroidetes. It also shows that a 40-d dry period with a single close-up diet reduces the shifts of rumen microbiota during the transition period. The sudden change in microbial composition during the most stressful time for dairy cows, at first week after calving, suggests the roles of host hormonal change along with diet in shaping the rumen microbiota composition. In addition, our data proposes a core function in the rumen that remains similar during the transition period even when the microbial composition changes. Moreover, individual differences were detected between animals within the same diet and treatment that suggests a core function between animals in addition to a core function between pre- and post-calving period.

CHAPTER SIX

6.1 GENERAL DISCUSSION

The most interesting finding in our study was that cows in second and subsequent parities have different responses to changing the dry period from 60-d with a far-off and a close up diet (CONV) to 40-d with only the close-up diet (SHORT). Cows on the SHORT treatment received the close-up diet almost 19 days longer than cows on the CONV treatment, which resulted in the provision of almost 30 percent more energy compared to cows on CONV treatment during that time. In the first manuscript we studied the effects of this change in dry period management on milk production, DMI and energy balance. We hypothesized that a SHORT treatment increases feed intake before and after calving, which consequently reduces negative energy balance after calving and enhances milk production in dairy cows compared to the CONV treatment. In contrast to our hypothesis, the SHORT treatment did not increase DMI before calving and after calving. After calving, interaction of parity and treatment affected the response of dairy cows as PAR 3+ cows especially on the SHORT treatment had lower feed intake compared to other cows. The negative impacts of excessive energy intake on PAR 3+ cows was also observed in milk production, as according to AUC calculation they produced 400 kg less milk in the first 16 wk of lactation compared to other cows. Lower milk production resulted in lower protein yields in this group of animals. In addition, we observed that cows on the SHORT treatment, regardless of their parity, had higher milk SCC compared to cows on the CONV treatment, probably because there was not enough time for intermammary infusion antibiotics to act.

Although increased feed intake during the prepartum period has been suggested to increase feed intakes during the postcalving period, providing excessive energy before calving has detrimental impacts on dairy cows health and performance (Overton and Waldron, 2004). A point that should be considered is that cows in second and subsequent parities respond differently to prepartum energy intake and length of dry period as second parity cows are still growing while cows in third parity and older are mature.

In the second manuscript we hypothesized that the SHORT treatment improves energy availability, decreases lipolysis in adipose tissues and ketogenesis, and also reduces accumulation of triacylglyceride (TAG) in the liver in dairy cows during early lactation compared to the CONV treatment. We also hypothesized that these changes are affected by parity as growing animals deposit less fat in the body and have lower mobilization of NEFA to liver and consequently lower liver TAG in early lactation. In contrast we observed that cows on the SHORT treatment had higher concentrations of NEFA in blood and tended to have higher liver TAG immediately after calving compared to cows on the CONV treatment. In agreement to our hypothesis, these effects tended to be greater in third and later parity cows compared to second parity cows. These differences between treatments are likely due to the higher energy intakes of the cows on the SHORT treatment compared to those on the CONV treatment between 40 and 21 d before calving, which may have led to comparatively more fat deposition in the cows on the SHORT treatment during the dry period and more lipolysis during early lactation as higher fat available during the negative energy balance, the higher lipolysis will happen in adipose tissues in early lactation. We discussed that PAR 2 cows on the SHORT treatment had a lower A: P ratio after calving due to higher concentrations of propionate that may have resulted in a higher availability of energy in this group of animals as propionate is the main precursor for gluconeogenesis in ruminants. In our study we observed higher concentrations of total rumen VFA across 3 wk before and also 9 wk after calving in PAR 2 cows compared to PAR 3+ cows.

We assume that the higher total VFA in PAR 2 cows might be due to lower rumen volume, lesser rumination efficiency, or lower passage rate (Dado and Allen, 1994; Bowman et al. 2003). However, it is still difficult to explain the reasons behind the higher VFA concentration in PAR 2 cows especially since we did not measure rumen volume, rumination efficiency and passage rate. The rumen pH was another parameter that was similar between treatments and parities across weeks before and after calving. Blood glucose concentrations were higher in PAR 3+ cows on the SHORT treatment during the first 3 wk of lactation compared to PAR 2 cows on the same treatment and cows on the CONV treatment. We assume that this is due to almost 67% higher blood NEFA concentrations in these animals that resulted in glucose sparing for lactose synthesis and milk production (Grummer and Carroll, 1991). In addition, the lower milk production in PAR 3+ cows on the SHORT treatment might be another reason for higher glucose level in cows on the SHORT treatment across the first 3 wk of lactation, as glucose was not used for high amount of lactose synthesis in milk. Providing excess energy during the dry period in the cows on the SHORT treatment likely resulted in deposition of more fat in this group of animals compared to others. Adipose tissues in cows with higher deposited fat are less responsive to glucose and insulin (Rukkwamsuk et al. 1999), which results in higher mobilization of NEFA similar to what we observed in cows on the SHORT treatment after calving. The liver TAG contents reflect the concentration of NEFA in serum (Vazquezanon et al. 1994; Bobe et al. 2004). Liver has a limited capacity to oxidize fatty acids or export them as very low-density lipoproteins (Van den Top et al. 1995). Fatty liver occurs when these capacities are exceeded, (Grummer, 1993). Higher concentrations of NEFA in cows on the SHORT treatment, therefore, resulted in higher concentration of TAG in liver of cows on this treatment after calving, and especially at first week of lactation when cows on the SHORT treatment experienced severe fatty acid accumulation in the liver. Accumulation of TAG in liver, such as that observed in our study may interfere with metabolic functions of liver such as reducing ureagenesis and gluconeogenesis and also reducing the immune response of animal (Strang et al. 1998), but our results do not confirm this, as concentrations of urea and glucose in the plasma were not affected by dry period length during this time. Also, regardless of treatment, PAR 3+ cows experienced higher blood NEFA after calving compared to PAR 2 cows. Dairy cows reach the maturity size at their third parity, and before this time they are still growing (NRC, 2001). Hence, this parity effect may be explained by higher energy deposits in adipose tissue than in lean muscle tissue in the more mature PAR 3+ cows leading to more lipolysis and associated problems in early lactation (Demment and Van Soest, 1985; Owens et al. 1993; McNamara and Cronje, 2000; NRC, 2001).

In the third manuscript we demonstrated the effect of dry period management on liver gene expression. We hypothesized that the SHORT treatment stimulates expression of genes and accelerates liver adaptation to higher lipid metabolism and gluconeogenesis compared to the CONV treatment., Although the SHORT treatment increased the hepatic expression of some key genes involved in fatty acid oxidation and gluconeogenesis compared to the CONV treatment, it was not able to activate all genes in the network of these pathways and, consequently, increase fatty acid oxidation and/or gluconeogenesis. At first week of lactation, genes involved in regulation of plasma NEFA such as ANGPTL4 upregulated compared to 3 wks before calving in both treatments, which probably increased mobilization of NEFA from adipose tissues during this time. These mobilized fatty acids required to be oxidized or exported as VLDL from liver or used by other tissues as a source of energy. To this end, expression of three key genes in β-oxidation including ACSL1, CPT1B, and ACADVL increased at the first week after calving in both treatments compared to 3 wks before calving. However, other genes involved in β-oxidation, such as CD36, CYC and ACOX2, only upregulated in cows on the CONV treatment at this time. This probably reduced the capacity of β-oxidation in the liver of cows on the SHORT treatment. Additionally, the DGAT gene a key gene in the triglyceride synthesis, was upregulated in cows on the SHORT treatment. This upregulation, along with a lower oxidation capacity of the liver compared to cows on the CONV treatment, resulted in accumulation of TAG in hepatocytes in this group of animals. The expression patterns of genes involved in gluconeogenesis showed higher capacity of the SHORT treatment compared to the CONV treatment. However, probably the upregulation of PC along with no change in the expression of PCK1 (cytosolic PCK) and PCK2 (mitochondrial PCK), key genes in converting the oxaloacetate to pyruvate in both treatments resulted in using of the synthesized oxaloacetate for increasing the capacity of the tricarboxylic acid cycle rather than for gluconeogenesis.

In the fourth manuscript we discussed the effect of the SHORT treatment and the CONV treatment on the rumen microbiome profile. We hypothesized and proved that a SHORT treatment reduces the compositional shifts in the rumen microbiome in liquid phase during the transition from prepartum to postpartum compared to the CONV treatment. In this study we showed the rumen microbial population changes during the transition period, and we showed that dry period management as well as parity impacts the rumen bacterial profile. The two predominant phyla identified in this study were Firmicutes and Bacteroidetes. The relative Abundances of these two phyla changed during the transition period as in the CONV treatment the abundance of Bacteroidetes before calving, due to consuming a high-fiber diet during far-off was higher than that of Firmicutes (Brulc et al. 2009). After calving, as a result of more available fermentable carbohydrates in the rumen the relative abundances of Firmicutes and Bacteroidetes switched as the relative abundances of Firmicutes increased dramatically and that of Bacteroidetes dropped. However, in the SHORT treatment, due to utilizing a close-up diet during the entire dry period, the number of Firmicutes was higher than that of Bacteroidetes during both the pre- and post-calving periods. This confirmed our hypothesis that providing a 40-d dry period with a single close-up diet reduces the microbial shifts during the transition period. In addition, due to the longer period of available high-fermentable carbohydrates, a higher microbial richness was observed before calving in the SHORT treatment than in the CONV treatment, whereas after calving the same diet was fed to all cows. We also observed that the rumen microbiome in PAR 2 cows are more responsive to changes in diet compared to PAR 3+ cows, since PAR 3+ cows had similar richness during pre- and post-calving.

A point that needs to be discussed is the power of the test and the sample size. Before conducting the experiment, a determination of the required number of replicates per treatment was conducted. We conducted this calculation assuming that we wanted to find a difference of 3 kg/d in daily milk yield significant at the 0.05 level with a standard deviation of 2.8 kg and a power of 0.80. This resulted in a required number of replicates per treatment of 14. This was similar to other experiments on dry cow management on production and health of dairy cows (Bernier-Dodier et al., 2011). After analyzing the date from our study we observed that the standard deviation of the daily milk yield was higher than expected, which resulted in a lower statistical power. Also, we had to exclude some animals from our study due to illness. Also after analyzing the data, we realized that parity had a significant effect on the results. As a result, we grouped the animals into two parity groups (PAR 2 and PAR 3+) and included the parity effect in our statistical model. If we had not done so, then the standard deviation of the treatment means would have been much greater, and few treatment differences would have been found significant. Although the power of statistical tests reduced as a result of these factors, important variables such as liver TAG still had acceptable power of 0.7 when α was set as 0.1, which was similar to that achieved in similar studies (Bernier-Dodier et al., 2011).

We compared the effects of applying a 40-d dry period during which a single close-up diet was fed with a CONV dry period during which a far-off diets was fed for 40 days and a close-up diet was fed for 21 days on dairy cow performance, physiology and metabolism, rumen microbiology, and liver gene expression. Relationship between these areas helps in better understanding the mechanisms through which dry cow management affects production and health of dairy cows. We believe that the reason behind the differences between the two treatments is due to utilizing close-up diet 19 days longer in cows on the SHORT treatment. This higher energy intake likely resulted in relatively more deposition of fat in PAR 3+ cows on this treatment, whereas PAR 2 cows on this treatment likely used the extra energy more for growing. Feed intake decreases during the last three weeks of gestation by 39% and it drops dramatically by 89% within the last week before calving. It increases gradually during 3 to 4 wk after calving (Hayirli et al. 2002). Low feed intake during late gestation is probably as a result of hormonal changes, such as increased estrogen, and other lipolytic hormones like prolactin, somatotropin and placental lactogen (Grummer et al. 1990) and, consequently, increased mobilization of NEFA, and also reduced rumen capacity due to the growing fetus (Ingvartsen, 2006).

Low feed intake and energy deficit in late gestation and early lactation period make cow utilize their body fat stores as a source of energy and mobilize NEFA from adipose tissues to liver for β-oxidation. In the current study, feed intake in PAR 3+ cows on the SHORT treatment reduced more than other cows after calving. This reduction reflects the carry-over effects of extra energy consumed by this group of cows during the dry period as described by others (Grummer et al.

2004). In our study, mobilization of NEFA started to increase 2 wk before calving in all cows and reached a peak at the first week of lactation. However, the magnitude of this mobilization differed between treatments and parity groups. Overall, PAR 3+ cows experienced higher NEFA concentrations in their plasma compared to PAR 2 cows from 2 wk before until 3 wk after calving. This was most pronounced in PAR 3+ cows on the SHORT treatment, which resulted in higher NEFA concentrations after calving in this treatment compared to the CONV treatment. Increased mobilization of NEFA in PAR 3+ cows on the SHORT treatment reflects the higher availability of fat in adipose tissues in these cows (Rukkwamsuk et al. 1999; McNamara and Cronje, 2000). High concentrations of NEFA in blood during the transition period have consequences including: 1) reduction of the insulin sensitivity in adipose tissues and muscles by changing the membrane fluidity, rotation of membrane proteins and hormone receptors responses (Drackley, 1999), thereby causing more mobilization of NEFA; 2) a higher NEFA supply that can lead to glucose sparing (Grummer and Carroll, 1991); and 3) increased accumulation of TAG in hepatocytes (Grummer, 1993). In agreement, we observed that PAR 3+ cows on the SHORT treatment had the highest blood glucose concentrations across the 3 wk after calving. Although there may be several reasons for high plasma glucose concentrations in these cows, the comparatively low milk production in this group must be one of the main reasons for this. In addition, the higher liver TAG observed in cows on the SHORT treatment during the first week of lactation indicated that they experienced severe fatty livers, whereas cows on the CONV treatment had a mild stage of fatty liver.

Treatments affected the expression of key genes involved in glucose and fatty acid metabolisms. Mobilized fatty acids can act as ligands and activate transcriptional coactivators such as PGC1-α and consequently nuclear receptors PPARγ and PPARα that are essential for insulin sensitivity, glucogenesis and β-oxidation of fatty acids (Rosen and Spiegelman, 2001). In agreement, our study showed that the PGC1-α gene was upregulated in both treatments during the first wk of lactation compared to 3 wk before calving when NEFA concentration in plasma was at the peak. The somatotropic axis, including GH, GHR and IGF-1, also plays an important role in the metabolic adaptations in the liver during the transition period. Upregulation of GH increases the gluconeogenesis and also, by mediating IGF-1, increases the uptake of NEFA by adipose tissue (Renaville et al. 2002). As reported by others (McCabe et al. 2012; Khan et al. 2014), we observed that the expression of GHR and IGF-1 decreased at first wk of lactation compared to at 3 wk before calving in both treatments. This decrease probably resulted in a reduction in NEFA uptake and increased lipolysis activity in adipose tissues. In addition, hepatic ANGPTL4, that regulates lipolysis in adipose tissues and concentration of NEFA in blood (McCabe et al. 2012), upregulated in both treatments at 1 wk after compared to 3 wk before calving. All these changes help to explain the increased plasma NEFA at the first wk of lactation. In the liver NEFA is used either for fatty acid oxidation or TAG synthesis or for VLDL export. The liver of dairy cows has inherently limited capacity for exporting TAG through VLDL. As we showed, this probably is due to low expression of the SCD gene that is involved in fatty acid biosynthesis, mainly of oleic acid, which is essential for the biosynthesis and export of VLDL from the liver (Legrand et al. 1997). Therefore, when the amount of transferred NEFA exceeds the liver capacity for oxidation and export of TAG, synthesized TAG accumulates in hepatocytes, which results in fatty liver (Drackley, 1999). The severe fatty liver condition observed in cows on the SHORT treatment compared to a moderate condition of fatty liver in cows on the CONV treatment may be explained by a higher hepatic capacity of fatty acid oxidation in the CONV treatment. This was likely the result of higher expressions of PGC-1α, CYC1 (electron carrier involved in oxidation in mitochondria), ACOX2 (β-oxidation of branched fatty acids in peroxisomes), and CYP2E1 (lipid peroxidation) in the CONV treatment compared to the SHORT treatment. In addition, a higher mobilization of NEFA along with higher expression of DGAT (catalyzes the last step of TAG synthesis) and lower expression of ELOVL5 (which reduces the inhibitory effect of ELOVL5 on uptaking fatty acids and TAG synthesis) in the SHORT treatment likely increased the TAG synthesis in the liver of this group compared to the cows on the CONV treatment. Activation of PGC-1α also resulted in upregulation of FBP and G6PC, key enzymes in gluconeogenesis, in both treatments. The PCK gene is also a key gene in regulating the glucogenesis, which was not affected by treatment during the first wk after calving. The expression of PCK isoforms determines whether amino acids or lactate are used for gluconeogenesis. The aldolase A, fructose-bisphosphate (ALDOA) gene catalyzes the conversion of fructose-1,6-biphosphate to glyceraldehyde 3-phosphate and dihydroacetone phosphate. This gene functions both in glycolysis and gluconeogenesis, and its upregulation at the first week after calving in both treatments may result in either pathways. Upregulation of G6PC, FBP1, ALDOA, and PC in the SHORT treatment during first week of lactation compared to at 3 wk before calving may reflect the higher capacity of liver for gluconeogenesis in this group compared to the CONV treatment in which only the FBP1, ALDOA and PC upregulated at that time. In addition, the expression of the SLC2A2 gene that facilitates the transportation of glucose through plasma membranes down-regulated at week 1 after calving in the CONV compared to 3 wk before calving, whereas expressions remained unchanged in the SHORT treatment. This may also illustrate the higher hepatic capacity for gluconeogenesis of cows on the SHORT treatment. It is difficult to determine if the higher plasma glucose in the cows on the SHORT treatment was only due to a higher capacity for gluconeogenesis. Although key genes in gluconeogenesis pathway upregulated in the SHORT treatment, both isoforms of PCK remained unchanged during the first week of calving in both treatments, which probably lead to using oxaloacetate for increasing the oxidative capacity of the tricarboxylic acid cycle, rather than using it for gluconeogenesis.

Dry period management also affected the rumen microbiota profile. In a traditional 60-d dry period management, diet is switched 3 times from lactation to far-off, from far-off to close-up and again from close-up to a lactation diet. Diet is one of the most important factors that can shape the rumen microbiota, and in high-forage diets the abundance of fibrolytic bacteria increase whereas in high grain diets the abundance of amylolytic, lactate-utilizer and propionate-producing bacteria increase (Mohammed et al. 2012). The lactation diet had higher energy and protein contents compared to close-up diet and replacing it with a far-off diet with low energy and protein at the onset of dry period requires adaptation of rumen microbiota from high starch utilizers to high fibrolytic ones. By switching from the far-off to the close-up diet, rumen microbiota again need to adapt to a high grain diet. These changes in diets during a conventional 60-d dry period induce adaptation stresses to rumen microbiota that affects amount of feed intake in dairy cows especially during early lactation (Rastani et al. 2005; Wang et al. 2012). In contrast to a traditional 60-d dry period management, a short 40-d dry period with only a close-up diet reduces the changing in diets and rumen microbiota adaptation stresses. The attempt should be to reduce the fluctuations in populations of rumen microbiota and keep their population as similar as possible to that when the lactation diet is fed. In our study the predominant phyla were Firmicutes and Bacteroidetes. The ratio between these two phyla affects the harvest of energy as Firmicutes are more efficient in harvesting energy from the feed. The ratio of these two phyla is strongly correlated with milk fat yield in dairy cow (Jami et al. 2014). Firmicutes are more efficient in extracting energy from the feed materials (Turnbaugh et al. 2006). In our study, the increased abundance of this phylum compared to that of other bacterial phyla involved in carbohydrate metabolism after providing high-concentrate diet during the close-up and especially during the lactation period suggests a symbiotic adaptation between Firmicutes and host. As extracting more energy in a form of SCFA from the feed during the transition period is critical for dairy cows, which are in a shortfall of energy for milk production (Bell, 1995). A higher abundance of Firmicutes compared to that of Bacteroidetes and other phyla before calving in the SHORT treatment compared to the CONV treatment is probably due to consuming the close-up diet in this group for 40 days compared to only 21 days in the CONV treatment which provided enough time for this group of bacteria to grow. In our study, offering the close-up diet during the entire dry period shaped the rumen microbiota structure during the prepartum as similar as the postpartum rumen profile, after consuming the lactation diet. This finding confirmed our hypothesis that providing a 40-d dry period with a single close-up diet reduces the microbial shifts during the transition period.

CHAPTER SEVEN

CONCLUSIONS

Compared to a 60-d dry period with separate far-off and close-up periods, a 40-d dry period during which only a close up diet was fed reduced DMI, fat corrected milk yield and milk protein yield in early lactation in third-parity and older cows, but not in second-parity cows. These differences between parity groups were most likely due to higher fat deposition and associated problems in the third-parity and older cows compared to the second-parity cows, as a result of feeding a 19-d longer close-up diet in the SHORT treatment.

We observed that cows on the SHORT treatment had higher concentrations of NEFA and tended to have higher liver TAG immediately after calving compared to cows on the CONV treatment. These effects tended to be greater in third and later parity cows compared to second parity cows, likely due to the higher energy intakes of the cows on the SHORT treatment compared to those on the CONV treatment between 40 and 21 d before calving, which may have led to comparatively more fat deposition in the cows on the SHORT treatment during the dry period and more lipolysis during early lactation in these cows.

Higher plasma glucose concentrations were observed in third-parity and older cows on the SHORT treatment after calving that was likely due to comparatively lower milk yields and higher serum NEFA concentrations of these cows that likely contributed to glucose sparing.

Expression of key genes involved in β-oxidation such as ACSL1, CPT1B, CPT2, and ACADVL increased at the first week after calving compared to the 3 wk before calving in both treatments. The other genes involved in β-oxidation such as CD36, CYC and ACOX2 upregulated during this time in the CONV treatment, while they remained unchanged in the SHORT treatment. This probably resulted in lower hepatic β-oxidation capacity in the SHORT treatment compared to the CONV treatment. At first week after calving compared to 3 week before calving, expression of DGAT, a key gene in the triglyceride synthesis, increased in the SHORT treatment while it remained unchanged in the CONV treatment. Moreover, expression patterns of genes involved in VLDL synthesis in both treatments showed a low capacity of liver for exporting triglyceride from liver. Considering all together, the lower capacity of β-oxidation and higher triglyceride synthesis in the 40-d treatment along with low VLDL synthesis capacity probably resulted in accumulation of triglyceride in the liver in this treatment compared to the CONV treatment.

The expression patterns of genes involved in gluconeogenesis showed higher capacity of SHORT treatment compared to the CONV treatment, however, probably due to the absence of a change in expression of PCK1 and 2, the synthesized oxaloacetate was used for increasing the capacity of tricarboxylic acid cycle. Also, glycolysis reduced in both treatments after calving, while interestingly the capacity of glycogenesis increased.

Our study discriminates between the rumen microbiota of the SHORT and the CONV treatments during pre-calving time and shows that consumption of close-up diet for a longer time increases the relative abundance of Firmicutes and reduces the relative abundance of Bacteroidetes. It also shows that a 40-d dry period with a single close-up diet reduces the shifts of rumen microbiota during the transition period.

The sudden change in microbial composition in the most stressful time for the cow, at first week after calving, suggests the role of host stress hormones in shaping the rumen microbiota composition. Also, our data proposes a core function in the rumen that remains similar during the transition period even when the microbial composition changes.

Based on these results, a 40-d dry period management with only a close-up diet might be beneficial for second parity cows. However, this treatment may be detrimental for older cows due to excessive energy intake and fat deposition during the dry period

CHAPTER EIGHT

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