AN ABSTRACT OF THE DISSERTATION OF

Shana Salah Ali Jaaf for the degree of Doctor of Philosophy in Animal Sciences presented on December 3, 2019.

Title: Effect of Agronomically Selenium Biofortified Hay on Oxidative Status, Metabolic and Inflammatory Biomarkers, and Immune Response of Transition Primiparous Dairy Cows and Se Transfer and Glutathione Peroxidase Activity in their Calves.

Abstract approved:

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Massimo Bionaz

During the peripartum period (3 weeks before through 3 weeks after calving, a.k.a. “transition”), high producing dairy cows experience, among others, , oxidative stress and immune suppression that compromise performance and increased incidence of diseases.Among trace minerals, supplementation of Selenium (Se) can help to improve the transition by enhancing glutathione peroxidase (**GPx**) activity and boosting the immune system. Thus, cows during the transition period would benefit from a high amount of Se. Among ways to supplement Se to dairy cows, use of biofortified plants (i.e., the soil is fertilized by inorganic Se that is then incorporated in plant proteins as Se-methionine and Se-cysteine) has proven to be effective in increasing the Se status and improve animals’ health and performance, including their offspring, when fed topregnant beef cows at 2.5 % BW. Different from beef cows, high-producing dairy cows need a more precisely balanced ration that requires a more

complex diet; thus, it is not possible to feed only hay. Thus, *it remains to be determined* if feeding an increased level of Se biofortified hay can be effective in improving the Se status in peripartum high-producing dairy cows.

The present dissertation aimed to assess if feeding Se biofortified hay at a level that is more typical for a ration of high-producing dairy cows is an effective way to improve the transition from pregnancy to lactation in high-producing dairy cows. To accomplish the aim, we tested if supplementing high producing dairy cows with 1 kg/100 kg BW (1% BW) of Se biofortified hay was effective in improving 1) Se status (Part 1) and 2) the transition from pregnancy to lactation (Part 2). For the experiment, we used 10 Jersey and 8 Holstein pregnantdairy heifers that were supplemented with 1% BW of Se biofortified (TRT; n=9; 3.2 mg/kg DM Se) or non-biofortified (CTR; n=9; 0.4 mg/kg DM Se) alfalfa hay mixed with the TMR from approx. 40 days prior- to 2 weeks post-partum.

The objectives of Part 1 of the experiment were to assess if the aforementioned treatment improved 1) the Se concentration in whole blood, liver, milk, and colostrum; 2) the amount of Se that is transferred into the calves; and 3) the antioxidant activity of cows and calves through the determination of GPx activity. For Part 1, we hypothesized that supplementing primiparous dairy cows with a relatively low amount of Se biofortified hay during the dry period and early lactation enhances the Se concentration and antioxidant status in cows and their calves. Se concentration and other trace minerals in whole blood of cows and claves and liver, milk, and colostrum of cows were measured by using ICP-MS, and GPx activity in samples was measured via a commercial kit. Se concentration in blood was 2-fold higher ($P<0.05$) in TRT vs. CTR

(204.5 vs. 95.0 ng/ml) which resulted in higher Se in liver (1.24 vs 0.62 µg/g dry weight) and colostrum (99.1 vs. 27.2 ng/ml) but not milk. Higher GPx activity in plasma (92.8 vs. 77.9 nmol/min/ml) and erythrocytes (549.2 vs. 260.0 nmol/min/ml) were detected, but not in milk. GPx activity in plasma samples was also higher in TRT vs. CTR (92.8 vs. 77.9 nmol/min/ml) and erythrocytes (549.2 vs. 260.0 nmol/min/ml) but not in milk. Compared to CTR, calves from TRT had higher Se in the blood (215.5 vs. 161.22 ng/ml) but only a numerically (P=0.09) larger GPx activity in plasma. A positive correlation was detected between Se in blood and GPx activity in erythrocytes and plasma in cows. Our results proved that feeding pregnant primiparous dairy heifers with one % BW of Se biofortified alfalfa hay is an efficient way to improve Se status in cows and their calves. Se supplementation increased antioxidant activity via GPx in cows and, numerically, in calves. Feeding Se biofortified hay raised Se level in colostrum but not in milk.

Part 2 of the experiment aimed to examine the effect of the treatment on performance, metabolism, oxidative status, and immune response of transition primiparous dairy cows. We hypothesized that supplementing primiparous dairy cows with a relatively small amount of Se biofortified hay during the dry period and early lactation improves performance, metabolism, oxidative status, and immune response. Cows were monitored daily for health status, dry matter intake (DMI), activity, weekly for body weight, and body condition score (BCS). Blood samples were also collected to measure hematocrit (HMC) and metabolic, oxidative, and inflammatory biomarkers. Phagocytosis, white blood cell differential count, and carrageenan skin test (CST) were measured in primiparous cows. Milk yield and components, including fatty acid profile

(FA), were determined. Supplementation of primiparous cows with 1% BW of Se-biofortified hay did not affect milk yield or milk components, including fatty acid profile, body weight or DMI. Se biofortified hay affected only a few of the measured parameters in the blood. Albumin level increased, and haptoglobin and urea tended to be increased by supplementation of Se-biofortified hay, indicating a better liver status, especially post-partum. The treatment increased advanced oxidation protein products (AOPP), which is a marker of protein oxidation. An improved antioxidative function of albumin by Se-biofortified hay supplementation was supported by the negative correlation of AOPP with myeloperoxidase and parameters related to inflammation but a positive association with albumin. Se biofortified hay increased hematocrit indicating either a positive effect on erythropoiesis or in lifetime of erythrocytes that could be reduced around calving and/or per effect of severe inflammations. Treatment did not affect any of the measured parameters associated with the immune system. Feeding 1% BW of Se biofortified hay had little effect on metabolic, inflammatory, and oxidative status parameters with no effect on cow's performance or immune response. Supplementation with Se biofortified hay possibly enhanced liver function, promoted the antioxidant role of albumin, and improved level of red blood cells.

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Effect of Agronomically Selenium Biofortified Hay on Oxidative Status, Metabolic and Inflammatory Biomarkers, and Immune Response of Transition Primiparous Dairy Cows and Se Transfer and Glutathione Peroxidase Activity in their Calves

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APPROVED:

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Shana Salah Ali Jaaf, Author

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support, and persistence. And, to my two angels Ahmed and Ava, I wish they will be proud of me in the future.

CONTRIBUTION OF AUTHORS

Shana Salah Ali Jaaf organized and performed the experiment, collected specimens, analyzed samples (specifically she performed analysis of microminerals in blood, milk, and liver, measured glutathione peroxidase activity in plasma and whole blood, assessed concentration of immunoglobulins in blood and colostrum, performed white blood cells count, phagocytosis, and differential via flow cytometer), statistically analyzed data, and wrote the manuscripts.

Dr. Massimo Bionaz provided funding, designed the experiment, helped in data analysis, and helped writing and provide final editing of the manuscript.

Dr. Charles Estill contributed to the liver biopsy and the editing of the manuscript for Part 1 of the experiment.

Dr. Erminio Trevisi performed the analysis of the blood markers and contributed to the editing of the manuscript for Part 2 of the experiment.

TABLE OF CONTENTS

Page

CHAPTER 1 1

1.1. Introduction: transition period and associated problems and way of improving using dietary approaches 1

1.2. Chemical forms of selenium and selenoproteins 6

1.3. The biological function of selenium and selenoproteins 8

1.4. Intestinal absorption of Se, distribution, and metabolism in dairy cows 11

1.5. Effect of selenium forms on immune function and cows health 13

1.7. References 16

CHAPTER 2 23

ONE % BW OF SELENIUM BIOFORTIFIED ALFALFA IMPROVES SELENIUM STATUS AND GLUTATHIONE PEROXIDASE ACTIVITY IN PREIPARTUM DAIRY COWS AND THEIR CALVES 23

2.1 Summary 24

2.2 Introduction: 25

2.3 Materials and Methods 27

2.3.1 Animals, ration, and experimental design 27

2.3.2 Dry matter of the TMR 31

2.3.3 Collection of blood and milk 34

2.3.4 Liver tissue collection 34

2.3.5 Measurement of Se and other trace minerals 35

2.3.6 Glutathione peroxidase activity 36

2.3.7 Se balance and % transfer into calves and Se excretion into milk 37

2.3.8 Statistical analysis 37

TABLE OF CONTENTS (Continued)

Page

2.4 Results 38

2.4.1 Se balance 38

2.4.2 Level of Se 42

2.4.3 Other microminerals 44

2.4.4 GPx activity 44

2.4.5. Correlations 45

2.5 Discussion 47

2.5.1 One % BW of Se biofortified alfalfa improves the Se status in cows. 47

2.5.2 Se is efficiently transferred to colostrum but not milk in early post-partum cows 49

2.5.3 Colostrum is more effective than placenta in transferring Se in calves 51

2.5.4 GPx activity in improved in cows by feeding Se biofortified hay 51

2.5.5 Jersey and Holstein cows differ in the response to feeding Se biofortified hay 52

2.6 Limitations of the study 52

2.7 Conclusions 53

2.8 Acknowledgments 53

2.9 References 54

CHAPTER 3 57

SELENIUM BIOFORTIFIED ALFALFA HAY SUPPLEMENTED 57

TABLE OF CONTENTS (Continued)

Page

TO PRIMIPAROUS COWS DURING THE PERIPARTUM AFFECTS THE ANTIOXIDANT FUNCTION OF ALBUMIN BUT HAS LITTLE IMPACT ON METABOLIC AND IMMUNE FUNCTION 57

3.1 ABSTRACT 58

3.2 INTRODUCTION 60

3.3 MATERIAL AND METHODS 62

3.3.1 Animals, ration, and experimental design 62

3.3.2 Feed intake, body weight, and body condition score 63

3.3.3 Activity, milk yield, milk components, and milk fatty acids 63

3.3.4 Inflammatory and metabolic profile 63

3.3.5 Carrageenan skin test 64

3.3.6 White blood cells counting, phagocytosis, and differential 65

3.4 Statistical analysis 66

3.5 RESULTS 66

3.5.1 Cow's performance and milk composition 66

3.5.2 Metabolic, inflammation, oxidative, and immune-related parameters measured in plasma 67

3.5.2.1 Metabolic-related parameters 67

3.5.2.2 Inflammatory-related parameters 68

3.5.2.3 Liver health 68

3.5.2.4 Oxidative status. 68

3.5.2.5 Minerals. 68

3.5.2.6 Kidney function 69

TABLE OF CONTENTS (Continued)

Page

3.5.2.7 Other parameters. ……...…….……69

3.5.3 Immune-related parameters 69

3.6 DISCUSSION 69

3.6.1 Cow performance was not affected by Se biofortified hay 69

3.6.2 Se biofortified hay improves albumin production by the liver 70

3.6.3 Se biofortified hay does not affect the oxidative status and the immune system but might improve the antioxidant function of albumin 72

3.6.4 Se-biofortified hay affects mineral metabolism 74

3.6.5 Se biofortified hay improves erythropoiesis 75

3.7 Limitations of the study 75

3.8 SUMMARY AND CONCLUSIONS 76

3.9 ACKNOWLEDGMENTS 76

3.10 REFRENCES 78

CHAPTER 4 94

CONCLUSIONS AND FUTURE STUDIES 94

LIST OF FIGURES

Figure Page

Figure 2.1 Experimental design and samplings. The Selenium group received 1 kg/100 kg of body weight of Se-biofortified alfalfa (3.25 ppm of Se) from -40 to 14 day relative to parturition (or day in milk – DIM) while the Control group received alfalfa without biofortification (0.43 ppm of Se). GPx = measurement of glutathione peroxidase activity via a commercial kit. Microminerals were measured via IPC-MS.....30

Figure 2.2 The concentration of Se in whole blood (A), liver (C), and milk (D) and glutathione peroxidase (GPx) activity in erythrocytes (E), milk (F), and blood plasma (G) in Jersey (Jer; n=5/group) and Holstein (Hol; n=4/group) cows supplemented with 1% BW of Se biofortified alfalfa hay (Sel) or no biofortified alfalfa hay (Ctr) from 40 days prior expected parturition to 14 days after calving. Reported are also the level of Se in whole blood (B) and GPx activity in plasma (H) of their calves during the first 24 days after birth. Significant (P≤0.05) effects of Selenium (TRT), breed (B), and time (T) and their interactions are indicated by the acronyms in the graph. *Denotes difference between groups (P≤0.05) when TRT×Time P≤0.05; #Denotes difference between groups (P≤0.05) when Breed×Time P≤0.05.....43

Figure 3.1 Experimental design and samplings. The Selenium group received 1% BW of Se-biofortified alfalfa (3.25 ppm of Se) from -40 to 14 day relative to parturition while the Control group received alfalfa without biofortification (0.43 ppm of Se)..... 84

Figure 3.2 Weekly milk yield until 120 days post-calving in Jersey (Jer; n=5/group) and Holstein (Hol; n=4/group) primiparous cows supplemented with 1% BW of Se-biofortified alfalfa hay (Sel) or no biofortified alfalfa hay (CTR) from 40 days prior expected parturition to 14 days after calving. Reported in the graph is the P-value of the effect of treatment (*TRT*), Week, Breed, and their interactions. 86

Figure 3.3 Blood parameters that were significantly (p≤.05) affected by treatment (*TRT*) or the interaction between *TRT*, breed (Br), and Time in Jersey (Jer; n=5/group) and Holstein (Hol; n=4/group) primiparous cows supplemented with 1% BW of Se-biofortified alfalfa hay (Sel) or no biofortified alfalfa hay (CTR) from 40 days prior expected parturition to 14 days after calving. Reported in the graph are the P-value of the overall effect of treatment (*TRT*), Time, Breed, and their interactions. * and # denote difference with P≤0.05 between groups at each time point in Holstein and Jersey cows, respectively, when significant (P≤0.05) TRT×Br×Time was detected. @ denotes a significant effect in a time point when significant (P≤0.05) TRT×Time interaction was detected..... 87

Figure 3.4 Proc correlation (r above and P-value below) among measured parameters in blood. Red to pink shade denotes the level of significance for the correlations that are either positive (purple shade) or negative (green shade)……..……93

LIST OF TABLES

Tables Page

Table 1.1 Effect of supplementation of cattle with an organic and inorganic form of Se on Se level in blood and antioxidant indices and biochemical parameters..... 4

Table 1.2 Chemical forms of Se and percentage of the primary form..... 7

Table 1.3 Types of GPx in different tissue and its main function 10

Table 1.4 Effect of organic and inorganic form of Se on cow's inflammation parameters 15

Table 2.1 Chemical characteristics of alfalfa hays used for the present experiment..... 32

Table 2.2 Composition and chemical characteristics of the total mixed rations used in the present experiment..... 33

Table 2.3 Se balance estimated in Jersey and Holstein heifers supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior to expected parturition. 39

Table 2.4 Se balance estimated in Jersey and Holstein heifers supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior expected parturition. 40

Table 2.5 The overall concentration of Se and other trace minerals in whole blood, milk, and liver of Jersey and Holstein heifers supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior expected parturition to 14 days after calving. Reported is also the level of the microminerals in the whole blood of their offspring during the first 24 days after birth. 41

Table 2.6 The correlation coefficient between whole blood, plasma, liver, and milk concentrations of Se and GPx activity of Jersey and Holstein primiparous cows supplemented with 1% BW of Se-biofortified alfalfa hay or control alfalfa hay from 40 days prior expected parturition to 14 days after calving. Reported are also the correlation coefficient in plasma of their offspring during the first 24 days after birth..... 46

Table 3.1 Performance of Jersey (n=5/group) and Holstein (n=4/group) primiparous cows supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior expected parturition to 14 days after calving ... 88

Table 3.2 Plasma parameters in Jersey and Holstein primiparous cows supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior expected parturition to 14 days after calving..... 89

LIST OF TABLES (Continued)

Tables Page

Table 3.3 Carrageenan skin test (CST), phagocytosis, and WBC differential in Jersey and Holstein primiparous cows supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior expected parturition to14 days after calving 90

Table 3.4 Fatty acid profile (mg/100 mg) of Jersey and Holstein primiparous cows supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior expected parturition to 14 days after calving…...91

LIST OF APPENDICES

Appendix Page

APPENDIX I PAPER PUBLISHED: 2,4-THIAZOLIDINEDIONE IN WELL-FED LACTATING DAIRY COATS: I. EFFECT ON ADIPOSITY AND MILK FAT SYNTHESIS 96

1.1 Abstract 97

1.2 Introduction 97

1.3 Materials and Methods 100

1.3.1 Experimental design and animal management 102

1.3.2 Treatments 102

1.3.3 Measurements, sample collection, and blood metabolites 103

1.3.4 Adipose biopsy and histological analysis, and mammary epithelial cells isolation 104

1.3.5 Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR) 105

1.3.6 Statistical analysis 106

1.4 Results 107

1.4.1 Animal Performance 110

1.4.2 Blood metabolic parameters 110

1.4.3 Adipocytes size distribution 115

1.4.4 Gene expression 115

1.4.4.1 Subcutaneous adipose tissue 115

1.4.4.2 Mammary epithelial cells 117

LIST OF APPENDICES (Continued)

Appendix Page

1.4.5 Fatty acid profile in milk 122

1.5 Discussion 123

1.5.1 2,4-TZD induces greater glucose utilization in the adipose tissue but did not activate PPARγ 123

1.5.2 2,4-TZD does not affect milk fat synthesis 126

1.6 Conclusions 129

1.7 Supplementary Materials: 129

1.8 Author Contributions: 130

1.9 Funding: 130

1.10 Acknowledgments: 130

1.11 Conflicts of Interest: 130

1.12 Appendix A 130

1.12.1 Supplementary Materials and Methods 131

1.12.1.1 Animal management 131

1.12.1.2 Dry matter intake, Energy Corrected Milk (ECM), Dairy Efficiency (DE), and Body Condition Score (BCS) 131

1.12.1.3 Adipose biopsy 132

1.12.1.4 Histological analysis of the adipose tissue 132

1.12.1.5 Mammary epithelial cells isolation 133

1.12.1.7 RT-qPCR 134

1.12.1.8 Milking and milk sample analysis 136

1.12.1.9 Milk fatty acid analysis 136

1.12.1.10 Blood Metabolites 137

LIST OF APPENDICES (Continued)

Appendix Page

1.13 References 139

APPENDIX 2 PARAMETERS AND PROTOCOLS 156

Non-esterified fatty acids (NEFA in mmol / l): 161

β-hydroxybutyrate (βOHB mmol / l): 161

Glucose (mmol / l): 161

Total cholesterol (mmol / l): 161

Urea (mmol / l): 162

Ceruloplasmin (mol / l): 162

Haptoglobin (g / l): 162

Myeloperoxidase (U / L): 163

Albumins (g / l): 163

Paraoxonase (U / ml): 163

Aspartate-amino-transferase (AST / GOT) (U / l): 163

Gamma-Glutamyl transferase (GGT) (U / l): 163

Alkaline phosphatase (U / l): 164

Total bilirubin (µmol / l): 164

Total ROMs (mg H2O2 / 100 ml): 164

Thiol groups (SHp) (µmol / l): 164

FRAP (µm / l TE): 165

AOPP (Advanced Oxidation Protein Products) (µmol / l): 165

Calcium (mmol / l): 165

Magnesium (mmol / l): 166

Creatinine (µmol / l): 166

LIST OF APPENDICES (Continued)

Appendix Page

Total proteins (g / l): 166

Globulins (g / l): 167

APPENDIX 2 ABSTRACTS, PRESENTATIONS AND POSTERS 168

2018 American Dairy Science Association (ADSA) Annual Meeting Abstract: Effect of 2,4-thiazoldinedione treatment on milk fat synthesis in lactating dairy goats fed adequate vitamins supplementation 168

2018 American Dairy Science Association (ADSA) Annual Meeting Abstract: Effect of selenium-enriched hay on selenium concentration in blood and milk, immune function, and performance in dairy cows during transition period 170

2018 American Dairy Science Association (ADSA) Annual Meeting Abstract: Alfalfa enriched with se fed to dairy heifers affects the immune system but not growth performance in their offspring 172

2019 Pacific Northwest (PNW) Animal Nutrition Conference Graduate Student Competition Abstracts: Effect of agronomic selenium biofortified alfalfa hay on selenium status and glutathione peroxidase activity in transition dairy cows and their calves..... 174

2019 American Dairy Science Association (ADSA) Discover Conference Abstract: Selenium biofortified alfalfa hay supplemented during the peripartum in Holstein and Jersey cows improve antioxidant response and positively affected IgG in Jersey's calves. 176

2018 American Dairy Science Association (ADSA) Annual Meeting Presentation: Effect of 2,4 thiazolidinedione treatment on milk fat synthesis in lactating dairy goats fed adequate vitamins supplementation 178

2018 American Dairy Science Association (ADSA) Annual Meeting Presentation: Effect of selenium-enriched hay on selenium status, immune function, and performance in dairy cows during transition period..... 183

2018 American Dairy Science Association (ADSA) Annual Meeting Presentation: Calves born from cows fed with alfalfa enriched with selenium have higher Se in blood and higher phagocytosis..... 186

2018 4th Annual College of Agriculture Science (CAS) Experiential Expo Poster: Mucine 15 as alternative marker for isolation of secretory mammary epithelial cells from milk of bovine and goat for downstream studies om mammary biology and nutriogenomics ... 189

LIST OF APPENDICES (Continued)

Appendix Page

2019 Pacific Northwest (PNW) Animal Nutrition Conference Graduate Student Competition Poster: Effect of agronomic selenium biofortified alfalfa hay on selenium status and glutathione peroxidase activity in transition dairy cows and their calves..... 190

2019 American Dairy Science Association (ADSA) Discover Conference Poster: Selenium biofortified alfalfa hay supplemented during the peripartum in primiparous Holstein and Jersey cows affects liver function and oxidative status in cows and liver function in their calves..... 191

LIST OF APPENDIX FIGURES

Figure Page

Figure 1.1 *Experimental design*. Twelve Saanen dairy goats were randomly assigned to receive a daily injection of 8 mg/kg BW of 2,4-thiazolidinedione (TZD) in 10 mL saline or only saline (n=6/treatment) for 26 days. Kids (2 per goat) were kept nursing the goats for the first 10 days of the experiment. After 2 weeks of treatment the goats received in the right half of the mammary gland an intramammary infusion of 7×10^8 cfu of *Strep. uberis.* All the TZD and three CTR goats were euthanized at the end of the experiment. The whole experiment was used to generate data for Objective 1 (present manuscript) and for Objective 2 in companion manuscript (Rosa et al., 2019). 101

Figure 1.2 Milk yield (kg produced by the left mammary gland in the AM milking), energy corrected milk, dairy efficiency, daily dry matter intake, milk fat and milk yield (produced by the left mammary gland in the AM milking) in goats receiving daily intra-jugular injection of 2,4-thiazolidinedione (TZD) or saline (CTR). Arrows indicate time of kid removal and intramammary infusion of *Strep. Uberis* in the right half of the mammary gland (IMI). Letters in the graph denote significant (*P*<.05) effects of time (*T*), treatment (*Z*), and interactions (*ZxT*). The reported P-value is for tendencies. 108

Figure 1.3 Non-esterified fatty acids (NEFA), glucose, β-hydroxybutyrate (BHBA), and estimated fatty acids present in plasma (mM NEFA+3×mM TAG) in goats receiving a daily intra-jugular injection of 2,4-thiazolidinedione (TZD) or saline (CTR). Arrows indicate removal of kids and time of intramammary infusion of Strep. uberis in the right half of the mammary gland (IMI). Letters in the graph (a, b) denote significant effects of time (T), treatment (Z), and interactions (ZxT). The reported P-value is for tendencies. 114

Figure 1.4 Median area of adipocytes and frequency (%) in each specific range in area of adipocytes in subcutaneous tissue collected from the tail-head of goats receiving a daily intra-jugular injection of 2,4-thiazolidinedione (TZD) or saline (CTR). Arrows indicate removal of kids and time of intramammary infusion of Strep. uberis in the right half of the mammary gland (IMI). Letters in the graph (a, b) denote significant effects of time and interactions (ZxT). Asterisks (*) denote differences (P(0.05) between treatments in the same range of area of adipocytes. 116

Figure S1. Body weight and body condition score in goats receiving a daily intra-jugular injection of 2,4-thiazolidinedione (TZD) or saline (CTR). Arrows indicate time of kid removal and intramammary infusion of Strep. Uberis in the right half of the mammary gland (IMI). Letters in the graph denote significant (P<.05) effects of time (T), treatment (Z), and interactions (ZxT). The reported P-value is for tendencies. 143

LIST OF APPENDIX FIGURES (Continued)

Figure Page

Figure S2. Pearson correlation (r above and P-value below) among measured parameters in blood and milk (plus dry matter intake). Yellow to orange to red shade denotes the level of significance for the correlations that are either positive (green shade) or negative (blue shade) 144

Figure S3. Mean±SD of the percentage amount of the 46 detected fatty acids in milk of all milk samples in our experiment. The upper quadrant reports a representative GC chromatogram. 145

LIST OF APPENDIX TABLES

Table Page

Table 1.1 Milk components in lactating goats injected daily with 2,4-thiazolidinedione (TZD) or saline (CTR). 111

Table 1.2 Plasma concentration of metabolic parameters in dairy goats injected daily with 2,4-thiazolidinedione (TZD) or saline (CTR). 112

Table 1.3 mRNA abundance of selected genes in adipose tissue of goats treated with 2,4-thiazolidinedione (TZD) or saline (CTR). Samples were n≥5 for both groups, except for the day 26 (n= 3 for CTR; n=6 for TZD). 118

Table 1.4 Transcript abundance of selected genes normalized by the geometric mean of RPS9, UXT, and MRPL39 in the mammary epithelial cell (MEC) in goats receiving 2,4-thiazolidinedione (TZD) or saline (CTR). 120

Table S1. Nutrient Composition of the forages used during the experimental period. .. 146

Table S2. Rations fed to *Saanen* lactating goats during the experiment period based on body weight (BW) and milk initial milk yield. 147

Table S3. Composition of vitamin drench (amount per 10 mL solution) given to each goat daily 148

Table S4. Primer-pairs newly designed for the present experiment 149

Table S5. Fatty acid profile (%) in the milk of goats receiving daily intrajugular injection of 2,4-thiazolidinedione (TZD) or saline (CTR) 150

Table S6. Grams/milking of each fatty acid in milk of goats receiving a daily intrajugular injection of 2,4-thiazolidinedione (TZD) or saline (CTR) 153

Table 2.1 parameters measured in the first paper of Se experiment 156

Table 2.2 Parameters measured in the second paper of Se experiment. 156

CHAPTER 1

1.1. Introduction: transition period and associated problems and ways of improving them using dietary approaches

The transition period, defined as the time from 3 weeks prior to 3 weeks after parturition, is the most critical period in the life of a dairy cow (Grummer 1995; Drackley 1999; Esposito et al., 2014; Abuelo et al., 2015; Roche et al., 2017). Dairy cows are highly susceptible to metabolic and oxidative stress during the early post-partum period (Overton and Yasui 2014; Abuelo et al., 2015; Mordak and Anthony 2015).

The production of energy partly causes oxidative stress through aerobic metabolism. The higher requirements of oxygen during times of increased metabolic demands of early post-partum cows results in higher production of reactive oxygen species (**ROS**). The high demand for lactation drives the cow into a negative energy balance (Esposito et al., 2014; Mordak and Anthony 2015) also due to variations in hormones and a decrease in feed intake (Ingvartsen and Andersen 2000; O'Rourke 2009; Sordillo and Mavangira 2014). Thus, the cow's body starts to mobilize stored fats in the form of non-esterified fatty acids (**NEFAs**). NEFAs can be transformed into energy via mitochondrial beta-oxidation in a process that can leak electrons and leads to excess production of free radicals (Lorraine M. Sordillo & Aitken, 2009). During the transition period, dairy cows also experience inflammatory-like conditions that can compromise liver function (Bertoni et al., 2008; Trevisi et al., 2015). These changes increase the incidence of metabolic and other diseases, such as mastitis, metritis, milk fever, retained placenta, and ketosis (O'Rourke 2009; Sordillo and Mavangira 2014). Diseases associated with the transition period are very expensive to the dairy industry (Liang et al., 2017). Therefore, interventions

to mitigate the damage or prevent the diseases associated with early the post-partum period are a priority for the dairy industry.

Good management and nutrition are essential to improve the health of dairy cows during the transition period (Ingvartsen & Moyes, 2013). Nutritional deficiencies are one of the major causes of immune suppression, as demonstrated by the positive effect of various supplements on improving the immune function, especially in the early post-partum cows (Mordak & Anthony, 2015).

The performance of high producing dairy cattle can be enhanced by supplementing diets with optimal levels of micronutrients with antioxidant capabilities (Lorraine M. Sordillo & Aitken, 2009). Among various supplements, Selenium (Se) and Vitamin E are known to play an essential role in the immune system of early post-partum cows and can help reduce oxidative stress as well as the severity of several diseases, such as mastitis (Weiss and Hogan 2005; Zigo et al., 2014; Oltramari et al., 2014; Smith et al., 2016). Both Se and Vitamin E are essential for the antioxidant activity in cows to mitigate the negative effect of free radicals on DNA and polyunsaturated fatty acids present in the cell membrane (Koyuncu & Yerlikaya, 2007).

In particular, supplementation with Se is used to improve the performance of early post-partum cows and is often associated with better health and immune responses in dairy cows (Surai 2006; Surai et al., 2019). In **Table 1.1.** are summarized the effects of the organic and inorganic form of Se on cow's antioxidant indices and biochemical parameters. Most of the studies were carried out in Holstein cows and using selenized yeast. Sodium selenite and sodium selenate were used as an inorganic form of Se. The cows were mostly

in the mid-lactation period. Overall, all the study detected an increase in Se concentration and GPx activity in supplemented cows with Se vs. unsupplemented cows.

Table 1.1 Effect of supplementation of cattle with an organic and inorganic form of Se on Se level in blood and antioxidant indices and biochemical parameters

Ref.	Breed/parity	Stage of lactation	Type Se used	Dose Se	Time feeding	What measured	Results (only significant)
(Séboussi et al., 2016)	Holstein/ primiparous	Mid lactation	Na_2SeO_3[1], YSe, FSe (Alfalfa/timothy)	Se mg/kg DM CTR[2]= 0.12 Na_2SeO_3 = 0.80 YSe = 0.70 FSe = 0.79	43 days	Milk yield, blood and milk Se, Milk MDA[3],Plasma MDA, GPx, TrxR, Se excretion, retenstion, and absorption, DMI, ECM[19], and Milk component	YSe & FSe↑Se vs. Na_2SeO_3, FSe ↑Se vs. YSe, FSe ↓ Se excreted in feces and urea & ↑ retention & absoption vs. YSe
(Ortman & Pehrson, 1999)	Swedish Red and White dairy cows/ multiparous	Late lactation	Na_2SeO_3 Na_2SeO_4[4] YSe	Na_2SeO_3 Na_2SeO_4 YSe CTR= Unsupplemented	12 weeks	GPx	↑ GPx activity in TRT[5] vs. CTR
(Gong & Xiao, 2018)	Holstein/ multiparous	Peripartum (-21 to 21 DIM[6])	Na_2SeO_3 YSe	0.3 mg Se/kg DM[7]	42 days	Plasma or erythrocyte: ROS[8], H2O2, ·OH, MDA, α-tocopherol, GSH, GPx, SOD, CAT, T-AOC	YSe = ↑ plasma & WB Se, ↑GPx and GSH in RBC, ↑SOD, ↑CAT, ↑α-tocopherol, ↑GSH, ↑T-AOC ↓MDA, ↓ROS, ↓H_2O_2
(Hall et al., 2014)	Jersey/multiparous	Peripartum (-56 to 14 DIM)	YSe above requirements	CTR= 0 or 105 mg YSe/week, in addition to Na_2SeO_3= 0.3 mg Se/kg DM in TMR	8 weeks	Erythrocyte GPx, serum albumin, cholesterol, α-tocopherol, haptoglobin, , Ca, Mg, P, NEFA[9], SAA, and BHBA at 48 h, and 14 DIM	↑ SAA at 48 h, ↑Erythrocyte GPx and serum albumin at 14 days, ↓ serum cholesterol and ↑ α-tocopherol/cholesterol ratios at calving and 48 h vs. CTR
(Abuelo et al., 2016)	Holstein /multiparous	Peripartum (-14 to 7 DIM)	TRT injection of DL-α-tocopheryl acetate & Na_2SeO_3, CTR=isotonic saline	DL-alpha-tocopheryl acetate =6 mg/kg BW Na_2SeO_3= 0.06 mg/kg	21 days	GLU[10], insulin, NEFA, BHBA	GLU AUC insulin and NEFA ↑ elimination rates
(Oltramari et al., 2014)	Holstein Friesian and Brown Swiss/not reported the parity	Lactating cows (DIM not reported)	Na_2SeO_3; YSe	YSe= 278 mg.kg–1 DM of Sel-Plex® Alltech, 0.1% Se) and (0.617 mg/kg-1DM, 45% Se)	124 days	Milk yield Milk composition	YSe ↑ fat% vs. Na2SeO3
(Sun et al., 2018)	Holstein/ multiparous	Mid-lactation (141±27 DIM)	HMSeBA[12]; Na_2SeO_3	0.3 mg Se/kg DM	46 days	SOD, GPx, T-AOC, MAD, Milk yield, Protein yield, Fat yield, Lactose yield, Protein %, Fat %, Lactose %, TS %, SCC, 4% FCM[13], ECM, RT, and RR	HMSeBA ↑T-AOC, Milk yield, ↓ MAD, NO, H_2O_2, Fat% vs. Na_2SeO_3
(Gong et al., 2014)	Holstein/ multiparous	Mid-lactation (150±10 DIM)	Na_2SeO_3 YSe	0.3 mg Se/kg DM	60 days	ROS, GPx, TrxR, SelP[11], SOD, CAT, T-AOC, milk yield, milk Se, selenoprotein P, and MDA	YSe↑ GPx, TrxR (60 d), SelP(30 d), T-AOC (60 d), and ↓ MDA vs. Na_2SeO_3

Cont'd Table 1.1: Effect of supplementation of cattle with an organic and inorganic form of Se on Se level in blood and antioxidant indices and biochemical parameters

(Cun et al.,, 2015)	Unknown breed/primiparous	Mid-lactation (DIM not reported)	TWG [14] Na_2SeO_3	mg/kgDM CTR=0.35 TWG= 0.65 Na_2SeO_3=0.65	February to May 2012	Kg/d Milk Yield, Fat, Protein, and Lactose SCC	TWG↑ Milk Yields vs. Na_2SeO_3 & CTR, Na_2SeO_3↑ CTR TWG↑ lactose vs. Na_2SeO_3 and CTR
(Grilli et al., 2013)	Holstein/ primiparous	Mid-lactation (162±114 DIM)	CTR Na_2SeO_3 Lipid microencapsulated Na_2SeO_3 YSe	Mg Se/kg DM CTR =0.3 Na_2SeO_3 =0.3 or 0.5 YSe = 0.3 or 0.5	56 days	GPx	Not affected by TRT
(Ceballos-Marquez et al., 2010)	Chilean Holstein-Friesian heifers/ primiparous	-30 to	CTR $BaSeO_4$[15] YSe	no supplementary Se subcutaneous injection of $BaSeO_4$ 1 mg/kg BW 3 mg/heifer/d	60 days	GPx	$BaSeO_4$ ↑ GPx TRT
(Wei et al., 2019)	Holstein/ Multiparous	mid-lactation (153 ± 18) DIM	CTR Na_2SeO_3 HMSeBA[16]	mg/kg DM CTR= 0.06 Na_2SeO_3= 0.34 HMSeBA= 0.15, 0.33, and 0.52	10 weeks, with a pretrial period of 2 weeks	Rumen fermentation parameters: pH, NH_3-N, Total VFA[17], Molar proportion of VFA, Acetate, Propionate, Butyrate, Acetate: propionate Apparent nutrient digestibility and Se absorption: % of DM, OM, CP, NDF, ADF, Ether extract, Se	HMSeBA↑ linearly total VFA, molar proportions of propionate and butyrate, ↓ rumen, NH_3-N concentration, and acetate to propionate ratio. HMSeBA ↑linearly absorption of total Se, quadratically enhanced the digestibility of DM, OM, CP, NDF, and ADF.
(Khalili et al., 2019)	Holstein/ Multiparous	Peripartum (-21 to 21 DIM)	CTR Na_2SeO_3 YSe Se-Met [18]	mg/kg DM CTR= without Se supplementation Na_2SeO_3=0.50 YSe=0.50 Se-Met =0.50	-21 to 21 DIM	Serum Se, glucose, total protein, blood urea nitrogen (BUN), cholesterol, and triacylglycerol (TAG), globulin, BHBA, Triiodothyronine (T3) and thyroxine (T4), GPx, total antioxidant capacity, and milk Se.	Se-Y& Se-Met↑Se serum vs.CTR & Na_2SeO_3. Se-M↑ albumin & total protein.

Na_2SeO_3[1]: Sodium selenite; YSe: Selenized yeast; FSe: Fertilized Se; CTR [2]: Control; [3]MDA : Malondialdehyde; GPx :Glutathione peroxidase; TrxR :Thioredoxin reductase; Na_2SeO_4[4] : Sodium selenite; [5]TRT : Treatment; DIM[6]: Day in milking ; DM[7]: Dry Matter; ROS[8]: Reactive oxygen species; H2O2 : Hydrogen peroxide; ˙OH : Hydroxyl peroxide; GSH : Glutathione; SOD : Superoxide dismutase; CAT : Catalase; T-AOC : Total antioxidant capacity; NEFA[9] : NEFA; SAA : serum amyloid A; BHBA : Beta hydroxyl butyric acid; GLU[10] : Glucose; SelP[11]: Selenoprotein P; HMSeBA[12] : hydroxy-selenomethionine ; 4% FCM[13]: Fat corrected milk; ECM: Energy corrected milk; RT: rectal temperature; and RR: Respiratory rate; TWG[14]: Se-enriched tall wheatgrass hay; $BaSeO_4$[15]: barium selenite; HMSeBA[16]: Hydroxy-analog of selenomethionine; VFA[17]: Volatile fatty acid, Se-Met [18]: selenomethionine, ECM[19]= Energy corrected milk.

1.2. Chemical forms of selenium and selenoproteins

Humans and animals require Se for the function of several Se-dependent enzymes (selenoproteins) (Behne and Kyriakopoulos 2000; Zigo et al., 2014). Selenium is present in biological systems in the form of amino acids, such as selenocysteine and selenomethionine, as a part of selenoproteins. Se can replace sulfur in several amino acids due to the chemical similarity (Gropper & Smith, 2013). Dietary Se can be provided as an organic form, such as selenized yeast, or inorganic sources, such as sodium selenite (Na_2SeO_3) or sodium selenate (Na_2SeO_4) (Weiss & Hogan, 2005). Se occurs naturally in foods as selenomethionine or selenocysteine, which are the primary organic forms of Se (Weiss 2005; Huang et al., 2012; Wallace et al., 2017). **Table 1.2.** reports the various chemical forms of Se.

Table 1.2 Chemical forms of Se and percentage of the primary form.

Se Type	Form/composition	Ref.
1-Na Selenite	[1](Na_2SeO_3) (+4; inorganic, crystalline solid), soluble in water	https://pubchem.ncbi.nlm.nih.gov/compound/Disodium_selenite
2-Na Selenate	[2] (Na_2SeO_4) (+6; inorganic, white salt)	https://pubchem.ncbi.nlm.nih.gov/compound/Disodium_selenate
3-YSe	Organic, Seleno-AA [1](SeMet, 60-80%, and SeCys, 1-2 mg Se/g yeast). ($C_5H_{11}NO_2Se$; Transparent, hexagonal sheets or plates; metallic luster of crystals) SeMet (CH3 SeCH2 CH2 CH(NH2)C02 H) (Se -II) , SeCys (HSeCH2 CH(NH2)C02 H) (Se -II)	(Whanger, 2002) (Patching & Gardiner, 1999)
4-Se nanoparticles (SeNPs)	Inorganic, treated with a reagent or added to bacteria to convert them into an organic form of Se	(Wang et al., 2018)
5-Algae spirulina selenium	Organic; blue-green color Na_2SeO_3 (2% of total Se); organic Se comprised SeMet (approx. 18%), with the majority present in the form of two selenoproteins (20–30 kDa and 80 kDa).	(Cases et al., 2001)
6-Methyl-selenocysteine (SeMCYS)	Organic; $C_4H_9NO_2Se$ Major selenocompound in Se-enriched plants such as garlic, onions, broccoli, sprouts and wild leeks. In young S. pinnate leaves, SeMCYS (88%) and selenocystathionine (12%) Were revealed inside leaf edges, and hyperaccumulation in trichomes as organic forms SeMCYS (53%)	(Haug et al., 2007)
7-Agronomicallybiofortified selenium	Organic, Whanger, 2002, indicated in his review that Se-enriched diet such as Grassland Legume [1]SEM 51-70%, SeSys 19-39%, and SeMCYS 10-13%	(Whanger, 2002)

[1]Na_2SeO_3= Sodium selenite
[2] $Na_2SeO_{4=}$ Sodium selenate
[3]SeMet= Selenomethionine, SeCys= Selenocystine
[4]SeMCYS= Methyl-seleno cysteine

1.3. The biological function of selenium and selenoproteins

Se is an essential structural component of a specific kind of protein called selenoproteins [e.g., glutathione peroxidase (**GPx**) and thioredoxin reductases (**TrxR**)], which have important antioxidant and detoxification functions toward free radicals (Sordillo, 2013). The importance of Se was first known as a structural part of "selenoenzyme" in 1973 when Rotruck and collaborators discovered GPx and its primary role as an antioxidant to protect the cell membrane from free radicals during oxidative stress (Rotruck et al., 1973). GPx plays a pivotal role in the removal and detoxification of hydrogen and lipid peroxides. The oxidized glutathione (**GSSG**) is then converted to the reduced form by using NADPH + H^+ (Rotruck et al., 1973; Zigo et al., 2014).

Oxidative damages are caused by free radicals such as superoxides, hydroxyl radicals, nitric oxide, and hydroperoxides. These are generally produced during aerobic metabolism for the generation of energy (Rotruck et al., 1973). Free radicals or ROS are also produced by immune cells such as macrophage and neutrophils to kill phagocytized bacteria (Smith et al., 2016). Because free radicals are atoms/molecules that are missing an electron, they are highly unstable and active reactive compounds (Sordillo, 2013). Free radicals can react with fatty acids in cell membranes, making them less stable. They can also react with enzymes, inactivating them, and with nucleic acids, causing mutation (Smith et al., 2016). ROS may be involved in signal transduction pathways leading to an increase in the expression of cytokines, important for host defense against microorganisms (Sordillo, 2013). However, the production of free radicals beyond the antioxidant capability results in oxidative stress with consequent damage to the cell and loss of normal

cell functions (Sordillo, 2013). In **Table 1.3.** Are reported the main isoforms of GPx that are available in different tissues and their main function.

Table 1.3 Types of GPx in different tissue and its main function

Tissue	GPX activity	Primary GPX-forms	Function of main GPX	Ref.
Plasma	170.5 (nmol/mL*min) early lactation; 221.3 mid-lactation	[1]GPx3	GPx3 → detoxification lipid hydroperoxides in LDL (phospholipid) and [2]H_2O_2	(Cigliano et al., 2014) (Citation, 2000)→ Institute of Medicine 2000. Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids. Washington, DC: The National Academies Press. https://doi.org/10.17226/9810. Visit (Benstoem et al., 2015)
Whole Blood	52.6 (Units/ mg/Hgb)→ in bovine	[3]GPx1 and GPx3	GPx1→ detoxification H_2O_2 specifically by catalyzingthe reduction of hydrogen peroxide to H_2O, lipid peroxides.	(Koller et al., 1984) (Citation, 2000) (Hill et al., 1996)
Red Blood Cells	20.27 (U/g Hb)→ in human 5.4 U/ml→ in yearling cattle	GPx1 → human	Same	(Ozden et al., 2002) (Comp et al., 1978) (Citation, 2000) (Hill et al., 1996)
Liver	66 (µmoles NADPH oxidized • min -I • g protein -1) → dairy cows	GPx1 → human	Same	(Harrison & Conrad, 1984) (Citation, 2000)
Skeletal Muscle	549.82 U/g → cattle	GPx3 → human GPx1 → human	Same	(Łuszczak, Ziaja-sołtys, & Rzymowska, 2011) (El Haddad et al., 2012) (Lubos, Loscalzo, & Handy, 2011)
Heart Muscle	1390 U/g→ in yearling cattle	GPx1, GPx3, [4]GPx4 → human	Overexpression protect to the heart→ GPx1 Prevent LDL oxidation→ GPx2 Protect cellular lipid from oxidation damage, and mitochondrial Overoxidation protect from injury→ GPx3	(Comp et al., 1978) (Benstoem et al., 2015)
Milk	19.38 (nmol mL-1 per min) → in rat	Human GPx3	The same function above→ Most, if not all, GPx activity in milk is due to the plasma selenoprotein form of the enzyme→ in human	(Eslami et al., 2015) (Avissar et al., 1991)

[1]GPx3: Plasma glutathione peroxidase; [2]H_2O_2: Hydrogen peroxide; [3]GPx1: Cytosolic glutathione peroxidase; [4]GPx4: Phospholipid-hydroperoxide glutathione peroxidase.

1.4. Intestinal absorption of Se, distribution, and metabolism in dairy cows

Se is absorbed in the small intestine, and the type of chemical form of Se greatly influences the mechanism of its intestinal absorption (Mehdi et al., 2013; Zane Davis and Hall 2011). Organic forms of Se are less toxic compared to the inorganic form of Se (Robinson et al., 1978). The organic forms of Se, selenomethionine and selenocysteine, are absorbed in the small intestine by a Na-dependent transporter (active amino acid transport mechanisms) and are more bioavailable than selenite or selenate (Ammerman and Miller 1975; Vendeland et al., 1994). Selenomethionine has a higher absorption compared to selenocysteine (Gropper & Smith, 2013). The inorganic form of Se, selenite is absorbed by passive diffusion through the intestinal brush border membranes (Vendeland et al., 1994), whereas selenate is absorbed by a sodium co-transport system (Wolffram et al., 1988).

In the rumen, some of the selenite can be used to synthesize seleno-amino acids (predominantly SeCys) that are incorporated into microbial protein (Weiss, 2005). The highest proportion of selenite can be converted into low-molecular-weight forms of Se that are not well absorbed or used by the cow, and are mostly found in feces (Weiss, 2005). Besides, bacteria in the rumen can reduce selenate ($SeO4^{-2}$) through selenite ($SeO3^{-2}$) to elemental Se, which is less absorbed (Weiss, 2005). Sodium selenate is also incorporated into microbes in the rumen into selenocysteine and can be partly absorbed in the small intestine (Weiss, 2005). Also, sodium selenate is more absorbed compared to selenite (Weiss, 2005).

After absorption and once selenium enters the cell (either organic or inorganic Se), it can be used to make seleno-containing proteins. Once Se enters the intracellular Se pool,

selenate and selenite are converted into selenide, and then selenide is changed to selenophosphate via selenophosphate synthetase. Ingested selenite and selenate are metabolized directly to selenide (Papp et al., 2007). Selenomethionine enters the methionine pool in the body, and then methionine is metabolized via the transsulfuration pathway to release selenocysteine (Gropper & Smith, 2013). Selenocysteine is then broken down releasing a reduced form of Se (selenide) (Suzuki et al., 2007).

Selenide can be converted to excretory metabolites (methyl selenide) or metabolized to selenophosphate, a precursor of selenocysteine in selenoproteins and selenium in tRNA. The insertion of selenocysteine into selenoproteins during translation is directed by the presence of a selenocysteine-insertion sequence (SECIS) within selenoprotein mRNAs. The recognition of SECIS by the translational machinery results in the recruitment of specific translational factors that decode inframe UGA codons by inserting selenocysteine into elongating selenoproteins (Huang et al., 2012; Wrobel et al., 2016). Selenoamino acids are then transported to the liver and other tissues via circulating blood. Se distribution depends on its chemical form (Reilly 2006; Gropper and Smith 2013). In the liver organic and inorganic Se are metabolized and incorporated into selenoproteins such as selenoprotein P that transports Se to other tissues or are incorporated in other selenoproteins such as GPx, TrxR, and thyroid hormone deiodinases (Tapiero et al., 2003; Papp et al., 2007).

Various tissues have different concentrations of Se (Zane Davis & Hall, 2011). Whole blood contains the highest concentration of Se followed by kidney, liver, heart, and skeletal muscle (Muth, 1967). The peak selenium content in blood, liver, muscle, kidney, spleen, and lung is reached within 24 hours after an injection of ^{75}Se as selenite in ewes

(Muth, 1967). Least sensitive to Se alimentation and repletion are brain and testis (Brown & Burk, 1973). Brain, thymus, and reproductive organs are not as effective in accumulating Se, since they reach the maximal content of Se only after blood, liver, muscle, kidney, spleen, and lung reach the maximum level (Behne et al., 1996; Smith et al., 1979). Thus, Se concentration in blood and liver is the best indicator of Se status in animals, including dairy cows (Grace et al., 2001).

1.5. Effect of selenium forms on immune function and cows health

During the transition period, innate and acquired immune defenses decrease in dairy cattle (Mallard et al., 2010), increasing the incidence of diseases (Sharma et al., 2011; Esposito et al., 2014; Gong and Xiao 2018). Highly energy requirements during the transition period increases the release of free radicals (Celi, 2011). Studies indicated that some ROS are involved in signal transduction and regulate expression of genes that code for cytokines and regulate the production of eicosanoids and other immunoregulatory factors that are important for the immune defense against infections (Sordillo, 2013). Although free radicals are important in regulating genes related to the immune and inflammatory response, excessive production can be harmful to the cells and cause oxidative stress (Abuelo et al., 2015). Antioxidants can be generally defined as any substance that suppresses, prevents or removes free radicals. Antioxidants are also enzymes, such as GPx, SOD, Trx, and catalase (Papp et al., 2007). Most of these enzymes are dependent on Se; thus, Se is an important micronutrient that can help to prevent oxidative stress and redox processes in the cell, including immune cells. As a consequence, Se can improve the immune response and overall health of animals (Hoffmann & Berry,

2008). Se is incorporated into selenoproteins as selenocysteine residue, the Se-Cys also has antioxidant capacity (Tapiero et al., 2003).

Se functions in the antioxidant structure as an essential part of glutathione peroxidase enzymes. These enzymes take a role in destruction of hydrogen peroxide and lipid hydroperoxides to prevent oxidative stress. Previous findings indicate that the Se-dependent GPx1 is the main antioxidant enzyme protecting phagocytic cells from oxidative damage during the oxidative burst (Zoidis et al., 2018). Se supplementation in dairy cows can enhance blood neutrophils' ability to kill bacteria (Sordillo, 2013). Se supplementation enhances the chemotactic migration of neutrophils and macrophages toward the site of infection (Huang et al., 2012). Moreover, in that study, the neutrophils from cows fed Se-supplemented diets had a higher ability to kill bacteria compared to neutrophils from non-supplemented dairy cows.

Żarczyńska et al. (2012) also found that Se supplementation stimulates neutrophils migration to the mammary gland and decreases the incidence of mastitis. In **Table 1.4.** are summarized the effects of Se supplementation on the immune response of cattle. In most of the studies, a significant effect of Se on reducing somatic cell count (**SCC**) in milk was observed, supporting improvement in the health of the mammary gland. The relationship between Se supplementation and SCC is probably related to the positive effects of Se on immune functions (Gerloff, 1992).

Table 1.4 Effect of organic and inorganic form of Se on cow's immune system

Ref	Breed	DIM	Type Se	Dose Se	Time feeding	What measured	Results
(Séboussi et al., 2016)	Holstein	Mid lactating	[1]Na_2SeO_3 YSe FSe (Alfalfa/timothy)	Se mg/kg DM [2]CTR = 0.12 Na_2SeO_3 = 0.80 YSe = 0.70 FSe = 0.79	43 d	SCC in milk	↓SCC
(Weiss & Hogan, 2005)	Holstein	-60 to 30 DIM	YSe [3]Na_2SeO_4	0.3 mg/kg DM		Neutrophils in vitro kill assay	No [3]TRT effect
(Oltramari et al., 2014)	Holstein Friesian and Brown Swiss	124 days	Na_2SeO_3 YSe	YSe= 278 mg.kg–1 DM of Sel-Plex® Alltech, 0.1% Se) and (0.617 mg/kg-1 DM, 45% Se)	124 days	SCC	YSe ↑ SCC vs. Na_2SeO_3
(Gong et al., 2014)	Holstein	60 days	Na_2SeO_3 YSe	0.3 mg Se/kg DM	60 days	IgA, IgG, IgM, CD4, CD8, TNF-α, IL-2, IL-4, IL-6 and tumor necrosis factor.	YSe ↑IgA, soluble CD4/soluble CD8 (30 d), soluble (60), IL-1 vs. Na_2SeO_3
(Salman et al., 2013)	Holstein-Friesian	Drying off to 105 DIM	CTR Na_2SeO_3 YSe	0.18 mg Se/kg DM 6 mg Se/d during gestation and lactation	Drying off to 105 DIM	Lymphocyte subpopulations and phagocytosis activity of neutrophilic granulocytes in milk	No TRT effect
(Ceballos-Marquez et al., 2010)	Chilean Holstein-Friesian heifers	-30 to	CTR [4]$BaSeO_4$ barium selenate YSe	no supplementary Se subcutaneous injection of $BaSeO_4$ 1 mg/kg BW 3 mg/heifer/d	-30 to 30 DIM	SCC, intramammary infection (IMI)	TRT↓SCC, intramammary infection (IMI) TRT

Na_2SeO_3[1]: Sodium Na_2SeO_3[1]: Sodium selenite; YSe: Selenized yeast; FSe: Fertilized Se; CTR [2]: Control[3]; TRT: Treatment; $BaSeO_4$[4]: barium selenite.

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CHAPTER 2

SELENIUM BIOFORTIFIED ALFALFA HAY IMPROVES SELENIUM STATUS AND GLUTATHIONE PEROXIDASE ACTIVITY IN TRANSITION DAIRY COWS AND THEIR CALVES

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(Paper under review in the Journal of Dairy Research)

2.1 Summary

The study hypothesizes that feeding one %BW of Se biofortified alfalfa hay during the later dry period and early lactation improves selenium status and glutathione peroxidase activity in dairy cows and their calves. For this purpose, 10 Jersey and 8 Holstein primiparous dairy cows were supplemented with 1% BW of Se biofortified (TRT; n=9) or non-biofortified (CTR; n=9) alfalfa hay mixed with the TMR from 40 days prior- to 2 weeks post-partum. Se level in whole blood, liver, milk, and colostrum, the transfer of Se to calves, and the glutathione peroxidase (GPx) activity were assessed in both cows and their calves. TRT had 2-fold higher ($P<0.05$) Se in blood vs. CTR (204.5 vs. 95.0 ng/ml) that resulted in higher Se in liver (1.24 vs 0.62 µg/g dry weight) and colostrum (99.1 vs. 27.2 ng/ml) but not in milk and higher GPx activity in plasma (92.8 vs. 77.9 nmol/min/ml) and erythrocytes (549.2 vs. 260.0 nmol/min/ml) but not in milk. Compared to CTR, calves from TRT had higher Se in the blood (215.5 vs. 161.22 ng/ml) but only a numerically ($P=0.09$) larger GPx activity in plasma. A positive correlation was detected between Se in blood and GPx activity in erythrocytes and plasma in cows. Our results demonstrated that feeding pregnant primiparous dairy cows with 1%BW of Se biofortified alfalfa hay is an effective way to improve Se status in cows and their calves leading to the greater antioxidant activity via GPx in cows and, numerically, in calves. Feeding Se biofortified hay increased Se level in colostrum but not in milk.

Keywords: Selenium biofortified alfalfa hay; glutathione peroxidase; milk; primiparous dairy cows; calves

2.2 Introduction:

High producing dairy cows typically experience heightened oxidative stress during the early post-partum (Abuelo *et al.*, 2015) as an outcome of higher production of free radicals, generally as a consequence of aerobic metabolism (Mehdi *et al.*, 2013). Free radicals are also produced from immune cells, such as macrophages and neutrophils, to kill phagocytized bacteria (Sordillo, 2013). Oxidative stress is associated with increased incidences of diseases (Sordillo & Mavangira, 2014); therefore, it is beneficial to improve the anti-oxidative status of dairy cows.

Selenium (Se) is an integral component of selenoenzymes, such as glutathione peroxidase (GPx), which plays a significant role in the removal and detoxification of free radicals (Zigo *et al.*, 2014). Thus, supplementation of Se during the peripartum can be an effective means to decrease oxidative stress (Gong & Xiao, 2018). As an added benefit, Se supplementation also improves the immune function of the cows (Sordillo & Mavangira, 2014) and the amount of immunoglobulins in the colostrum, potentially benefitting the calves (Hall *et al.*, 2014a).

Due to its toxic properties when supplemented in excess, Se is the only micronutrient regulated as a feed additive by the FDA, setting the limit of Se supplementation in cattle to 0.3 mg/Kg DM (NRC, 2001). Se can be supplemented as an organic source, such as selenized yeast, or inorganic sources, such as selenite or selenate (Weiss, 2005). Organic Se has a higher bioavailability (>50%) compared with inorganic Se (45%) when fed to dairy cows (Séboussi *et al.*, 2016). Animals supplemented with organic Se, such as hydroxy-selenomethionine and selenized yeast, have a higher concentration of Se in whole blood and milk, and higher antioxidant capabilities, including

higher blood GPx activity, compared to cows supplemented with equal amounts of inorganic Se (Séboussi *et al.*, 2016; Sun *et al.*, 2018).

Biofortification of forages with Se is an effective approach to improve Se in the diet of beef cows (Hall *et al.*, 2013; Hall *et al.*, 2011; Novoselec *et al.*, 2018). Feeding Se biofortified forages successfully improve the Se status of pregnant beef cows and icreases the level of immunoglobulins in colostrum and the Se status and performance of their calves (Hall *et al.*, 2013; Wallace *et al.*, 2017; Ranches *et al.*, 2017).

The above studies were performed in beef animals and, except for one study (Ranches *et al.*, 2017), the researchers fed a relatively large amount of Se biofortified forages (up to 2.5% of BW). Unlike beef cattle, non-grazing high producing dairy cows require a more specialized ration with, in general, ≤60% forages. Furthermore, because of the need to balance the requirements of these animals, several types of forages are usually used in the formulation of the ration. Therefore, it is essential to demonstrate the effectiveness of using a relatively low amount of agronomically Se biofortified forage in the ration of high-producing dairy cows. In a prior study, a ration containing 25% DM of Se biofortified alfalfa/timothy silage improved the Se status of mid-lactation dairy cows (Séboussi *et al.*, 2016). However, an adequate amount of Se is even more crucial for peripartum cows, considering the high oxidative stress and the need to boost the immune status. Furthermore, it is also important to boost the immune status of the calves.

The objectives of the present study were to evaluate the effects of feeding 1% BW of Se biofortified alfalfa to dairy cows during the peripartum on 1) Se level in whole blood, liver, milk, and colostrum; 2) transfer of Se to calves; 3) and the antioxidant status of cows and calves via measurement of GPx activity. We hypothesize that supplementing dairy

cows with a relatively low amount of Se biofortified hay during the dry period and early lactation improves the Se and antioxidant status in cows and their calves.

2.3 Materials and Methods

2.3.1 Animals, ration, and experimental design

Experimental procedures used in this study were approved by the Institutional Animal Care and Use Committees (IACUC) of Oregon State University (protocol# 4894). The overall experimental design is shown in Figure 2.1.

Three different batches of alfalfa hay from a prior experiment conducted by Hall et al. (2013) were used for the present study. Two alfalfa hay batches obtained from fields fertilized with 45 and 90 g sodium selenite/ha were mixed in equal amounts to obtain alfalfa with 3.25 mg Se/kg DM and used for the treatment group. As a control, we used alfalfa hay containing 0.43 mg Se/kg DM, which was obtained from a field that was not fertilized with sodium selenite. Chemical analysis results of the three hays, obtained from a commercial laboratory (Dairy One Forage Testing Laboratory, Ithaca, NY) are reported in Table 2.1.

Ten Jersey and eight Holstein pregnant heifers from the Oregon State University Dairy Center were enrolled in the study. Around 45 days before expected parturition, the animals were moved into a pen equipped with free stalls bedded with mattress and sawdust, free access to water, and Calan gates. Due to the year-round herd calving, the low number of available animals, the use of two breeds in both treatments, and the feeding period of around 56 days (40 days before to two weeks post-partum), the entire experiment spanned nine months (from September 2017 to May 2018). Heifers were randomly assigned to two

groups blocked by breed and expected time of calving. Starting from 40 (38±5) days before expected calving, the heifers received either 1 kg DM/100 kg BW of Se-biofortified alfalfa hay (TRT; 5 Jersey and 4 Holstein heifers) or 1 kg of control hay (CTR; 5 Jersey and 4 Holstein heifers) mixed into their ration. All the heifers received the supplemental hay until 14 days in milk (DIM). One week before expected calving, animals were moved to a single calving pen where they continued to receive the lactation total mixed ration (TMR) and the alfalfa hay supplement. At 3 DIM, the animals were moved back into the Calan gate pen. Animals were milked twice a day, at 04:15 AM, and 2:15 PM and milk yield was recorded by the Afilab system (Afimilk, Israel). Heifers were monitored daily for health status, dry matter intake, milk yield, and weekly for body weight (BW).

During the dry and lactation period, heifers received *ad libitum* total mixed ration (TMR) formulated for dry and lactating cows, respectively. TMR was provided twice a day, approximately at 07:30 AM and 04:30 PM. Before feeding, TMR was mixed by hand with the chopped Se-biofortified alfalfa hay or the control alfalfa hay for each animal. Samples of TMR were collected once a month during the trial and preserved at -20°C until analysis. Except for Se, the chemical analysis of the TMR was performed by a commercial laboratory (Dairy One Forage Testing Laboratory, Ithaca, NY). The composition and chemical analysis of the TMR, including the analysis for Se, are reported in Table 2.2**.** Cows received an individual commercial Trace Mineral Salt Brick (cat#270220, American Stockman) without Se that was inserted into the Calan gate using a commercial plastic adaptor.

Calves born from the heifers enrolled in the experiment received colostrum from their mothers 1-6 hours after birth (depending on whether the calf was born during a

milking shift). They received 3 quarts of colostrum for their first feeding and 2 quarts of colostrum for their second feeding. Both feedings occurred within the first 24 hours after parturition. From day 2 to weaning, all calves were fed raw cow milk collected from the bulk tank (2 quarts twice daily until 2 wks and then 3 quarts tiuce daily). Calves also received 2 quarts of a commercial electrolites starting from 5 days post-calving. Furthermore, *ad libitum* access to a starter concentrate was provided starting at 1-day post-birth to all calves. Blood samples from calves were collected at 1 and 24-days post-birth. Calves were kept in individual hutches for the entire experimental period.

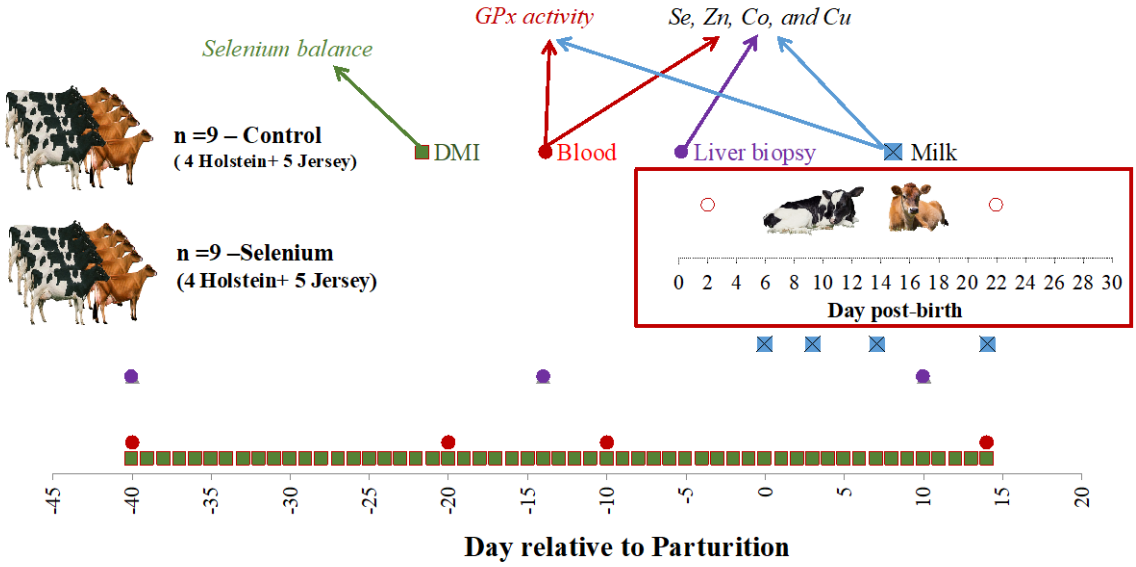

Figure 2.1 Experimental design and samplings. The Selenium group received 1 kg/100 kg of body weight of Se-biofortified alfalfa (3.25 ppm of Se) from -40 to 14 day relative to parturition (or day in milk – DIM) while the Control group received alfalfa without biofortification (0.43 ppm of Se). GPx = measurement of glutathione peroxidase activity via a commercial kit. Microminerals were measured via IPC-MS.

2.3.2 Dry matter of the TMR

Dry matter of the TMR and residuals after feeding was measured weekly. Briefly, approx. 200 g of the sample was put in a glass container of known weight (Tare) and precisely weighed (W_1) and put in the microwave at high power for 8 min. Then it was mixed by hand and put back in the microwave for 4 min at high power; sample weight was then recorded (W_2).

The % dry matter was = (W_2-Tare)/ (W_1-Tare) × 100.

Table 2.1 Chemical characteristics of alfalfa hays used for the present experiment

	Control Hay	Medium Se hay	High Se hay
DM, %	88.6	89.6	88.9
Nutrient, DM basis			
CP, %	21.8	19.8	20.8
ADF, %	31.6	35.9	31.9
NDF, %	37.9	44.2	40.4
NFC, %	28.3	24.0	26.8
NEL, Mcal/kg	0.63	0.60	0.62
Relative Feed Value	158	128	148
Minerals, DM basis			
Ca, %	1.52	1.35	1.42
P, %	0.22	0.22	0.24
Mg, %	0.38	0.37	0.24
K, %	1.96	1.97	1.95
Na, %	0.092	0.094	0.097
S, %	0.34	0.31	0.33
Fe, ppm	258	723	305
Zn, ppm	17	16	17
Cu, ppm	11	10	10
Mn, ppm	42	51	46
Mo, ppm	< 0.1	< 0.1	< 0.1
Se, ppm	0.43	2.2	4.4

Table 2.2 Composition and chemical characteristics of the total mixed rations used in the present experiment

Item	Dry cows	Lactating cows
Ingredient, %		
Alfalfa	-	13.6
Corn Silage	40	39.6
Grass Silage	45	26.1
Grass Hay	15	-
Corn/Barley	-	14.4
Soybean Meal	-	5.6
Mineral Supplement1	-	0.7
Dry Matter (DM)2	41.40± 3.68	50.00±3.96
NEL; Mcal/kg of DM	1.54±0.00	1.67±0.01
Chemical composition, % DM		
Crude protein	9.00±0.14	18.3±0.99
NDF	52.3±0.8	32.5±4.0
ADF	30.1±0.3	19.6±3.3
Calcium	0.33±0.02	0.59±0.09
Phosphorus	0.24±0.01	0.42±0.08
Magnesium	0.19±0.01	0.25±0.03
Potassium	1.44±0.47	1.87±0.50
Sodium	0.08±0.07	0.06±0.01
Sulfur	0.14±0.01	0.22±0.01
Iron (ppm)	1021±238	536±133
Zinc (ppm)	28.5±0.7	44.0±21.2
Copper (ppm)	6.00±1.41	11.50±4.9
Manganese (ppm)	140.5±33.2	58.5±7.8
Molybdenum (ppm)	1.00±0.00	1.15±0.21
Selenium (ppm)	0.26±0.17	0.99±0.37

[1] Wilbur-Ellis Feed, LLC, OR (Cat# 1187036). It contains (as %DM) 17.60-21.00 % Ca, 7% P, 8% Mg, 1.65% S, 20-24 ppm Se, 440,000 IU/Kg DM Vitamin A. In addition to the mineral provided via TMR cows also received an individual mineral block without Selenium (Cat#90013, Stockman Trace Mineralized & Salt Brick). The indicated content included a minimum guarantee of 98% NaCl, 4000 ppm Zn, 1600 ppm Fe, 1200 ppm Mn, 260 ppm Cu, 100 ppm I, and 40 ppm Co.

[2]The data are mean±SD of monthly TMR samples collected during the trial

2.3.3 Collection of blood and milk

Blood samples were collected from cows at -40, -20, -10, and 14 DIM, and from calves at 2 and 24 days after birth to measure GPx activity, Se and other trace minerals. Blood samples from cows and calves were collected from the jugular vein into Na-heparin tubes (Cat# 6102751; Becton Dickinson, Franklin Lakes, NJ) before the morning feeding to measure GPx activity. Blood samples were also collected into EDTA tubes (Cat# 455036, Greiner bio-one. Monroe, NC 28110, USA) to measure Se and other trace minerals.

Colostrum and milk samples were collected during the morning milking at 1, 3, 7, and 14 DIM in a 15-ml plastic tube (Cat# 525-0400. VWR. USA) and immediately put on ice for transport to the laboratory. Plasma, serum, whole blood, colostrum, and milk samples were preserved at -20°C until analyses.

2.3.4 Liver tissue collection

Liver biopsies were performed at at 40 and 10 days before expected parturition and at 10 DIM. Ultrasound (Ibex Pro, E.I. Medical., Loveland, CO) was used to determine the liver location at the right 10^{th} intercostal space at the level of the greater trochanter. The biopsy area was clipped and scrubbed with 7.5% povidone-iodine (Cat# 055479, VetUS) and 70% ethanol. Following infiltration of a local anesthetic (lidocaine 2%, Cat# 002468, VetUS) and intravenous injection of 10 mg xylazine sterile solution (20 mg/ml) (Cat# 4811, Akorn, Inc., Decatur, IL62522), a small incision (approx. 0.5-1.0cm) was made in the skin at the right 10^{th} intercostal space. The biopsy instrument was inserted through the body wall, introduced into the liver parenchyma, and a liver sample was collected.

Following the biopsy, the cutaneous incision was closed using a wound stapler (Cat# 8535, USA). Liver samples were immediately put in 2 ml cryotubes (Cat# 10018-760, VWR), flash-frozen in liquid nitrogen, and stored at -80°C until analysis. Before the biopsy and the day after the surgical intervention, the rectal temperature of the animals was measured to check for possible inflammation or health issues.

2.3.5 Measurement of Se and other trace minerals

Se, Zinc (Zn), Cobalt (Co), and Copper (Cu) were measured by using 0.5 ml of whole blood, colostrum, and milk. Samples were added into screw cap glass vials with 2 ml of home-distilled concentrated nitric acid, 1 ml hydrogen peroxide (Cat# 0000191085, Avantor, USA), and with 0.1 ml of indium (200 ppb) as an internal standard. Vials were capped and heated at 60°C for approx. 2 hours. Then, 2 ml of concentrated HNO_3 and 0.5 ml of H_2O_2 were added. Samples were heated at 80°C until a clear digest was obtained, then they were uncovered and left to evaporate completely. The pellet was dissolved in 5 ml of 1% HNO_3. Finally, 1 ml of the sample was diluted with 4 ml of 1% HNO_3 before analysis.

Liver samples were freeze-dried (model # HR9000-AL, Harvest Right). The dried liver was precisely weighed and placed into screw cap glass vials. One ml of HNO_3 with 0.1 ml of indium as an internal standard was added in four consecutive bouts of 0.25 ml to the sample and allowed reacting. Samples were covered and heated overnight at 60°C. Two ml of H_2O_2 was added to the samples in 0.5 ml increments. Vials were capped and heated overnight at 60°C, then uncapped and allowed complete evaporation in a fume hood. The

pellet was re-dissolved in 8 ml of 1% HNO_3. Following this, 1.6 ml of solution was diluted with 3.3 ml of 1% of HNO_3 before analysis. The same protocol was used for the Se extraction from the TMR samples with the exception that freeze-dried samples were ground using a SPEX 6700 freezer Mill (Model#6700-115. Industries Edison, N.J., USA) before extraction.

Trace minerals were measured using an Elemental X-series 2 Inductively coupled plasma mass spectrometry (Cat# 01957C, Thermo Scientific USA) with a collision cell as previously described (Pérez-Rodríguez et al., 2017; Memon et al., 2007). Briefly, samples were free aspirated through a Teflon nebulizer into a Peltier cooled cyclonic spray chamber. Analytes were measured in 3 runs of 120 sweeps per run with peak dwell times of 10 ms. Intensity fluctuations throughout the run were normalized using Indium (Cat# 13846, Alfa Aesar Specpure). Final data was corrected using a 5-point standard curve for each element using purified standards for ICP-MS available from the Keck Collaboratory for all microminerals except Se, which was purchased (Cat# 82026-056, VWR Chemicals, USA).

2.3.6 Glutathione peroxidase activity

GPx activity was assessed using a commercial kit (cat# 703102-480, Cayman Chemical, USA) in erythrocyte lysate from whole blood samples of heifers and calves collected in Na-heparin tubes (Cat# 6102751, 10 ml; Becton Dickinson, Franklin Lakes, NJ) at 40 (39±5), -20(±3), 7, and 14 DIM. Blood samples were centrifuged in a Beckman Coulter centrifuge (Cat# 392187, Allegra X-22R Centrifuge, Beckman Coulter, INC) using 50 ml tubes (Cat#525-0402, VWR, USA) at 3000×*g* for 15 minutes at 4°C. The plasma

was collected in 1.7 ml Eppendorf (Cat# 22-281, Genesee Scientific) and stored at -80°C until analysis. The buffy layer was discarded, and four volumes of ice-cold distilled water were added, followed by centrifugation at 10,000×*g* for 15 min at 4°C. The supernatant (erythrocyte lysate) was collected in 1.7 ml Eppendorf (Cat# 22-281, Genesee Scientific) and frozen at -80°C until analysis. The GPx assay was run in duplicate in a 96 well plate (Cat#400012). The absorbance was read at 340 nm using Molecular Devices SpectraMax plus Microplate Spectrophotometer 384.

2.3.7 Se balance and % transfer into calves and Se excretion into milk

The balance of Se was estimated by using the calculated amount of Se fed via TMR and hay corrected by the residuals and two estimated requirements: 1) NRC recommendation (i.e., 0.3 mg of Se/kg DM) or 2) losses (as % of Se intake) from a prior experiment (Séboussi *et al.*, 2016) plus the calculated Se excretion in milk by using the Se concentration measured in milk × daily milk yield, and the accumulation of Se in the fetus as suggested by NRC (0.055 mg/d) (NRC, 2001). The % of Se transferred to calves was calculated as mg/L Se in whole blood of calf × estimated blood volume of calf/ mg/L Se in whole blood of dam × estimated blood volume of the dam, following as previous estimate of transfer of immunoglobulins (Hall *et al.*, 2014a).

2.3.8 Statistical analysis

Outlier data were checked using PROC REG of SAS 9.4 (SAS Institute, Inc., Cary, NC, USA) before statistical analysis. Data with a studentized t >3.0 were removed. Data were analyzed by GLIMMIX of SAS with the fixed effect of treatment, breed, time, and

their interactions with the cow (or calf) as random effect using the Spatial Power covariate model with homogeneous variance. PROC CORR was used for correlation analysis among the variables. Significance was declared with a P-value≤0.05 and tendency with a P-value >0.05 and ≤0.10.

2.4 Results

2.4.1 Se balance

The results are presented in Table 2.3. Cows in our study received between 33 and 37% of Se-biofortified or control alfalfa hay in their diet (as DM). The combination of TMR plus alfalfa hay provided between 3.3 and 3.7 mg Se/d in CTR and 14 and 17 mg Se/d in TRT during the dry period and between 8.6 and 10.4 mg Se/d in CTR and 19 and 25 mg Se/d in TRT. Based on the calculated requirements, following the NRC recommendation (NRC, 2001) or losses estimated using a previous work (Séboussi *et al.*, 2016), the Se balance was positive in all cases. A larger positive Se balance was detected in TRT vs. CTR. The Se balance was more positive when using NRC losses vs. the ones from Séboussi et al. (2016) due to higher estimated losses in the latter (Table 2.4).

Table 2.3 Se balance estimated in Jersey and Holstein pregnanct heifers supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior expected parturitionto 14 days post-partum.

	Control		Selenium			P-value[1]					
	Holstein	***Jersey***	***Holstein***	***Jersey***	***SEM***	***TRT***	***Breed***	***T***	***T×B***	***T×TRT***	***T×B×T***
% experimental hay in the ration (DM)											
Overall	36.2	33.7	35.9	34.7	*2.10*	0.85	0.36	<0.01	0.76	0.35	0.96
Dry	37.3	34.1	36.7	35.3	*1.95*	0.86	0.23	0.22	0.64	0.05	0.41
Lactation	34.1	32.9	34.3	33.4	*3.60*	0.91	0.76	0.01	0.97	0.01	0.98
Se fed (mg/d)											
Overall	5.80	4.96	19.5	16.0	*0.50*	<0.01	<0.01	<0.01	0.01	0.76	0.61
Dry	3.76	3.32	17.2	14.3	*0.47*	<0.01	<0.01	0.11	0.02	<0.01	0.29
Lactation	10.4	8.6	24.7	19.8	*0.93*	<0.01	<0.01	<0.01	0.11	0.93	0.15
mg Se/kg DM											
Overall	0.47	0.47	1.53	1.50	*0.03*	<0.01	0.58	<0.01	0.61	<0.01	0.30
Dry	0.32	0.32	1.43	1.37	*0.04*	<0.01	0.40	0.01	0.41	0.05	0.34
Lactation	0.78	0.78	1.77	1.80	*0.05*	<0.01	0.76	0.48	0.69	0.03	0.29
μg Se/BW[2]											
Overall	10.7	10.7	35.3	34.2	*1.25*	<0.01	0.65	<0.01	0.66	0.55	0.87
Dry	6.55	6.85	30.6	29.9	*1.10*	<0.01	0.87	0.04	0.64	<0.01	0.54
Lactation	20.1	19.3	45.9	43.9	*2.40*	<0.01	0.54	<0.01	0.80	0.89	0.08
Se balance (fed-losses; mg/d)											
NRC[3]											
Overall	2.14	1.81	15.7	12.8	*0.36*	<0.01	<0.01	<0.01	0.01	0.64	0.68
Dry	0.22	0.23	13.5	11.1	*0.37*	<0.01	<0.01	<0.01	<0.01	0.01	0.47
Lactation	6.43	5.32	20.4	16.5	*0.65*	<0.01	<0.01	0.01	0.04	0.96	0.16
Estimated[4]											
Overall	2.07	1.70	8.18	6.65	*0.20*	<0.01	<0.01	<0.01	<0.01	0.26	0.48
Dry	1.37	1.21	7.35	6.13	*0.20*	<0.01	<0.01	0.10	0.01	<0.01	0.30
Lactation	3.63	2.80	10.0	7.82	*0.33*	<0.01	<0.01	0.14	0.05	0.53	0.23

[1]TRT=effect of supplementing 1% BW of Se-biofortified hay (i.e., Selenium) vs. control hay; T×B= TRT × Breed interaction; T×B×T = TRT × Breed × Time interaction

[2]μg Se/BW=All mg Se intake/BW*1000 (from mg to μg) *0.5 (assuming 50% absorption)

[3]Balance of Se calculated using the requirements according to NRC 2001(i.e., 0.3 mg/kg DM)

[4]Balance of Se calculated using the requirements calculated using the urine losses (6.1% of Se intake in CTR and 13.1% of Se intake in cows receiving forage with high Se), feces losses (56.0% of Se intake in CTR and 43.8% of Se intake in cows receiving forage with high Se), and milk as calculated using our data or Se requirement for conceptus as indicated by NRC (0.055 mg/d) (Séboussi et al., 2016).

Table 2.4 Se from hay, estimated Se absorbed, and estimated Se requirements in pregnant Jersey and Holstein heifers supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior to expected parturition.

	Control		**Selenium**		***P-value*[1]**						
	Holstein	***Jersey***	***Holstein***	***Jersey***	***SEM***	***TRT***	***Breed***	***T***	***TxB***	***TxTRT***	***TxBxT***
Se from Hay mg/d											
Overall	2.03	1.79	16.39	13.68	*0.31*	<0.01	<0.01	0.95	<0.01	0.62	0.98
Dry	2.04	1.79	16.25	13.66	*0.36*	<0.01	<0.01	0.93	<0.01	0.65	0.92
Lactation	2.03	1.78	16.72	13.73	*0.31*	<0.01	<0.01	0.90	<0.01	0.62	0.89
Se absorbed mg/d											
Overall	2.55	2.18	10.97	9.01	*0.26*	<0.01	<0.01	<0.01	<0.01	<0.01	0.36
Dry	1.65	1.46	9.66	8.06	*0.26*	<0.01	<0.01	0.08	0.01	<0.01	0.32
Lactation	4.57	3.77	13.86	11.13	*0.44*	<0.01	<0.01	<0.01	0.04	0.91	0.14
NRC[2] mg/d											
Overall	3.69	3.14	3.83	3.24	*0.20*	0.54	0.01	<0.01	0.92	0.78	0.69
Dry	3.58	3.09	3.67	3.20	*0.20*	0.62	0.03	0.04	0.96	0.26	0.18
Lactation	3.94	3.25	4.20	3.34	*0.29*	0.54	0.01	0.01	0.76	0.63	0.68
Estimated[3] mg/d											
Overall	3.73	3.25	11.34	9.38	*0.31*	<0.01	<0.01	<0.01	0.02	0.93	0.80
Dry	2.39	2.12	9.84	8.21	*0.27*	<0.01	<0.01	0.11	0.02	<0.01	0.28
Lactation	6.75	5.77	14.65	11.97	*0.64*	<0.01	0.01	<0.01	0.18	0.95	0.19

[1]TRT=effect of supplementing 1% BW of Se-biofortified hay (i.e., Selenium) vs. control hay; T×B= TRT × Breed interaction; T×B×T = TRT × Breed × Time interaction

[2]Requirements according to NRC 0.3 mg/kg DM

[3]Balance of Se calculated using the requirements calculated using the urine losses (6.1% of Se intake in CTR and 13.1% of Se intake in cows receiving forage with high Se), feces losses (56.0% of Se intake in CTR and 43.8% of Se intake in cows receiving forage with high Se), and milk as calculated using our data or Se requirement for conceptus as indicated by NRC (0.055 mg/d) (Séboussi et al., 2016),

Table 2.5 Overall concentration of Se and other trace minerals in whole blood, milk, and liver of Jersey and Holstein pregnant heifers supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior expected parturition to 14 days after calving. Reported is also the level of the microminerals in the whole blood of their offspring during the first 24 days after birth.

Micromineral	Control		Selenium			P-value[1]			
	Holstein	*Jersey*	*Holstein*	*Jersey*	*SEM*	*TRT*	*Breed*	*TxB*	*TxBxT*
Whole blood cows, ng/ml									
Se	122.8	98.3	171.0	175.5	*10.30*	<.01	0.40	0.31	0.62
Co	3.64	1.92	1.64	1.44	*0.91*	0.22	0.34	0.45	0.34
Cu	1276.5	887.7	812.4	745.5	*147.8*	0.60	0.80	0.28	0.74
Zn	1154.8	840.3	1466.2	849.7	*267.5*	0.32	0.02	0.35	0.50
Whole blood calves, ng/ml									
Se	158.9	163.5	196.3	234.7	*13.22*	<0.01	0.19	0.29	0.43
Co	2.78	1.55	1.69	1.61	*0.29*	0.13	0.05	0.10	0.29
Cu	668.6	679.8	769.1	727.5	*70.90*	0.36	0.84	0.74	0.24
Zn	1170.8	1022.1	1229.4	994.7	*110.9*	0.87	0.09	0.66	0.52
Liver cows, µg/g									
Se	0.74	0.51	1.34	1.13	*14.25*	<0.01	0.05	0.91	0.149
Co	0.78	0.39	0.49	0.44	0.01	<0.01	<0.01	<0.01	0.18
Cu	35.8	46.3	42.3	28.5	*88.4*	0.58	0.87	0.24	0.12
Zn	73.2	55.7	63.8	49.5	*13.6*	0.62	0.32	0.92	0.85
Colostrum/Milk, ng/ml									
Se	19.8	25.3	42.6	54.1	*4.48*	<0.01	0.08	0.53	0.71
Co	7.39	9.39	6.87	14.20	*4.34*	0.65	0.33	0.58	0.63
Cu	259.2	273.1	258.1	380.9	*68.7*	0.48	0.37	0.47	0.84
Zn	2971.6	3201.8	2982.3	2843.9	*413.1*	0.69	0.91	0.67	0.99
Se transfer into calves[2], %	8.05	9.90	6.32	6.02	*0.87*	*<0.01*	*0.42*	*0.26*	

[1]TRT=effect of supplementing 1% BW of Se-biofortified hay (i.e., Selenium) vs. control hay; T×B= TRT × Breed interaction; T×B×T = TRT × Breed × Time interaction

[2]Efficiency of transferred Se into calves (%)= Se concentration in whole blood of calf (mg/L) × 9.71% of BW of the calf (kg) / Se concentration in whole blood of dam (mg/L) × 9.71% of BW of dam (kg).

2.4.2 Level of Se

TRT had a significant increase in Se level in blood vs. CTR reaching a plateau after 20 days of supplementation with no differences between breeds (Figure 1A). In CTR, the level of Se in blood tended to decrease during the first 20 days of the experiment (from 144.9 at -40 DIM to 95.0 ng/ml at -10 DIM; P=0.08).

The level of Se in plasma of calves born from cows in the TRT group was higher compared to calves born from cows in the CTR group up to 24 days post-birth (Figure 1B). Overall, Jersey's calves had higher amounts of Se in blood during the early post-birth vs. Holstein's calves. However, the difference disappeared at 24 days post-birth due to an increase of Se in Holstein's calves (Figure 1B).

The level of Se in the liver increased in TRT from -40 to -10 DIM reaching >2-fold higher level in TRT vs. CTR (Figure 2.2C and Table 2.5). The rate of increase of Se in the liver was faster in Jersey compared to Holstein cows reaching the plateau at -10 DIM, with a decline afterward. In comparison, the increase was continuous until the end of the trial for Holstein cows (Figure 1C).

Se in colostrum was >3-fold higher in TRT vs. CTR, but differences disappeared quickly during the first week of lactation, and no differences were observed in milk at 14 DIM (Figure 2.2D and Table 2.5). Among cows in TRT, Jersey had a numerically larger overall Se in milk compared to Holstein cows (39.7 vs. 31.2 ng/ml; P=0.06). The efficiency in transferring Se into the calf was lower in TRT vs. CTR (6.1 vs. 8.5%; Table 2.5).

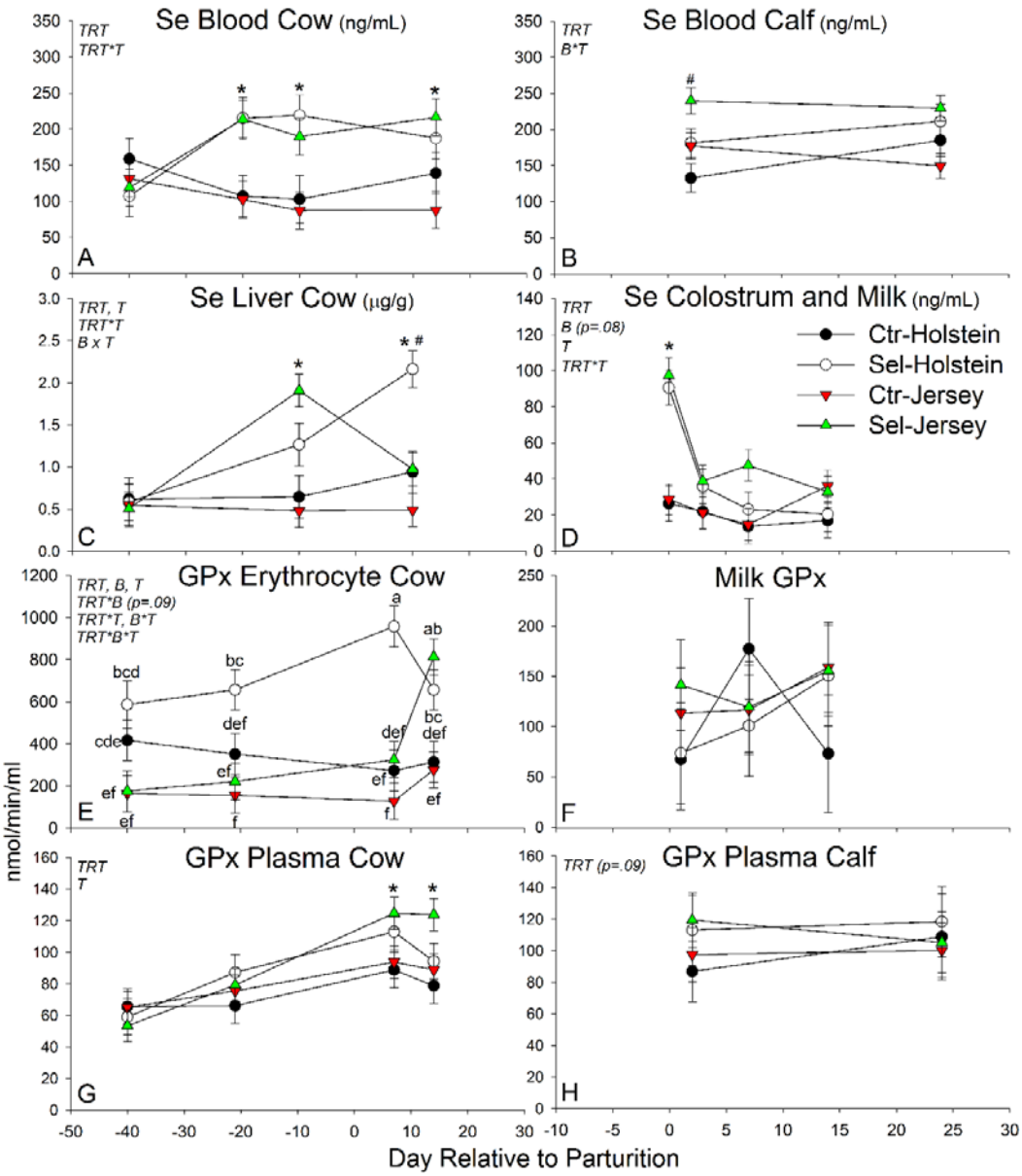

Figure 2.2 The concentration of Se in whole blood (A), liver (C), and milk (D) and glutathione peroxidase (GPx) activity in erythrocytes (E), milk (F), and blood plasma (G) in Jersey (Jer; n=5/group) and Holstein (Hol; n=4/group) cows supplemented with 1% BW of Se biofortified alfalfa hay (Sel) or no biofortified alfalfa hay (Ctr) from 40 days prior expected parturition to 14 days after calving. Reported are also the level of Se in whole blood (B) and GPx activity in plasma (H) of their calves during the first 24 days after birth. Significant (P≤0.05) effects of Selenium (TRT), breed (B), and time (T) and their interactions are indicated by the acronyms in the graph. *Denotes difference between groups (P≤0.05) when TRT×Time P≤0.05; #Denotes difference between groups (P≤0.05) when Breed×Time P≤0.05.

2.4.3 Other microminerals

The concentration of Co, Cu, and Zn in the whole blood in both cows and calves and in milk was not affected by the supplementation of Se biofortified hay (Table 2.5). There was a higher Zn concentration in the blood of Holstein relative to Jersey animals in both cows and calves (Table 2.5). Holsteins cows had higher Co in the liver while Holstein calves had higher Co in blood compared to Jersey cows. In the liver, the level of Co was lower in TRT vs. CTR only in Holstein cows (Table 2.5).

2.4.4 GPx activity

The GPx activity in erythrocytes was >2-fold higher in Holstein than in Jersey cows (Figure 1E). TRT had higher GPx activity in erythrocytes vs. CTR (549.2 vs. 260.0 nmol/min/ml) with a more rapid response in Holstein vs. Jersey cows reaching a peak at 7 DIM but with a decrease afterward. Jersey cows had a rapid increase in GPx activity in erythrocytes from 7 to 14 DIM (Figure 1E). GPx activity in milk was not affected by feeding Se biofortified alfalfa or the breed of the cows (Figure 1F). Compared to CTR, TRT had higher GPx activity in plasma (77.9 vs. 92.8 nmol/min/ml), which was more apparent during lactation, especially due to higher values in Jersey vs. Holstein cows (Figure 1G). The activity of GPx in the plasma of the calves born from cows in the TRT group tended to be higher compared with calves born from CTR group (114.1 vs. 98.4 nmol/min/ml; $P=0.09$; Figure 1H).

2.4.5. Correlations

A positive correlation was detected between Se level in blood and GPx activity in the erythrocytes of the cows (Table 2.6**)**. Se in blood was also positively associated with the level of Se in the liver and tended (P=0.06) to be associated with the level of Se in milk. Se in milk was positively associated with the level of Se and GPx activity of the calves' plasma but was not associated with the activity of GPx in milk. There were no associations between the Se concentration in the blood of cows and calves; however, a positive association was detected between the level of Se in the liver of cows and the level of Se in the blood of calves.

Table 2.6 The correlation coefficient between whole blood, plasma, liver, and milk concentrations of Se and GPx activity of Jersey and Holstein primiparous cows supplemented with 1% BW of Se-biofortified alfalfa hay or control alfalfa hay from 40 days prior expected parturition to 14 days after calving. Reported are also the correlation coefficient in plasma of their offspring during the first 24 days after birth.

	Cows					Calves plasma	
	Se		*GPx*			*GPx*	*Se*
	Liver	*Milk*	*Erythrocytes*	*Plasma*	*Milk*	*Plasma*	*Plasma*
Se Cow Blood	[1]*0.32*	[2]*0.33*	*0.40*	*0.27*	0.01	0.27	0.16
P-value	*0.03*	*0.06*	*<0.01*	*0.06*	*0.98*	*0.31*	*0.55*
Se Cow Liver		*0.34*	*0.43*	*0.60*	-0.08	0.35	*0.52*
P-value		*0.06*	*0.02*	*<0.01*	*0.65*	*0.20*	*0.04*
Se Cow Milk			-0.03	0.11	-0.08	*0.48*	*0.61*
P-value			*0.86*	*0.55*	*0.57*	*0.05*	*0.01*
Erythrocytes Cow				*0.31*	0.06	.	.
P-value				*0.01*	*0.72*	.	.
Plasma Cow GPx					-0.12	.	.
P-value					*0.51*	.	.
Milk Cow GPx						0.14	*0.44*
P-value						*0.61*	*0.07*
Calves Plasma GPx							0.08
P-value							*0.66*

[1]Bold and italic value= significant effect; [2]Italic value=tendency effect

2.5 Discussion

2.5.1 One % BW of Se biofortified alfalfa improves the Se status in cows.

The cows enrolled in our experiment had an adequate level of whole blood Se just before starting the trial (128.6±46.9 ng/ml; mean±SD), with only two cows (one Holstein and one Jersey) with a level of Se <100 ng/ml, considered to be marginal (Dargatz & Ross, 1996). During the trial, the animals did not receive any Se supplementation. That can explain the numerical decrease in Se in blood of CTR cows during the first 40 days of the trial, where cows in the CTR had a level of Se of 93.0±19.4 ng/ml, with 7 out of 9 cows with levels <100 ng/ml. Thus, control animals in the close-up group can be considered marginally deficient for Se despite having a calculated positive Se balance. This observation indicates that classical means to calculate Se requirements are inadequate. In our case, we did not measure real Se losses, but we used previously published data (Séboussi *et al.*, 2016) to provide a more realistic estimate of the Se requirement. Even with that approach, we failed to capture the real requirement of Se.

The supplementation with 1% BW of Se biofortified alfalfa hay was sufficient to double the level of Se in whole blood within a relatively short period of supplementation (20 days), reaching a plateau in whole blood considered high adequate (214.3±48.0 ng/ml) (Dargatz & Ross, 1996). We estimated that dry and lactating cows in the TRT group received between 14 and 17 mg and between 19 and 25 mg of Se/d, respectively.

Pregnant beef cows receiving 28 mg of Se/d via Se biofortified hay had a linear increase in the amount of Se in whole blood, reaching approximately 250 ng/ml after four weeks and >300 ng/ml after 10 weeks of feeding (Wallace *et al.*, 2017). In our case, we did

not observe a linear increase; rather, the cows reached a plateau in 3 weeks with values slightly lower than those detected in beef cows after 4 weeks of treatment. Similar to findings in beef cows, Jersey cows receiving 15 mg/d of Se-yeast for 8 weeks prior parturition had a 1.6-fold increase of Se in whole blood compared to control cows on the day of calving, reaching >380 ng/ml (Hall *et al.*, 2014b). In a study conducted in Holstein cows (Ran *et al.*, 2010), the supplementation of 5 mg/d of Se-yeast increased the level of Se and reached a relatively high level after three months of supplementation (179.8±10.9 in whole blood) with a numerical increase following up to 4 months of supplementation (187.8±8.4 ng/ml). In another study, lactating multiparous Holstein cows received a ration with 0.15 or 0.4 mg of Se-yeast/kg of DM for five weeks. All cows, including cows not receiving Se supplementation, had >200 ng of Se/ml in whole blood. The authors detected a linear increase of blood Se due to doses of Se in the ration (Juniper *et al.*, 2011). In that experiment, cows receiving 0.4 mg of Se/kg of DM had >250 ng/ml of Se in whole blood. In our experiment, control cows received around 0.8 mg of Se/kg of DM while treated cows received approx. 1.7 mg of Se/kg DM. Despite this, the level of Se in all our animals did not reach 250 ng/ml. In a more recent study, Holstein cows in late lactation fed a ration containing up to 25% of alfalfa/timothy silage with 1.97 mg of Se/Kg of DM for 43 days (total of 17.3 mg of Se/day) reached a level of Se in blood similar to our study (Séboussi *et al.*, 2016). However, in our study, Holstein cows during early postpartum received an average of 24.7 mg of Se/day, 42.7% more Se compared to cows in that study.

Se concentrations in the liver can range between 1.2 and 2.0 μg/g of dry weight for all species regardless of age (Stowe & Herdt, 1992). In our experiment, TRT cows had a

substantial increase in the level of Se in the liver, reaching >1.2 μg/g after 30 days of Se supplementation compared to CTR (0.6 μg/g). Our data is somewhat similar to a prior study where beef cows treated for 112 days with 0.5 mg/kg DM of Se-yeast had higher Se in the liver (1.6 μg/g) compared to non-supplemented cows (0.64 μg/g) (Davis *et al.*, 2008).

None of the cows in our study had health issues despite being fed a very large amount of organic Se. This observation supports prior data indicating that supplementing dairy cows with organic Se is effective and non-toxic in their study (Séboussi *et al.*, 2016). Synergism in the absorption of Se and other trace minerals exists, but also some interference in intestinal absorption exists (Van Campen, 2018; Spears, 2003). Our data did not indicate any synergism or interference in the intestinal absorption of measured trace minerals, only a significant for lower Co in TRT Holstein cows vs. TRT Jersey cows.

Overall, the above data indicated some marginal differences of our data compared to prior data, likely due to differences in the age of animals (primiparous vs. pluriparous), baseline Se status, and/or feeding method. However, our data confirmed the efficacy of using Se biofortified hay to improve Se status in dairy cows.

2.5.2 Se is efficiently transferred to colostrum but not milk in early post-partum cows

Using Se biofortification can be a good strategy to increase dietary Se level in livestock products, including milk (Lyons *et al.*, 2003). Se levels in milk have been implicated as a means to assess the Se status in cows (Wichtel *et al.*, 2004). According to the references provided (Wichtel *et al.*, 2004), the level of Se in the colostrum in our cows was more than adequate while the Se in milk would be considered marginal, even those in

the TRT group. Thus, our data indicated a high transfer of Se in colostrum but a poor transfer of Se to milk.

Higher transfer of Se in colostrum relative to milk was also observed in beef (Slavik *et al.*, 2008; Ranches *et al.*, 2017) and dairy cows (Salman *et al.*, 2013). The high Se level in colostrum compared to milk is likely due to high amounts of selenoproteins, as observed in humans, where Se level decreases as lactation progresses (Dorea, 2002). However, the lack of difference in Se content of milk between TRT and CTR in our study is different than prior reports (Juniper *et al.*, 2006; Séboussi *et al.*, 2016; Ran *et al.*, 2010). Those studies were conducted in mid and late lactating dairy cows, whereas out study evaluated early lactating cows. Our data are similar to other studies where a lack of Se enrichment in milk was detected in early post-partum dairy cows supplemented with Se (Salman *et al.*, 2013).

Se supplementation is associated with an increased amount of Se in various tissues, but, as observed previously in human, does not appear to be highly associated with Se level in milk (Bianchi *et al.*, 1999). In goats, the Se level in milk increases from early to late lactation (Rozenská *et al.*, 2013). The reason for the higher content of Se in milk of late vs. early lactating goats, the enrichment of Se in milk of mid or late lactating cows fed higher level of Se, and the lack of Se enrichment in milk in early post-partum cows in our study, may be explained by a higher expression of the main Se-methionine transporters (i.e., *SLC3A1*) in mammary glands during late vs. early lactation, as observed in pigs (Chen *et al.*, 2018). To our knowledge, the expression of *SLC3A1* in mammary tissue in bovine through lactation has not been assessed.

2.5.3 Colostrum is more effective than placenta in transferring Se in calves

In ruminants, the transfer of nutrients across the placenta or the mammary gland into the fetuses and newborns respectively, is very important for proper development and growth (Abdelrahman & Kincaid, 1995). The transfer of Se is known to be more efficient via the placenta than through the milk (Dargatz & Ross, 1996). In our experiment, the calves received colostrum from their mother; thus, the Se level in the blood of calves just after birth can be considered an accumulated transfer from the placenta and colostrum. However, the lack of association between the level of Se in the blood of cows and calves and the high association of Se in calves' blood with the level of Se in colostrum indicate a more important role of Se transfer via the colostrum compared to the placenta.

2.5.4 GPx activity is improved in cows by feeding Se biofortified hay

GPx activity is known to decrease around parturition with the nadir at calving (Gong & Xiao, 2016; Cigliano *et al.*, 2014). The pattern of GPx activity in CTR in our experiment is consistent with such a pattern. However, in TRT, the level of GPx activity increased, avoiding the decrease at calving, consistent with prior findings (Ran *et al.*, 2010; Hall *et al.*, 2014b). Thus, our data support an improvement of the anti-oxidative capacity of cows supplemented with Se biofortified hay.

Our study indicates that icreasing the Se level in cows increases the Se in plasma of their calves early post-birth but only numerically augments the GPx activity measured in calves' plasma. A prior study detected a positive effect of erythrocyte GPx and blood Se

in calves; however, the effect disappeared after the colostral phase (Rowntree *et al.*, 2004). Thus, our data do not allow concluding if feeding Se biofortified hay to cows benefits the anti-oxidative status of their calves. However, different than the prior study (Rowntree *et al.*, 2004), we only measured GPx activity in plasma of calves.

2.5.5 Jersey and Holstein cows differ in response to feeding Se biofortified hay

Research comparing Holstein and Jersey cows with regard to trace mineral supplementation are sparse. However, most dairy research involves Holstein cows. Our data indicated that the response to supplementation with Se biofortified alfalfa differs somewhat between the breeds. It appears that Jersey tend to accumulate more Se pre-partum while releasing it fast, especially from the liver, early post-partum. Holstein cows instead appear to store more the Se in liver post-partum releasing it less for other uses, such as GPx activity. The reason is unclear for such difference; however, prior experiments in peripartum cows indicate breed-specific differences in response to various dietary supplements (Guretzky *et al.*, 2006; Drackley *et al.*, 2001).

2.6 Limitations of the study

Our study presents several limitations. It is well-known that multiparous dairy cows are more subjected to oxidative stress compared to primiparous cows (Bühler *et al.*, 2018); thus, findings from our study should be confirmed in pluriparous dairy cows. However, our study was aimed to determine the feasibility of feeding a relatively low amount of Se biofortified hay using a low number of animals; thus, the use of primiparous cows was a

safeguard to minimize diseases that could have compromised the study. The use of two breeds, although providing a window of possible differences in response to Se, especially GPx activity, has reduced the statistical power, especially considering the low number of animals used. The lack of whole blood GPx activity in calves also has limited our ability to make clear conclusions about the anti-oxidative status of dairy calves. Measurements of other anti-oxidative parameters on the plasma could help overcome this limitation.

2.7 Conclusions

Despite the above limitations, our data demonstrate that supplementing a relatively low amount of Se biofortified alfalfa hay in peripartum dairy cows is an effective method to increase Se concentration in cows and their calves, improving the anti-oxidative status of the cows. Colostrum is an effective means to transfer selenium to calves, but early post-partum cows have a very poor transfer of Se into milk. Our study revealed some differences in response to Se biofortified hay between Jersey and Holstein cows that deserve further investigations.

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CHAPTER 3

SELENIUM BIOFORTIFIED ALFALFA HAY SUPPLEMENTED TO PRIMIPAROUS COWS DURING THE PERIPARTUM AFFECTS THE ANTIOXIDANT FUNCTION OF ALBUMIN BUT HAS LITTLE IMPACT ON METABOLIC AND IMMUNE FUNCTION

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3.1 Abstract

The transition from pregnancy to lactation is the most critical period in high producing dairy cows. During that time, the indidence of diseases worsen due also to higher oxidative stress and depressed immune function. Selenium (Se) is a trace mineral that plays an important role in anti-oxidative response and immune function in dairy cows. We hypothesize that supplementing dairy cows with a relatively small amount of Se biofortified hay during the dry period and early lactation improves performance, metabolism, oxidative status, and immune response. To test this hypothesis, 10 Jersey and 8 Holstein primiparous dairy cows were supplemented with 1% BW of Se biofortified (TRT; n=9; 3.2 mg/kg DM Se) or non-biofortified (CTR; n=9; 0.4 mg/kg DM Se) alfalfa hay mixed with the TMR from approx. 40 days prior- to 2 weeks post-partum. Primiparous cows were monitored daily for dry matter intake, activity, and weekly for body weight and body condition score. Immune response parameters were measured, such as carrageenan skin test and phagocytosis. Milk yield and components, including fatty acid profile, were measured. Blood samples were also collected to measure metabolic, oxidative, and inflammatory biomarkers and blood hematocrit.

Supplementation of Se-biofortified hay did not affect milk yield or milk components, including fatty acid profiling, body weight, or DMI. Supplementation of Se biofortified hay affected only a few of the measured parameters in the blood. Albumin was increased, and haptoglobin and urea tended to be increased by the treatment, indicating a better liver status, especially post-partum. Despite higher glutathione peroxidase activity, we did not detect any effect on the measured parameters related to the oxidative status, except advanced oxidation protein products (AOPP) that were increased by the treatment. The negative correlation of AOPP with

myeloperoxidase and parameters related to inflammation but a positive association with albumin suggests an improved antioxidative function of albumin by Se biofortified hay supplementation. Se biofortified hay increased hematocrit, indicating a positive effect on erythropoiesis and/or preservation of red blood cells. Treatment did not affect any of the measured parameters associated with the immune system and oxidative stress. Feeding 1% BW of Se biofortified hay had little effect on metabolic, inflammatory, and oxidative status parameters with no effect on cow's performance or immune response. Supplementation with Se biofortified hay possibly enhanced liver function, promoted the antioxidant role of albumin, and improved viability/formation of red blood cells.

Keywords: Selenium, biofortified hay, blood parameter, milk yield, immune response, primiparous cows

3.2 Introduction

The most critical period in the life of a dairy cow is the "transition period," which is defined as three weeks before and three weeks after parturition (Drackley, 1999). During that time, high-producing dairy cows undergo many complex physiological and metabolic changes (Sordillo and Aitken, 2009). The high nutritional requirements of lactation, together with the inability to eat enough, drive the cow into a negative energy balance that elicits the mobilization of non-esterified fatty acids **(NEFA)** from the adipose tissue. A large amount of NEFA can lead to excess reactive oxygen species **(ROS)** during the process of being converted to energy in the mitochondria (Sordillo and Aitken, 2009; Mordak and Anthony, 2015; Abuelo et al., 2016). ROS are not only produced by high NEFA oxidation but also by active phagocytes (Knight, 2000). An imbalance between the high production of ROS and low antioxidant defenses generates oxidative stress (Trevisan et al., 2001). Early post-partum dairy cows also experience inflammatory-like conditions that can negatively affect the ability of the liver to face the ensuing large metabolic and immune challenges (Trevisi et al., 2015; Bertoni et al., 2008). All the above cause an increase in the incidence of diseases, such as mastitis, metritis, retained placenta, and ketosis (Sordillo and Mavangira, 2014; O'Rourke, 2009).

The metabolic and oxidative stress experienced by dairy cows during the transition period negatively impact the immune system. However, immune suppression early post-partum in dairy cows is also highly determined by nutritional deficiencies, as demonstrated by the positive effects on the immune system by various supplements during this stage of lactation (Spears, 2000; Ingvartsen and Moyes, 2013). Among trace minerals, selenium **(Se)** supplementation can enhance simultaneously the antioxidative status and the innate and adaptive immune responses (Gill and

Walker, 2008; Brummer, 2012; Hall et al., 2014; Mehdi and Dufrasne, 2016; Gong & Xiao, 2018). Se supplementation can also affect overall inflammatory response and metabolism. Supplementation of Jersey dairy cows with Se-yeast during the dry period increased serum amyloid A and decreased cholesterol 48 h postpartum and increased serum albumin 14 days post-partum (Hall et al., 2014).

Se is very low in forages produced in several parts of the world, including NW US, due to the low Se availability in the soil (USGS, 2018). Thus, cows do not receive an adequate amount of Se from forages, and supplementation is required. It is difficult to reach the requirements of Se in dairy cows using inorganic Se supplementation. That is mostly due to the acute toxicity of high doses of inorganic Se. For this reason, Se is the only micronutrient regulated as a feed additive by the FDA that sets the limit of Se supplementation in cattle to 0.3 mg/Kg dry matter (ppm) in various supplements with a maximum of 3 mg/head per day (NRC, 2001).

Compared to inorganic Se (i.e., selenate and selenite), the organic Se is less toxic to cattle and can be used as a supplement to match the requirement of Se in this species (Salman et al., 2013; Oltramari et al., 2014). For this reason, agronomic biofortification of forages with Se can be an excellent alternative to supplement Se in high producing dairy cows fed forages grown in areas with low Se in the soil (Hall et al., 2011; Hall et al., 2013; Novoselec et al., 2018).

In our prior study, feeding dairy cows during the last 40 days of pregnancy until 14 days in milk with a relatively low amount of Se biofortified hay (1 kg DM/100 kg BW) improved the Se status and the glutathione peroxidase activity in whole blood (see Chapter 2 of the dissertation). However, the effect of Se biofortified alfalfa on metabolism, overall oxidative status, immune status, and performance of the animals was not assessed.

Based on prior observations of the effect of organic Se in dairy cows as mentioned above, we hypothesize that supplementing dairy cows with a relatively small amount of Se biofortified hay during the dry period and early lactation improves performance, metabolism, oxidative status, and immune response. The objective of the present study was to evaluate the effect of feeding 1 % of BW of Se biofortified alfalfa to dairy cows during the dry period and early lactation on blood biomarkers, performance, and immune status.

3.3 Materials and Methods

3.3.1 Animals, ration, and experimental design

Experimental procedures used in this study were approved by the Institutional Animal Care and Use Committees **(IACUC)** of Oregon State University (protocol# 4894). The overall experimental design is shown in **Figure 3.1**. Details of the experiments are reported in Chapter 2. Briefly, ten Jersey and eight Holstein primiparous cows kept in a pen equipped with Calan gates received either 1 kg /100 kg body weight **(BW)** of Se biofortified alfalfa hay (**TRT**; 3.25 mg Se/kg DM) or control hay (**CTR**; 0.43 mg Se/kg DM) starting at 40 day (39±5) from expected parturition to 14 days in milk **(DIM)**. Animals were milked and fed with ad-libitum total mixed ration (**TMR)** twice daily. Before feeding, TMR was mixed by hand with the chopped Se biofortified alfalfa hay or the control alfalfa hay. Details of the feeding and composition and chemical analysis of the feedstuffs are reported in Chapter 2.

3.3.2 Feed intake, body weight, and body condition score

Dry matter intake **(DMI)** was recorded daily by measuring feed provided and residuals. The BW was measured once a week with an Afimilk walk-in scale. The body condition score **(BCS)** was measured every week by three different people, using a 1 to 5 scale.

3.3.3 Activity, milk yield, milk components, and milk fatty acids

The activity of the animals, milk yield, and milk conductivity were measured by the Afimilk system (Afimilk, Israel). Milk samples were collected in tubes containing bronopol during morning milking at 3, 7, and 14 DIM and stored at 4°C until analysis at Willamette DHIA laboratory (Salem, OR). Milk fatty acid analysis was performed as previously described (Jaaf et al., 2019) using behenic acid as the internal standard (100 µl of a 0.2 mg/mL solution, Cat#1161, Matreya, LLC).

3.3.4 Inflammatory and metabolic profile

Blood samples were collected before the morning feeding from the jugular vein into evacuated tubes with Na-heparin (Cat# 366480, 10 mL; Becton Dickinson, Franklin Lakes, NJ) at -40 (-39±5), -30 (-27±4), -10 (-10±3), 1, 4, 7, 10 DIM. Aliquots of plasma were sent to the Department of Animal Sciences, Food and Nutrition (DIANA), Università Cattolica del Sacro Cuore, Piacenza, Italy for metabolic and inflammatory profiling. Blood samples were processed and analyzed following the methods described by Calamari et al. (2016) if not differently specified. Energy, protein, and mineral metabolism were profiled measuring the concentration of glucose, cholesterol, NEFA, beta-hydroxybutyric acid **(BHBA)**, urea, creatinine, and total protein,

globulin, Ca, and Mg. Positive acute-phase proteins such as haptoglobin and ceruloplasmin and negative acute-phase proteins such as albumin and paraoxonase were used to evaluate the inflammatory response. The paraoxonase was analyzed with a method described previously (Bionaz et al., 2007). Moreover, myeloperoxidase **(MPO)** was also determined to assess the activity of neutrophils, using a method described previously (Bradley et al., 1982). Liver activity and health were assessed, measuring total bilirubin, aspartate aminotransferase, γ-glutamyl transferase, and alkaline phosphatase **(ALP)**. The oxidative stress was monitored by measuring total reactive oxygen metabolites, thiolic groups, ferric reducing antioxidant power, and advanced oxidation protein products **(AOPP)** (Hanasand et al., 2012).

3.3.5 Carrageenan skin test

The carrageenan skin test for the assessment of the *in vivo* immune response (mostly neutrophil migration) was done as described in Trevisi et al. (2015) at -50, -21, -3, and 7 DIM. Briefly, sterile carrageenan (κ-Carrageenan, Cat# IC10488680, VWR, USA) was dissolved in sterile saline (Cat# VINV-SALN-1000, Henry Schein, USA) to obtain a 0.7% solution. The CST was administered by injecting 0.6 mL of carrageenan solution under the skin (equal to 4.2 mg of carrageenan for each treatment) on the shoulder region of the cows. The injection was made alternatively in the left and right shoulder, the site of treatment was different for each infusion and the area of injection was marked by clipping the hair. The skin thickness was measured using a skinfold caliper (Cat# 470119-588, VWR, USA) immediately before carrageenan injection (0 days), then at 2 and nine days after the injection. The overall response was calculated as the area

under the curve of the thickness measured at day 2 and day 9, subtracting the thickness measured at day 0.

3.3.6 White blood cells counting, phagocytosis, and differential

Total white blood cells (**WBC**) were counted using the Leuko-tic blue kit (Cat#4013-0006/-0007/-0008, bioanalytical, Germany). PHrodo BioParticles Phagocytosis kit containing green *S. aureus* bioparticles (Cat# P35382, Life Technologies) was used to measure the phagocytosis by WBC according to the manufacturer's protocol. At the end of the protocol, cells were stained with primary antibodies CAM36A (IgG anti-CD14; Cat# 6-9-03) and CH138A (IgM; neutrophils marker; Cat# 2001) from the Washington State University Monoclonal Antibody Center, Pullman WA. Allophycocyanin goat anti-mouse IgG (Cat# M30005, Caltag Laboratories) and R-Phycoerythrin goat anti-mouse IgM (Cat# 662587, Invitrogen) were used as secondary antibodies. Briefly, samples were centrifuged at 350×g for 5 minutes at 20°C, and the pellet resuspended in 200 µL of appropriate primary antibody solution and incubated for 1 hour on ice. After incubation, samples were centrifuged at 1000×g for 5 minutes at 20°C and the pellet resuspended in 200 µL of appropriate secondary antibody solution and incubated for 30 min on ice. After incubation, samples were centrifuged at 1000×g for 5 minutes at 20°C and the pellet resuspended in 1 mL of formalin (Cat# 170191, Fisher chemicals, USA) + 2‰ DAPI solution and incubated for 30 min at room temperature. Finally, all the samples were centrifuged at 1000×g for 5 minutes at 20°C, resuspended in 500 µL of PBS, filtered using a cheese-cloth, and loaded on a 96 well flat-bottomed plate (Cat#655 180, Cellstar, USA) for flow cytometric assay using a

Beckman Coulter CytoFLEX. Flow cytometery data for phagocytosis and differential were analyzed as previously described (Rosa et al., 2017).

3.4 Statistical analysis

Milk production was analyzed as daily milk yield during the first 14 DIM and as a weekly average for the first 120 DIM. For data obtained at -40 DIM (baseline) for each breed separately, the data points for each cow were arithmetically corrected by the difference between TRT and CTR at -40 DIM as previously described (Rosa et al., 2017) to obtain the same mean between groups at -40 DIM but keep the differences between breeds. After the correction, the -40 DIM was removed from the model. For all parameters, outlier data were checked using PROC REG of SAS (v9.4, SAS Institute, Inc., Cary, NC, USA) before statistical analysis. Data with a studentized t >3.0 were removed. Data were analyzed by GLIMMIX of SAS with the fixed effect of treatment, breed, time and their interactions with the cow as a random effect using the default covariate model. The Kenward-Roger degrees of freedom approximation was used. For milk fatty acid analysis, a Proc GLM of SAS was used. PROC CORR was used for correlation analysis among the variables. Differences were considered significant with $P \leq 0.05$ and with a tendency with $P \leq 0.10$.

3.5 Results

3.5.1 Cow's performance and milk composition

Performance data are reported in **Table 3.1**. None of the cow's performance data were affected by treatment. Several differences were observed between the two breeds, some expected,

such as DMI, BW, and yield of milk (all higher in Holstein vs. Jersey cows), but some were somewhat unexpected, such as the higher BCS, somatic cell count **(SCC)**, milk urea **(MUN)** (Knowlton et al., 2010), and activity in Jersey compared to Holstein cows. The DMI, DMI/BW, and milk yield during the first 120 DIM (**Figure 3.2**) had a tendency (P=0.07) for a TRT×Time or TRT×Breed×Time interaction mostly driven by an increase in Holstein cows in TRT vs. CTR. A tendency (P=0.08) for a full interaction for % milk protein was detected due to a numerical decrease in cows fed Se biofortified hay vs. CTR in Holstein cows and a numerical increase in TRT vs. CTR in Jersey cows.

Feeding 1% BW of Se biofortified hay was associated with a decrease in the concentration of few unsaturated fatty acids in milk fat such as C18:3n3, C18:4n3, and C20:3t and increased the concentration of only two unsaturated fatty acids, i.e., C16:1cis10, and C20:1n9 (**Table 3.4**). Breed had a larger effect on the fatty acid composition of the milk fat, especially the unsaturated fatty acids. We detected higher concentrations in Holsteins of C12:1, C14:1cis9, C14:0hydroxy, C16:1trans, C16:1cis10, C18:1tr11, C18:3n3, C20:1n9, and Δ9 C14:1 while higher concentrations of C14:1c11, C16:1cis7, C16:1cis9, C18:2c9t12, C18:2t9c12, C19:0, C20:3t, Δ9 C16:1 was detected in Jersey vs. Holstein cows.

3.5.2 Metabolic, inflammatory, oxidative, and immune-related parameters measured in plasma

The summary of all the parameters measured in plasma is reported in **Table 3.2**. The temporal pattern of parameters with a significant effect is shown in **Figure 3.3**.

3.5.2.1 Metabolic-related parameters. None of the measured parameters related to metabolism were significantly affected by feeding 1% BW of Se biofortified hay in primiparous cows. We

detected a tendency (P=0.06) for higher blood urea in TRT vs. CTR cows. Holstein cows had an overall higher cholesterol concentration in plasma compared to Jersey cows.

3.5.2.2 Inflammatory-related parameters. Among the various inflammatory-related parameters measured, we detected significant TRT×Time and TRT×Breed×Time interactions for the negative acute-phase protein albumin (**Figure 3.3A**). There was significantly higher albumin at 14 DIM in TRT vs. CTR, mostly driven by Holstein cows. A tendency for a full interaction (P=0.06) was detected for the positive acute-phase protein haptoglobin, but no clear effect of treatment in any of the time points evaluated was observed. (**Figure 3.3B**). We also detected a tendency (P=0.10) for higher MPO in TRT vs. CTR.

3.5.2.3 Liver health. Among parameters related to liver function and health, only ALP was affected by the full interaction between TRT×Breed×Time, but in none of the evaluated time points there was a significant difference between treatments (**Figure 3.3C**).

3.5.2.4 Oxidative status. Of all parameters measured related to the oxidative status, only plasma concentration of AOPP was overall higher in TRT vs. CTR cows. The AOPP was also higher in Holstein vs. Jersey cows (**Figure 3.3D**). The AOPP:albumin ratio, as an index of protein oxidation (Celi et al., 2012), was higher in TRT vs. CTR and was also higher in Holstein vs. Jersey cows.

3.5.2.5 Minerals. Plasma concentration of Ca and Mg was affected by a full interaction of TRT×Breed×Time. Ca (**Figure 3.3E**) and estimated free Ca were higher in TRT vs. CTR in Jersey cows at -10 DIM while Ca was higher in TRT vs. CTR in Holstein cows at 14 DIM. Mg was decreased by Se biofortified hay prior parturition only in Holstein cows (**Figure 3.3F**; P<0.08). Mg was also overall lower in Holstein vs. Jersey cows.

3.5.2.6 Kidney function. As expected by the higher body weight, creatinine was higher in Holstein compared to Jersey cows. The urea:creatinine ratio as an index of kidney function was higher in TRT vs. CTR cows and was higher in Jersey than Holstein cows.

3.5.2.7 Other parameters. The hematocrit was overall higher in TRT vs. CTR cows with a tendency (P=0.07) to be higher in Holstein vs. Jersey cows (**Figure 3.3G**). The concentration of blood total protein tended (P=0.08) to be increased by the treatment and was higher in Holstein vs. Jersey cows, while globulin tended (P=0.06) to be differentially affected by the treatment based on breed (i.e., increased numerically in Jersey cows while decreased in Holstein cows fed Se biofortified hay vs. control).

3.5.3 Immune-related parameters

None of the measured immune-related parameters, including phagocytosis, WBC differential, and carrageenan skin test were affected by the treatment (**Table 3.3**); however, the WBC count was significantly higher in Holstein vs. Jersey cows and was affected by the full interaction, due to a sudden drop early post-partum with a quick increase in Holstein cows in the treatment group compared to the other groups (**Figure 3.3H**).

3.6 Discussions

3.6.1 Cow performance was not affected by Se biofortified hay

In our experiment, we expected a significant effect of Se biofortified hay supplementation on the milk yield. Previous studies on ewes indicated a positive effect of Se supplementation during pregnancy on mammary gland growth, development, and vascularity (Swanson et al., 2008;

Vonnahme et al., 2011; Davis & Collier, 1985) that can increase milk production. In our study, we detected only a tendency for increased milk production during the first 120 days of lactation in primiparous Holstein cows and not in Jersey cows. Our finding is similar to prior studies where Se supplementation did not affect milk yield or milk components (Gong et al., 2014; Séboussi et al., 2016). Different than those prior studies and our study, Oltramari et al. (2014) observed a significant increase in fat % in cows supplemented with Se yeast compared to unsupplemented cows. The lack of effects on milk components in our study can also be due to the short period assessed post-partum (up to 14 DIM).

Se supplementation positively affects the proportion of polyunsaturated fatty acids **(PUFA)** in the milk of cows (Ran et al., 2010) and humans (Dodge et al., 1999). It is thought that the effect of Se on PUFA is due to its role via glutathione peroxidase enzyme **(GPx)**. The GPx can prevent the oxidation of PUFA by free radicals (Zoidis et al., 2018). Contrary to the above experiment, we detected a decrease of several PUFA in milk fat in Se-biofortified hay treated cows. However, our prior data indicated that feeding Se biofortified hay does not affect milk GPx (Chapter 2).

3.6.2 Se biofortified hay improves albumin production by the liver

In our experiment, supplementation of dairy cows with Se biofortified hay improved albumin concentration early post-partum. Similar results were detected previously in Jersey cows supplemented with Se-yeast during the last eight weeks of gestation (Hall et al., 2014) and in Holstein cows supplemented with selenomethionine from 21 days prepartum to 21 days post-partum (Khalili et al., 2019).

The reason for the consistent higher serum albumin when organic Se is provided to cattle is unclear. Albumin is a negative acute-phase protein and an indicator of liver function (Gruys et

al., 2005). Albumin concentrations in serum and hepatic synthesis of albumin decreases with inflammation or when liver function is depressed and higher albumin concentration during the first week after calving is associated with better health status (Trevisi et al., 2012). In our experiment, supplementing primiparous dairy cows with 1% of Se biofortified hay have likely improved liver production of albumin during the transition period.

Urea concentration in plasma is the result of the balance between urea input (i.e., produced by the liver and rumen utilization and absorption of urea) and output (i.e., elimination by the kidneys and passive loss via feces, sweat, and milk) (Weiner et al., 2015). Hence, increased plasma urea can be caused by increased urea production, decreased urea elimination, or a combination of the two (Higgins, 2016). We can exclude the increase of urea by protein intake considering that there was no difference in DMI between groups. We can also eliminate any kidney problem, considering that we detected higher urea:creatine ratio as index of kidney filtration (Walker et al., 1990) in cows supplemented with Se biofortified hay. Urea in plasma can also increase as a consequence of higher muscle proteolysis (Schutz, 2011). This does not appear to be the case due to the lack of any difference in body weight and creatinine levels between treatment groups. Thus, the only explanation for the almost significant higher urea in the blood remains an improved hepatic urea cycle. An increase in blood urea concentration in Se supplemented animals was also observed in rats supplemented with inorganic Se (Abdo, 1994) and in 1 year old Angus steers fed organic Se (Jia et al., 2019) but not in multiparous dairy cows (Kamada, 2017; Khalili et al.,2019). The discrepancy between studies with ruminants might be due to the age of the animals since we used primiparous cows that are closer to age with the yearling steers.

Overall, the higher albumin and urea concentration appears to support a liver in better condition in cows treated with Se biofortified hay. However, our data do not allow us to conclude if this translates into a more functional liver.

3.6.3 Se biofortified hay does not affect the oxidative status and the immune system but might improve the antioxidant function of albumin

During the transition period, dairy cows are highly subjected to oxidative stress. This is caused by an imbalance between the production of ROS and the ability to detoxify the reactive intermediates or to repair the resulting damage (Hanasand et al., 2012). It has been observed previously that supplementation of dairy cows with the organic form of Se during the transition period enhances the anti-oxidative response and the immune function of dairy cows (Surai et al., 2019). One of the main antioxidant enzymes in the blood of cows is the Se-dependent GPx that acts to remove free radicals normally produced by cells (Sordillo, 2013). The activity of GPx was increased significantly in erythrocytes and plasma of the cows supplemented with Se biofortified hay compared to control cows in our experiment, indicating an enhanced antioxidant response (Chapter 2). However, none of the additional oxidative status parameters measured were affected by the treatment with the exception of the AOPP.

AOPP is a marker of protein oxidation that was first described in the plasma of uraemic human patients (Witko-Sarsat et al., 1996), and it has been recognized as a marker of inflammation in several diseases in humans (Witko-Sarsat et al., 1998). Higher AOPP in plasma was also observed in dairy cows experiencing grade 2 endometritis (Gabai et al., 2019). AOPP are products of proteins exposed to free radicals, and they are formed by the reaction between plasma proteins and chlorinated oxidants mediated by the neutrophil enzyme MPO (Celi, 2011; Bordignon et al.,

2014). AOPP can also trigger the oxidative burst and the synthesis of inflammatory cytokines in neutrophils and monocytes (Arsat et al., 2003). Increased chemotaxis and respiratory burst of neutrophils was detected in sheep supplemented with Se nanoparticles compared to sodium selenite (Kojouri et al., 2012). Unfortunately, we did not measure respiratory burst capacity in neutrophils in our experiment, but MPO tended to be higher in cows supplemented with Se biofortified hay. We detected a negative correlation between AOPP and MPO and AOPP:albumin and MPO ($r < -0.45$; $P < .0001$; Suppl. File 1). Both data do not support a role of MPO in determining the level of AOPP in our study. In humans, a positive correlation between levels of AOPP and MPO was observed in plasma but only in patients with kidney failure and not in healthy patients (Capeillère-Blandin et al., 2006). Our primiparous cows were, as expected, all healthy with no kidney problems, as indicated by the higher urea:creatinine ratio in TRT vs. CTR cows.

The above data do not explain the higher AOPP detected in animals treated with Se biofortified hay compared to control cows. Interestingly, plasma level of AOPP in our study was not correlated (Suppl. File 1) with any of the immune-related parameters and negatively correlated ($P < .001$) with plasma GPx ($r = -0.46$), total ROM ($r = -0.41$), inflammatory parameters haptoglobin and ceruloplasmin ($r = -0.38$), NEFA (-0.36), BHBA ($r = -0.31$) or indexes of poor liver activity/health such as AST/GOT ($r = -0.47$) and total bilirubin ($r = -0.28$). The plasma level of AOPP was instead positively correlated ($r > 0.32$; $P < .0001$) with parameters related to low inflammation and/or good liver function such as albumin, cholesterol, and paraoxonase (Bionaz et al., 2007). Thus, considering the above, it appears that a higher level of AOPP in Se biofortified hay fed cows could be considered a positive outcome.

Serum albumin is one of the major antioxidant proteins in the blood (Gutteridge, 1986). Interestingly, the result of albumin oxidation is AOPP (Anraku et al. 2013). The AOPP produced from oxidized albumin bind to high-density lipoprotein scavenger receptor and are primarily eliminated by the liver and the spleen (Iwao et al., 2006). Thus, the higher AOPP can partly be explained by the higher albumin levels in cows fed Se biofortified hay. That is also supported by the aforementioned positive correlation between plasma levels of albumin and AOPP.

The antioxidant function of albumin is due to the amino acids present in its structure (Taverna et al., 2013). In albumin, methionine (0.8% of all amino acids) and cysteine (5.8%) account for up to 80% of its total antioxidant activity (Bourdon et al., 2005). The thiol group Cys34 in albumin that acts as a scavenger for reactive oxygen and nitrogen species accounts for 80% of the reduced thiols in human plasma. In our study, levels of thiol groups and albumin were correlated with $r = 0.60$ ($P<.0001$).

3.6.4 Se-biofortified hay affects mineral metabolism

The higher amount of serum Ca in Se biofortified hay treated cows can partly be driven by the known relationship between albumin and Ca since Ca ions (approx. 50% of the total serum Ca) is bound in blood to albumin (Pal et al., 2016). This is of importance, considering that this is the active form of Ca, that when decreased induces the release of parathyroid hormone via the Ca^{2+} sensing receptor, controlling Ca homeostasis (Martín-Tereso and Martens, 2014). Ca homeostasis prepartum is of paramount importance in dairy cows, especially Jersey cows, which are known to be more at risk of this post-partum hypocalcemia. Probably even more importantfor the prevention of hypocalcemia is the prepartum level of Mg in plasma. Magnesium, as a cofactor in parathyroid

hormone action, is required for the efficient absorption and resorption of calcium; hence, cows that have low blood Mg around calving are more prone to get milk fever (Roche et al., 2017). Our data indicated lower Mg in Holstein cows fed Se biofortified hay. However, none of our cows had milk fever.

3.6.5 Se biofortified hay improves red blood cells

The hematocrit was positively affected by feeding Se biofortified hay in our study. While there are no previous data concerning cows, Kaushal et al. (2010) reported that mice supplemented with Se had higher hematocrit. Se has a role in regulating red blood cells homeostasis by mitigating oxidative stress-dependent modulation of genes that affect the differentiation of erythroid progenitors (Kaushal et al., 2010). Rotruck et al., (1972) also suggested that dietary Se, which functions as an antioxidant through selenoproteins, prevents the erythrocyte lysis and the formation of methemoglobin as observed *in vitro*. Thus, it is possible that higher Se and GPx (see Chapter 2) improved erythropoiesis and/or increased erythrocyte lifespan in our study.

3.7 Limitations of the study

Our study presents several limitations. The number of animals used was low. Another limitation was the use of primiparous instead of multiparous cows. It is well-known that primiparous cows are less susceptible to certain metabolic diseases compared to multiparous cows. Thus, it is possible that a beneficial effect of the Se supplementation was not detected due to the enrollment of animal that are prone to remain healthy. The short term of data collection after parturition (14 days), especially milk components, was also a limitation of the study. That is

suggested by the tendency for higher milk yield in Holstein cows when observed for 120 days post-partum.

3.8 Summary and Conclusions

The present study revealed that feeding primiparous cows with a relatively low amount of Se biofortified hay did not influence cow's milk yield or composition, including the fatty acid profile of the butterfat. The treatment also had little effect on metabolic and inflammatory parameters. Furthermore, despite higher GPx detected in our prior analysis (Chapter 2), no other measured parameters related to antioxidant response were affected. However, data allowed us to infer that supplementation with Se biofortified hay in primiparous cows likely improved liver function, promoted the antioxidant function of albumin, and improved erythropoiesis and/or viability of red blood cells. All those outcomes need to be further validated, and, as indicated above, a lack of stronger effect might be due to the use of primiparous cows and the low number of animals. It appears sensible to test further the effect of Se biofortified hay in pluriparous cows that are known to be more metabolically and immunologically challenged. If a new experiment should be performed, our data indicate that the respiratory burst should be inserted among the measured parameters.

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Figure Caption

Figure 3.1 Experimental design and samplings. The Selenium group received 1% BW of Se-biofortified alfalfa (3.25 ppm of Se) from -40 to 14 day relative to parturition while the Control group received alfalfa without biofortification (0.43 ppm of Se).

Figure 3.2 Weekly milk yield until 120 days post-calving in Jersey (Jer; n=5/group) and Holstein (Hol; n=4/group) primiparous cows supplemented with 1% BW of Se-biofortified alfalfa hay (Sel) or no biofortified alfalfa hay (CTR) from 40 days prior expected parturition to 14 days after calving. Reported in the graph is the P-value of the effect of treatment (*TRT*), Week, Breed, and their interactions.

Figure 3.3 Blood parameters that were significantly ($p \leq .05$) affected by treatment (*TRT*) or the interaction between *TRT*, breed (Br), and Time in Jersey (Jer; n=5/group) and Holstein (Hol; n=4/group) primiparous cows supplemented with 1% BW of Se-biofortified alfalfa hay (Sel) or no biofortified alfalfa hay (CTR) from 40 days prior expected parturition to 14 days after calving. Reported in the graph are the P-value of the overall effect of treatment (*TRT*), Time, Breed, and their interactions. * and # denote difference with $P \leq 0.05$ between groups at each time point in Holstein and Jersey cows, respectively, when significant ($P \leq 0.05$) TRT×Br×Time was detected. @ denotes a significant effect in a time point when significant ($P \leq 0.05$) TRT×Time interaction was detected.

Figures

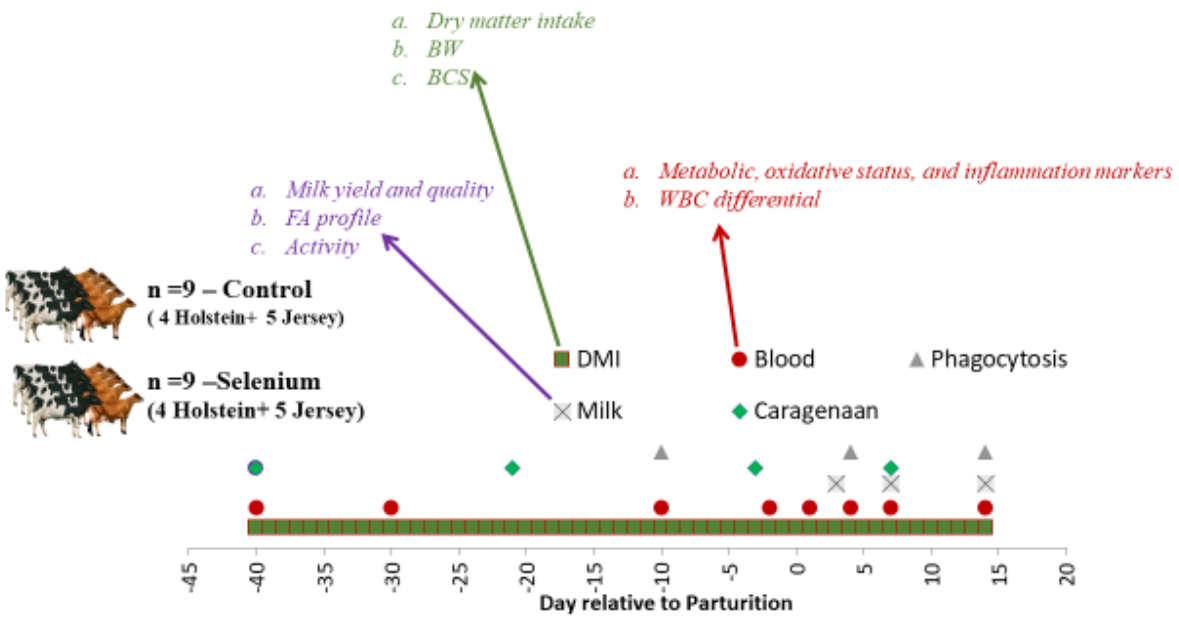

Figure 3.1

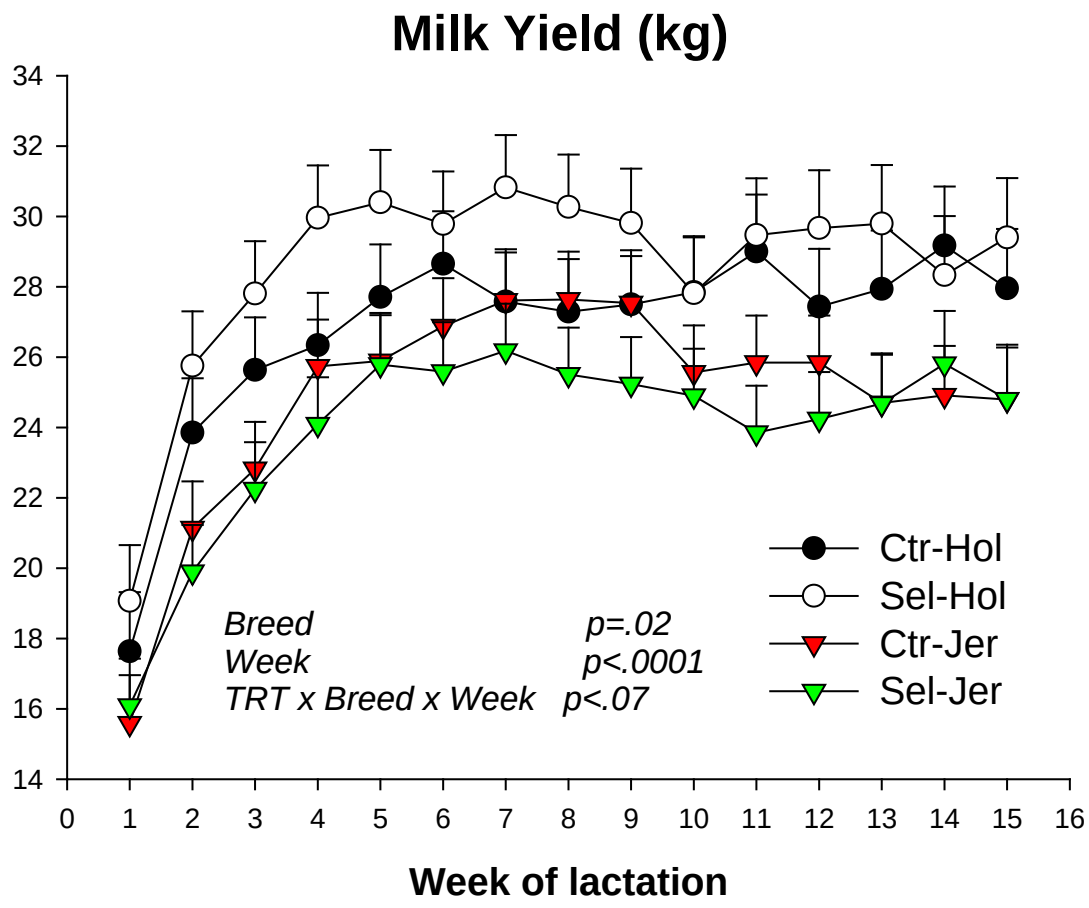
Milk Yield (kg)
34
32
30
28
26
24
22
20
18
16
14
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Week of lactation
Ctr-Hol
Sel-Hol
Ctr-Jer
Sel-Jer
Breed
Week
TRT x Breed x Week
p=.02
p<.0001
p<.07

Figure 3.2

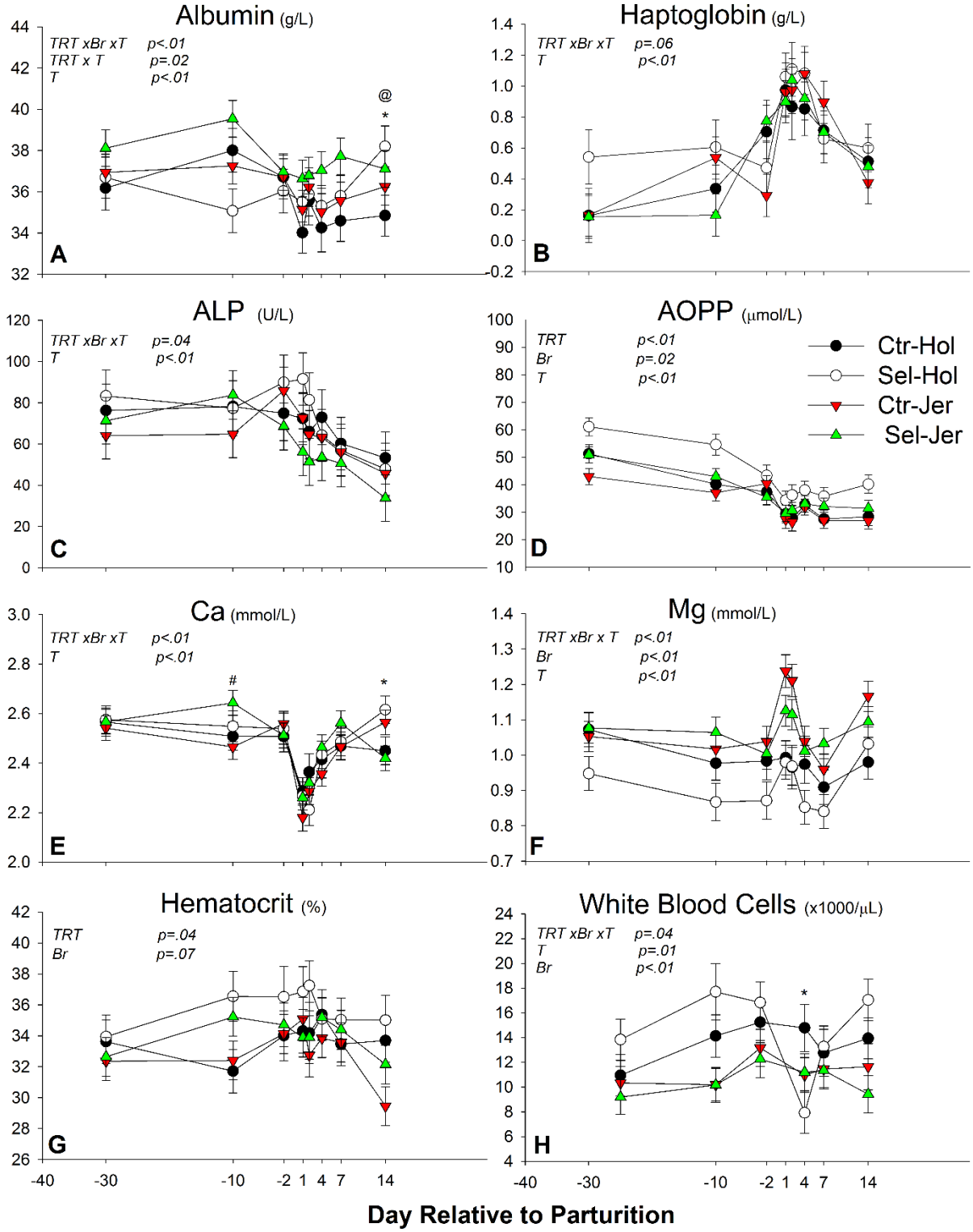
Albumin (g/L)
TRT xBr xT p<.01
TRT x T p=.02
T p<.01
A
Haptoglobin (g/L)
TRT xBr xT p=.06
T p<.01
B
ALP (U/L)
TRT xBr xT p=.04
T p<.01
C
AOPP (µmol/L)
TRT p<.01
Br p=.02
T p<.01
Ctr-Hol
Sel-Hol
Ctr-Jer
Sel-Jer
D
Ca (mmol/L)
TRT xBr xT p<.01
T p<.01
E
Mg (mmol/L)
TRT xBr x T p<.01
Br p<.01
T p<.01
F
Hematocrit (%)
TRT p=.04
Br p=.07
G
White Blood Cells (x1000/µL)
TRT xBr xT p=.04
T p=.01
Br p<.01
H
-40 -30 -10 -2 1 4 7 14
Day Relative to Parturition

Figure 3.3

TABLE

Table 3.1 Performance of Jersey (n=5/group) and Holstein (n=4/group) primiparous cows supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior expected parturition to 14 days after calving

Parameter	Control		Selenium			P-value[1]						
	Holstein	*Jersey*	*Holstein*	*Jersey*	*SEM*	*TRT*	*Br*	*T*	*TRTxBr*	*TRTxT*	*BrxT*	*TRTxBrxT*
BW kg	548.6	469.1	554.7	463.1	*19.8*	0.99	<0.01	<0.01	0.75	0.12	0.57	0.20
BCS	2.97	3.18	2.94	3.21	*0.09*	0.96	0.02	0.03	0.72	0.24	0.87	0.72
DMI kg/d	12.2	11.3	12.9	11.2	*0.31*	0.28	<0.01	<0.01	0.18	0.07	<0.01	0.56
DMI/BW %	2.24	2.37	2.32	2.39	*0.07*	0.45	0.16	<0.01	0.67	0.07	<0.01	0.64
Milk Yield Kg/d[2]	21.3	19.1	22.8	17.7	*1.75*	0.96	0.03	0.01	0.34	0.15	0.50	0.70
Milk Yield Kg/d[3]	27.0	25.0	28.5	24.0	*1.19*	0.79	0.02	0.01	0.34	0.82	0.46	0.07
ECM kg/d[4]	17.4	19.5	21.2	18.5	*2.19*	0.49	0.88	<0.01	0.24	0.49	0.64	0.99
FCM kg/d[5]	16.0	19.0	20.2	18.6	*2.63*	0.42	0.77	<0.01	0.33	0.47	0.45	0.90
FPCM kg/d[6]	17.9	19.8	21.5	18.9	*2.28*	0.49	0.84	<0.01	0.28	0.49	0.65	0.99
Dairy Efficiency[7]	1.27	1.52	1.45	1.48	*0.09*	0.44	0.11	<0.01	0.21	0.57	0.69	0.19
Milk composition[8]												
Fat %	2.79	3.83	3.13	3.86	*0.51*	0.70	0.08	0.02	0.75	0.64	0.01	0.66
Protein %	3.94	3.73	3.73	3.82	*0.22*	0.78	0.78	<0.01	0.49	0.34	0.69	0.08
Lactose %	4.37	4.52	4.65	4.47	*0.12*	0.32	0.90	0.19	0.17	0.90	0.62	0.48
SNF %	9.33	9.57	9.43	9.33	*0.22*	0.79	0.77	<0.01	0.49	0.23	0.54	0.03
SCC (log_2)	7.03	7.57	5.07	7.71	*0.71*	0.20	0.04	<0.01	0.14	0.50	0.83	0.29
MUNN mg/dl	13.1	16.5	13.6	17.1	*1.45*	0.70	0.02	0.13	0.95	0.68	0.29	0.19
Fat g/d	502.7	755.3	728.1	750.2	*145.2*	0.40	0.30	<0.01	0.38	0.33	0.21	0.72
Protein g/d	832.4	777.2	854.5	663.7	*60.6*	0.36	0.02	0.17	0.19	0.44	0.57	0.37
Lactose g/d	976.9	876.6	1071.1	788.2	*81.2*	0.96	0.01	<0.01	0.20	0.17	0.39	0.62
SNF g/d	2021.0	1846.7	2166.9	1637.0	*151.1*	0.80	0.01	<0.01	0.18	0.23	0.51	0.45
MUNN g/d	2.78	2.89	3.32	3.14	*0.34*	0.17	0.90	0.01	0.60	0.45	0.72	0.58
Conductivity mS	8.95	8.93	8.97	9.05	0.18	0.71	0.89	<0.01	0.80	0.97	0.93	0.73
Activity steps/h	119.8	152.4	127.2	147.8	*8.0*	0.89	0.02	0.10	0.54	0.93	0.62	0.68

[1]TRT = Treatment; Br = Breed; T = Time

[2]Up to14 day post-partum with daily milk yield

[3]Up to 120 days post-partum with daily milk yield averaged weekly

[4]Energy Corrected Milk = kg milk*(38.3×kg fat+24.2×kg protein+15.71×kg lactose+20.7)/3140 (Sjaunja et al., 1991).

[5]Fat-corrected Milk = 0.4× kg milk + 15 × kg fat (Gaafar et al., 2010).

[6] Fat and Protein corrected Milk = [0.337 + 0.116 × fat (%) + 0.06 × protein (%)] × kg milk (van Hoeij et al., 2017).

[7] FCM/DMI

[8] SNF = Solid Non Fat; SCC = Somatic Cell Count in 10^3 cell/mL; MUNN = Milk Urea Nitrogen

Table 3.2 Plasma parameters in Jersey and Holstein primiparous cows supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior expected parturition to 14 days after calving

Parameter[1]	Control		Selenium		P-value[2]					
	Holstein	*Jersey*	*Holstein*	*Jersey*	*SEM*	*TRT*	*Br*	*TRTxT*	*TRTxBr*	*TRTxBrxT*
Metabolic										
NEFA mmol/l	0.48	0.61	0.58	0.53	0.12	0.92	0.71	0.93	0.44	0.65
NEFA/Albumin[3]	0.91	1.18	1.07	0.80	0.18	0.51	0.67	0.96	0.14	0.77
BHBA mmol/l	0.57	0.65	0.60	0.70	0.08	0.65	0.28	0.31	0.88	0.45
Glucose mmol/l	4.36	4.24	4.36	4.22	0.11	0.90	0.23	0.22	0.90	0.79
Cholesterol mmol/l	2.54	1.96	2.65	1.99	0.24	0.77	0.02	0.97	0.85	0.61
Urea mmol/l	5.24	5.17	5.75	6.11	0.37	0.06	0.67	0.56	0.54	0.98
Inflammatory										
Ceruloplasmin µmol/l	2.52	2.90	2.65	2.96	0.30	0.73	0.23	0.64	0.90	0.61
Haptoglobin g/l	0.64	0.66	0.77	0.67	0.09	0.42	0.65	0.76	0.50	0.06
Myeloperoxidase U/L	402.9	394.0	468.1	426.2	29.1	0.10	0.37	0.79	0.56	0.46
Albumin g/l	35.5	36.1	36.1	37.5	0.8	0.26	0.22	0.02	0.62	<0.01
Paraoxonase U/ml	73.5	74.1	79.2	74.8	5.4	0.54	0.70	0.61	0.63	0.94
Liver function										
AST/GOT U/L	99.4	98.4	99.2	92.8	4.4	0.50	0.39	0.99	0.53	0.93
GGT U/L	22.3	22.0	23.5	21.4	1.7	0.83	0.46	0.11	0.58	0.97
ALP U/L	69.3	64.6	74.1	58.6	11.2	0.95	0.36	0.36	0.62	0.05
Bilirubin µmol/l	4.16	3.52	4.56	3.41	0.64	0.81	0.16	0.82	0.67	0.94
Oxidative status										
ROMt mg H_2O_2/100 ml	14.8	14.6	15.3	15.5	1.1	0.51	0.98	0.72	0.81	0.77
Thiol Groups µmol/l	335.3	352.7	357.3	359.9	14.4	0.30	0.47	0.19	0.60	0.65
FRAP µmol/l	149.4	139.4	134.8	132.5	8.7	0.22	0.47	0.38	0.65	0.53
AOPP µmol/l	34.3	32.5	43.0	35.8	1.9	<0.01	0.02	0.34	0.15	0.96
Minerals and others										
Hematocrit V/V%	33.8	32.9	35.8	34.0	0.7	0.04	0.07	0.62	0.51	0.85
Ca mmol/l	2.45	2.43	2.46	2.47	0.04	0.44	0.90	0.39	0.70	<0.01
Free Ca mmol/l[4]	1.21	1.19	1.21	1.20	0.01	0.79	0.36	0.36	0.71	0.03
Mg mmol/l	0.98	1.09	0.92	1.07	0.03	0.20	<0.01	0.72	0.58	<0.01
Creatinine µmol/l	100.2	86.1	94.9	88.2	2.5	0.52	<0.01	0.32	0.13	0.21
Urea/Creatinine	52.3	59.7	60.2	69.3	3.63	0.02	0.03	0.46	0.80	0.98
Total protein g/l	74.9	71.0	76.7	74.5	1.5	0.08	0.04	0.78	0.55	0.61
Globulin g/l	39.0	34.8	35.3	37.0	1.5	0.61	0.41	0.33	0.06	0.60
Albumin/Globulin	0.94	1.06	1.04	1.02	0.05	0.56	0.36	0.36	0.18	0.50
AOPP/Albumin	0.97	0.91	1.15	0.94	0.05	0.05	0.01	0.67	0.17	0.94

[1]NEFA= Non-esterified fatty acids; BHBA: β- hydroxybutyric acid; AST/GOT: Aspartate amino-transferase; GGT: γ-glutamyl transferase; ALP: Alkaline phosphatase; ROMt: Total reactive oxygen metabolites; FRAP: Ferric reducing antioxidant power; AOPP: Advanced oxidation protein products

[2]TRT = Treatment; Br = Breed; T = Time; [3]NEFA/Albumin ratio = mM NEFA/ mM Albumin → (Albumin g/L/(Albumin MW [66,463] × 1000))

[4]Free calcium was calculated accoding to Mir et al., (2016) using the Orrell adjusted Ca as follow (Free Ca = (Ca mmol/l + 0.0176 * (34- albumin g/L)/2

Table 3.3 Carrageenan skin test (CST), phagocytosis, and WBC differential in Jersey and Holstein primiparous cows supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior expected parturition to14 days after calving

Parameters	Control		Selenium			P-value[1]					
	Holstein	*Jersey*	*Holstein*	*Jersey*	*SEM**	*TRT*	*Br*	*TRTxT*	*TRTxBr*	*BrxT*	*TRTxBrxT*
CST											
Day 2 mm	1.13	1.30	1.29	1.46	*0.30*	0.57	0.54	0.81	0.99	0.92	0.97
Day 9 mm	1.82	1.57	1.63	2.07	*0.35*	0.63	0.77	0.71	0.30	0.56	0.34
AUC[2]	11.7	11.7	11.6	13.6	*2.06*	0.65	0.59	0.83	0.61	0.93	0.83
Phagocytosis (%)											
Leukocytes	25.4	20.1	34.8	21.5	*7.11*	0.36	0.13	0.82	0.50	0.73	0.41
Granulocyte[3]	50.4	40.9	71.2	43.7	*14.2*	0.32	0.13	0.60	0.45	0.92	0.48
Monocytes[4]	78.8	75.6	78.2	68.9	*16.3*	0.80	0.66	0.33	0.83	0.01	0.61
PMN+Monocytes[5]	50.1	39.1	67.5	40.8	*11.4*	0.34	0.07	0.58	0.43	0.51	0.19
Differential (%)											
Granulocytes[3]	31.6	33.7	35.3	33.1	*4.60*	0.69	0.99	0.56	0.59	0.34	0.84
PMN+Monocyte[5]	47.5	51.5	55.2	46.4	*4.91*	0.77	0.58	0.97	0.15	0.54	0.94
Lymphocytes[5]	52.5	48.6	45.1	53.5	*4.94*	0.77	0.60	0.96	0.17	0.51	0.96
Monocytes[4]	1.56	0.76	0.56	1.82	*0.92*	0.97	0.78	0.25	0.22	0.30	0.76
WBC ×1000/µl[6]											
Total[#]	13.6	11.3	14.4	10.6	*1.4*	0.50	<0.01	0.19	0.87	0.06	0.02
Granulocytes[3]	4.18	3.80	3.08	3.34	*0.78*	0.21	0.92	0.99	0.60	0.38	0.21
PMN+Monocyte[5]	5.64	4.85	5.44	4.54	*0.76*	0.67	0.17	0.64	0.92	<0.01	0.54
Lymphocytes[5]	6.92	5.50	4.34	5.58	*1.32*	0.26	0.93	0.84	0.24	0.09	0.55

[1]TRT = Treatment; Br = Breed; T = Time
[2]Area under the curve of skin thickness from day 0 to 9.
[3]Cells positive to CH138A antibody
[4]Cells positive to CAM36 antibody
[5]Cells gated manually
[6]Absolute number of each category of leukocytes using differential (%)
[#]The WBC total count was measured during the whole trial while the flow cytometer analysis was performed only at -10, 4, and 14 DIM. The total WBC was also corrected by the baseline.
*Highest SEM

Table 3.4 Fatty acid profile (mg/100 mg) of Jersey and Holstein primiparous cows supplemented with 1% BW of Se-biofortified alfalfa hay (Selenium) or no biofortified alfalfa hay (Control) from 40 days prior expected parturition to 14 days after calving

Fatty acid	Control		Selenium				P-value[1]		
mg/100 mg	*Holstein*	*Jersey*	*Holstein*	*Jersey*	*SEM*	*TRT*	*Br*	*TRTxBr*	*TRTxBrxT*
C4:0	2.98	2.73	2.56	2.55	0.21	0.61	0.53	0.78	0.44
C6:0	2.14	1.83	2.15	2.22	0.22	0.5	0.58	0.9	0.86
C8:0	1.11	0.95	1.2	1.26	0.15	0.28	0.76	0.75	0.63
C10:0	1.94	1.61	2.21	2.15	0.32	0.2	0.55	0.73	0.66
C11:0	0.11	0.11	0.16	0.13	0.02	0.2	0.52	0.56	0.89
C12:0	2.10	1.81	2.33	2.26	0.33	0.18	0.59	0.74	0.72
C12:1	0.07	0.03	0.06	0.05	0.01	0.29	0.04	0.4	0.29
C13:0	0.13	0.1	0.11	0.11	0.01	0.41	0.12	0.71	0.37
C14:0	7.66	6.68	8.29	7.66	0.81	0.36	0.34	0.71	0.88
C14:1cis9	0.45	0.32	0.45	0.34	0.04	0.84	0.01	0.67	1.00
C14:1c11	0.30	0.42	0.39	0.42	0.03	0.24	0.04	0.76	0.51
C14:0hydroxy	1.04	0.82	1.02	0.87	0.06	0.74	0.01	0.49	0.99
C15:1	0.34	0.28	0.29	0.3	0.02	0.26	0.18	0.71	0.62
C15:0iso	0.23	0.19	0.21	0.19	0.02	0.42	0.16	0.48	0.91
C16:0	23.16	23.21	23.83	23.32	0.81	0.45	0.78	0.53	0.47
C16:0iso	0.03	0.02	0.03	0.02	0.01	0.88	0.64	0.53	0.77
C16:1trans	0.46	0.14	0.45	0.14	0.06	0.20	<.0001	0.42	0.14
C16:1cis7	5.47	6.86	5.65	6.33	0.48	0.48	0.05	0.56	0.59
C16:1cis9	1.65	2.09	1.71	1.94	0.16	0.5	0.05	0.55	0.64
C16:1cis10	0.06	0.03	0.09	0.05	0.02	0.03	0.04	0.32	0.11
C17:0	0.03	0.1	0.03	0.1	0.02	0.82	<.001	0.17	0.58
C17:1cis10	0.41	0.48	0.41	0.43	0.05	0.67	0.34	0.55	0.85
C18:0	12.55	11.77	11.87	11.67	0.52	0.06	0.36	0.35	0.40
C18:0iso	3.19	2.96	2.9	2.76	0.13	0.16	0.15	0.98	0.28
C18:0anteiso	0.12	0.15	0.08	0.10	0.03	0.39	0.38	0.54	0.68
C18:1tr910	0.35	0.33	0.24	0.33	0.03	0.4	0.34	0.24	0.23
C18:1tr11	1.45	0.53	1.47	1.3	0.14	0.44	<.001	0.09	0.54
C18:1t12	0.17	0.72	0.14	0.02	0.15	0.40	0.18	0.58	0.54
C18:1t14	0.5	0.30	0.45	0.44	0.06	0.57	0.13	0.92	0.25
C18:1cis9	20.78	23.75	20.91	21.95	1.83	0.66	0.29	0.64	0.86
C18:1cis11	0.74	0.97	0.72	0.93	0.07	0.35	0.01	0.46	0.70
C18:1cis12	0.19	0.19	0.19	0.18	0.01	0.43	0.62	0.98	0.41
C18:1c14	0.27	0.29	0.31	0.25	0.02	0.23	0.38	0.51	0.90
C18:1cisomer	0.63	0.82	0.67	0.6	0.09	0.31	0.51	0.57	0.42
C18:1c15	0.04	0.11	0.04	0.03	0.03	0.32	0.31	0.7	0.20
C18:2	1.87	1.84	1.88	1.76	0.08	0.44	0.36	0.44	0.98

C18:2c9t12	0.04	0.13	0.03	0.05	0.03	0.6	0.06	0.61	0.37
C18:2t9c12	0.06	0.07	0.06	0.07	0.02	0.29	0.54	0.39	0.53
C18:3t9c12t15	0.03	0.06	0.03	0.06	0.01	0.59	0.08	0.42	0.37
C18:3n3	0.62	0.55	0.61	0.53	0.03	0.04	0.03	0.38	0.38
C18:3CLA	1.28	1.65	1.50	1.89	0.18	0.32	0.05	0.03	0.61
C18:4n3	0.08	0.09	0.07	0.07	0.02	0.99	0.98	0.21	0.02
C19:0	0.06	0.16	0.06	0.15	0.03	0.59	0.01	0.19	0.43
C19:1	0.13	0.15	0.13	0.13	0.01	0.1	0.64	0.58	0.39
C20:0	0.16	0.16	0.16	0.15	0.02	0.56	0.66	0.38	0.75
C20:1n9	0.07	0.07	0.08	0.06	0.01	0.02	0.73	0.2	0.6
C20:3t	0.04	0.05	0.02	0.06	0.01	0.06	0.11	0.21	0.35
C20:3n3	0.5	0.36	0.41	0.43	0.05	0.36	0.27	0.41	0.39
C26:0	0.16	0.08	0.14	0.11	0.02	0.74	0.01	0.14	0.14
Denovo	36.7	34.68	38.28	37.42	2.24	0.3	0.53	0.71	0.88
Preformed	61.24	64.45	60.54	61.52	2.5	0.21	0.41	0.7	0.72
Bacteria	5.57	5.31	5.16	5.05	0.17	0.11	0.30	0.78	0.68
Δ9 C18[1]	1.68	2.05	1.75	1.91	0.19	0.65	0.17	0.44	0.83
Δ9 C16[1]	0.07	0.09	0.07	0.08	0.01	0.47	0.07	0.44	0.49
Δ9 C14[1]	0.06	0.05	0.06	0.04	0.003	0.23	<.01	0.96	0.55
Δ9[1]	0.57	0.68	0.57	0.62	0.06	0.77	0.26	0.54	0.92
Saturated	54.29	51.29	55.09	53.84	2.39	0.56	0.39	0.51	0.79
Unsaturated	38.63	43.39	39.14	40.8	2.62	0.45	0.24	0.51	0.95
PUFA	4.52	4.80	4.61	4.92	0.3	0.39	0.34	0.17	0.70

[1]Delta 9 desaturase indexes were calculated as follow:
Δ9 C14 = cis9 C14:1/(C14:0 + cis9 C14:1)
Δ9 C16 = cis9 C16:1/(C16:0 + cis9 C16:1)
Δ9 C18 = cis9 C18:1/(C18:0 + cis9 C18:1)
Δ9 C18:1 = cis9 trans12 C18:2/(trans12 C18:1 + cis9 trans12 C18:2)
Δ9 desaturation = sum of C14, C16, C18, trans12 C18:1 cis9/(sum of C14:0, C16:0, C18:0, trans12 C18:1 + sum of C14, C16, C18, trans12 C18:1 cis9)

Figure 3.4 Proc correlation (r above and P-value below) among measured parameters in blood. Red to pink shade denotes the level of significance for the correlations that are either positive (purple shade) or negative (green shade)

CHAPTER 4
CONCLUSIONS AND FUTURE STUDIES

The data presented in this dissertation allow us to conclude that supplementing primiparous dairy cows with a relatively low amount of Se biofortified alfalfa hay is effective in improving the Se status of cows and calves with a minor, although interesting, effect on metabolism, immune, and oxidative status. In particular, the first part of the experiment allows us to conclude that the treatment has a significant role in improving Se concentration in the liver and blood, enhances the amount of Se that is transferred into the calves and increases the activity of GPx in cows but does not increase the level of Se in milk. The second part of the experiment allows us to conclude that the treatment appeared to have no effect on cows' performance and minimal effect on metabolic, immune, oxidative, and inflammatory parameters. In particular, this minimal effect encompassed an improved liver function, and increased antioxidant capacity of albumin, and enhanced erythropoiesis/red blood cell viability.

All of the above observations require further studies. This is even more important considering the disclosed limitations, in particular, the use of primiparous cows, the inclusion of two different breeds, and the low number of animals. It appears sensible to repeat the experiment in multiparous dairy cows that are known to be more metabolically and immunologically challenged. If a new experiment should be conducted, the following improvement of the experimental design should be made: 1) use of only a single breed of multiparous cows (or more cows in each breed to increase the statistical power), 2) gene expression of the mammary tissue should be assessed in order to figure out the reason

behind the low transfer of Se into the milk, maybe considering also different stages of lactation, and 3) the respiratory burst of leukocytes should be inserted among the measured parameters. Furthermore, in the future, it should be interesting to investigate in more detail the significance of improved antioxidant activity of albumin by organic Se supplementation.

APPENDIX I PAPER PUBLICATION

2,4-THIAZOLIDINEDIONE IN WELL-FED LACTATING DAIRY COATS: I. EFFECT ON ADIPOSITY AND MILK FAT SYNTHESIS

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1.1 Abstract

Background: In a prior experiment, treatment of goats with the putative PPARγ agonist 2,4-thiazolidinedione (2,4-TZD) did not affect milk fat or expression of milk-fat related genes. The lack of response was possibly due to deficiency of vitamin A and/or a poor body condition of the animals. In the present experiment, we tested the hypothesis that PPARγ activation affects milk fat synthesis in goats with a good body condition and receiving adequate levels of vitamin A. *Methods*: Lactating goats receiving a diet that met NRC requirements, including vitamin A, were injected with 8 mg/kg BW of 2,4-TZD (*n* = 6) or saline (*n* = 6; CTR) daily for 26 days. Blood metabolic profiling and milk yield and components were measured including fatty acid profile. Expression of genes related to glucose and lipid metabolism was measured in adipose tissue and mammary epithelial cells (MEC). The size of adipocytes was assessed by histological analysis. *Results*: NEFA, BHBA, and fatty acids available in plasma decreased while glucose increased in 2,4-TZD vs. CTR. The size of cells and expression of insulin signaling and glucose metabolism-related genes were larger in 2,4-TZD vs. CTR in adipose tissue. In MEC, expression of *SCD1* and desaturation of stearate was lower in 2,4-TZD vs. CTR. *Conclusions*: Overall data revealed a lack of PPARγ activation by 2,4-TZD and no effect on milk fat synthesis despite a strong anti-lipolysis effect on adipose tissue.

Keywords: 2,4-thiazolidinedione; goat; milk fat; metabolism

1.2 Introduction

Butterfat is important for milk quality, especially for taste and flavor. Butterfat is also one of the most important components in the calculation of milk prices received by

dairy farmers in the US. Milk fat content is highly affected by the composition of the diet, especially dietary fat and fiber.

The main components of dietary fat are long-chain fatty acids (**LCFA**). Those are known to have a nutrigenomic effect through modulation of transcription factors, especially peroxisome proliferator-activated receptors (**PPARs**) (Bionaz et al., 2013). Among the three known PPAR isotypes, the PPARγ in mammary tissue of ruminants has been the most studied and preliminary evidence supported a role of it on the regulation of milk fat synthesis (Bionaz et al., 2015). Several subsequent *in vitro* studies performed on goat and bovine mammary epithelial cells confirmed the original observation (Chen et al., 2015, Liu et al., 2016, Shi et al., 2016).

Several *in vivo* studies were carried out using PPARγ agonists on dairy ruminants. The treatment of dairy cows with 2,4-thiazolidinedione (**2,4-TZD**), a putative PPARγ agonist, during the prepartum period, did not affect milk fat percentage and did not affect or numerically decreased milk fat yield in early post-partum cows (Smith et al., 2009). Peripartum cows treated with the synthetic PPARγ agonist pioglitazone had a significant decrease in milk fat percentage and yield compared to cows not treated with pioglitazone (Yousefi et al., 2016). In both studies, the large decrease in plasma non-esterified fatty acids (**NEFA**) might partly explain the decrease in milk fat synthesis detected. In both studies, the decrease of plasma NEFA compared to the control group was between 23 to 28%, while the decrease in milk fat yield was between 8 and 11%. Plasma NEFA is an important source of preformed LCFA for milk fat synthesis, and a positive relation exists between plasma NEFA and milk fat concentration (Palmquist, 2006). Results from the

above *in vivo* studies did not provide support for a positive effect of PPARγ activation on milk fat yield, but there was the confounding effect of the large decrease in NEFA, especially during the early post-partum.

In a recent study by our laboratory, we treated dairy goats in mid-late lactation with TZD. Despite not having observed a decrease in NEFA, 2,4-TZD did not increase milk fat yield but tended to prevent milk fat decreased after induction of mammary infection (Rosa et al., 2017). Furthermore, we did not observe any large effect of 2,4-TZD on the expression of classical PPARγ target genes in mammary epithelial cells (**MEC**) or adipose tissue, indicating that 2,4-TZD is, at best, a weak PPARγ agonist. These results confirmed the lack of effects of 2,4-TZD on expression of PPARγ target genes in adipose tissue of dairy cows during the close-up period (Schoenberg et al., 2011); however, another study detected a change in expression of several genes in adipose tissue after treatment with 2,4-TZD in non-pregnant dry cows (Hosseini et al., 2015). Similarly, in a recent study carried out in sheep treatment with 2,4-TZD to prevent milk fat depression by conjugated linoleic acid (Sandri et al., 2017). The same study detected a positive effect of 2,4-TZD on the expression of *PPARG* in mammary tissue and on lipogenic genes in the adipose tissue.

In the prior goat study performed in our laboratory (Rosa et al., 2017), the animals were not in optimal body condition and the hay-based diet was not supplemented adequately with vitamins, especially vitamin A. Vitamin A plays an important role in PPARγ activation, due to the agonistic effect of its metabolite 9-*cis*-retinoic acid on retinoic-X-receptor, the essential heterodimer of PPARs. The essential role of 9-*cis*-

retinoic acid on the activation of PPARs by 2,4-TZD was supported by several *in vitro* studies carried out in bovine and caprine mammary cells (Bionaz et al., 2015).

For the above reasons, it remained to be determined if PPARγ plays a role in controlling milk fat synthesis *in vivo*. Therefore, it is necessary to repeat the prior experiment (Rosa et al., 2017) by using dairy goats with good initial body condition and a balanced ration including the levels of vitamin A. Therefore, our hypothesis is that continuous activation of PPARγ by 2,4-TZD increases milk fat synthesis in dairy goats in good body condition and supplemented with an adequate amounts of vitamin A. The objective of the present study, which is part of a larger study, is to assess if the activation of PPARγ by 2,4-TZD increases milk fat synthesis.

1.3 Materials and Methods

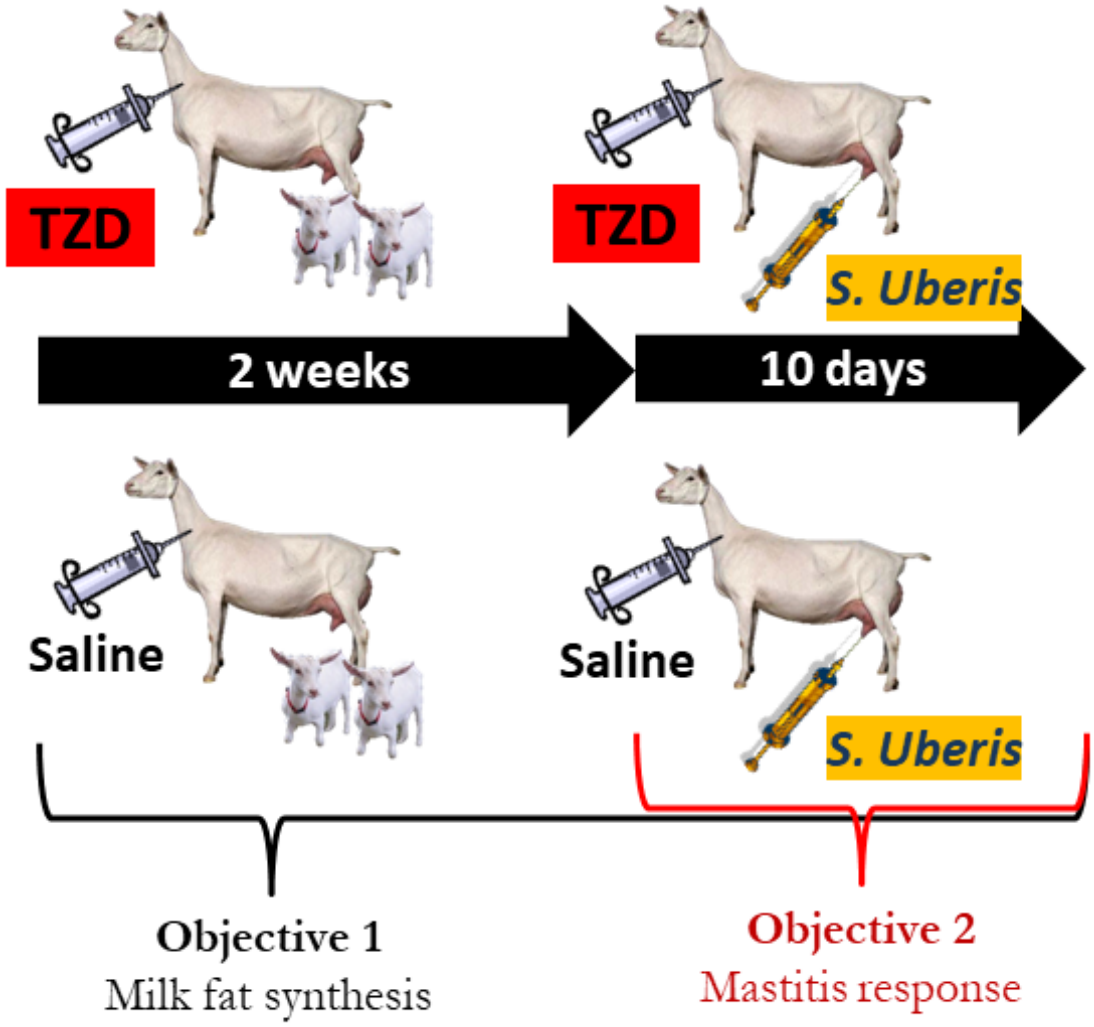

Figure 1.1 Experimental design. Twelve Saanen dairy goats were randomly assigned to receive a daily injection of 8 mg/kg BW of 2,4-thiazolidinedione (TZD) in 10 mL saline or only saline (n=6/treatment) for 26 days. Kids (2 per goat) were kept nursing the goats for the first 10 days of the experiment. After 2 weeks of treatment, the goats received in the right half of the mammary gland an intramammary infusion of 7×108 CFU of Strep. uberis. All the TZD and three CTR goats were euthanized at the end of the experiment. The whole experiment was used to generate data for Objective 1 (present manuscript) and for Objective 2 in companion manuscript (Rosa et al., 2019).

1.3.1 Experimental design and animal management

Figure 1.1 depicts the whole study encompassing two objectives, one pertinent to the present manuscript and the other related to the companion manuscript (Rosa et al., 2019). For the study, we used 12 lactating Saanen goats (mean±SD) 52.6±16.2 days in milk, 69.2±7.1 kg of body weight (**BW**), 2.6±0.2 body condition score (**BCS**), 1.7±0.6 kids and negative to milk bacterial analysis. For the experiment, goats were housed in individual pens and randomly assigned to treatment groups after blocking for body weight (**BW**), milk yield, and milk components. Goats were fed twice a day as described in detail in **Appendix A**. Dry matter, and chemical composition of individual forages were determined by standard wet chemistry techniques at a commercial laboratory (Dairy One Forage Testing Laboratory, USA) (**Table S1**). The ration amount fed was calculated individually for each goat using BW and milk yield (National Research Council, 2007) (**Table S2**). Animals were drenched once daily to supply the level of vitamins required as described in **Appendix A.** See **Table S3** for each component of the drench and the total amount of each vitamin provided.

1.3.2 Treatments

After the adaptation period, the goats started to receive at 1000 h daily injections *via* jugular vein of 8 mg/kg of BW of 2,4-thiazolidinedione (**2,4-TZD**; SC-216281, Santa Cruz Biotechnology, USA) in 10 mL sterile physiological saline (002479, Henry Schein, Dublin, OH) (n=6; **TZD**) throughout the whole study (26 days). A control group (n=6; **CTR**) received 10 mL of sterile physiological saline without 2,4-TZD. Considering a BW

of 70 kg and a molecular weight of 117.13 g/mole, it was estimated that the maximum concentration in blood of 2,4-TZD after injection was approx. 190 mM, a dose known to activate PPAR *in vitro* in the presence of 10 μM of 9-*cis*-retinoic acid (Bionaz et al., 2015). An intramammary infusion with *Streptococcus uberis* in the right half of the mammary gland was performed in all goats on day 15 of 2,4-TZD treatment (see companion papers for details (Rosa et al., 2019)). The 2,4-TZD is not a compound approved by FDA for use with animals or human, but it is the backbone molecules used for the production of rosiglitazone and other FDA-approved compounds used to treat type II diabetes in human (Quinn et al., 2008, Jain et al., 2013).

1.3.3 Measurements, sample collection, and blood metabolites

Details are available in **Appendix A.** Briefly, during the first 11 days of the experiment, while goats were nursed by the kids, milk yield was measured on 0, 2, 5, and 10 days of treatment by removing the kids at 2000 h and immediately milked. The goats without the kids were milk again 12h later, where we recorded milk yield and collected milk samples. After the kids were weaned (d 11), goats were milked twice a day, and milk yield was measured at each milking (0600 and 1800 h) until the end of the trial. For the present manuscript, only the milk from the left quarter [i.e., quarter that did not receive the bacteria to induce infection, see (Rosa et al., 2019)] during the morning milking was used to determine the amount of milk and milk composition. Milk samples were collected for analysis of somatic cells count (**SCC**), lactose, fat, protein, and solids non-fat (**SNF**) just before 2,4-TZD injection (time 0) and then at 2, 5, 15, 16, 17, 20, 21, and 22 day of 2,4-

TZD treatment. At 0, 2, 5, and 15 days of 2,4-TZD treatment additional samples were collected in 15 mL sterile tubes for fatty acid analysis. Details on dry matter intake (**DMI**), energy-corrected milk (**ECM**), dairy efficiency (**DE**), body condition score (**BCS**), and milk fatty acid analysis are available in **Appendix A**.

Blood samples were collected prior to treatment (time 0) and then on day 7, 9, 15, 16, 17, 18, 22, and 26 for analysis of glucose, cholesterol, urea, creatinine, NEFA, triacylglycerol (**TAG**), and β-hydroxybutyric acid (**BHBA**) (see intra- and inter-assay variation in (Calamari et al., 2016, Rosa et al., 2019)). The analyses were performed following the procedures described previously (Bionaz et al., 2007, Calamari et al., 2016) using a clinical auto-analyzer (ILAB 650, Instrumentation). To check for replicability, a duplicate was performed for up to 10% of the samples selected randomly. Samples that are evident outliers or have dubious data were re-analyzed. The calculation of fatty acids available in blood was performed as mM of NEFA + 3×mM of TAG. The percent variation of available fatty acids in blood was calculated as percentage variation compared to time 0 in each goat.

1.3.4 Adipose biopsy and histological analysis, and mammary epithelial cells isolation

Subcutaneous adipose tissue was collected by biopsy as previously described (Rosa et al., 2017) from alternate sides of the tail-head at around 1000 h and samples were flash-frozen in liquid nitrogen. The biopsy was performed the day prior to starting 2,4-TZD and after 13 days of treatment. Adipose tissue was also collected after euthanasia (d 26 days of 2,4-TZD injection) for all 6 goats receiving 2,4-TZD and 3 random CTR goats. Euthanasia

was performed using pentobarbital. MEC was isolated from 250 mL of milk using magnetic sorting on day four of treatment. Samples Details on Materials and Methods used for histological analysis of the adipose tissue and MEC isolation are available in **Appendix A.** The KingFisher protocol file for MEC isolation is available in **Appendix B** and the pipeline for CellProfiler is available in **Appendix C.**

1.3.5 Reverse Transcriptase Quantitative Polymerase Chain Reaction (**RT-qPCR**)

RNA was extracted from adipose tissue, and MEC and RT-qPCR was performed as described in detail in **Appendix A**. The purity of RNA (OD260/280) was 1.84±0.13 for MEC and 1.90±0.12 for adipose tissue (mean±SD). The RNA integrity number (RIN) was assessed by electrophoretic analysis using a 2100 Bioanalyzer Instruments (Agilent, USA) at the Center for Genome Research and Biocomputing, Oregon State University. The RIN values were (mean±SD) 5.5±0.25 for adipose tissue samples and 5.6±1.5 for MEC samples (5.0±1.3 for negative MEC and 6.3±1.4 for positive MEC; P=.10).

The target genes selected to be evaluated in both MEC and adipose tissue were PPARγ (*PPARG*), lipoprotein lipase (*LPL*), stearoyl-CoA desaturase 1*(SCD1)*, sterol regulatory element-binding factor 1 (*SREBF1*), acetyl-CoA carboxylase alpha (*ACACA*), very low-density lipoprotein receptor *(VLDLR)*, fatty acid translocase *(CD36)*, and long-chain fatty acid transport protein 6 *(SLC27A6)*. In adipose tissue, we also measured transcription of insulin receptor substrate 1 *(IRS1)*, insulin receptor *(INSR)*, fatty acid synthase (*FASN*), pyruvate dehydrogenase kinase 4 (*PDK4*), phosphoenolpyruvate carboxykinase 1 (*PCK1*), glycerol-3-phosphate dehydrogenase 1 (*GPD1*), and solute

carrier family 2 (facilitated glucose transporter) member 4 *(SLC2A4)*. For MEC we also measured transcription of interleukin 8 (*IL8*), fatty acid-binding protein 3 (*FABP3*), cytokeratin 8 *(KRT8)*, nuclear factor (erythroid-derived 2)-like 2 *(NFE2L2)*, nuclear respiratory factor 1 *(NRF1)*, kappa-casein *(CSN3)*, and lactalbumin alpha *(LALBA)*. If not already designed (Rosa et al., 2017), primer-pairs were designed as previously described (Rosa et al., 2017). Details of amplicons validation and selection of internal control genes are available in **Appendix A.** Details of primer pairs not previously published are available in **Table S4**.

1.3.6 Statistical analysis

For each goat, an arithmetical correction was performed to obtain the same average between groups at baseline, as described previously (Rosa et al., 2017). Prior to statistical analysis, data were checked for outliers using PROC REG of SAS 9.4 (SAS Institute, Inc., Cary, NC, USA). Data with a studentized t >3.0 were removed. Data were analyzed with the PROC GLIMMIX. Fixed effects in the model were treatment and time and treatment x time interaction as main effects (or treatment and cell and their interaction for gene expression of MEC) and goat as random effect. The best covariance structure, either the spatial power, autoregressive (1), or autoregressive (1) with heterogeneous variance, for each parameter was selected using the lowest Akaike's information criterion. Statistical significance and tendencies were declared at $P \leq .05$ and $P \leq .10$, respectively. For FA analysis, a Proc GLM of SAS was used. Correlations between metabolic parameters and

milk related parameters were performed using Proc Corr of SAS. Syntaxes for SAS analysis are available in **Appendix A**.

1.4 Results

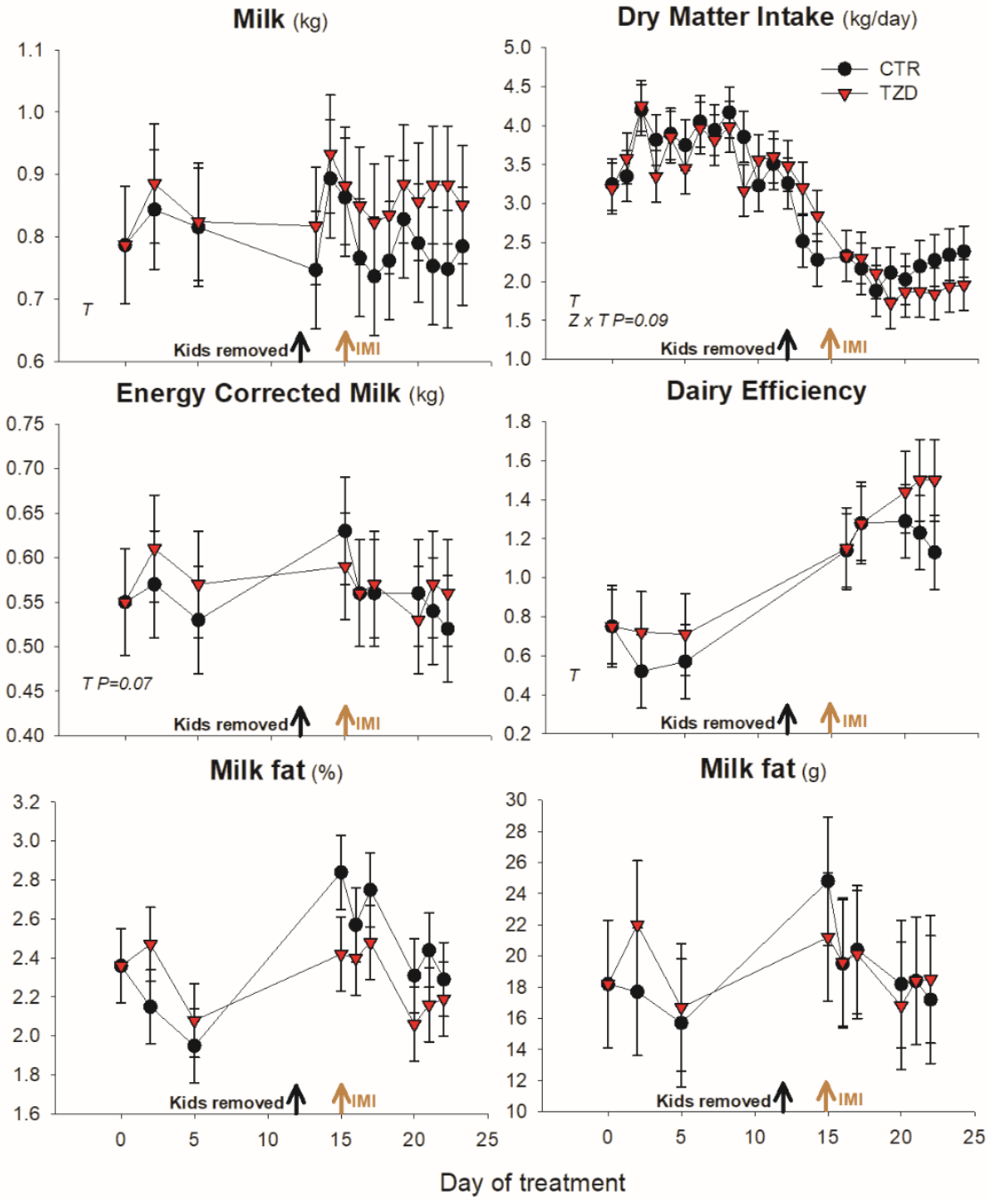

Figure 1.2 Milk yield (kg produced by the left mammary gland in the AM milking), energy corrected milk, dairy efficiency, daily dry matter intake, milk fat and milk yield (produced by the left mammary gland in the AM milking) in goats receiving daily intra-jugular injection of 2,4-thiazolidinedione (TZD) or saline (CTR). Arrows indicate time of kid removal and intramammary infusion of *Strep. Uberis* in the right half of the mammary

gland (IMI). Letters in the graph denote significant ($P<.05$) effects of time (*T*), treatment (*Z*), and interactions (*ZxT*). The reported P-value is for tendencies.

1.4.1 Animal Performance

Dry matter intake had a tendency (P=.09) to be affected by treatment × time with a numerical greater feed intake in TZD just prior to IMI and a numerically lower feed intake in TZD vs. CTR after IMI (**Figure 1.2**). Treatment did not affect milk yield, DMI, DE and ECM (**Figure 1.2**). Similarly, TZD compared to CTR had no significant difference in body weight, body condition score (**Figure S1**), milk fat (**Figure 1.2**), or any of the other milk components (**Table 1.1**).

1.4.2 Blood metabolic parameters

Table 1.1 Milk components in lactating goats injected daily with 2,4-thiazolidinedione (TZD) or saline (CTR).

Parameter		**Day of treatment**									**SEM**	**P-value[1]**		
		0	2	5	15	16	17	20	21	22		Z	Time	Z×T
Protein %	CTR	2.32	2.29	2.22	2.32	2.35	2.28	2.41	2.41	2.39	0.09	0.30	0.10	0.78
	TZD	2.32	2.26	2.19	2.18	2.18	2.17	2.21	2.21	2.19	0.09			
Protein g[2]	CTR	18.3	19.4	18.2	20.3	18.2	17.0	19.2	18.4	18.1	2.5	0.98	<0.05	0.76
	TZD	18.3	20.4	17.9	18.9	18.2	17.5	18.4	19.2	19.0	2.5			
Lactose %	CTR	4.45	4.47	4.42	4.38	4.40	4.35	4.34	4.35	4.31	0.07	0.45	<0.05	0.23
	TZD	4.45	4.68	4.51	4.33	4.37	4.41	4.42	4.39	4.37	0.07			
Lactose g[2]	CTR	34.9	37.2	36.0	37.8	33.7	32.1	34.2	32.7	32.2	4.3	0.61	<0.05	0.61
	TZD	34.9	40.3	36.6	37.3	36.4	35.5	36.9	38.1	37.9	4.3			
SNF[3] %	CTR	7.66	7.61	7.55	7.53	7.51	7.45	7.48	7.51	7.46	0.31	0.98	0.44	0.63
	TZD	7.66	7.87	7.61	7.42	7.44	7.46	7.46	7.43	7.40	0.31			
SNF g[2]	CTR	60.2	64.6	61.5	65.4	57.8	55.1	59.2	56.8	56.0	7.6	0.69	<0.05	0.67
	TZD	60.2	69.3	62.0	64.1	62.1	60.2	62.1	64.5	64.2	7.6			

[1]Z= 2,4-thiazolidinedione effect, T=Time, Z×T=2,4-thiazolidinedione × time effect.
[2]grams produced by the left mammary gland in the AM milking
[3]Solid non-fat

Table 1.2 Plasma concentration of metabolic parameters in dairy goats injected daily with 2,4-thiazolidinedione (TZD) or saline (CTR).

Parameter		**Day of treatment**									**SEM**	**P-value[1]**		
		0	7	9	15	16	17	18	21	26		Z	Time	Z×T
Cholesterol, mM	CTR	2.55	2.30	2.27	2.18	2.28	2.31	2.33	2.40	2.32	0.24	0.38	0.18	0.70
	TZD	2.55	2.73	2.56	2.40	2.51	2.61	2.61	2.62	2.61	0.24			
ΔFA, %[2]	CTR	0.0	5.1	-6.8	5.0	3.8	0.8	9.1	7.7	-6.6	8.5	0.06	0.88	0.90
	TZD	0.0	-13.2	-14.5	-9.4	-4.9	-8.1	-9.7	-17.2	-12.0	8.5			
TAG, mM	CTR	0.14	0.15	0.16	0.19	0.17	0.18	0.19	0.19	0.13	0.02	0.89	0.30	0.82
	TZD	0.14	0.14	0.15	0.19	0.19	0.19	0.18	0.16	0.15	0.02			
Creatinine, µM	CTR	71.9	77.3	77.1	71.9	72.6	70.2	70.4	72.2	75.8	2.7	0.98	<0.05	0.94
	TZD	71.9	76.6	77.6	70.7	72.2	71.7	70.6	72.4	76.1	2.7			
Urea, mM	CTR	6.73	6.32	4.59	5.97	6.81	5.61	6.54	6.45	8.05	0.45	0.25	<0.05	0.19
	TZD	6.73	6.40	5.16	5.29	6.08	5.08	5.61	5.71	6.22	0.45			

[1]Z= 2,4-thiazolidinedione effect, T=Time, Z×T=2,4-thiazolidinedione × time effect.
[2]Percentage variation of total available fatty acids (as mM of NEFA + [3 × mM of TAG]) compared to time 0

2,4-TZD treatment significantly decreased NEFA, BHBA, and fatty acids available in plasma while increased glucose in the blood (**Figure 1.3**; **Table 1.2**). Other parameters measured in plasma were not affected by 2,4-TZD treatment (**Table 1.2**).

Results of correlation analysis between metabolic parameters, DMI, and milk-related parameters are available in **Figure S2**. Milk production was positively associated with DMI, glucose, and cholesterol level in blood but was negatively associated with level of urea in blood. Milk fat percentage was negatively associated with glucose and cholesterol in blood but was not associated with level of NEFA or BHBA.

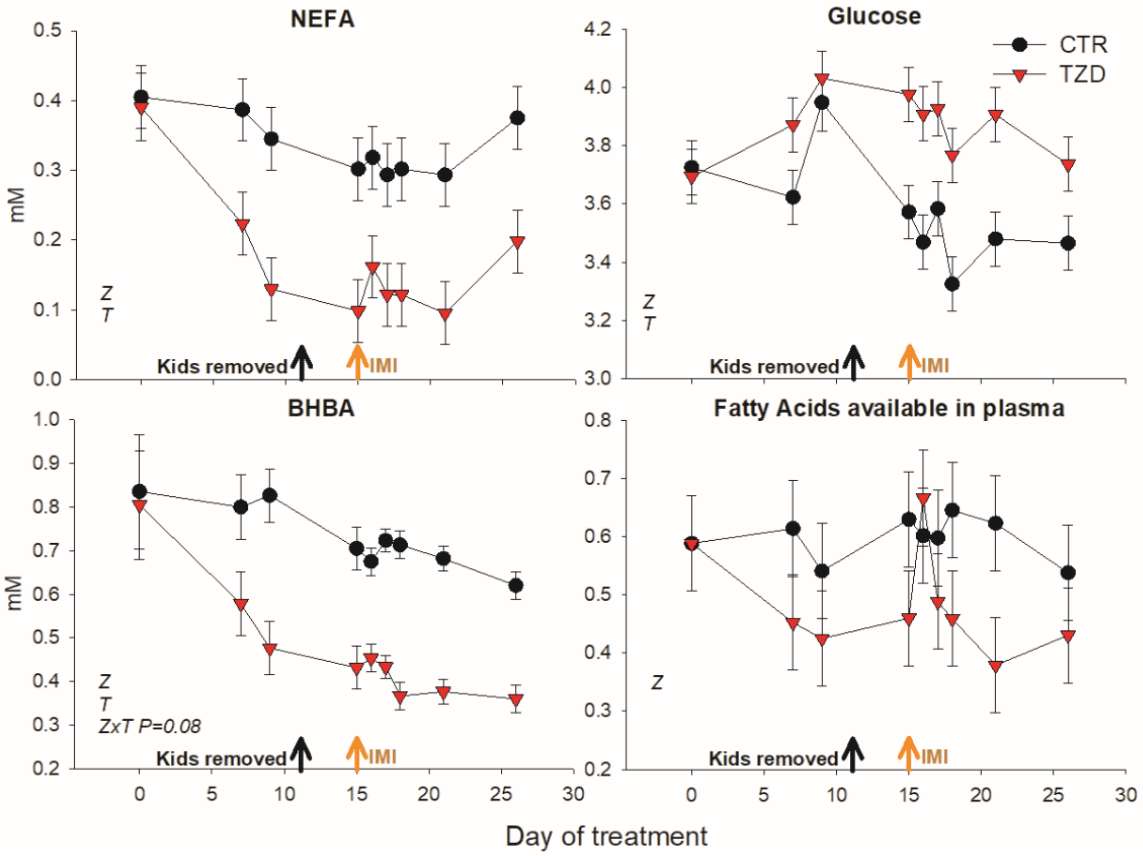

Figure 1.3 Non-esterified fatty acids (NEFA), glucose, β-hydroxybutyrate (BHBA), and estimated fatty acids present in plasma (mM NEFA+3×mM TAG) in goats receiving a daily intra-jugular injection of 2,4-thiazolidinedione (TZD) or saline (CTR). Arrows indicate removal of kids and time of intramammary infusion of *Strep. uberis* in the right half of the mammary gland (IMI). Letters in the graph (a, b) denote significant effects of time (T), treatment (Z), and interactions (ZxT). The reported P-value is for tendencies.

1.4.3 Adipocytes size distribution

The adipocyte area was affected by treatment × time (**Figure 1.4**). 2,4-TZD treatment had a significant increase in median area of adipocytes from 13 to 26 days of treatment while no changes were detected for the CTR goats. The effect was due to an increased frequency of large adipocytes in TZD vs. CTR goats, whereas the frequency of small-to-medium size adipocytes decreased (**Figure 1.4**).

1.4.4 Gene expression

1.4.4.1 Subcutaneous adipose tissue

Relative to CTR, the 2,4-TZD treatment decreased the expression of *CD36*, coding for a major protein involved in LCFA import, but increased the expression of *GAPDH* and *IRS1*, tended to increase the transcript abundance of *SLC2A4*, and tended to decrease the expression of *YWHAZ* (**Table 1.3).** Among transcripts associated with glyceroneogenesis we only detected a tendency (treatment × time P=0.08) for higher expression of *PDK4* in TZD vs. CTR at the end of the trial.

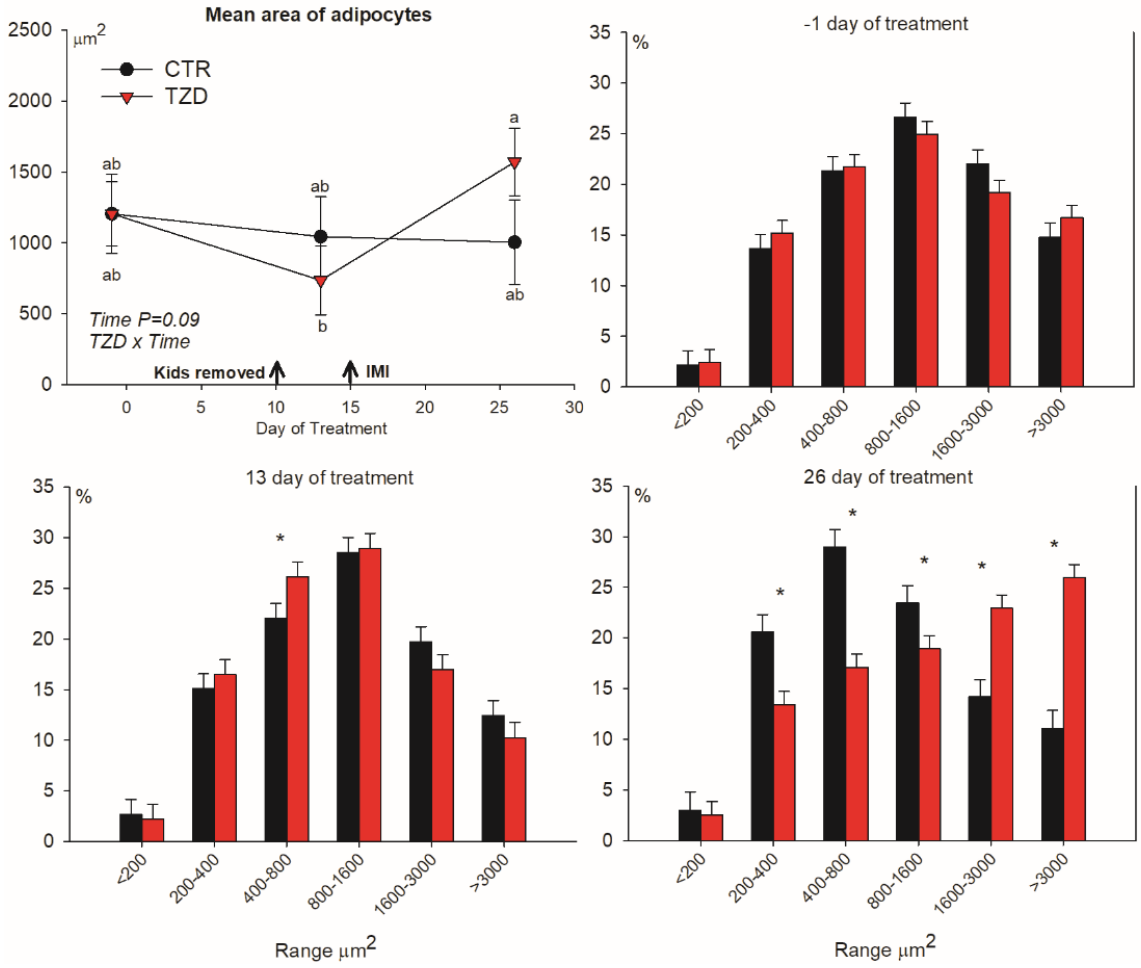

Figure 1.4 Median area of adipocytes and frequency (%) in each specific range in the area of adipocytes in subcutaneous tissue collected from the tail-head of goats receiving daily intra-jugular injection of 2,4-thiazolidinedione (TZD) or saline (CTR). Arrows indicate removal of kids and time of intramammary infusion of *Strep. uberis* in the right half of the mammary gland (IMI). Letters in the graph (a, b) denote significant effects of time and interactions (ZxT). Asterisks (*) denote differences ($P \leq 0.05$) between treatments in the same range of area of adipocytes.

1.4.4.2 Mammary epithelial cells

Table 1.4 reports the results for the abundance of transcripts in mammary epithelial cells. The isolation of cells using cytokeratin 8 antibodies significantly enriched cells expressing the specific epithelial marker *KRT8*, however, contrary to expectations, the expression of mammary-specific genes such as *CSN3*, *LALBA*, and *FABP3* was higher in negative- than positive-cytokeratin 8 isolated cells.

Table 1.3 mRNA abundance of selected genes in adipose tissue of goats treated with 2,4-thiazolidinedione (TZD) or saline (CTR). Samples were n≥5 for both groups, except for the day 26 (n= 3 for CTR; n=6 for TZD).

		Time			SEM[1]	P-value[2]		
Gene		**-1**	**13**	**26**		**Z**	**Time**	**ZxT**
Fatty acids import and de novo fatty acid synthesis								
CD36	CTR	7169.8	6987.6	6233.3	681.3	0.03	0.03	0.03
	TZD	7169.8	3362.5	5781.9				
LPL	CTR	122.2	130.5	244.6	52.1	0.62	0.04	0.90
	TZD	122.2	118.0	206.7				
SLC27A6	CTR	0.09	0.12	0.11	0.03	0.84	0.15	0.94
	TZD	0.09	0.14	0.11				
VLDLR	CTR	2.3	2.0	1.0	0.9	0.67	0.48	0.25
	TZD	2.3	1.1	2.7				
ACACA	CTR	37.6	26.6	48.5	24.3	0.33	0.51	0.14
	TZD	37.6	88.6	45.6				
FASN	CTR	473.3	47.7	58.6	439.9	0.13	0.29	0.18
	TZD	473.3	163.1	1328.2				
SCD1	CTR	275.2	500.8	410.9	333.4	0.67	0.16	0.54
	TZD	275.2	917.0	329.3				
Insulin signal and glucose metabolism								
GAPDH	CTR	159.2	125.6	91.2	41.7	0.01	0.25	0.08
	TZD	159.2	271.2	195.9				
INSR	CTR	7.8	5.2	3.7	1.9	0.12	0.37	0.21
	TZD	7.8	5.8	9.4				
IRS1	CTR	9.2	6.8	4.6	2.0	0.04	0.42	0.05
	TZD	9.2	7.4	12.5				
SLC2A4	CTR	4.5	5.6	5.1	1.6	0.09	0.10	0.40
	TZD	4.5	8.9	7.3				
YWHAZ	CTR	167.5	191.8	149.0	23.9	0.07	0.50	0.04
	TZD	167.5	101.4	150.1				
Glyceroneogenesis								
PCK1	CTR	8.0	9.8	15.0	4.2	0.67	0.01	0.47
	TZD	8.0	8.7	21.0				
PDK4	CTR	1.8	1.2	1.1	0.4	0.66	0.03	0.08
	TZD	1.8	0.8	2.1				
GPD1	CTR	71.5	109.1	90.8	33.9	0.45	0.07	0.71
	TZD	71.5	145.8	116.4				
Transcription regulation								
PPARG	CTR	43.5	41.7	49.5	9.6	0.81	0.19	0.70

	TZD	43.5	37.3	58.3				
SREBF1	CTR	16.1	19.3	23.0	16.7	0.61	0.38	0.61
	TZD	16.1	14.8	44.7				

[1]The highest SEM is shown; [2]Z= 2,4-thiazolidinedione effect, T=Time, Z×T=2,4-thiazolidinedione × time effect.

Table 1.4 Transcript abundance of selected genes normalized by the geometric mean of *RPS9*, *UXT*, and *MRPL39* in the mammary epithelial cell (MEC) in goats receiving 2,4-thiazolidinedione (TZD) or saline (CTR).

		Cell[1]			P-value[2]		
Gene		Neg	Pos	SEM	Z	Cell	Z×Cell
Mammary epithelial-specific genes							
CSN3	CTR	6324	3877	875	0.46	<0.05	0.42
	TZD	5050	3326	911			
FABP3	CTR	20.0	8.5	3.0	0.57	<0.05	0.41
	TZD	16.1	7.8	3.2			
LALBA	CTR	1341	578	233.4	0.56	<0.05	0.56
	TZD	1065	501	251.7			
KRT8 (Ln)[3]	CTR	1.78	2.50	0.27	0.30	<0.05	0.64
	TZD	1.48	2.03	0.29			
Fatty acid transport and synthesis							
LPL	CTR	9.68	4.87	1.52	0.70	<0.05	0.77
	TZD	10.8	5.19	1.68			
CD36	CTR	1300	979	104	0.30	<0.05	0.87
	TZD	1445	1094	115			
VLDLR (Ln)	CTR	0.57	0.84	0.13	0.21	<0.05	0.31
	TZD	0.43	0.53	0.14			
ACACA	CTR	0.29	0.58	0.09	0.99	0.09	0.10
	TZD	0.43	0.43	0.10			
SCD1	CTR	5.84	5.03	0.68	0.06	0.02	0.53
	TZD	4.19	2.89	0.71			
Transcriptional regulation							
NFE2L2	CTR	51.2	62.0	12.3	0.30	<0.05	0.50
	TZD	66.4	83.7	12.6			
NRF1	CTR	0.15	0.17	0.03	0.69	0.50	0.91
	TZD	0.17	0.18	0.03			
PPARG (Ln)	CTR	-2.73	-1.94	0.33	0.38	<0.05	0.50
	TZD	-2.20	-1.71	0.35			
SREBF1	CTR	0.85	1.17	0.13	0.45	<0.05	0.79
	TZD	0.74	1.01	0.14			
Other							
GAPDH (Ln)	CTR	3.63	4.07	0.31	0.39	<0.05	0.47
	TZD	3.85	4.56	0.33			
IL8 (Ln)	CTR	4.8	4.9	0.6	0.31	0.17	0.40
	TZD	5.5	5.9	0.6			
YWHAZ	CTR	31.6	40.7	8.3	0.64	0.19	0.61

	TZD	39.1	43.3	8.3

[1]Pos= cell positively isolated using cytokeratin 8; Neg = the cells that remained after the isolation of cytokeratin+ cells

[2]Z= 2,4-thiazolidinedione effect, T=Time, Z×T=2,4-thiazolidinedione × time effect.

[3]**data were transformed prior statistical analysis and showed as Ln=natural logarithm**

Almost all transcripts measured were significantly affected by the cell isolation, with *PPARG, GAPDH, NFE2L2, VLDLR,* and *SREBF1* being more abundant in cytokeratin 8-positive vs. cytokeratin 8-negative cells and *LPL, CD36, and SCD1* were more abundant in cytokeratin 8-negative vs. cytokeratin 8-positive cells. None of the measured transcripts were significantly affected by 2,4-TZD treatment except *SCD1*, where 2,4-TZD treatment tended (*P*=.06) to decrease its expression in both cell types, and *ACACA*, which expression tended to be increased (P=.10) by 2,4-TZD treatment in cytokeratin 8-negative cells.

1.4.5 Fatty acid profile in milk

We detected 46 fatty acids in goat milk (**Figure S3**). 2,4-TZD treatment significantly (*P<.05*) increased the percentage in milk of saturated fatty acids stearic acid (C18:0) and behenic acid (C22:0) and several unsaturated fatty acids, including *cis-7* C14:1, *cis9* C16:1, (unspecified) *trans* C16:1, *trans-9, cis-12, cis-15* C18:3 (**Table S5**). Furthermore, *all-trans* linolenic acid was increased by 2,4-TZD treatment, but the same treatment decreased the proportion of *cis-12* C18:1 and C19:1 (**Table S5**). A significant or a tendency for treatment × time interaction for the percentage of *cis9* C14:1, *trans-16* C18:1, and C20:4*n6* was detected, whereas a larger proportion over time was detected in TZD vs. CTR (**Table S5**).

When considering the g of FA/milking, only a numerically higher behenic acid (C22:0; *P=.10*) in TZD vs. CTR goats were detected (**Table S7 in Supplementary Materials**). Among all desaturation indexes calculated, the Δ9 18:1 desaturation index was significantly lower in TZD vs. CTR (**Tables S6 and S7**).

1.5 Discussion

1.5.1 2,4-TZD induces greater glucose utilization in the adipose tissue but did not activate PPARγ

Thiazolidinedione family of molecules, including rosiglitazone and pioglitazone, are potent activators of PPARγ, which is a key player in insulin sensitivity, glucose metabolism, lipid homeostasis, and adipogenesis (Boden et al., 2003, Varga et al., 2011, Cui et al., 2017). In dairy cows, the use of thiazolidinedione molecules during the perinatal period, including 2,4-TZD and pioglitazone, consistently decrease blood NEFA in early lactation (Smith et al., 2007, Yousefi et al., 2016), except when used in non-pregnant dry cows (Hosseini et al., 2015). The decrease of NEFA by thiazolidinedione molecules is consequence of a positive effect on insulin sensitivity (Quinn et al., 2008). We did not measure insulin sensitivity in the present experiment, but data allow inferring a large insulin sensitivity in TZD vs. CTR goats. The improved insulin sensitivity in TZD vs. CTR goats is supported by large decrease of NEFA and, in adipose tissue, an upregulation of *IRS1*, a gene related to insulin signaling (Guo, 2014), a tendency for a higher expression of *SLC2A4*, coding for the insulin-regulated glucose transporter, and larger transcription of *GAPDH*, a gene positively induced by insulin in rodents (Alexander et al., 1988). Increased insulin sensitivity by 2,4-TZD treatment was also suggested by data from our prior experiment (Rosa et al., 2017). The likely increase of insulin sensitivity in goats treated with 2,4-TZD in the present experiment is also supported by an increased size of adipocytes, considering that insulin induces lipogenesis (Boden et al., 2003).

Different compared to our prior experiment (Rosa et al., 2017), in the present experiment blood glucose was increased by 2,4-TZD treatment, prior corroborating observations in dairy cows (Smith et al., 2007, Hosseini et al., 2015, Yousefi et al., 2016). It is unclear the reason for the increased glucose in the blood by 2,4-TZD despite a possible larger insulin sensitivity; however, the larger concentration of glucose together with gene expression data (*SLC2A4* and *GAPDH*) indicated a larger import and utilization of glucose by the adipose tissue in TZD vs. CTR goats.

Glucose in adipose tissue of ruminants can be used for fatty acid synthesis, including the provision of NADPH, even though acetate is the main source (Nafikov and Beitz, 2007). Larger glucose in plasma after treatment with 4 mg 2,4-TZD/kg BW was also detected in early post-partum dairy cows (Smith et al., 2009) or non-pregnant dry dairy cows (Hosseini et al., 2015). The use of pioglitazone pre-partum, but not post-partum, also increased glucose levels in dairy cows (Yousefi et al., 2016). These data contrast to what generally observed in humans since thiazolidinedione molecules are classical glucose-lowering drugs. It is possible that the lower plasma NEFA by thiazolidinedione positively affected liver gluconeogenesis (Adewuyi et al., 2005), increasing glucose in plasma. In the experiment by Smith and collaborators (Smith et al., 2009), 2,4-TZD tended to lower lactose yield. Similar to Yousefi and collaborators (Yousefi et al., 2016), we did not observe any effect of 2,4-TZD on lactose yield in our experiment. The above data indicate that a decrease in utilization of glucose by the mammary gland is not the main cause of the observed increase in hematic glucose.

Association of *GAPDH* expression/activity with adipogenesis has been documented in rodents (Dugail et al., 1988). The reason for such association remains elusive; however, the reaction of this enzyme is reversible producing dihydroxyacetone phosphate that is then used to produce glyceraldehyde 3-phosphate via *GPD1* through the glyceroneogenesis pathway (Hanson and Reshef, 2003). Glyceroneogenesis in adipose tissue increases in rodent pre-adipocytes treated with rosiglitazone or pioglitazone via induction of expression of phosphoenolpyruvate carboxykinase (Tordjman et al., 2003). However, 2,4-TZD failed to affect the expression of *PCK1* in our experiment, similar to data in bovine (Hosseini et al., 2015).

Our original hypothesis was that 2,4-TZD would have activated PPARγ in well-fed animals. Data indicated that 2,4-TZD failed to activate PPARγ in the adipose tissue. The classical PPARγ target gene *LPL* or previously indicated target genes, such as *SREBF1* or *SCD1* (Bionaz et al., 2013), were not affected by 2,4-TZD treatment, confirming prior data in cows (Schoenberg and Overton, 2011) and goats (Rosa et al., 2017). Thus, we cannot attribute the decrease in NEFA and the effect on glucose metabolism-related genes in the adipose tissue to PPARγ activation. Further support for inadequate activation of PPARγ by 2,4-TZD is provided by the lack of increase in the frequency of newly formed small adipocytes compared to CTR since PPARγ is a key player in adipogenesis (Cui et al., 2017).

The tendency for transcription of *PDK4* to be increased by 2,4-TZD in the adipose tissue in our experiment is of interest. This enzyme plays a crucial role in glucose metabolism and overall energy homeostasis by inhibiting pyruvate dehydrogenase

providing pyruvate for the formation of oxaloacetate that can be used for glyceroneogenesis (Zhang et al., 2014). Furthermore, it is a well-established PPARβ/δ target genes in monogastric animals (Zhang et al., 2014) and dairy cows (Lohakare et al., 2018). Among PPAR isotypes, activation of PPARβ/δ induces a reduction of NEFA in porcine (Yu et al., 2010). It is possible that 2,4-TZD activated PPARβ/δ in the adipose tissue in our experiment. However, 2,4-TZD did not affect the expression of *PDK4* in liver (see companion paper (Rosa et al., 2019)).

Overall, our data suggested 2,4-TZD increased the use of glucose by the adipose tissue, likely for glyceroneogenesis and, maybe *de novo* fatty acid synthesis. The latter is partly supported by an increase in the size of adipocytes together with a numerically larger expression of *FASN* and a concomitant decreased expression of *CD36*, important for preformed fatty acid import (Bionaz and Loor, 2008). However, the effect of 2,4-TZD on the adipose tissue appears to be PPARγ-independent. Although not possible to support the present data, other mechanisms for the effect of 2,4-TZD on adipose tissue, such as effect on mitochondria, activation of AMPK and/or heat shock response, are possible as previously reviewed (Feinstein et al., 2005).

1.5.2 2,4-TZD does not affect milk fat synthesis

Our original hypothesis was that 2,4-TZD would have increased milk fat synthesis by activating PPARγ. We did not observe any effect of 2,4-TZD on milk fat synthesis. The lack of effect on milk fat synthesis might be indicating a positive effect of 2,4-TZD on milk fat synthesis, considering the large decrease of LCFA available for the mammary gland.

Based on our estimates, the decrease in NEFA lowered the available LCFA for the mammary gland, including the ones from the triglycerides present in the VLDL, up to 17%. Milk fat is synthesized with about 50% of fatty acids coming from the preformed LCFA present in blood (Palmquist, 2006). A positive association between NEFA in plasma and milk fat has been reported in cows (Palmquist, 2006). That appeared to be the case from prior studies in periparturient dairy cows where a decrease in milk fat was detected as consequence of lowered NEFA by 2,4-TZD or pioglitazone treatment (Smith et al., 2009, Yousefi et al., 2016). NEFA concentration is however high during early post-partum (reaching values >1 mM), and the uptake of NEFA by the mammary gland from the blood is highly dependent on its concentration (Miller et al., 1991). Despite the above data, it has been determined that more than 98% of stearate in milk fat comes from the blood TAG-rich lipoproteins (Palmquist, 2006). Thus, a change in NEFA might not have affected milk fat in our experiment due to the relatively low NEFA (<0.5 mM) as consequence of the goats being in mid-lactation stage. The lack of any association between NEFA and percentage of milk fat in our experiment corroborates the above findings.

We observed only a minor effect of 2,4-TZD on the fatty acid composition of the milk, including LCFA, with potentially positive effects on human health. Among saturated fatty acids affected by 2,4-TZD in our study, stearic acid (C18:0) and behenic acid (C22:0) are known to decrease LDL or total cholesterol in humans and mice (Mensink, 2005, Moreira et al., 2017). Among unsaturated LCFA, proportionally more abundant in TZD vs. CTR goats, punicic acid (C18:3c9,t11,c13) can aid in combating metabolic syndrome (Shabbir et al., 2017) and n-7 trans-palmitoleate, abundant in dairy products (possible the

major isomers in our trans-palmitoleate) has been associated with a preventive role in diabetes (Mozaffarian et al., 2013). The origin of this fatty acid is unclear; however, it is.

The above data indicated that the lowered NEFA did not affect the yield of milk fat and only slightly changed milk fat composition. Thus, the data indicated no effect of 2,4-TZD on milk fat synthesis. Gene expression data in MEC support such conclusion. Despite the fact that the use of cytokeratin 8 antibody did not allow to enrich milk-secreting MEC from somatic cells, data in both cell types isolated none of the PPARγ target genes were affected by 2,4-TZD, corroborating our prior finding (Rosa et al., 2017). 2,4-TZD also did not affect the expression of *NFE2L2* and *NRF1,* coding for transcription factors involved in mitochondrial biogenesis and antioxidant response (Merry and Ristow, 2016) and previously shown to be up-regulated by pioglitazone in bone marrow neuroblasts (Wang et al., 2017).

Unexpectedly, 2,4-TZD treatment decreased the expression of *SCD1* in MEC. This gene codes for a protein that plays a key role in milk fat synthesis (Bionaz and Loor, 2008). Inhibition of this protein is associated with a decrease in milk fat synthesis, and its expression is consistently decreased during milk fat depression, as previously reviewed (Palmquist, 2006). The decrease in the expression of *SCD1* in our experiment also translated into a decreased desaturation of stearate to oleic acid. Our data, however, indicated that SCD1 activity is not essential to maintain overall milk fat synthesis in goats, which is contrary to previous reports (Palmquist, 2006, Bionaz and Loor, 2008) but support conclusions from a prior study in dairy cows (Griinari et al., 2000).

1.6 Conclusions

Despite having animals in good body condition and fed an adequate level of vitamin A, we failed to detect any increase in milk fat synthesis or expression of related genes in MEC by 2,4-TZD. The TZD treatment had however a large effect on the adipose tissue considering the decreased level of NEFA in blood and increased adipocyte size while affecting the expression of genes related to insulin signaling and glucose metabolism. Our data indicated that 2,4-TZD has a strong effect on adipose tissue but does not activate PPARγ. This finding undermined the possibility of testing our original hypothesis.

1.7 Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, File S1, CellProfiler pipeline for adipocytes size, File S2. KingFisher protocol file for MEC isolation, Table S1. Nutrient composition of the forages, Table S2. Rations fed, Table S3. Composition of vitamin drenches, Table S4. Primer-pairs, Table S5. Fatty acid profile (%) in milk, Table S6. Grams/milking of each fatty acid in milk, Figure S1. Body weight and body condition score, Figure S2. Pearson correlation (r above and P-value below) among measured parameters in blood and milk (plus dry matter intake). Yellow to orange to red shade denotes the level of significance for the correlations that are either positive (green shade) or negative (blue shade), Figure S3. Percentage of the 46 detected fatty acids in goat milk in our experiment. The upper quadrant reports a representative GC chromatogram.

1.8 Author Contributions: Conceptualization, Charles Estill and Massimo Bionaz; Data curation, Shana Jaaf, Erminio Trevisi and Massimo Bionaz; Formal analysis, Shana Jaaf, Fernanda Rosa, Johan Osorio and Massimo Bionaz; Funding acquisition, Charles Estill and Massimo Bionaz; Investigation, Fernanda Rosa, Misagh Moridi, Johan Osorio, Jayant Lohakare and Massimo Bionaz; Methodology, Shana Jaaf, Fernanda Rosa, Johan Osorio, Erminio Trevisi, Shelby Filley, Gita Cherian and Massimo Bionaz; Project administration, Massimo Bionaz; Resources, Erminio Trevisi, Shelby Filley, Gita Cherian and Charles Estill; Supervision, Charles Estill and Massimo Bionaz; Validation, Shana Jaaf, Fernanda Rosa, Johan Osorio, Erminio Trevisi and Massimo Bionaz; Visualization, Massimo Bionaz; Writing – original draft, Shana Jaaf, Johan Osorio and Massimo Bionaz; Writing – review & editing, Fernanda Rosa, Misagh Moridi, Jayant Lohakare, Erminio Trevisi, Shelby Filley, Gita Cherian, Charles Estill and Massimo Bionaz

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1.11 Conflicts of Interest: The authors declare no conflict of interest.

1.12 Appendix A

1.12.1 Supplementary Materials and Methods

1.12.1.1 Animal management

The goats were housed in individual pens at the Hogg Metabolism Barn at Oregon State University for the experiment. Goats were allowed ten days of adaptation to the new environment prior to starting the experiment. Each goat was nursed by 2 kids during the first 11 days of the experiment, except for one goat (in TZD group) which had only one kid, due to the unwillingness of the goat to nurse other kids.

Goats were fed twice a day at 0700 and 1900 h with orchard grass hay and alfalfa hay. The ration was supplemented in the morning feeding with a commercial grain goat mix (Kountry Buffet, CHS Inc., USA) and a mineral mix (SWEETLIX® Caprine Magnum-Milk Mineral, USA). Goats also received 1 cup of the commercial grain during milking. Vitamins requirements (IU/d) ranged from 10700 to 16000 for vitamin A, from 2700 to 4000 for vitamin D, and from 330 to 500 for vitamin E. The drench was a mixture of a commercial water-soluble premix of vitamins and electrolytes (Durvet, MO, USA) plus vitamin D and E (generously provided by CHS Inc., Tangent, OR, USA). The vitamins were suspended in 50 mL water and drenched by using a 60 mL syringe.

1.12.1.2 *Dry matter intake, Energy Corrected Milk (ECM), Dairy Efficiency (DE), and Body Condition Score (BCS)*

The DMI during the first 11 days of treatments also included the feed consumed by kids. The weights of the kids nursing the CTR and TZD goats was not different (14.0±2.8

kg in CTR vs. 13.4±5.3 kg in TZD at 1 day prior starting the treatment and 16.8±3.0 kg in CTR vs. 16.1±5.4 kg in TZD when weaned, in both cases the *P*-value was >0.71).

Kilograms of ECM were calculated (Sjaunja et al., 1990) only for the morning milking of the left mammary gland as follow:

Kg milk × ((38.3 × Fat g/kg + 24.2 × protein g/kg + 16.54 × lactose g/kg + 20.7)/3140).

The DE was calculated as a kilogram of ECM produced in one milking by the left half of the mammary gland/ ¼ daily kg DMI.

The BCS was assessed using a 1 to 5 scale, as previously described (Villaquiran et al., 2004).

1.12.1.3 Adipose biopsy

Subcutaneous adipose tissue was collected by biopsy as previously described (Rosa et al., 2017) from alternate sides of the tail-head at around 1000 h, and samples were flash-frozen in liquid nitrogen.

1.12.1.4 Histological analysis of the adipose tissue

Adipose tissue samples were sent to the Oregon Veterinary Diagnostic Laboratory for histological preparation. Adipose tissue was fixed in 10% neutral buffered formalin, processed on Tissue-Tek VIP5 Tissue Processor (Sakura, USA), and embedded in Paraffin Type 9 (Richard-Allen Scientific, USA). Tissue sections were cut at 4-5 microns on a Microm HM355S microtome and mounted on glass microscope slides. Slides were stained with a standard hematoxylin and eosin stain using the Thermo Shandon GLX Linear Slide

Stainer (Thermo Scientific, USA). Slides were imaged with a 10X magnification objective using a Leica DM6000 microscope equipped with a DFC295 digital color camera (Leica Microsystems Inc., USA). The analysis of adipocytes size was performed using CellProfiler v.2.2.0 software (Kamentsky et al., 2011). Besides the average area of adipocytes, the frequency of various ranges of area of adipocytes was measured to estimate the formation of new adipocytes (i.e., small size) and formation of large adipocytes, i.e., accumulation of triglycerides (CellProfiler pipeline is available in **File S1**).

1.12.1.5 Mammary epithelial cells isolation

Milk samples were collected in 250 mL DNase/RNase free sterile tubes (430776, VWR, USA) and immediately placed on ice (4°C) until isolation (~1 hr). Tubes were centrifuged at 1500×g for 10 min to pellet the cells. Cells were washed twice with 12 mL of sterile phosphate-buffered saline (**PBS**, PBL06-500ML, VWR, USA) and centrifuged at 1000×g for 5 min. Before the last wash the cells were passed through a cell strainer (BD Falcon– 70μm nylon, 732-2758, VWR, USA). The final pellet was resuspended in 1 mL of PBS solution plus 0.1% BSA (AK8917-0100, Akron, USA) and transferred to a 1.5 mL tube pre-wetted with the PBS+0.1% BSA solution. Cell isolation was performed using the KingFisher Duo (5400100, Thermo Scientific, USA) following the manufacturer's instructions for Thermo Scientific Dynabeads isolation with some modifications. In brief, 1 mL solution of cells was transferred in a well of a sterile KingFisher Flex Microtiter Deepwell 96 plate (95040460, Thermo Scientific, USA). In a new well, 2 μL of the

antibody against the epithelial-specific marker cytokeratin 8 (MA1-19037, Thermo Scientific, USA) was added in 1 mL PBS+0.1% BSA solution. Twenty-five microlitermicroliter of Dynabeads® Pan Mouse IgG (11041, Thermo Scientific, USA) were added into an empty well of the plate diluted in 1 mL PBS+0.1% BSA. The isolation steps consisted of washing the Dynabeads®, followed by the conjugation of the antibody with the Dynabeads® by incubation for 30 min at 6°C, 20 min incubation with the cells, and two washing steps. The KingFisher protocol file is available in **File S2**. We obtained $36.6\times10^6\pm74.3\times10^6$ total cells from 250 ml milk (146,550 MEC/mL of milk). Cells positively isolated by the magnetic beads and cells remaining after isolation were transferred to 1.5 ml tubes, pelleted by centrifugation at 1000×*g* at 4 °C for 5 min, and re-suspended in 1 mL of cold TRIzol® (15596018, Ambion, USA). The samples were stored at -80°C until RNA extraction.

1.12.1.7 RT-qPCR

Approx. 200 mg of frozen adipose tissue samples were weighed and immediately placed into 1200 µL ice-cold TRIzol® in a 2 ml RNase/DNase free tubes (Metal Bead beating tubes, 13117-50, Qiagen, USA) and 1 bead (69989, 5mm, Qiagen, USA) per tube was added using the bead dispenser (69965, Qiagen). Tubes were loaded into a Bullet Blender Next Advance (Laboratory Instruments, USA), and samples were homogenized twice for 1 min at maximum speed with 1 min resting on ice. RNA was extracted following a previously published protocol (Bionaz and Loor, 2007) and RNA cleaning was performed using RNeasy Plus Mini Kit (74104, Qiagen) for MEC and RNeasy Plus Lipid Tissue Mini

Kit (74804, Qiagen) for adipose samples following the manufacturer's instructions without DNA digestion. SpectraMax® Plus 384 (Molecular Devices, USA) was used to measure RNA concentration.

Amplicons not previously validated were cleaned with DNA Clean & Concentrator kit (11-302C, Zymo Research, USA) and sequenced at the CGRB. The sequencing product was confirmed through BLASTN (megablast) at the *National Center for Biotechnology Information*.

The cDNA synthesis and the RTqPCR were performed as previously described (Rosa et al., 2017) by using 90 ng of RNA for each 20 μl reaction for MEC and 80 ng for adipose tissue. The PCR analysis was performed using Power SYBR® Green PCR Master Mix (4368706, Thermo Scientific, USA) in a 7900HT PCR system (Applied Biosystems, USA) and final RTqPCR data were obtained by using LinRegPCR (Ramakers et al., 2003). The genes ribosomal protein 9 (*RPS9*), ubiquitously-expressed transcript (*UXT*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (*YWHAZ*), mitochondrial ribosomal protein L39 (*MRPL39*), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were tested as potential internal control genes (ICG) using geNorm (Vandesompele et al., 2002). The reliability of the selected ICG and the stability of the normalization factor through the calculation of the pairwise variation V (the lower, the better) were assessed. The most stable normalization was achieved using the geometric mean of *RPS9*, *UXT*, and *MRPL39* in both tissues with a V-value of 0.122 for adipose samples and 0.146 for MEC.

The intra-assay variation for qPCR data was (% coefficient of variation between technical replicates) <10% (geometrical mean 5.5%).

1.12.1.8 Milking and milk sample analysis

Goats were milked by using a portable milking machine with two claws. Teats of the goats were pre-dipped before milking and post-dipped after milking using a 0.5% iodine solution. Milk samples were collected in 50 mL tubes with a preservative (Bronopol) and shipped for analysis using NIR at Willamette National Dairy Herd Information Association (Salem, OR).

1.12.1.9 Milk fatty acid analysis

FIM-FAME-6 mixture was used as an external standard (33mg/ml, 2009, Matreya, USA). The external standard was used to build a 5-point standard curve for each of the annotated compounds for the calculation of the absolute amount of each detected fatty acid (quantity of the fatty acids not present in the external standard was determined using annotated fatty acids with same/similar carbon length and degree of saturation). Final fatty acid data were obtained as a percentage of total measured fatty acids and g of each fatty acid in milk/day. The latter was obtained by using the intercept and slope of the standard curve for each of fatty acid using the external standard (linear regression ≥0.99) corrected by the internal standard (to obtain the mg fatty acid/mL of milk) and multiplied by milk produced by the left mammary gland (kg/d) for each goat. Lipids were extracted from milk,

and fatty acid profiling was performed using gas chromatography as previously described (Oeffner et al., 2013).

1.12.1.10 Blood Metabolites

Blood samples were collected prior to the morning feeding from the jugular vein using 20-gauge BD Vacutainer needles and tubes, one containing serum clot activator and the other sodium heparin (BD Vacutainer®, Becton Dickinson, USA). After blood collection, tubes containing sodium heparin were placed on ice (4°C), while tubes with clot activator were kept at room temperature (~30min) until centrifugation. Serum and plasma were obtained by centrifugation at 1000×*g* for 15 minutes. Aliquots of serum and plasma were frozen (-80°C) until further analysis.

Aliquots of plasma and serum were shipped on dry ice to the Department of Animal Sciences, Food and Nutrition, Universitá Cattolica del Sacro Cuore, Piacenza, Italy, for metabolic profiling. The intra- and inter-assay variations for the parameters measured in plasma are available elsewhere (Calamari et al., 2016, Rosa et al., 2019).

1.12.2 Statistical analysis

Prog Reg of SAS to determine outliers

```
proc reg lineprinter;
    model Parameter = Time/r;
    run;
```

Prog GLIMMIX of SAS with AR(1)

```
proc glimmix;
class TRT Time Goat;
model Parameter= TRT|Time /ddfm=kr ;
```

random Time/Residual subject=Goat type=ar(1);

lsmeans TRT|Time /diff lines;

Run;

Prog GLIMMIX of SAS with ARH(1)

proc glimmix;

class TRT Time Goat;

model Parameter= TRT|Time /ddfm=kr ;

random Time/Residual subject=Goat type=arH(1);

lsmeans TRT|Time /diff lines;

Run;

Prog GLIMMIX of SAS with Spatial Power

proc glimmix;

class TRT Time Goat;

model Parameter = TRT|Time /ddfm=kr;

random Time/residual subject=Goat type=sp(pow)(Time);

lsmeans TRT|Time /diff lines;

Run;

Prog GLM of SAS for fatty acid analysis

proc glm;

class TRT Time Goat;

model FA1, FA2, FA3, …= TRT|Time;

lsmeans TRT|Time/stderr diff lines;

means TRT|Time/t lines;

run;

Prog CORR of SAS for correlation analysis

proc corr;

run;

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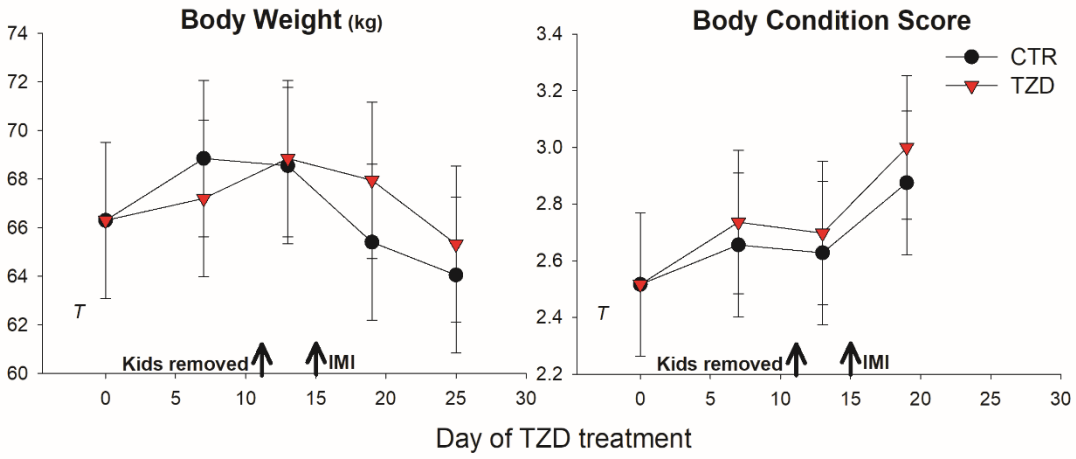
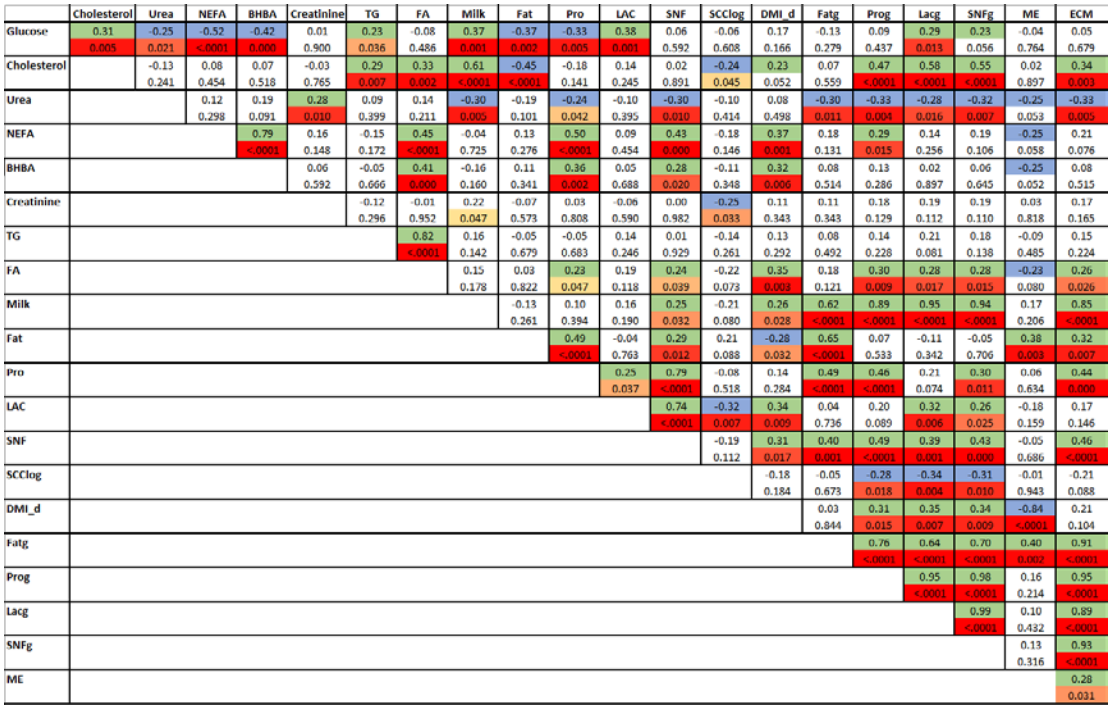


Figure S1. Body weight and body condition score in goats receiving a daily intra-jugular injection of 2,4-thiazolidinedione (TZD) or saline (CTR). Arrows indicate time of kid removal and intramammary infusion of *Strep. Uberis* in the right half of the mammary gland (IMI). Letters in the graph denote significant ($P<.05$) effects of time (*T*), treatment (*Z*), and interactions (*ZxT*). The reported P-value is for tendencies.



	Cholesterol	Urea	NEFA	BHBA	Creatinine	TG	FA	Milk	Fat	Pro	LAC	SNF	SCClog	DMI_d	Fatg	Prog	Lacg	SNFg	ME	ECM
Glucose	0.31	-0.25	-0.52	-0.42	0.01	0.23	-0.08	0.37	-0.37	-0.33	0.38	0.06	-0.06	0.17	-0.13	0.09	0.29	0.23	-0.04	0.05
	0.005	0.021	<.0001	0.000	0.900	0.036	0.486	0.001	0.002	0.005	0.001	0.592	0.608	0.166	0.279	0.437	0.013	0.056	0.764	0.679
Cholesterol		-0.13	0.08	0.07	-0.03	0.29	0.33	0.61	-0.45	-0.18	0.14	0.02	-0.24	0.23	0.07	0.47	0.58	0.55	0.02	0.34
		0.241	0.454	0.518	0.765	0.007	0.002	<.0001	<.0001	0.141	0.245	0.891	0.045	0.052	0.559	<.0001	<.0001	<.0001	0.897	0.003
Urea			0.12	0.19	0.28	0.09	0.14	-0.30	-0.19	-0.24	-0.10	-0.30	-0.10	0.08	-0.30	-0.33	-0.28	-0.32	-0.25	-0.33
			0.298	0.091	0.010	0.399	0.211	0.005	0.101	0.042	0.395	0.010	0.414	0.498	0.011	0.004	0.016	0.007	0.053	0.009
NEFA				0.79	0.16	-0.15	0.45	-0.04	0.13	0.50	0.09	0.43	-0.18	0.37	0.18	0.29	0.14	0.19	-0.25	0.21
				<.0001	0.148	0.172	<.0001	0.725	0.276	<.0001	0.454	0.000	0.146	0.001	0.131	0.015	0.256	0.106	0.058	0.076
BHBA					0.06	-0.05	0.41	-0.16	0.11	0.36	0.05	0.28	-0.11	0.32	0.08	0.13	0.02	0.06	-0.25	0.08
					0.592	0.666	0.000	0.160	0.341	0.002	0.688	0.020	0.348	0.006	0.514	0.286	0.897	0.645	0.052	0.515
Creatinine						-0.12	-0.01	0.22	-0.07	0.03	-0.06	0.00	-0.25	0.11	0.11	0.18	0.19	0.19	0.03	0.17
						0.296	0.952	0.047	0.573	0.808	0.590	0.982	0.033	0.343	0.343	0.129	0.112	0.110	0.818	0.165
TG							0.82	0.16	-0.05	-0.05	0.14	0.01	-0.14	0.13	0.08	0.14	0.21	0.18	-0.09	0.15
							<.0001	0.142	0.679	0.683	0.246	0.929	0.261	0.292	0.492	0.228	0.081	0.138	0.485	0.224
FA								0.15	0.03	0.23	0.19	0.24	-0.22	0.35	0.18	0.30	0.28	0.28	-0.23	0.26
								0.178	0.822	0.047	0.118	0.039	0.073	0.003	0.121	0.009	0.017	0.015	0.080	0.026
Milk									-0.13	0.10	0.16	0.25	-0.21	0.26	0.62	0.89	0.95	0.94	0.17	0.85
									0.261	0.394	0.190	0.032	0.080	0.028	<.0001	<.0001	<.0001	<.0001	0.206	<.0001
Fat										0.49	-0.04	0.29	0.21	-0.28	0.65	0.07	-0.11	-0.05	0.38	0.32
										<.0001	0.763	0.012	0.088	0.032	<.0001	0.533	0.342	0.706	0.003	0.007
Pro											0.25	0.79	-0.08	0.14	0.49	0.46	0.21	0.30	0.06	0.44
											0.037	<.0001	0.518	0.284	<.0001	<.0001	0.074	0.011	0.634	0.000
LAC												0.74	-0.32	0.34	0.04	0.20	0.32	0.26	-0.18	0.17
												<.0001	0.007	0.009	0.736	0.089	0.006	0.025	0.159	0.146
SNF													-0.19	0.31	0.40	0.49	0.39	0.43	-0.05	0.46
													0.112	0.017	0.001	<.0001	0.001	0.000	0.686	<.0001
SCClog														-0.18	-0.05	-0.28	-0.34	-0.31	-0.01	-0.21
														0.184	0.673	0.018	0.004	0.010	0.943	0.088
DMI_d															0.03	0.31	0.35	0.34	-0.84	0.21
															0.844	0.015	0.007	0.009	<.0001	0.104
Fatg																0.76	0.64	0.70	0.40	0.91
																<.0001	<.0001	<.0001	0.002	<.0001
Prog																	0.95	0.98	0.16	0.95
																	<.0001	<.0001	0.214	<.0001
Lacg																		0.99	0.10	0.89
																		<.0001	0.432	<.0001
SNFg																			0.13	0.93
																			0.316	<.0001
ME																				0.28
																				0.031

Figure S2. Pearson correlation (r above and P-value below) among measured parameters in blood and milk (plus dry matter intake). Yellow to orange to red shade denotes the level of significance for the correlations that are either positive (green shade) or negative (blue shade)

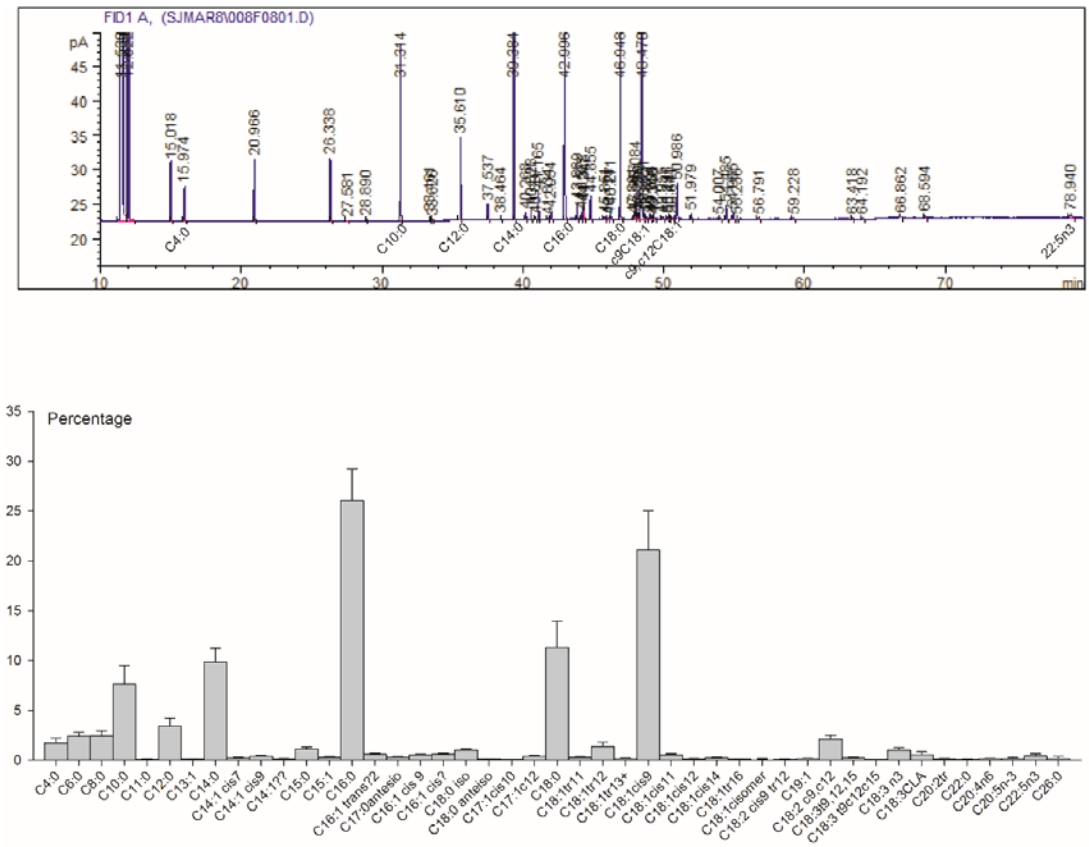


Figure S3. Mean±SD of the percentage amount of the 46 detected fatty acids in milk of all milk samples in our experiment. The upper quadrant reports a representative GC chromatogram.

Table S1. Nutrient Composition of the forages used during the experimental period.

Chemical analysis		Alfalfa hay	Orchard Grass hay
Dry Matter (DM)	%	89.7	91.4
NE_L (Mcal/Lb)	% DM	0.69	0.53
Crude Protein (CP)	% DM	23.2	12.2
Adjusted crude protein	% DM	22.6	12.2
Soluble Protein	% CP	46.0	26.0
ADF	% DM	25.7	37.0
aNDF	% DM	33.2	60.0
NFC	% DM	27.3	18.6
TDN	% DM	65.0	59.0
Calcium	% DM	0.97	0.31
Phosphorus	% DM	0.24	0.20
Magnesium	% DM	0.51	0.24
Potassium	% DM	2.64	2.28
Sodium	% DM	0.39	0.05
Sulfur	% DM	0.34	0.20
Iron	ppm	1460	223
Zinc	ppm	20	18
Copper	ppm	8	8
Manganese	ppm	48	112
Molybdenum	ppm	5	0.4

Table S2. Rations fed to *Saanen* lactating goats during the experiment period based on body weight (BW) and milk initial milk yield.

Goats features							
BW (kg)	60	70	70	80	80	90	90
Milk yield (kg/d)	2.1-3.2	2.1-3.2	3.5-4.8	2.1-3.2	3.5-4.8	2.1-3.2	3.5-4.8
Feed components	***Daily Feed (kg head^{-1}, DM basis)***						
Alfalfa Hay	0.5	0.5	1.2	0.5	1.5	0.6	1.0
Orchardgrass Hay	1.6	1.8	1.2	2.2	2.0	2.4	2.8
Kountry Buffet	0.5	0.6	0.5	0.5	0.5	0.5	0.5
SweetLix Minerals (g/d)	14.2	14.2	14.2	14.2	14.2	14.2	14.2

Table S3. Composition of vitamin drench (amount per 10 mL solution) given to each goat daily.

Component	Daily g per goat	Vitamin	IU supl.[4]
Vitamin D (CHS[1])	0. 2	A	17,000
Vitamin E (CHS[2])	12.5	D	7,000
Vitamins and Electrolytes mix (Durvet[3])	7.5	E	1,700

[1]Contains 12,000 IU/Kg (CHS, MN, USA)
[2]Contains 227,000 IU/Kg (CHS, MN, USA)
[3]Contains a maximum of 37% of sodium, and a minimum of 3% of potassium, 2,267,965 IU/kg of Vitamin A, 907,186 IU/kg of Vitamin D3, 907 IU/kg of Vitamin E, 2 mg/kg of Vitamin B12, 907 mg/kg of menadione, 680 mg/kg of riboflavin, 1,134 mg/kg of d-pantothenic acid, and 227 mg/kg of thiamine hydrochloride, 2,268 mg/kg of niacin, 3,402 mg/kg of ascorbic acid, 227 mg/kg of pyridoxine hydrochloride and 59 mg/kg of folic acid (Durvet, MO, USA).
[4]IU/day supplemented by the drench

Table S4. Primer-pairs newly designed for the present experiment

Symbol	NCBI Acc.	Start	Primer	Amp size	Amplicon sequence
CD36	NM_001285578.1	F-1243	TTTGGCTTAATGAGACTGGTACCA	90	TAAAGCGGATGTTCAGAAGCAAGTGACGGGGAAAATAAAC CTCCTTGGCCTGGA
.	.	R-1332	CCAGGCCAAGGAGGTTTATTT		
FABP3	NM_001285701.1	F-69	CACTCGGTGTCGGTTTTGCT	120	TTGACAGCCTACCACAATCATCGAAGTGAATGGGGACACA GTCATCATAAAAACACAAAGCACCTTCAAGAACACAGAGA A
.	.	R-188	TCTCTGTGTTCTTGAAGGTGCTTT		
INSR	XM_018051134.1	F-751	CAGAAAGTGTGTCCGACTATCTGTAAG	199	ATGGGCTGCACTCTGAGGGTCTCTGCTGCCACAGCGAGTGC TTGGGCAACTGCTCAGAGCCCGACGACCCCACCAAGTGCG TGGCCTGTCGCAACTTCTACCTGGACGGCAGGTGCGTGGA GACCTGCCCGCCCCCTTACTACCACTTCCAAGACTGGCGCT GCGTGA
.	.	R-949	TCACGCAGCGCCAGTCT		
IRS1	XM_018058864.1	F-1475	TCCATCCCCATGCCTTCTT	100	GCTTCGCAAGCCCCGTCAGTCTGTCGTCCAGCAGCACGAGT GGCCACGGCTCCACCTCAGACTGCCA
.	.	R-1574	GGCAGTCTGAGGTGGAGCC		
KRT8	XM_005679931.1	F-612	GGCTGGTGGAGGACTTCAAG	105	GACTAGGATCACAGCGCACAGACATGGAGAATGAATTTGT CATCATCAAGAAGGATGTGGATAGAAGCTTACAAAA
.	.	R-716	TGTAAGCTTCATCCACATCCTTCTT		
NFE2L2	NM_001314327.1	F-419	CATTCCCAAAGCAGATGATTTG	91	TATGCATGCAGCTTTTGGCAGAGACATTCCCGTTTGTAGAT GACAATGAGGTTTCTTCAGA
.	.	R-509	CGAAGAAACCTCATTGTCATCTACA		
NRF1	XM_018046932.1	F-4072	CGGGCCCTTGCTAAACCT	126	aAgttTGgtGCcaCTGGCTttcccAGCtaaAATGATGCtcTCCttgCcAC CTcCATcACCCCTTtcTtTcCTTTTGTTAGGgTTAtggA
.	.	R-4197	CCTAACCCTAACAAAAGGAAAGAAAG		
SLC27A6	XM_018050707.1	F-1124	ACTGAGCACAGCAACTGATTGTC	116	CCATGCTATCTCAACACCAAGTCTCCTTATCTTTATATTTTT ACTTCTGGAACAACAGGTCTACCAAAAGCAGCTAA
.	.	R-1239	TAGCTGCTTTTGGTAGACCTGTTGT		
SLC2A4	NM_001314227.1	F-1051	TCAATGCGGTTTTCTATTATTCGA	80	TTGAGTGCAGGGTAGAGAACCAGCCTATGCCACCATCGGA GCCA
.	.	R-1130	GGCTCCGATGGTGGCATA		
VLDLR	XM_018052030.1	F-1332	CAGTAGGCAAAGAGCCGAGTCT	110	gaCGAgaatCAGGAAGAtTGGCTTGGAGAGGAAAGAATATAT CCAACTAGTTGAACAGCTGCGAAACACTGTGA
.	.	R-1441	CACAGTGTTTCGCAGCTGTTC		

Table S5. Fatty acid profile (%) in milk of goats receiving daily intrajugular injection of 2,4-thiazolidinedione (TZD) or saline (CTR).

Fatty Acid%		Day of TZD injection					*P-value*		
		0	**2**	**5**	**15**	**SEM**	***TZD***	***Time***	***Z x T***
C4:0	**CTR**	1.76	1.40	1.57	1.88	0.17	*0.47*	*<0.01*	*0.80*
	TZD	1.76	1.34	1.77	2.06				
C6:0	**CTR**	2.51	2.13	2.46	2.79	0.12	*0.52*	*<0.01*	*0.70*
	TZD	2.51	2.38	2.51	2.72				
C8:0	**CTR**	2.42	2.12	2.65	2.86	0.18	*0.95*	*<0.01*	*0.99*
	TZD	2.42	2.17	2.61	2.83				
C10:0	**CTR**	7.28	6.30	8.13	9.47	0.57	*0.98*	*<0.01*	*0.99*
	TZD	7.28	6.37	8.06	9.52				
C11:0	**CTR**	0.09	0.08	0.13	0.14	0.01	*0.97*	*<0.01*	*0.47*
	TZD	0.09	0.08	0.11	0.16				
C12:0	**CTR**	2.99	2.94	3.81	4.43	0.25	*0.35*	*<0.01*	*0.92*
	TZD	2.99	2.84	3.49	4.18				
C13:1	**CTR**	0.13	0.13	0.14	0.11	0.01	*0.92*	*0.57*	*0.37*
	TZD	0.13	0.12	0.13	0.13				
C14:0	**CTR**	9.25	9.28	10.8	11.0	0.04	*0.59*	*<0.01*	*0.67*
	TZD	9.25	9.48	10.0	10.9				
C14:1cis7	**CTR**	0.25	0.25	0.24	0.19	0.01	*0.03*	*<0.01*	*0.30*
	TZD	0.25	0.27	0.26	0.24				
C14:1cis9	**CTR**	0.41	0.43	0.45	0.37	0.03	*0.44*	*0.39*	*0.10*
	TZD	0.41	0.38	0.47	0.49				
C14:1??	**CTR**	0.10	0.07	0.13	0.14	0.02	*0.62*	*0.08*	*0.89*
	TZD	0.10	0.08	0.11	0.11				
C15:0	**CTR**	1.06	1.10	1.20	1.17	0.06	*0.09*	*0.01*	*0.44*
	TZD	1.06	1.18	1.24	1.36				
C15:1	**CTR**	0.30	0.36	0.38	0.24	0.03	*0.57*	*0.05*	*0.20*
	TZD	0.30	0.34	0.35	0.34				
C16:0	**CTR**	25.9	26.1	27.6	29.2	1.11	*0.56*	*<0.01*	*0.76*
	TZD	25.9	25.4	25.9	29.7				
C16:1cis9	**CTR**	0.53	0.56	0.52	0.38	0.03	*0.05*	*<0.01*	*0.42*
	TZD	0.53	0.60	0.55	0.48				
C16:1cis?	**CTR**	0.53	0.68	0.58	0.58	0.05	*0.73*	*0.13*	*0.98*
	TZD	0.53	0.67	0.58	0.54				
C16:1trans?	**CTR**	0.66	0.70	0.59	0.40	0.03	*<0.01*	*<0.01*	*0.34*
	TZD	0.66	0.75	0.70	0.50				
C17:0anteiso	**CTR**	0.32	0.36	0.34	0.32	0.02	*0.76*	*0.10*	*0.84*
	TZD	0.32	0.34	0.37	0.32				

C17:1cis12	**CTR**	0.34	0.44	0.35	0.25	0.04	*0.99*	*<0.01*	*0.92*
	TZD	0.34	0.44	0.38	0.22				
C18:0	**CTR**	12.6	12.1	9.62	8.01	0.75	*0.06*	*<0.01*	*0.72*
	TZD	12.6	13.8	11.0	9.18				
C18:0iso	**CTR**	0.95	1.06	0.95	0.77	0.04	*0.55*	*<0.01*	*0.52*
	TZD	0.95	1.01	0.99	0.85				
C18:1cis?	**CTR**	0.14	0.13	0.15	0.17	0.02	*0.19*	*0.54*	*0.21*
	TZD	0.14	0.14	0.11	0.13				
C18:1cis9	**CTR**	21.1	23.8	18.7	17.8	1.32	*0.67*	*<0.01*	*0.51*
	TZD	21.1	22.5	20.4	15.7				
C18:1cis11	**CTR**	0.51	0.57	0.47	0.47	0.04	*0.51*	*0.01*	*0.48*
	TZD	0.51	0.58	0.48	0.37				
C18:1cis12	**CTR**	0.12	0.13	0.10	0.15	0.15	*<0.01*	*0.07*	*0.23*
	TZD	0.12	0.10	0.07	0.08				
C18:1cis14	**CTR**	0.25	0.22	0.20	0.28	0.03	*0.06*	*0.03*	*0.37*
	TZD	0.25	0.22	0.14	0.20				
C18:1tr11	**CTR**	0.25	0.29	0.26	0.23	0.02	*0.58*	*0.03*	*0.76*
	TZD	0.25	0.33	0.24	0.25				
C18:1tr12	**CTR**	1.65	1.43	1.41	1.11	0.16	*0.11*	*0.18*	*0.69*
	TZD	1.65	1.66	1.58	1.49				
C18:1tr13	**CTR**	0.14	0.16	0.12	0.16	0.03	*0.53*	*0.68*	*0.90*
	TZD	0.14	0.13	0.12	0.15				
C18:1tr16	**CTR**	0.13	0.12	0.11	0.03	0.01	*0.22*	*<0.01*	*0.08*
	TZD	0.13	0.12	0.10	0.08				
C18:2cis9cis12	**CTR**	2.05	2.17	1.95	1.67	0.13	*0.65*	*<0.01*	*0.95*
	TZD	2.05	2.03	1.93	1.65				
C18:2cis9tr12	**CTR**	0.10	0.09	0.12	0.12	0.02	*0.88*	*0.63*	*0.94*
	TZD	0.10	0.10	0.11	0.10				
C18:3n3	**CTR**	0.85	0.89	0.92	0.77	0.08	*0.68*	*0.09*	*0.87*
	TZD	0.85	0.91	0.91	0.66				
C18:3tr9cis12cis15	**CTR**	0.07	0.07	0.03	0.04	0.01	*0.04*	*<0.01*	*0.49*
	TZD	0.07	0.10	0.06	0.05				
C18:3CLA	**CTR**	0.63	0.49	0.51	0.58	0.10	*0.45*	*0.23*	*0.92*
	TZD	0.63	0.35	0.46	0.56				
C18:3tr9tr2tr5	**CTR**	0.30	0.27	0.24	0.21	0.02	*<0.01*	*<0.01*	*0.25*
	TZD	0.30	0.30	0.28	0.28				
C19:1	**CTR**	0.12	0.14	0.16	0.14	0.01	*0.03*	*0.40*	*0.57*
	TZD	0.12	0.11	0.12	0.11				
C20:2tr	**CTR**	0.09	0.18	0.12	0.11	0.03	*0.33*	*0.07*	*0.86*
	TZD	0.09	0.14	0.12	0.08				

C20:4n6	**CTR**	0.15	0.16	0.15	0.12	0.01	*0.25*	*0.30*	*0.07*
	TZD	0.15	0.14	0.17	0.16				
C20:5n3	**CTR**	0.15	0.10	0.16	0.12	0.02	*0.15*	*0.14*	*0.54*
	TZD	0.15	0.08	0.10	0.09				
C22:0	**CTR**	0.11	0.10	0.10	0.09	0.01	*<0.01*	*0.93*	*0.25*
	TZD	0.11	0.13	0.12	0.13				
C22:5n3	**CTR**	0.45	0.30	0.37	0.42	0.06	*0.56*	*0.34*	*0.89*
	TZD	0.45	0.37	0.43	0.40				
C26:0	**CTR**	0.08	0.11	0.12	0.10	0.02	*0.51*	*0.55*	*0.87*
	TZD	0.08	0.11	0.09	0.09				
Denovo	**CTR**	40.8	38.8	45.5	48.3	1.60	*0.57*	*<0.01*	*0.83*
	TZD	40.8	38.1	43.2	48.7				
Preformed	**CTR**	59.2	61.2	54.4	51.7	1.61	*0.57*	*<0.01*	*0.83*
	TZD	59.2	61.9	56.8	51.3				
Saturated	**CTR**	66.0	63.8	68.9	71.2	1.69	*0.98*	*<0.01*	*0.75*
	TZD	66.0	63.3	66.9	72.8				
Unsaturated	**CTR**	33.8	36.0	31.0	28.3	1.70	*0.95*	*<0.01*	*0.79*
	TZD	33.8	35.6	33.0	27.1				
Δ9 C14[1]	**CTR**	0.46	0.53	0.41	0.39	0.04	*0.02*	*0.02*	*0.32*
	TZD	0.46	0.61	0.57	0.45				
Δ9 C16[1]	**CTR**	0.02	0.02	0.02	0.01	0.002	*0.42*	*<0.01*	*0.69*
	TZD	0.02	0.02	0.02	0.02				
Δ9 C18[1]	**CTR**	0.63	0.66	0.66	0.69	0.02	*0.05*	*0.09*	*0.42*
	TZD	0.63	0.62	0.65	0.64				
Δ9 C18 tr12[1]	**CTR**	0.44	0.39	0.57	0.40	0.05	*0.39*	*0.02*	*0.56*
	TZD	0.44	0.44	0.54	0.49				
Δ9 desaturation[1]	**CTR**	0.31	0.34	0.29	0.27	0.02	*0.67*	*<0.01*	*0.48*
	TZD	0.31	0.32	0.31	0.25				

[1]Delta 9 desaturase indexes were calculated as follow:
Δ9 C14 = cis9 C14:1/(C14:0 + cis9 C14:1)
Δ9 C16 = cis9 C16:1/(C16:0 + cis9 C16:1)
Δ9 C18 = cis9 C18:1/(C18:0 + cis9 C18:1)
Δ9 C18:1 = cis9 trans12 C18:2/(trans12 C18:1 + cis9 trans12 C18:2)
Δ9 desaturation = sum of C14, C16, C18, trans12 C18:1 cis9/(sum of C14:0, C16:0, C18:0, trans12 C18:1 + sum of C14, C16, C18, trans12 C18:1 cis9)

Table S6. Grams/milking of each fatty acid in milk of goats receiving a daily intrajugular injection of 2,4-thiazolidinedione (TZD) or saline (CTR).

Fatty Acid	Treatment	Day of TZD injection				SEM	*P-value*		
		0	**2**	**5**	**15**		***TZD***	***Time***	***Z × T***
C4:0	**CTR**	1.38	1.23	1.13	1.71	0.19	*0.36*	*0.14*	*0.65*
	TZD	1.38	0.96	1.22	1.37				
C6:0	**CTR**	1.70	1.55	1.43	1.90	0.22	*0.27*	*0.30*	*0.84*
	TZD	1.70	1.30	1.35	1.54				
C8:0	**CTR**	1.79	1.66	1.62	2.08	0.25	*0.37*	*0.41*	*0.93*
	TZD	1.79	1.45	1.49	1.76				
C10:0	**CTR**	6.77	6.23	6.59	8.54	0.94	*0.50*	*0.17*	*0.96*
	TZD	6.77	5.90	5.98	7.62				
C12:0	**CTR**	3.25	3.29	3.36	4.23	0.45	*0.48*	*0.19*	*0.95*
	TZD	3.25	3.17	2.93	3.84				
C13:1	**CTR**	0.07	0.07	0.06	0.04	0.02	*0.52*	*0.29*	*0.61*
	TZD	0.08	0.04	0.04	0.05				
C14:0	**CTR**	10.8	11.2	10.7	12.6	1.27	*0.42*	*0.57*	*0.91*
	TZD	10.8	11.0	9.5	11.1				
C14:1cis7	**CTR**	0.25	0.26	0.21	0.20	0.03	*0.90*	*0.21*	*0.99*
	TZD	0.25	0.26	0.21	0.20				
C14:1cis9	**CTR**	0.38	0.35	0.36	0.37	0.06	*0.95*	*0.83*	*0.97*
	TZD	0.38	0.34	0.34	0.40				
C14:1??	**CTR**	0.08	0.06	0.08	0.11	0.03	*0.91*	*0.76*	*0.31*
	TZD	0.08	0.13	0.06	0.07				
C15:0	**CTR**	0.96	1.09	0.93	1.02	0.11	*0.82*	*0.43*	*0.99*
	TZD	0.96	1.13	0.92	1.07				
C15:1	**CTR**	0.37	0.44	0.39	0.34	0.05	*0.20*	*0.93*	*0.54*
	TZD	0.37	0.30	0.34	0.34				
C16:0	**CTR**	19.8	20.5	18.1	22.4	2.07	*0.35*	*0.33*	*0.88*
	TZD	19.8	19.8	16.3	19.1				
C16:1cis9	**CTR**	0.58	0.69	0.53	0.50	0.07	*0.46*	*0.11*	*0.96*
	TZD	0.58	0.61	0.50	0.47				
C16:1cis?	**CTR**	0.62	0.48	0.61	0.68	0.10	*0.75*	*0.88*	*0.68*
	TZD	0.62	0.59	0.55	0.53				
C16:1trans?	**CTR**	0.71	0.77	0.61	0.55	0.07	*0.82*	*0.03*	*0.99*
	TZD	0.71	0.75	0.61	0.52				
C17:0	**CTR**	0.32	0.37	0.32	0.33	0.08	*0.94*	*<0.01*	*0.65*
	TZD	0.32	0.38	0.34	0.29				
C17:0anteiso	**CTR**	0.40	0.44	0.39	0.42	0.04	*0.39*	*0.65*	*0.92*
	TZD	0.40	0.42	0.37	0.36				
C17:1cis12	**CTR**	0.56	0.67	0.55	0.52	0.07	*0.31*	*0.13*	*0.83*
	TZD	0.56	0.61	0.52	0.39				

C17:1cis10	**CTR**	0.19	0.26	0.18	0.04	0.04	*0.37*	*<0.01*	*0.86*
	TZD	0.20	0.25	0.23	0.10				
C18:0	**CTR**	12.8	12.8	9.8	10.1	1.52	*0.52*	*0.03*	*0.91*
	TZD	12.8	12.7	9.0	8.1				
C18:0iso	**CTR**	1.13	1.24	1.04	1.04	0.13	*0.34*	*0.31*	*0.92*
	TZD	1.13	1.13	0.97	0.87				
C18:1cis9	**CTR**	19.1	21.5	16.3	17.8	2.09	*0.13*	*0.06*	*0.60*
	TZD	19.1	18.7	14.9	12.3				
C18:1cis11	**CTR**	0.67	0.45	0.63	0.67	0.09	*0.75*	*0.46*	*0.62*
	TZD	0.67	0.56	0.57	0.52				
C18:1cis12	**CTR**	0.32	0.35	0.28	0.37	0.03	*0.27*	*0.28*	*0.63*
	TZD	0.32	0.33	0.27	0.29				
C18:1cis14	**CTR**	0.44	0.44	0.41	0.48	0.04	*0.15*	*0.36*	*0.71*
	TZD	0.44	0.42	0.33	0.38				
C18:1*cis*isomer	**CTR**	0.33	0.20	0.33	0.36	0.04	*0.88*	*0.17*	*0.56*
	TZD	0.33	0.27	0.29	0.31				
C18:1tr11	**CTR**	0.43	0.49	0.42	0.44	0.04	*0.74*	*0.13*	*0.71*
	TZD	0.43	0.54	0.38	0.39				
C18:1tr12	**CTR**	1.56	1.15	1.28	1.26	0.23	*0.75*	*0.50*	*0.92*
	TZD	1.56	1.43	1.28	1.20				
C18:1tr13	**CTR**	0.30	0.37	0.29	0.33	0.04	*0.39*	*0.82*	*0.81*
	TZD	0.30	0.28	0.28	0.31				
C18:1tr16	**CTR**	0.30	0.31	0.26	0.08	0.04	*0.14*	*<0.01*	*0.17*
	TZD	0.30	0.31	0.27	0.24				
C18:2cis9cis12	**CTR**	2.25	2.40	2.04	2.08	0.22	*0.11*	*0.16*	*0.79*
	TZD	2.25	2.08	1.74	1.64				
C18:2cis9tr12	**CTR**	0.27	0.28	0.28	0.29	0.03	*0.89*	*0.96*	*0.95*
	TZD	0.27	0.29	0.27	0.27				
C18:3n3	**CTR**	1.03	1.09	1.01	1.01	0.12	*0.36*	*0.76*	*0.93*
	TZD	1.03	1.02	0.92	0.86				
C18:3tr9cis12cis15	**CTR**	0.19	0.22	0.10	0.15	0.05	*0.34*	*0.09*	*0.80*
	TZD	0.19	0.31	0.15	0.15				
C18:3tr9tr2tr15	**CTR**	0.43	0.44	0.38	0.34	0.04	*0.50*	*0.23*	*0.91*
	TZD	0.43	0.47	0.38	0.40				
C18:3CLA	**CTR**	0.61	0.59	0.54	0.64	0.07	*0.19*	*0.42*	*0.77*
	TZD	0.61	0.45	0.51	0.55				
C19:1	**CTR**	0.30	0.33	0.32	0.33	0.03	*0.67*	*0.64*	*0.80*
	TZD	0.30	0.36	0.30	0.29				
C20:2tr	**CTR**	0.27	0.35	0.29	0.29	0.05	*0.53*	*0.17*	*0.83*
	TZD	0.27	0.37	0.23	0.24				
C20:4n6	**CTR**	0.33	0.36	0.33	0.29	0.03	*0.70*	*0.72*	*0.86*
	TZD	0.33	0.35	0.33	0.33				
C20:5n3	**CTR**	0.36	0.35	0.37	0.32	0.05	*0.80*	*0.90*	*0.92*

	TZD	0.36	0.36	0.32	0.33				
C22:0	**CTR**	0.29	0.31	0.29	0.22	0.04	*0.10*	*0.54*	*0.54*
	TZD	0.29	0.37	0.31	0.33				
C22:5n3	**CTR**	0.62	0.54	0.53	0.77	0.09	*0.50*	*0.52*	*0.47*
	TZD	0.62	0.58	0.54	0.54				
C26:0	**CTR**	0.17	0.26	0.24	0.55	0.10	*0.18*	*0.35*	*0.19*
	TZD	0.19	0.27	0.18	0.16				
De novo	**CTR**	37.3	37.0	35.5	43.8	4.30	*0.40*	*0.40*	*0.93*
	TZD	37.3	35.4	32.0	38.2				
Preformed	**CTR**	58.4	61.6	50.6	55.2	5.96	*0.29*	*0.16*	*0.82*
	TZD	58.4	58.2	46.5	44.0				
Saturated	**CTR**	59.8	60.0	54.3	65.4	6.58	*0.40*	*0.49*	*0.90*
	TZD	59.8	58.1	49.1	56.0				
Unsaturated	**CTR**	35.8	38.4	31.5	33.1	3.67	*0.25*	*0.15*	*0.81*
	TZD	35.8	35.3	29.2	26.1				
Δ9 C14	**CTR**	0.034	0.029	0.031	0.024	0.003	*0.11*	*0.43*	*0.29*
	TZD	0.034	0.029	0.036	0.035				
Δ9 C16	**CTR**	0.028	0.033	0.028	0.020	0.002	*0.46*	*<0.01*	*0.47*
	TZD	0.028	0.031	0.031	0.024				
Δ9 C18	**CTR**	0.61	0.64	0.64	0.67	0.02	*0.04*	*0.10*	*0.39*
	TZD	0.61	0.60	0.63	0.62				
Δ9 C18:1	**CTR**	0.16	0.29	0.21	0.23	0.03	*0.27*	*0.08*	*0.83*
	TZD	0.16	0.23	0.20	0.19				
Δ9 desaturation	**CTR**	0.31	0.34	0.29	0.28	0.02	*0.46*	*<0.01*	*0.35*
	TZD	0.31	0.31	0.31	0.26				

APPENDIX 2 PARAMETERS AND PROTOCOLS

Table 2.1 parameters measured in the first paper of Se experiment.

Samples	Parameters/Unit	Methods	Significant
Various tissues	Se, Zn, Co, Cu ng/ml	Elemental X-series 2 Inductively coupled (ICP-MS) plasma mass spectrometry with a collision cell	Level of Se and other microminerals
Erythrocyte, plasma, milk	GPx activity nmol/min/ml	Glutathione peroxidase assay kit- The GPX activity is rate-limiting the rate of decrease in absorbance at 340 nm.	Index of antioxidative status

Table 2.2 Parameters measured in the second paper of Se experiment.

Tissue	[1]Parameters/Unit	Methods	Significant
Milk	Fatty acid profile	Gas chromatography	Determination of de novo and diet-derived fatty acids in milk.
Skin	CST	Subcutaneous injection of sterile carrageenan skin thickness measured by a skinfold caliper	Assessment of in vivo neutrophils migration to the site of infection
Inflammatory and metabolic profile assay			
Metabolic parameters			
Plasma	NEFA mmol/l	The ILAB 650 automated analyzer (Instrumentation Laboratory) Enzymatic-colorimetric WAKO kit	A marker of negative energy balance (NEB)
Plasma	BHBA mmol/l	Kit Randox (enzyme kinetic) The enzyme hydroxy-butyrate-dehydrogenase (HBDH) catalyzes the oxidation of hydroxybutyrate to acetate.	Secondary marker of negative energy balance, produced by the rumen and liver. Marker of incomplete oxidation of NEFA by the liver
Plasma	Glucose mmol/l	The ILAB 650 automated analyzer enzymatic determination kit with oxidase [Glucose Oxidase (GOD) and Peroxidase (POD)]	Glucose level is highly regulated, and slight changes are mostly due to large uptake (e.g., mammary gland) or large production (e.g., liver). Differences might be seeing when animals have insulin resistance
Plasma	Cholesterol mmol/l	The ILAB 650 automated analyzer, kit (enzymatic-colorimetric). The method is based on the cleavage of cholesterol esters present in the plasma in cholesterol and fatty acids due to cholesterol esterase (CHOE)	A marker of lipid dietary content (when high) and liver activity (when low)
Plasma	Urea mmol/l	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). Kit for enzymatic determination by urease and glutamate-dehydrogenase (GLDH).	A marker of kidney and liver health. Increased plasma urea can be caused by increased urea production, decreased urea elimination, or a combination of the two

Inflammatory parameters			
Plasma	Ceruloplasmin µmol/l	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). It is based on the coloring that originates from the oxidation of the paraphenylenediamine garden, operated by ceruloplasmin.	Positive acute-phase proteins, produced from the liver. It exhibits a copper-dependent oxidase activity, which is associated with possible oxidation of Fe^{2+} (ferrous iron) into Fe^{3+} (ferric iron), therefore assisting in its transport in the plasma in association with transferrin, which can carry iron only in the ferric state. Oxidizes iron, facilitating for ferritin, inhibiting microbe iron intake.
Plasma	Haptoglobin g/l	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). The determination is carried out by a colorimetric test by using reagent (methemoglobin, physiological solution, distilled H2O, guaiacol, and H2O2).	Positive acute-phase proteins, produced in a liver. Binds hemoglobin, inhibiting microbe iron uptake, and prevents kidney damage.
Plasma	Myeloperoxidase U/L	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). The determination of myeloperoxidase (MPO) was performed by adapting the colorimetric method of Bradley et al. (1982) to the ILAB 650 automatic analyzer.	Effective in killing various microorganisms. Polymorphonuclear leukocytes (PMN) are the main source of this enzyme.
Plasma	Albumin g/l	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). The determination of the albumins is carried out by exploiting the color variation due to the formation of a complex between the albumins and the bromocresol green (IL kit).	A negative acute-phase proteins produced from a liver. High concentration in plasma an indicator of liver activity. Regulating osmotic pressure and serving as a carrier for substances in plasma. Albumin's main function also antioxidant properties.
Plasma	Paraoxonase U/ml	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). The method is of the kinetic-photometric type and is based on the measurement of the hydrolysis of paraoxon by arylialkyl phosphatase, also called paraoxonase, with the formation of p-nitrophenol.	Anti-inflammatory, anti-oxidative, anti-atherogenic, anti-diabetic, anti-microbial, and organophosphate-hydrolyzing properties. Marker of liver activity
Liver health			
Plasma	AST/GOT U/L	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). The IL kit, of enzymatic type, uses aspartate aminotransferase and malate dehydrogenase	Enzymes found mainly in the liver, but also found in red blood cells, heart cells, muscle tissue and other organs, respectively. Its levels are a valuable aid primarily in the diagnosis of liver disease.

Plasma	GGT U/L	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). The method is based on the reaction, catalyzed by γ - glutamyl-transferase, between L- γ - glutamyl-3-carboxy-4-nitroanilide and glycylglycine.	Elevated levels may be due to liver diseases, such as hepatitis or cirrhosis
Plasma	ALP U/L	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). IL (enzymatic) nitrophenol kit. Alkaline phosphatase catalyzes the dephosphorylation of p-nitrophenyl phosphate (pNPP) into p-nitrophenol which absorbs light at 405 nm.	The highest concentrations of ALP are present in the cells that comprise bone and the liver. Elevated levels of ALP in the blood are most commonly caused by liver disease or bone disorders.
Plasma	Bilirubin µmol/l	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). kit for colorimetric determination with diazotization. The total bilirubin present in the sample is directly proportional to the amount of azobilirubin that is formed following the reaction, in the presence of lithium dodecyl sulfate, between the same total bilirubin and diazotized sulphanilic acid.	An orange-yellow pigment formed in the liver by the breakdown of hemoglobin and excreted in bile. Elevated levels may indicate liver damage or disease. Index of liver function as clearance of bilirubin is a function of the liver
Oxidative status			
Plasma	ROMt mg H_2O_2/100 ml	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). The reactive oxygen metabolites are determined using a colorimetric kit (DIACRON, Grosseto). ROMs are a variety of free radicals characterized by extreme chemical reactivity that form, in plasma and cells, derivatives that are just as reactive. The principle of determining total ROMs consists of a spectrophotometric measurement at 510 nm of a colored complex that is formed following the reaction of these derivatives with a suitably buffered chromogen.	A marker of oxidative stress
Plasma	SHp µmol/l	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). Diacron kit (colorimetric). The principle of the test is based on the formation of a colored complex, as a product of the reaction between sulfhydryl groups and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) in a special buffer solution (pH 7.6)	Antioxidant function→ reduced function
Plasma	FRAP µmol/l	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). The determination of FRAP (ferric reducing antioxidant power) was performed by adapting the colorimetric method of Benzie and Strain (1999) to the automatic analyzer	A marker of plasma antioxidant activity

		ILAB 650 (Instrumentation Laboratory, Lexington, MA).	
Plasma	AOPP µmol/l	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). The AOPPs were determined according to the method of Hanasand et al. (2012) adapted to the ILAB650 automatic analyzer (Instrumentation Laboratory, Lexington, MA) by using citric acid.	A marker of protein oxidation. It can trigger the oxidative burst and the synthesis of inflammatory cytokines in neutrophils and monocytes. It can be also a final product of antioxidant function of albumin.
Minerals and others			
Plasma	Ca mmol/l	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). IL kit. The colorimetric method is based on the formation of a colored complex deriving from the reaction in an alkaline environment between calcium and cresophthalin complexone.	It is an index of Ca dietary intake but most importantly of Ca metabolism and regulation. A marker of metabolic disorder especially in high producing dairy cows that high susceptible to milk fever.
Plasma	Mg mmol/l	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). IL kit. Enzymatic kinetic analysis in which the Magnesium (Mg) contained in the sample reacts with adenosine 5 'triphosphate (ATP) to form the Mg-ATP complex that converts glucose into glucose-6-phosphate (G-6-P) in the presence of glucokinase (GlcK) and forms the Mg-ADP complex.	Index of Mg intake. Low Mg can be problematic, such as tetany. It important for the efficient absorption and resorption of calcium to decrease milk fever.
Plasma	Creatinine µmol/l	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). Kit IL (colorimetric). The method is based on the reaction between picric acid and creatinine in an alkaline environment. The amount of colored complex formed, with maximum absorption at 510 nm, is related to the concentration of creatinine	A breakdown product of creatine phosphate in muscle, and is usually produced at a fairly constant rate by the body→ excreted in urine. A marker of healthy kidney and a marker of muscle mass
Plasma	Total protein g/l	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). The colorimetric method is based on the reaction in a strongly alkaline solution between polypeptides containing at least two peptide bonds and biuret and copper tartrate. The product of the reaction is a stable complex of violet color which absorbs light at 550 nm.	Used to calculate globulin
Plasma	Globulin g/l	The ILAB 650 automated analyzer (Instrumentation Laboratory, Lexington, MA). globulins are not the result of an analysis but the result of a calculation. The value is obtained by deducting the value in albumins from the total proteins.	Transport proteins. Has vital role in natural and acquired immunity to infection. Inhibition of an enzyme that digests protein.

[1] CST= Carrageenan skin test; NEFA= Non-esterified fatty acids; BHBA: β- hydroxyl butyric acid; AST: Aspartate aminotransferase; GGT: γ-glutamyl transferase; ALP: Alkaline phosphatase; ROMt: Total reactive oxygen metabolites; SHp: Thiolic groups; FRAP: Ferric reducing antioxidant power; AOPP: Advanced oxidation protein products

Non-esterified fatty acids (NEFA in mmol / l):

Enzymatic-colorimetric WAKO kit. From the reaction, catalyzed by Acyl-CoA synthase (ACS), between non-esterified plasma fatty acids and CoenzymeA (CoA), in the presence of adenosine triphosphate (ATP) and magnesium ions, adenosine monophosphate, and pyrophosphate, Acyl-CoA is released. This is then oxidized by acyl-CoA oxidase (ACOD) with release of hydrogen peroxide, which, in the presence of peroxidase (POD), favors the condensation between 3-methyl-N-ethyl-N- (β-hydroxyethyl) -aniline (MEHA) and 4-aminoantipyrine to form a colored complex with maximum absorbance at 546 nm.

β-hydroxybutyrate (βOHB mmol / l):

Kit Randox (enzyme kinetic). The enzyme hydroxy-butyrate-dehydrogenase (HBDH) catalyzes the oxidation of-hydroxy butyrate to acetate acetate. The consequent reduction of NAD to NADH leads to an increase in absorbance at 340 nm.

Glucose (mmol / l):

Enzymatic determination kit with oxidase [Glucose Oxidase (GOD) and Peroxidase (POD)]. The action of glucose oxidase catalyzes the oxidation of glucose to gluconic acid and H2O2. Peroxidase catalyzes the reaction of 2 molecules of H2O2 with one of phenol and one of 4-aminoantipyrine to quinoneimina (red dye) and 4 molecules of H2O. The formation of the dye and the consequent increase in absorbance recorded at 510 nm is proportional to the concentration of glucose present in the sample.

Total cholesterol (mmol / l):

IL kit (enzymatic-colorimetric). The method is based on the cleavage of cholesterol esters present in the plasma in cholesterol and fatty acids due to cholesterol esterase (CHOE). From the reaction, catalyzed by cholesterol oxidase (CHOD) between free cholesterol and oxygen in the air, colest-4-en-3-one and hydrogen peroxide are formed. The hydrogen peroxide, in a peroxidase-catalyzed reaction (POD), reacts with 4-aminoantipyrine (4-AAP) and phenol to give water and quinoneimin, a colored compound detectable at 510 nm.

Urea (mmol / l):

IL kit for enzymatic determination by urease and glutamate-dehydrogenase (GLDH). Ammonia originating from the hydrolysis of urea by the action of urease is used by GLDH in the conversion of α-oxoglutarate to L-glutamate. In this reaction, the amount of NADH that is oxidized, measured in terms of absorbance reduction at 340 nm, is proportional to the concentration of urea.

Ceruloplasmin (mol / l):

Based on the coloring that originates from the oxidation of the paraphenylenediamine garden, operated by ceruloplasmin.

Haptoglobin (g / l):

The determination is carried out by a colorimetric test by using reagent (methemoglobin, physiological solution, distilled H2O, guaiacol, and H2O2). The change in absorbance at 450 nm is proportional to the concentration of haptoglobin present in the sample.

Myeloperoxidase (U / L):

The determination of myeloperoxidase (MPO) was performed by adapting the colorimetric method of Bradley et al. (1982) to the ILAB 650 automatic analyzer. The method is based on the reaction of the myeloperoxidase contained in the plasma sample with the hydrogen peroxide with the formation of H2O and O-. O-dianisidine dihydrochloride, an H donor, reacts with O-liberating H2O and a colored compound.

Albumins (g / l):

the determination of the albumins is carried out by exploiting the color variation due to the formation of a complex between the albumins and the bromocresol green (IL kit).

Paraoxonase (U / ml):

Kinetic-photometric type and is based on the measurement of the hydrolysis of paraoxon by arylialkyl phosphatase also called paraoxonase, with the formation of p-nitrophenol. For the correction of the results, 3 known value standards are used: SeraChem Control Level 1 (Instrumentation Laboratory, Milan, Italy), Randox (Randox Laboratories LTD, UK) and bovine plasma pool.

Aspartate-amino-transferase (AST / GOT) (U / l):

The IL kit, of enzymatic type, uses aspartate aminotransferase and malate dehydrogenase. Therefore, ultimately, to AST.

Gamma-Glutamyl transferase (GGT) (U / l):

Kinetic analysis, according to the Szazs / Persijn method. The method is based on the reaction, catalyzed by γ-glutamyl-transferase, between L- γ -glutamyl-3-carboxy-4-nitroanilide and glycylglycine. The 5-amino-2-nitrobenzoate, colored product of the

reaction, determines a color change read at 405 nm. The speed with which the intensity of staining increases is proportional to the amount of γ -glutamyl transferase present in the plasma.

Alkaline phosphatase (U / l):

IL (enzymatic) nitrofenol kit. Alkaline phosphatase catalyzes the dephosphorylation of p-nitrophenyl phosphate (pNPP) into p-nitrophenol which absorbs light at 405 nm.

Total bilirubin (µmol / l):

kit for colorimetric determination with diazotation. The total bilirubin present in the sample is directly proportional to the amount of azobilirubin that is formed following the reaction, in the presence of lithium dodecyl sulfate, between the same total bilirubin and diazotized sulphanilic acid.

Total ROMs (mg H2O2 / 100 ml):

The reactive oxygen metabolites are determined using a colorimetric kit (DIACRON, Grosseto). ROMs are a variety of free radicals characterized by extreme chemical reactivity that form, in plasma and cells, derivatives that are just as reactive. The principle of determining total ROMs consists of a spectrophotometric measurement at 510 nm of a colored complex that is formed following the reaction of these derivatives with a suitably buffered chromogen.

Thiol groups (SHp) (µmol / l):

Diacron kit (colorimetric). The principle of the test is based on the formation of a colored complex, as a product of the reaction between sulfhydryl groups and 5,5-dithiobis-

2-nitrobenzoic acid (DTNB) in a special buffer solution (pH 7.6). The intensity of the instrumentally detected color is directly proportional to the concentration of the thiol groups.

FRAP (µm / l TE): The determination of FRAP (ferric reducing antioxidant power) was performed by adapting the colorimetric method of Benzie and Strain (1999) to the automatic analyzer ILAB 650 (Instrumentation Laboratory, Lexington, MA). Automated test measuring the ferric reducing ability of plasma. Ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration. Absorbance changes are linear over a wide concentration range with antioxidant mixtures, including plasma, and with solutions containing one antioxidant in purified form.

AOPP (Advanced Oxidation Protein Products) (µmol / l):

The AOPPs were determined according to the method of Hanasand et al. (2012) adapted to the ILAB650 automatic analyzer (Instrumentation Laboratory, Lexington, MA). Plasma was diluted with citric acid, and AOPP measured as absorbance at 340 nm.

Calcium (mmol / l):

IL kit. The colorimetric method is based on the formation of a colored complex deriving from the reaction in an alkaline environment between calcium and cresophthalin complexone. The intensity of the color produced is proportional to the amount of calcium present in the sample. Magnesium interferences are prevented by the selective complexing capacity of 8-oxyquinoline.

Magnesium (mmol / l):

IL kit. Enzymatic kinetic analysis in which the Magnesium (Mg) contained in the sample reacts with adenosine 5 'triphosphate (ATP) to form the Mg-ATP complex that converts glucose into glucose-6-phosphate (G-6-P) in the presence of glucokinase (GlcK) and forms the Mg-ADP complex. The Mg ions dissociating from the Mg-ADP complex again form the Mg-ATP complex that is used repeatedly for the reaction. The reaction kinetics depends on the concentration of Mg in the sample. At the same time, G-6-P reacts with nicotinamine adenine dinucleotide phosphate (NADP) in the presence of glucose 6 phosphate dehydrogenase (G-6-PDH) and 6-phosphogluconic acid and NADPH. The rate of increase in absorbance due to the formation of NADHP is directly proportional to the concentration of Mg in the sample.

Creatinine (µmol / l):

Kit IL (colorimetric). The method is based on the reaction between picric acid and creatinine in an alkaline environment. The amount of colored complex formed, with maximum absorption at 510 nm, is related to the concentration of creatinine.

Total proteins (g / l):

IL kit. The colorimetric method is based on the reaction in a strongly alkaline solution between polypeptides containing at least two peptide bonds and biuret and copper tartrate. The product of the reaction is a stable complex of violet color which absorbs light at 550 nm.

Globulins (g / l):

globulins are not the result of an analysis but the result of a calculation. The value is obtained by deducting the value in albumins from the total proteins.

APPENDIX 2 ABSTRACTS, PPRESENTATIONS AND POSTERS

2018 American Dairy Science Association (ADSA) Annual Meeting Abstract

EFFECT OF 2,4-THIAZOLDINEDIONE TREATMENT ON MILK FAT SYNTHESIS IN LACTATING DAIRY GOATS FED ADEQUATE VITAMINS SUPPLEMENTATION

Shana Jaaf, Fernanda Rosa, Misagh Moridi , Johan Osorio, Erminio Trevisi, Shelby Filley, Gita Cherian, and Massimo Bionaz

In vitro studies support the role of Peroxisome Proliferator-activated Receptor gamma (PPARγ) in the regulation of milk fat synthesis in goats and cows. This has not been proved in an *in vivo* study. In a prior *in vivo* experiment, daily injection of the putative PPARγ agonist 2,4-thiazolidinedione (TZD) in dairy goats did not affect milk fat or expression of milk-fat related genes. The lack of response was explained by a potential deficiency of vitamin A and/or a less than adequate body condition of the animals. In the present experiment, we tested the hypothesis that PPARγ activation affects milk fat synthesis in goats with a good body condition and receiving adequate level of vitamin A. Saanen multiparous goats in early-mid lactation received a diet that met NRC requirements, including vitamin A, and were injected daily with 8 mg/kg BW of TZD (n=6) or saline (n=6; CTRL) for 3 weeks. Blood samples were collected to measure 20 parameters related to metabolism and inflammation, milk yield, milk components including fatty acid profile, expression of fatty acid metabolism-related genes via RTqPCR in adipose tissue and mammary epithelial cells (MEC) isolated from milk, and adipocytes size by histological analysis. Data were analyzed by GLIMMIX of SAS with treatment (TZD) and Time and

TZD×Time interaction as main effects and goat as random effect. The NEFA, NEFA/Albumin, BHBA, and fatty acids available in plasma were significantly decreased ($P<0.05$) while glucose significantly increased ($P<0.05$) in TZD vs. CTR. SCC was higher in TZD vs. CTR goats, but milk fat % was not affected. In adipose tissue, TZD vs. CTR increased the expression of *SLC2A4* and genes related to de novo fatty acid synthesis such as, *SREBF1* ($P=0.06$), *GAPDH* ($P\leq0.02$) and *FASN* ($P=0.06$) while decreased the expression of *YWHAZ* ($P\leq0.04$), that plays, among other, an anti-insulin signaling role,. In MEC we only detected a lower expression of the delta 9 desaturases *SCD1* ($P=0.06$) in TZD vs. CTR. Very few fatty acids in the milk were affected by TZD treatment with an increase in proportion of long and very long-chain fatty acids of dietary origin in TZD vs. CTR, such as C16:t1, C18:0, several 18:1 isomers, all-trans C18:3, and C22:0 ($P<0.05$). No other effects were observed. Results confirmed a mild-to-no effect of TZD on milk fat synthesis and a lack of any transcriptomic effect in MEC but an increase in de-novo related genes in adipose tissue; however, milk fat production was maintained despite a lower availability of NEFA.

Keyword: PPARγ; TZD; milk fat synthesis; NEFA

2018 American Dairy Science Association (ADSA) Annual Meeting Abstract

EFFECT OF SELENIUM-ENRICHED HAY ON SELENIUM CONCENTRATION IN BLOOD AND MILK, IMMUNE FUNCTION, AND PERFORMANCE IN DAIRY COWS DURING TRANSITION PERIOD

Shana Jaaf, Matteo Mezzetti, Angela Krueger, Brandon Batty, Jennifer Belveal, Michele Premi, Janelle Foster, Erminio Trevisi, Gerd Bobe, Charles Estill, and Massimo Bionaz

Dairy cows during the transition from pregnancy to lactation are more subjected to diseases due to metabolic, oxidative, and inflammatory stresses and immune suppression. Se is trace minerals that play important roles in anti-oxidative response and immune function in dairy cows. Thus, feeding organic Se to dairy cows during pregnancy can improve their immune system and the amount of Se in milk. To test the hypothesis, we used 12 primiparous cows (6 Holsteins and 6 Jerseys) fed ad libitum with a TMR based on grass silage (0.14 mg Se/kg DM). Cows were homogeneously divided into two groups to be supplemented from 40 days prior parturition to 2 weeks post-partum with alfalfa hay in reason of 1 kg/100 kg of BW mixed with the TMR. TRT group was supplemented with alfalfa enriched with Se (3.2 mg/kg DM), and the CTR group received alfalfa with low Se (0.4 mg/kg DM). Heifers were monitored daily for health status, dry matter intake, activity, and milk yield and weekly for body weight and body condition score. A carrageenan skin test was performed throughout the experiment. Whole blood was used to determine Se and other trace minerals by ICP-MS. White blood cells count and blood hematocrit were assessed. Phagocytosis and leukocytes differential were measured by flow cytometer. Milk yield and components were measured at each milking using Afimilk system and samples

were collected to assess milk composition by DHIA lab. Level of Se, Zn, Cu, and Co were measured in milk by ICP-MS. Data were analyzed by GLIMMIX of SAS with the fixed effect of treatment, breed, time and their interactions and cows as random effect. After 4 weeks into the trial, Se concentration in blood increased 2-fold ($P<0.0001$) in TRT vs. CTR (2.0 vs. 1.0 ppm) which results in higher ($P<0.01$) Se in milk (0.42 vs. 0.25 ppm). Milk yield was higher in TRT vs. CTR (20.2 vs. 18.2 kg/d, $P < 0.05$). No other differences were detected. Our results point out that providing dairy heifers with a relatively low amount of Se-enriched hay is an effective way to increase Se in blood and milk. Se supplementation improved milk yield but had no effect on the function of the immune system in primiparous cows during the transition period.

Keywords: Selenium; immune response; performance; dairy heifers

2018 American Dairy Science Association (ADSA) Annual Meeting Abstract

ALFALFA ENRICHED WITH SE FED TO DAIRY HEIFERS AFFECT THE IMMUNE SYSTEM BUT NOT GROWTH PERFORMANCE IN THEIR OFFSPRING

[2]Matteo Mezzetti, [1]Shana Jaaf, [2]Michele Premi, [1]Angela Krueger, [1]Brandon Batty, [1]Jennifer Belveal, [2]Erminio Trevisi, [1]Gerd Bobe, and [1]Massimo Bionaz

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Selenium is known to affect immune response in dairy cows, and his availability at body level affect his concentration in milk and colostrum. Previous studies have suggested that the effect of selenium on cows could be reflected in their offspring. This study investigated the hypothesis that a higher Se level in cows might improve their calves' immune system, leading to better growth performances. Ten dairy calves born from 4 Holsteins and 6 Jerseys heifers supplemented with 1 kg/100 kg of BW of Alfalfa enriched with Se (3.2 mg/kg DM) or control Alfalfa (0.4 mg Se/kg DM) received their mother's colostrum and were monitored for body weight (**BW**) at calving, average daily gain (**ADG**) and health status. Blood samples were collected at 1, 4 and 24 days to check Se level using mass-spectrometry and white blood cell count (**WBCC**) using a commercial kit. Granulocytes and monocytes % and their phagocytosis were determined using a commercial kit in combination with flow cytometric analysis and granulocytes- and monocytes-specific antibodies. Data were analyzed using proc GLM (ADG, health status)

and Glimmix (BW and blood parameters) of SAS. BW, ADG, health status, WBCC and leukocytes differential in calves were not affected by the Se treatment of the cows. Higher rates of phagocytosis were detected in calves born from cows receiving Alfalfa enriched with Se in comparison to control Alfalfa both in total cells ($p < 0.05$) in granulocytes ($p < 0.1$) and in monocytes ($p < 0.1$). The greater phagocytosis of calves born from cows receiving the Alfalfa enriched with Se indicates an improvement of their immune system in comparison to control. The higher phagocytosis was observed during the most critical phase of the calves' life when the adaptive immune system is developing. Our data indicate that providing organic Se in pregnant cows can partly improve the immune system of the calves. More analyses are underway to further assess the immune and metabolic status of calves.

Keywords: Selenium; immune response; calves

2019 Pacific Northwest (PNW) Animal Nutrition Conference Graduate Student Competition Abstracts

Effect of agronomic selenium biofortified alfalfa hay on selenium status and glutathione peroxidase activity in transition dairy cows and their calves

Shana Jaaf, Brandon Batty, Angela Krueger, Bobe Gerd, Charels Estill, and Massimo Bionaz

Animal and Rangeland Sciences, Oregon State University, 112 Withycombe Hall, 97331, Corvallis, USA

Selenium (Se) is an important trace mineral for humans and livestock. Se is an essential micronutrient for the antioxidant glutathione peroxidase (GPx), which is important to protect the cell from free radicals. Oxidative stress is particularly acute during early post-partum in dairy cows. Supplementation of selenium in pregnant cows can improve the Se status of their offspring, bursting their antioxidant capability and, thus, fostering better health and growth performance. Se supplementation in ruminants is particularly important in region of low Se in soil, such as Oregon; however, inorganic Se supplementation is limited by FDA. Thus, feeding agronomic Se biofortified alfalfa hay to dairy heifers during pregnancy can improve their Se status, antioxidant activity, and the amount of Se transfer into the calves. To test the hypothesis, we used 18 primiparous cows (8 Holsteins and 10 Jerseys) fed ad libitum with a TMR based on grass silage (0.14 mg Se/kg DM). Cows were blocked by breed and randomly assigned to two groups. One group received from 40 days prior parturition to 2 weeks post-partum 1 kg agronomic Se biofortified alfalfa hay (3.2 mg/kg DM)/100 kg of BW mixed with the TMR (TRT). A

group received alfalfa with low Se (0.4 mg/kg DM; CTR). Whole blood in cows and their calves and liver and milk samples of cows were used to determine Se and other trace minerals by ICP-MS. A plasma of cows and their calves, erythrocyte, and milk samples of cows were used to measure GPx activity by using a commercial assay kit. DMI and milk yielded were measured daily. Data were analyzed by GLIMMIX of SAS with the fixed effect of treatment, breed, time and their interactions and cows as random effect. PROC CORR was used to find the correlation between the variances.

After 4 weeks into the trial, Se concentration in blood increased 2-fold (P<0. 0001) in TRT vs. CTR (204.49±19.19 vs. 95.01±20.90 ng/ml) that results in higher (P<0.0001) Se in liver (1137.09±68.70 vs 619.10±73.45 mg/g), Se in milk (48.3415±3.40 vs. 22.52±3.3562 ng/ml), and Se in blood of calves (215.50±10.92 vs 161.22±10.92). GPx activity increased in plasma (92.84±3.44 vs. 77.88±3.36 nmol/min/mL) and erythrocyte of cows (549.23± 35.32 vs. 260.02± 34.15 nmol/min/mL) by Se biofortified hay, while only a numerical increase was detected in their calves and no effect was detected in milk GPx. Our results point out that feeding pregnant dairy heifers with a relatively low amount of Se-fortified alfalfa hay is an effective way to increase Se in blood, liver, and milk, which is, in turn, improved antioxidant activity and the amount of Se that transferred into the calves.

Keywords: Agronomic Se; Selenium concentration blood; GPx; liver; Milk; dairy heifers; Calves

2019 American Dairy Science Association (ADSA) Discover Conference Abstract

Selenium biofortified alfalfa hay supplemented during the peripartum in Holstein and Jersey cows improves antioxidant response and positively affected IgG in Jersey's calves.

Shana Jaaf[1], Matteo Mezzetti[2], Angela Krueger[1], Brandon Batty[1], Michele Premi[2], Francesca Rose Perrotta[1], Janelle Foster[1], Melanie Hanlon[1], Erminio Trevisi[2], and Massimo Bionaz[1]

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[2]*Department of Animal Sciences, Food and Nutrition (DIANA), Università Cattolica del Sacro Cuore, Via Emilia Parmense, 84, 29122 Piacenza PC, Italy*

Abstract:

Selenium (Se) plays essential roles in anti-oxidative response and immune functions in dairy cows and their calves, as demonstrated by improved Se and IgG status in calves by feeding dairy cows with selenized yeast during the end of pregnancy. We hypothesized that feeding Se-biofortified forage to dairy cows during pregnancy can improve their antioxidative status and the immunoglobulin level in calves. To test this hypothesis, we used 18 primiparous cows (8 Holsteins and 10 Jerseys) fed ad libitum with a total mixed ration (TMR). Cows were homogeneously split into two groups to be supplemented with agronomic Se biofortified alfalfa hay (TRT, 3.2 mg Se/kg DM) or control hay (CTR, 0.4 mg Se/kg DM) in reason of 1 kg/100 kg of BW mixed with the TMR

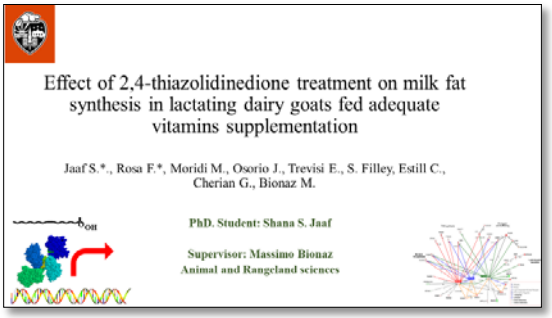
from 40 days prior parturition to 2 weeks post-partum. Blood samples from cows and calves were collected to assess metabolic and inflammatory biomarkers. IgG and IgA were measured in colostrum samples and calve's a serum.

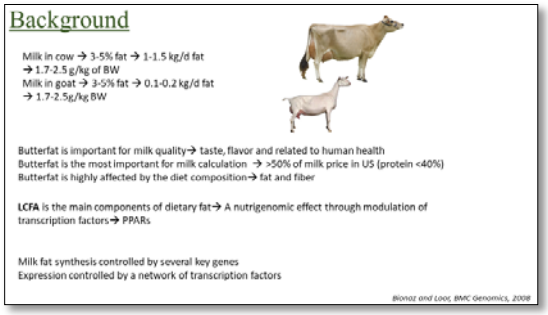
Advanced oxidation protein products were higher in TRT vs. CTR (40.3 vs. 35.1 µmol/l, P=0.01), and higher in Holsteins than Jerseys (40.5 vs. 34.9 µmol/l, P=0.01). Urea was higher in TRT vs. CTR (5.6 vs. 4.9 mmol/l, P= 0.04*).* TRT×Breed×Time interaction *(P<0.05)* was detected for albumin, Ca, and Mg due to a positive response to Se in Jerseys and negative to Holsteins. Calves born from heifers supplemented with Se-biofortified hay had significantly higher haptoglobin concentration (0.49 vs. 0.33 g/l, P=0.02) and higher concentration in Holsteins compared to Jerseys (0.48 vs. 0.34 g/l, P=0.03). A significant TRT×Breed×Time interaction *(P≤0.05)* was detected for ceruloplasmin, paraoxonase, alkaline phosphatase, and IgG concentration in calve's serum. In all cases, Jersey's calves had a better response due to Se-supplementation of their dams compared to Holstein's calves.

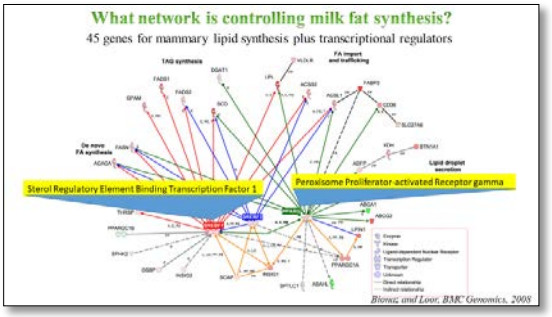
Our results demonstrated that feeding pregnant dairy heifers with a relatively low amount of Se-biofortified alfalfa hay during the dry period helps to ameliorate the antioxidant response but had little effect on other inflammatory and metabolic parameters. Jersey's calves appear to benefit more than Holstein calves to the Se-supplementation of their mothers.

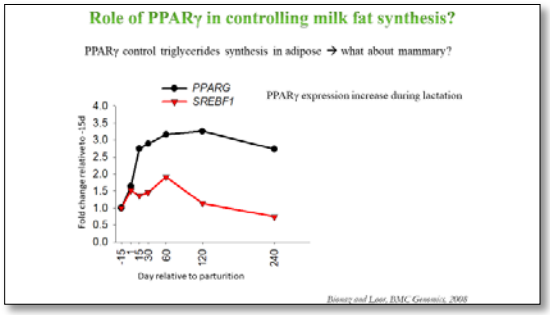
Keywords: Selenium biofortified hay; blood parameters; dairy cows; Calves

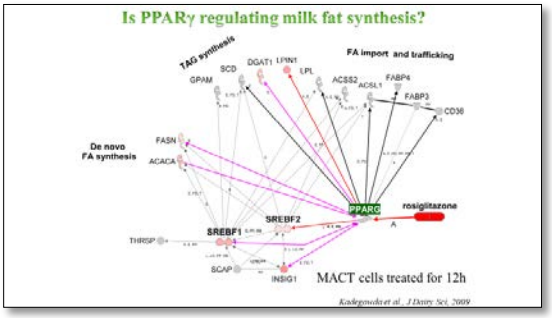
2018 American Dairy Science Association (ADSA) Annual Meeting Presentation

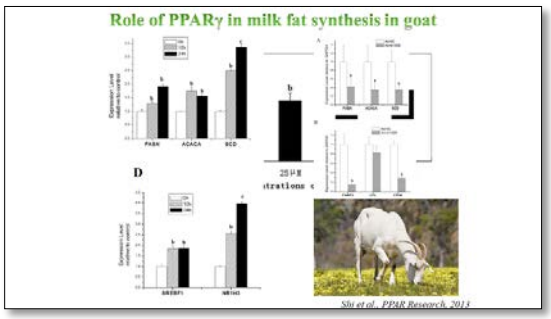






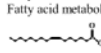




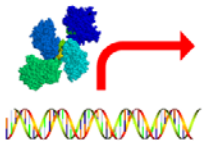


Peroxisome proliferator-activated receptors (PPARs)?

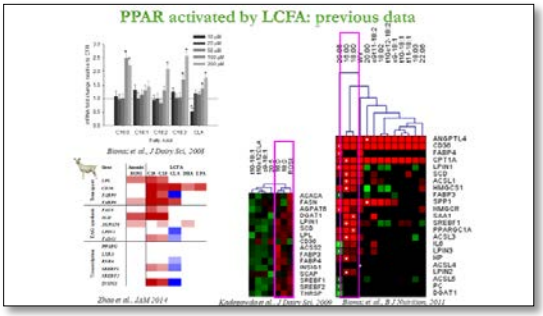
Fatty acids
Fatty acid metabolites



PPAR·RXR

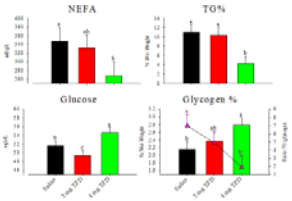


PPAR activated by LCFA: previous data

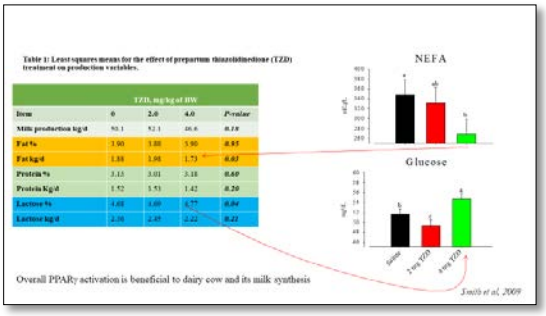


PPARγ role on whole body metabolism

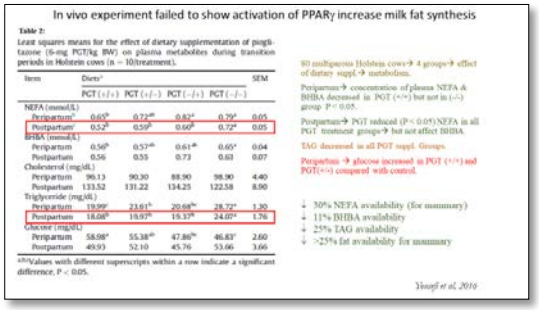
Prepartum cows (n=36) injected daily with Thiazolidinedione, 21d before parturation → better metabolic response post-partum

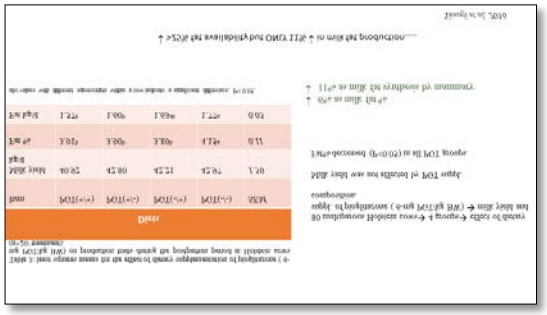


Smith et al., J Dairy Sci, 2009

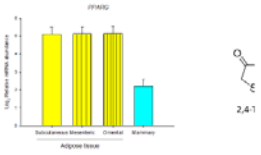


In vivo experiment failed to show activation of PPARγ increase milk fat synthesis





Competition with adipose tissue?



2,4-T

Bionaz et al., PPAR Res., 2013

Hypothesis:

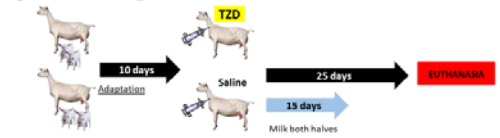
Activation of *PPARγ* by 2,4-TZD affect fat synthesis in goats fed adequate vitamin A supplementation

Objective :

Evaluate the effect of 2,4-TZD on milk composition and blood parameters in lactating goats

Material and Methods

Experimental design



12 lactating goats
n=6/group
2 kids/goat

TZD = 2,4-thiazolidinedione

Grant from Oregon Beef Council

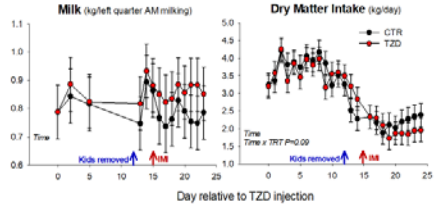
Used the healthy left halve for the last 10 days of experiment

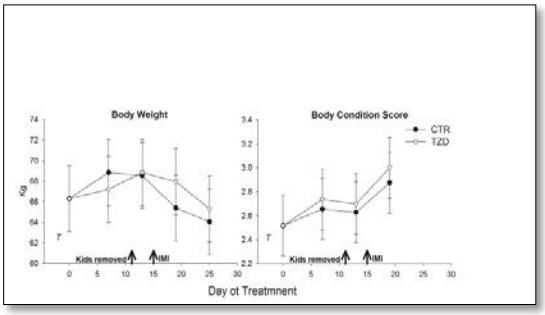
Material and Methods

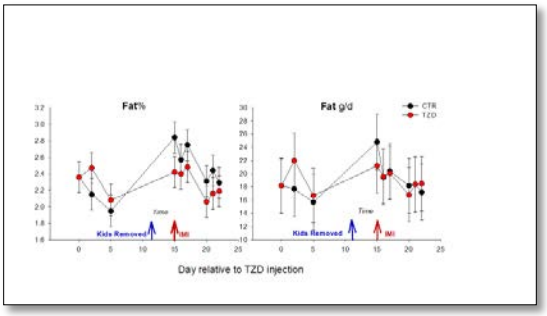
- DMI daily, BW, and BCS
- Milk yield, and composition (Fat, Protein, lactose, SCC, SNF)→ DHIA
- Fatty acid profile in milk→ GC
- Mammary Epithelial cells → gene expression
- Adipose biopsy→ gene expression & histology
- Blood samples for Metabolic parameters (NEFA, BHBA, glucose, Plasma FA, urea)
- Daily drench of Vitamins (A+E+D)

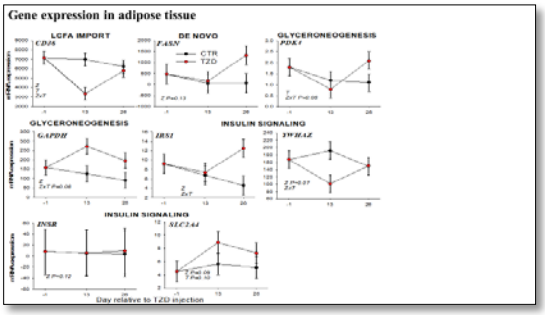
- GLIMMIX of SAS → Treatment (CTR, TZD), and Time and TRT x Time interaction as main effects. Goat = random effect
  Significance: Tukey's-adjusted $P<0.05$.

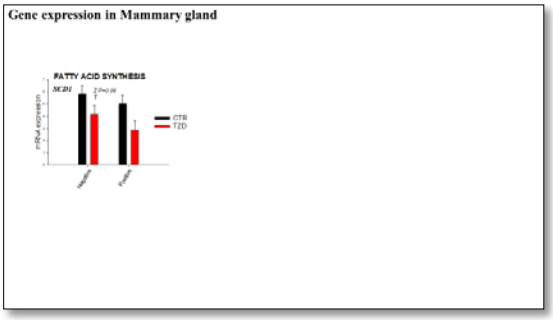
Results:

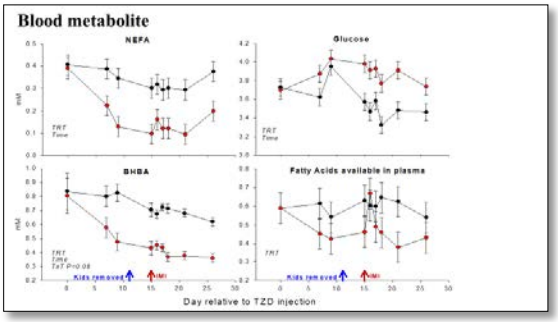


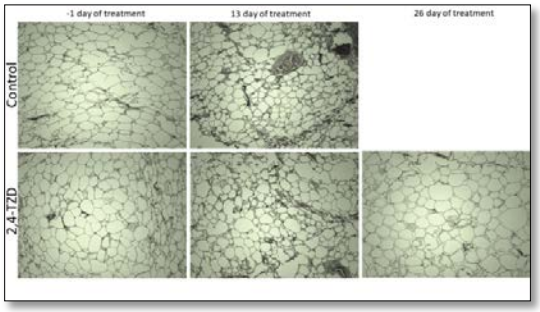

Body Weight
Body Condition Score
CTR
TZD
Kg
Kids removed
IMI
Day ot Treatmnent

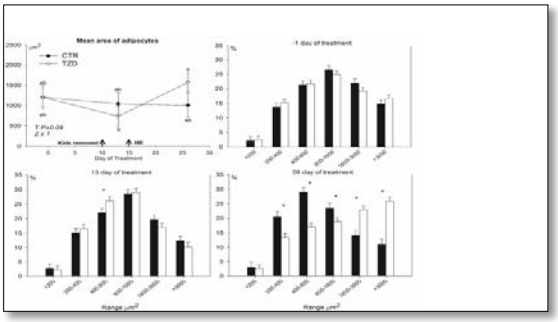

Fat%
Fat g/d
CTR
TZD
Time
Kids Removed
IMI
Day relative to TZD injection


Gene expression in adipose tissue
LGFA IMPORT
CD36
DE NOVO
FASN
GLYCERONEOGENESIS
FOXA1
GLYCERONEOGENESIS
GAPDH
INSULIN SIGNALING
IRS1
PPARA
INSULIN SIGNALING
INSR
SLC2A4
CTR
TZD
Day relative to TZD injection


Gene expression in Mammary gland
FATTY ACID SYNTHESIS
SCD1
CTR
TZD


Blood metabolite
NEFA
Glucose
BHBA
Fatty Acids available in plasma
mM
Kids removed
IMI
Day relative to TZD injection


-1 day of treatment
13 day of treatment
26 day of treatment
Control
2,4-TZD



Conclusions

- We failed to detect any increase in milk fat synthesis or expression of related genes in MEC by TZD.
- Strong improvement in metabolism through decreasing the level of NEFA in blood, increasing adipocyte size by affecting the expression of genes related to insulin signaling and glucose metabolism
- Our data clearly indicated that TZD did not activate PPARγ. These results indicate that other more potent PPARγ agonists, such as rosiglitazone should be used to test the original hypothesis.

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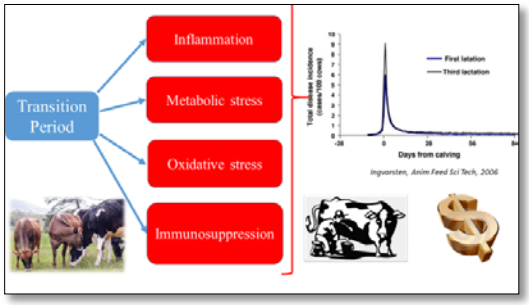
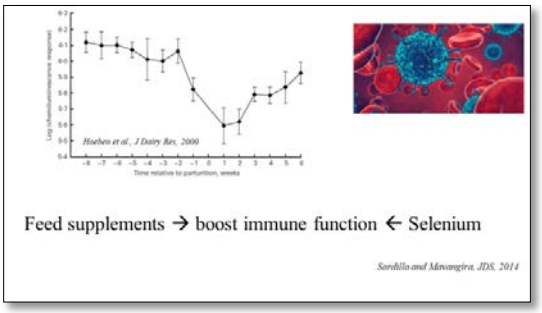
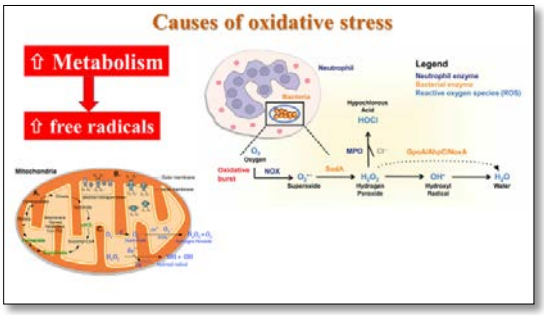
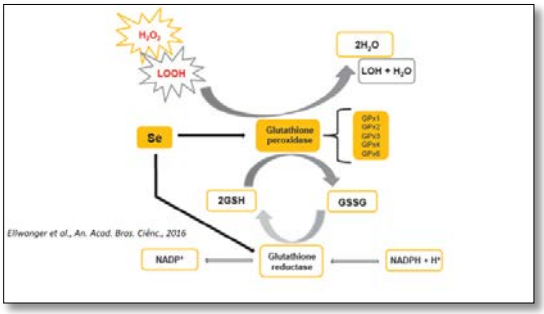
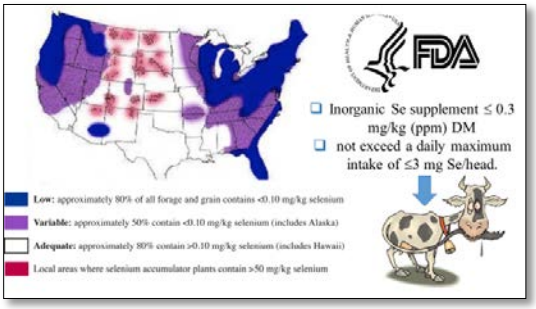
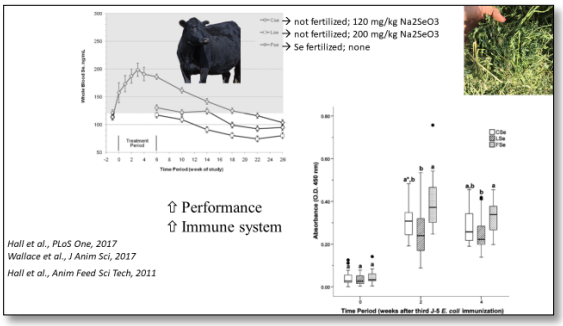










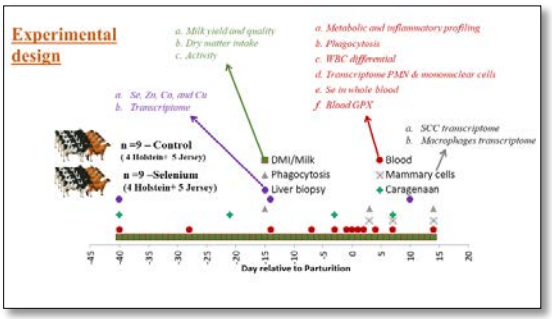


Objectives:

Evaluate the effect of feeding selenium-biofortified alfalfa hay to dairy cows during transition period on selenium status, immune function, and performance

Hypothesis:

Feeding Se-enriched hay during pregnancy improves performance and the immune system in transition dairy cows

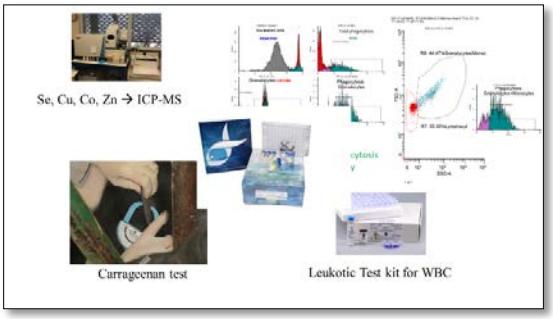


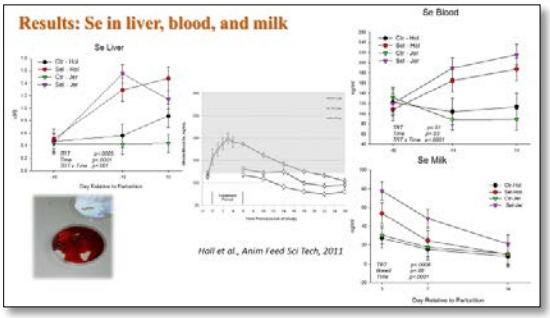
Materials and Methods

- Primiparous:
  - ❑ TRT = 1 kg DM/100 kg BW (3.2 mg Se/kg DM)
  - ❑ CTR = 1 kg DM/100 kg BW (0.4 mg Se/kg DM)
  - ❑ TMR→ Corn Silage, glover, haylage, Alfalfa
- BW and BCS
- Milk (DHIA) → Selenium
- Calan gate→ individually

Statistical analysis

GLIMMIX (SAS) TRT, Time, Breed, and interactions.
Cow=random Significant $P \leq 0.05$

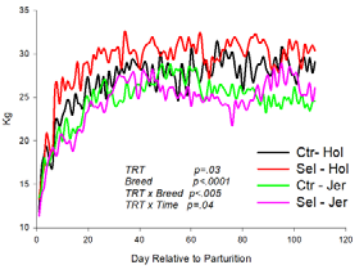




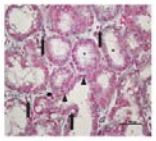
Results: milk yield

⇑ Milk yield:

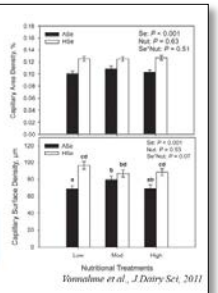
- Ewes supplemented with high Se *(Meyer et al., 2011)*.
- Yeast or fertilizer Se *(Sahoussi et al., 2016)*



Supranutritional Se → ⇑ mammary vascularity in ewe

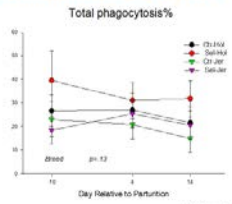


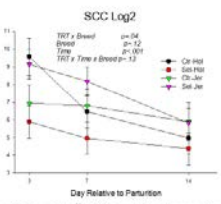
- Adequate Se (ASe; 9.5 µg /kg of body weight)
- High Se (HSe; 81.8 µg /kg of body weight)



Vonnahme et al., J Dairy Sci, 2011

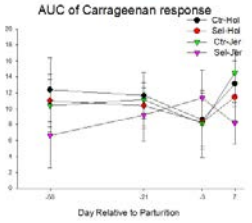
Results: immune response

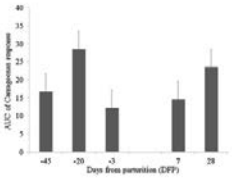




Dietary Se associated with ⇓ SCC and incidence of mastitis *(Weiss et al., 1990)*

Results: Carrageenan skin test





Juhan et al., Vet Immun and Immunopath, 2015

Conclusions

- **Relative low amount of Se-enriched hay → effective**
- **Se supplementation improved milk yield**
- **No effect on the function of the immune system**

Questions?

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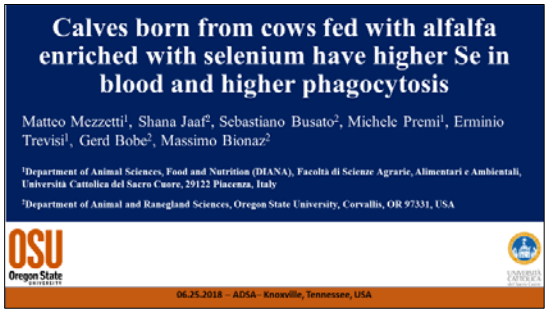
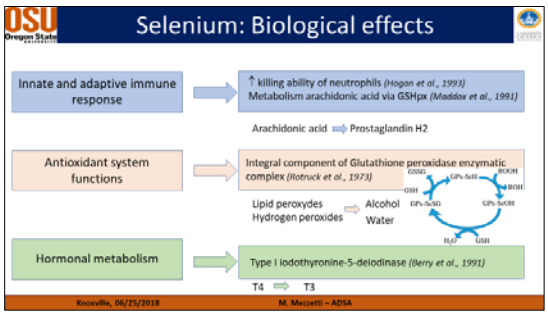
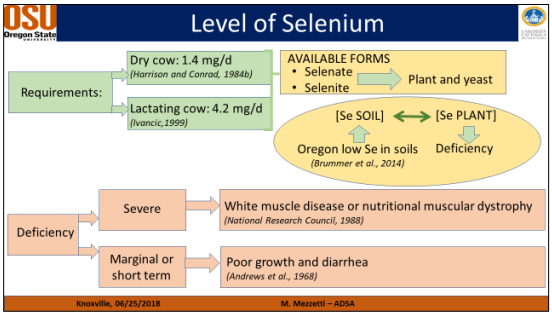
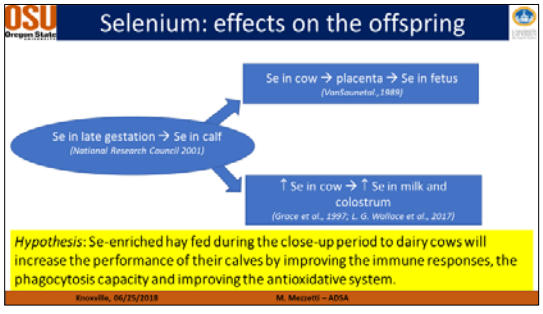
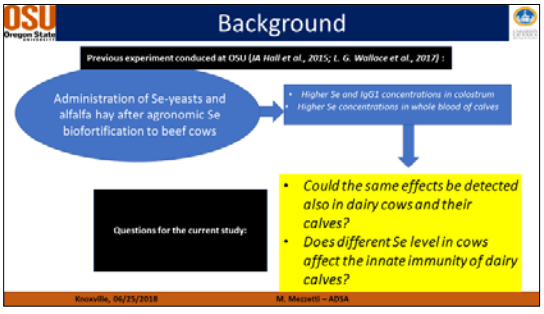


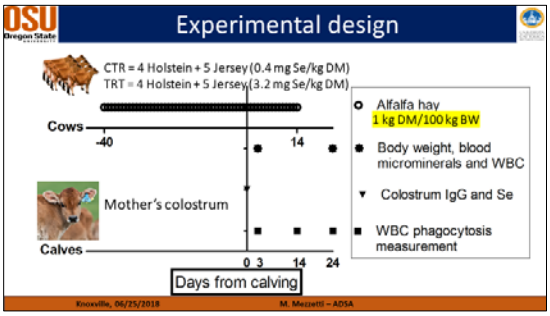


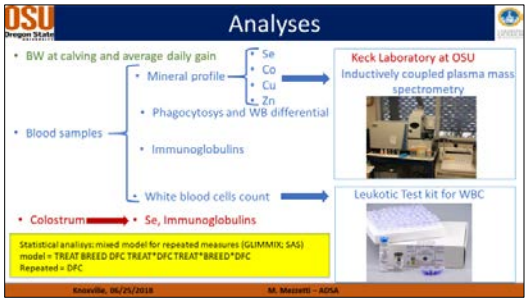


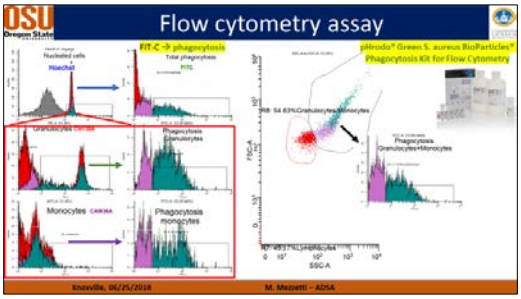




MATERIALS AND METHODS

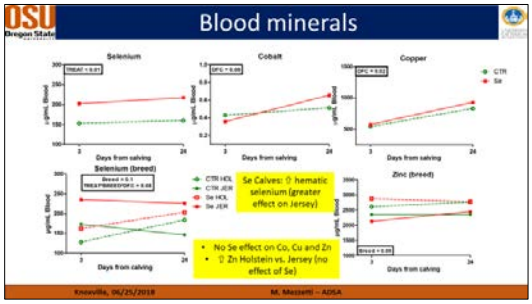


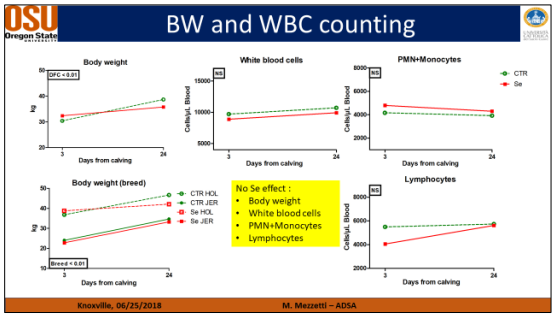


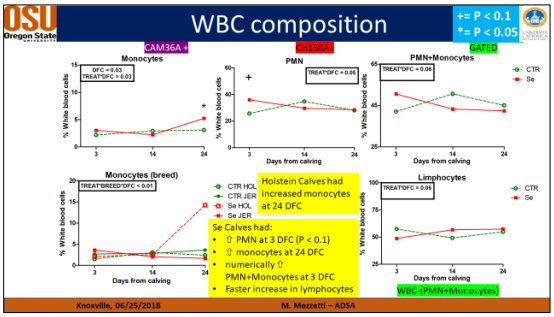


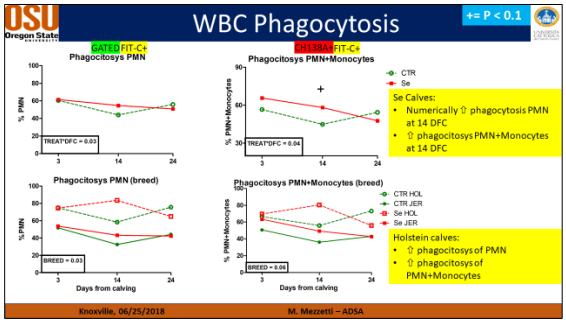
RESULTS

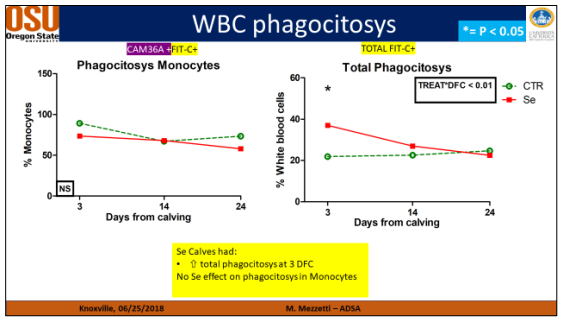
06.25.2018 – ADSA– Knoxville, Tennessee, USA

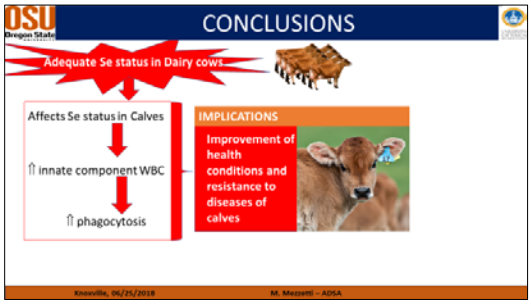



BW and WBC counting
Body weight
White blood cells
PMN+Monocytes
Body weight (breed)
Lymphocytes
Days from calving
CTR
Se
CTR HOL
CTR JER
Se HOL
Se JER
No Se effect :
Body weight
White blood cells
PMN+Monocytes
Lymphocytes
Knoxville, 06/25/2018
M. Mezzetti – ADSA


WBC composition
+= P < 0.1
*= P < 0.05
CAM36A +
CH138A-
GATED
Monocytes
PMN
PMN+Monocytes
Monocytes (breed)
Lymphocytes
% White blood cells
Days from calving
CTR
Se
CTR HOL
CTR JER
Se HOL
Se JER
Holstein Calves had increased monocytes at 24 DFC
Se Calves had:
⇧ PMN at 3 DFC (P < 0.1)
⇧ monocytes at 24 DFC
numerically ⇧ PMN+Monocytes at 3 DFC
Faster increase in lymphocytes
WBC-(PMN+Monocytes)
Knoxville, 06/25/2018
M. Mezzetti – ADSA

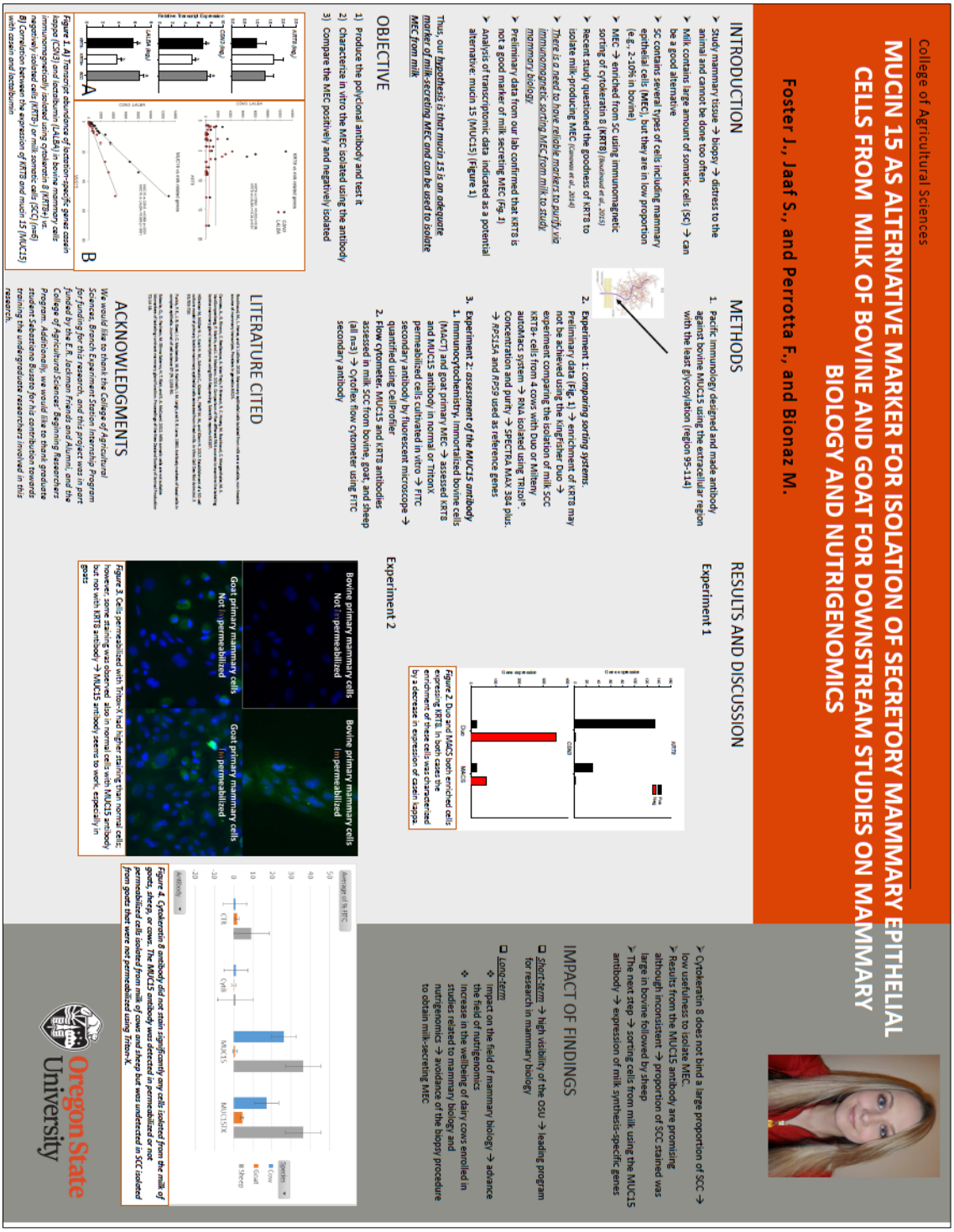

WBC Phagocytosis
+= P < 0.1
GATED FIT-C+
CH138A- FIT-C+
Phagocytosys PMN
Phagocytosys PMN+Monocytes
Phagocytosys PMN (breed)
Phagocytosys PMN+Monocytes (breed)
% PMN
% PMN+Monocytes
Days from calving
CTR
Se
CTR HOL
CTR JER
Se HOL
Se JER
Se Calves:
Numerically ⇧ phagocytosis PMN at 14 DFC
⇧ phagocytosys PMN+Monocytes at 14 DFC
Holstein calves:
⇧ phagocytosys of PMN
⇧ phagocytosys of PMN+Monocytes
Knoxville, 06/25/2018
M. Mezzetti – ADSA


WBC phagocitosys
*= P < 0.05
CAM36A- FIT-C+
TOTAL FIT-C+
Phagocitosys Monocytes
Total Phagocitosys
% Monocytes
% White blood cells
Days from calving
CTR
Se
Se Calves had:
⇧ total phagocitosys at 3 DFC
No Se effect on phagocitosys in Monocytes
Knoxville, 06/25/2018
M. Mezzetti – ADSA


CONCLUSIONS
Adequate Se status in Dairy cows
Affects Se status in Calves
⇧ innate component WBC
⇧ phagocytosis
IMPLICATIONS
Improvement of health conditions and resistance to diseases of calves
Knoxville, 06/25/2018
M. Mezzetti – ADSA

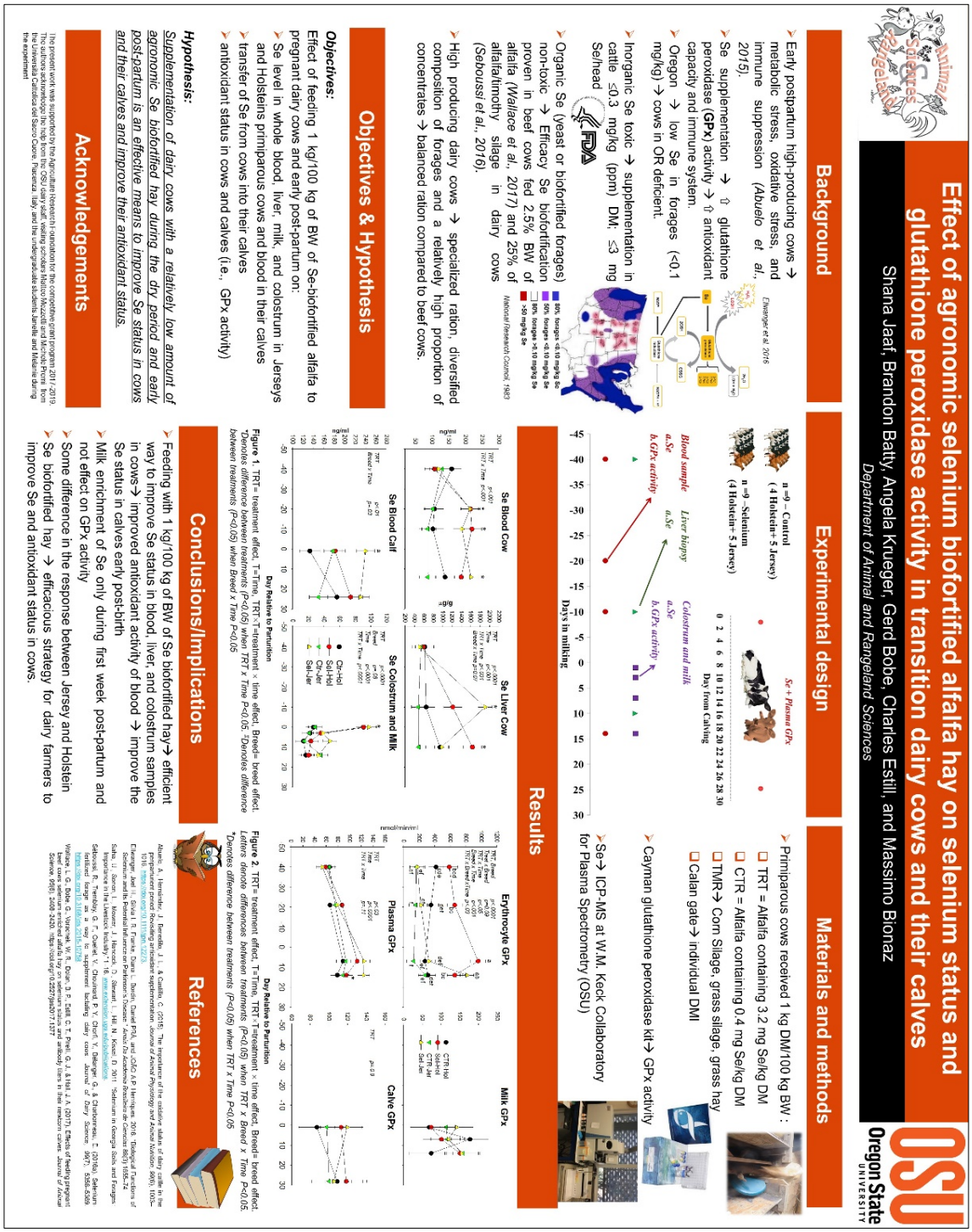
2018 4th Annual College of Agriculture Science (CAS) Experiential Expo Poster

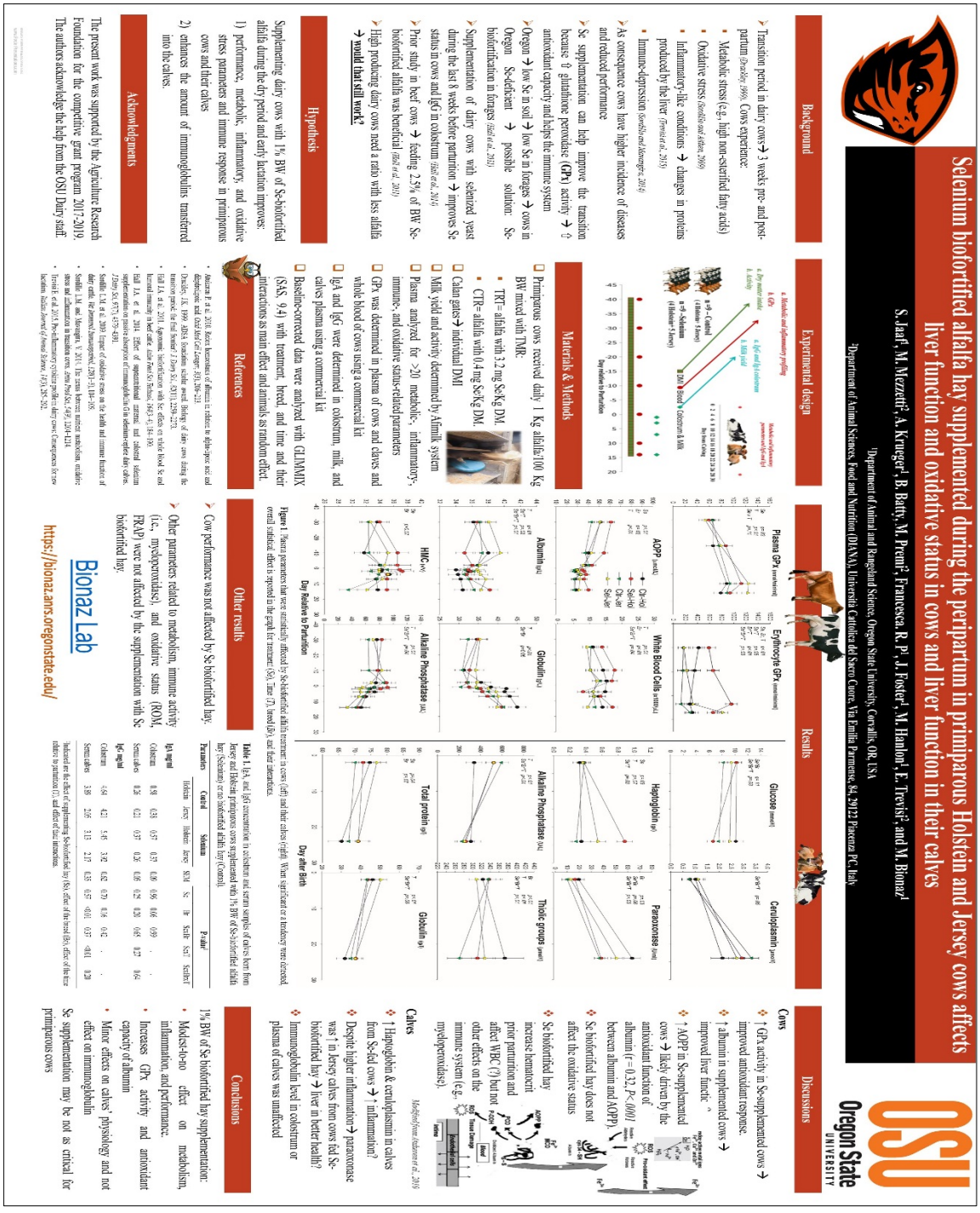
MUCINE 15 AS ALTERNATIVE MARKER FOR ISOLATION OF SECRETORY MAMMARY EPITHELIAL CELLS FROM MILK OF BOVINE AND GOAT FOR DOWNSTREAM STUDIES OM MAMMARY BIOLOGY AND NUTRIOGENOMICS



2019 Pacific Northwest (PNW) Animal Nutrition Conference Graduate Student Competition Poster

EFFECT OF AGRONOMIC SELENIUM BIOFORTIFIED ALFALFA HAY ON SELENIUM STATUS AND GLUTATHIONE PEROXIDASE ACTIVITY IN TRANSITION DAIRY COWS AND THEIR CALVES



2019 American Dairy Science Association (ADSA) Discover Conference Poster

SELENIUM BIOFORTIFIED ALFALFA HAY SUPPLEMENTED DURING THE PERIPARTUM IN PRIMIPAROUS HOLSTEIN AND JERSEY COWS AFFECTS LIVER FUNCTION AND OXIDATIVE STATUS IN COWS AND LIVER FUNCTION IN THEIR CALVES